Article

Impact of High-Moisture Ear Corn on Antioxidant Capacity, Immunity, Rumen Fermentation, and Microbial Diversity in Pluriparous Dairy Cows

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Abstract: Due to the increasing costs of livestock farming, it is important to find cost-effective alternatives of feed stuffs. This study investigated the effects of high-moisture ear corn (HMEC) feeding on the production performance, serum antioxidant capacity, immunity, and ruminal fermentation and microbiome of dairy cows. Thirty pluriparous Chinese Holstein cows were randomly allocated to two groups: steam-flaked corn (SFC) and HMEC (replacement of 2 kg equal dry matter SFC) and fed for a 60 day trial. The results showed replacing SFC with HMEC significantly increased dry matter intake, milk yield, and 4% fat-corrected milk yield ($p < 0.05$). Serum levels of superoxide dismutase, glutathione peroxidase, and immunoglobulins G, M, and A were significantly higher, and those of creatinine and cholesterol were significantly lower, in the HMEC group than in the SFC group ($p < 0.05$). HMEC also significantly increased total volatile fatty acid and acetate ($p < 0.05$) concentrations. In both groups, the dominant phyla of ruminal bacteria were Bacteroidetes, Firmicutes, and Actinobacteria, and the dominant genera were *Prevotella*, *NK4A214-group*, and *Succiniclasticum*. *Mogibacterium*, *Eubacterium nodatum group*, *norank-f-Lachnospiraceae*, and *Eubacterium brachy group* were significantly enriched in the ruminal fluid of HMEC-group cows ($p < 0.05$). In conclusion, replacing SFC with HMEC improved production performance, antioxidant capacity, and immunity, while regulating both ruminal fermentation and the composition of the ruminal microbiome in dairy cows.

Keywords: high-moisture ear corn; rumen fermentation; rumen microbiome; dairy cows; antioxidant capacity; immunity

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1. Introduction

Corn is a widely used energy source in dairy cow diets, and is commonly processed for storage using methods such as grinding and steam flaking. Steam-flaked corn (SFC), while improving starch digestibility, increases processing costs. The rapid starch degradation of SFC in the rumen can increase the risk of ruminal acidosis [1]. In contrast, high-moisture ear corn (HMEC) contains relatively high levels of neutral detergent fiber (NDF), which may reduce the risk of acidosis in the rumen [2]. HMEC refers to a high-starch feed material obtained from corn cobs and kernels with a moisture content ranging from 28% to 33%, undergoing crush and subsequent fermentation in an anaerobic environment for a duration of over 60 days. Research has shown that feeding dairy cows with HMEC can improve starch digestibility, increase microbial protein production, and enhance ammonia nitrogen utilization efficiency, resulting in better production performance [3,4]. Additionally, the fermentation process has a protective effect on the β-carotene content of corn [5]. This

phenomenon potentially contributes to the immune-enhancing attributes of HMEC. Wet storage of HMEC leads to the production of organic acids, including lactic acid and acetic acid [6]. These acids lower the pH of the storage environment, inhibiting the growth of bacteria and preventing mold, thereby reducing nutrient loss. When the pH drops below 4.0, the activities of yeast, mold, aerobic bacteria, and other microorganisms are inhibited, creating a stable environment that facilitates long-term preservation of the raw materials [7].

In recent years, the dairy farming industry has faced challenges, including high feeding costs, limited forage energy, and the need for more efficient livestock farming practices [8]. The search for sustainable and cost-effective feed sources has led to the exploration of alternative ingredients for livestock diets. The innovations in alternative feed sources offer opportunities for improved productivity and resource management [9]. As a result, HMEC feed has gained popularity because its use of cobs can increase biomass production and lower the cost with respect to SFC. However, little research has been conducted regarding the effects of HMEC being added to the diet of dairy cows in China. Ruminal microbes play crucial roles in nutrient digestion, production performance, and overall animal health in ruminants. Although diet substantially influences the modulation of the microbial population and fermentation capacity in the rumen [10], there have been no research reports on the influence of HMEC on ruminal microorganisms.

As the HEMC and SFC are basically of the same origin with different processing methods, this study evaluated the effects of replacing dietary SFC with HMEC on the production performance, serum antioxidant capacity, immunity, and ruminal fermentation and microbiomes of Chinese Holstein dairy cows. The results of this study should establish a theoretical foundation for widespread use of HMEC in the dairy cows.

2. Materials and Methods

2.1. Diet and Animal Management

The Ethics Committee on Animal Use at Beijing University of Agriculture approved the procedures for animal care and handling necessary for this experiment (Beijing, China) (approval number: BUA2023072).

The HMEC was manufactured by Qiushi Grain Trade Company (Xingtai, China) using LAB fermentation additives (Lactobacillus plantarum and Lactobacillus brucei both at an application rate of 1×10^6 cfu/g of fresh matter) from NDS Science and Technology Development Co., Ltd. (Beijing, China). The animal trial was carried out at the Fouao Dairy Farm (Hengshui, China). Thirty pluriparous Chinese Holstein dairy cows (production—38.97 ± 3.20 kg milk/day; days in milk—129.00 ± 3.95; parity—2.80 ± 1.06) were used. The cattle were randomly separated into two feeding groups: SFC and HMEC, in which HMEC replaced 2 kg SFC on an equal dry matter basis. The diets were prepared following the guidelines established by the National Academies of Sciences, Engineering and Medicine (NASEM) [11]. The composition and nutrient content of the diets are provided in Table 1. The study included a 14 day adaptation period followed by a 60 day formal observation phase. Throughout the experimental period, all animals were provided ad libitum access to both feed and water.

Table 1. Ingredients and nutritional content of diets (%DM) with high-moisture ear corn (HMEC) and steam-flaked corn (SFC).

Items	Treatment	
	SFC	HMEC
Corn silage	16.76	16.76
Wheat silage	6.51	6.51
Alfalfa hay	10.74	10.74
Oat grass	1.68	1.68
Whole cotton seed	4.47	4.47
Brewers grains	11.52	11.52
High-moisture ear corn	--	6.58

Table 1. *Cont.*

Items	Treatment	
	SFC	**HMEC**
Steam-flaked corn	6.58	--
Corn grain	19.30	19.30
Soy bean meal	10.99	10.99
Expanded soybean	0.72	0.72
Canola meal	3.19	3.19
DDGS	3.99	3.99
Premix [1]	1.27	1.27
Bypass fat	2.27	2.27
Total	100.00	100.00
Nutrient levels		
DM	50.10	50.10
CP	16.92	16.93
EE	5.36	5.43
ADF	15.66	15.62
NDF	27.90	28.78
Starch	26.97	25.68
Ca	0.69	0.69
P	0.38	0.39
NE_L/(MJ/kg) [2]	6.70	6.71

[1] Premix per kg includes: vitamin E (2000 IU), vitamin D (40 KIU), vitamin A (200 KIU), zinc (1000 mg), manganese (800 mg), copper (180 mg), iodine (15 mg), and selenium (10 mg). [2] The net energy requirement for lactation (NE_L) was calculated according to the National Academies of Sciences, Engineering and Medicine (2021) [11], while the remaining values were measured. ADF, acid detergent fiber; Ca, calcium; CP, crude protein; DDGS, distillers dried grains with solubles; EE, ether extract; NDF, neutral detergent fiber; P, phosphorus.

2.2. Chemical Analysis

Forage samples were air dried at 65 °C until they reached a constant weight, and then ground to achieve a consistency suitable for analysis. The analysis encompassed the determination of various components, including the following: dry matter (DM), ether extract (EE), crude ash (Ash), crude protein (CP), acid detergent fiber (ADF), and NDF, following the established protocols outlined by the Association of Official Agricultural Chemists [12]. Phosphorus (P) and calcium (Ca) content were quantified using previously established methods described by Wang et al. [13].

2.3. Dry Matter Intake, Milk and Composition

Feed intake and milk yield were documented on a daily basis. To guarantee complete intake, the dairy cows were provided with unrestricted access to the diet. To calculate dry matter intake (DMI), feed quantities for stall barn cows were measured, alongside the remaining feed, three times a week and finally averaged.

Samples of 50 mL raw milk were collected in the morning, afternoon, and evening milking on the 1st, 30th, and 60th day, with a ratio of 4:3:3 ($v/v/v$). The mixed milk was preserved at 4 °C using a saturated potassium dichromate solution. Within 2 to 4 days after sampling, a Delta FT-A multifunctional dairy analyzer (PerkinElmer, Waltham, MA, USA) was used to determine concentrations of milk fat, protein, lactose, total solids, urea, and somatic cell count. To calculate feed efficiency and 4% fat-corrected milk (FCM) the following formulae were employed:

$$\text{Feed efficiency} = 4\%\ \text{FCM}/\text{DMI};$$

$$4\%\ \text{FCM (kg/d)} = (0.4 + (0.15 \times \text{milk fat (\%)})) \times \text{milk yield (kg/d)}$$

2.4. Blood Sampling and Analysis

During the experimental period, jugular vein blood samples were drawn from all dairy cows every 14 days, before morning milking. Ten mL Vacutainer tubes without any

additives (Shandong Aosaite Medical Instrument Co., Ltd., Heze, China) were utilized, resulting in the extraction of approximately 8 mL blood from each cow. The blood samples were centrifuged at 3000× *g* for 20 min at 4 °C, and the resulting serum was transferred into 2-mL tubes. The serum samples were then stored at −20 °C until analysis.

The glucose, cholesterol, albumin, urea, creatinine, and triglyceride (TG) of serum were assessed using an automated biochemical analyzer (TBA-120FR, Toshiba Ltd., Tokyo, Japan). The concentrations of various serum biomarkers, including total glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), total antioxidant capacity (T-AOC), immunoglobulin (Ig)M, IgG, IgA, and tumor necrosis factor-α (TNF-α) were evaluated using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's guidelines.

2.5. Ruminal Fermentation Analysis

On the 60th day of the experiment, approximately 150 mL of ruminal fluid sample was collected from each cow using an oral stomach tube. To minimize contamination from the oral saliva, the initial 50 mL of each collected sample was discarded. The pH in the remaining ruminal fluid was promptly measured using a pH-100 Portable Acidity Meter (Shanghai Lichen-BX Instrument Technology Co., Ltd., Shanghai, China), and the samples were then filtered through 4 layers of medical gauze and kept at −80 °C until analysis. For analysis, the frozen ruminal fluid samples were thawed and then subjected to centrifugation at 4 °C for 15 min at 5400× *g*. The resulting clear liquid was employed to determine the levels of volatile fatty acids (VFAs) and ammonia nitrogen (NH_3-N). Ruminal NH_3-N concentrations were determined following the colorimetric method outlined by Broderick et al. [14], and VFA concentrations were quantified using gas chromatography [15].

2.6. High-Throughput Sequencing

Following the instructions provided with e E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA), total DNA extraction of rumen bacteria was performed. Assessments of the concentration and quality of the extracted DNA were made using a NanoDrop 2000 ultra-micro spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the DNA was further evaluated by subjecting it to 1% agarose gel electrophoresis for 20 min under a voltage of 5 V/cm. The V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR). The primers used for high-throughput sequencing were 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) [16]. The prepared clone libraries were then subjected to Illumina MiSeq sequencing technology for high-throughput sequencing (Illumina Corporation, San Diego, CA, USA) to obtain sequence data.

2.7. Bioinformatics Analysis

Data processing and analysis were supported by the MiSeq Bioinformatics Cloud platform. Raw data obtained from MiSeq sequencing were spliced by pair-end double-end sequence splicing (FLASH V 1.2.11) according to the overlap relationship [17]. The sequences were subjected to quality control and screening Fastp (V 0.19.6) software. Sample identification was accomplished by utilizing the barcodes and primers located at both ends of the sequences, followed by appropriate adjustment of sequence orientation [18]. Using the Usearch software (V 11) platform, the sequences were screened, and operational taxonomic unit (OTU) statistics were performed. The sequences were clustered on the basis of 97% similarity and subsequently classified and annotated using the RDP Classifier Bayesian algorithm (V 2.13) and the Silva 138 16S rRNA database. The community composition of the samples was analyzed at each classification level. Alpha diversity (including the Simpson, Shannon, Chao 1, and ACE indices) was computed using QIIME (V 1.9.1) software. Non-metric multidimensional scaling (NMDS) was employed to visualize and compare the beta-diversity of the ruminal microbial community structures of the two groups. Principal coordinate analysis (PCoA) was used to derive coordinates for visualization of complex

multidimensional data [19]. Venn diagrams were used to analyze common and specific OTUs between groups. Linear discriminant analysis effect size (LEfSe) was employed to identify variations in dominant microbial communities between the groups, using selection conditions of $p < 0.05$ and linear discriminant analysis (LDA) scores > 2. To assess the relationships between different phenotypic indices and the microbiome, Spearman correlation heatmaps were constructed.

2.8. Statistical Analysis

Data on milk yield and composition, DMI, and serum indices were analyzed using the MIXED procedure in SAS (ver. 9.4, SAS Institute Inc., Cary, NC, USA). The statistical model included treatment, sampling date, and the interaction between treatment and sampling time point as the fixed effects, sampling date as the repeated measurement, and animal as the random effect. Ruminal fermentation parameters and alpha-diversity indices were analyzed using the one-way ANOVA model. Data are expressed as the mean ± standard error of the mean (SEM). Treatment effects were considered significant at $p < 0.05$, and trends at $0.05 \leq p < 0.10$ were discussed.

3. Results

3.1. DMI, Milk, and Composition

Table 2 illustrates the effects of HMEC on DMI, milk yield, and milk composition in dairy cows. Notably, the HMEC group exhibited significantly higher DMI ($p < 0.05$), milk yield ($p < 0.01$), and 4% FCM ($p < 0.05$) compared with the SFC group. Further, there were trends towards increased feed efficiency and milk protein production ($p = 0.079$ and $p = 0.097$, respectively). Conversely, no significant between-group differences were observed in terms of milk fat, lactose, total solids, and milk urea nitrogen concentrations, or somatic cell count ($p > 0.05$).

Table 2. Comparison of dry matter intake (DMI), and milk yield and composition of milk between the SFC and HMEC groups.

Items	Treatment		SEM	*p*-Value		
	SFC	HMEC		Treatment	Time	Treatment × Time
Milk yield (kg/d)	38.21 [b]	40.22 [a]	0.29	<0.01	0.02	0.09
DMI (kg/d)	24.37 [b]	25.36 [a]	0.38	0.01	0.66	0.89
4% FCM (kg/d)	34.62 [b]	38.99 [a]	1.54	0.01	0.93	0.99
Feed efficiency	1.42	1.54	0.12	0.08	0.93	0.99
Fat (%)	3.75	3.90	0.26	0.13	0.93	0.99
Protein (%)	3.27	3.50	0.14	0.09	0.95	0.60
Lactose (%)	5.34	5.31	0.06	0.61	0.59	0.16
Total solid (%)	13.01	13.23	0.39	0.58	0.98	0.72
Somatic cell count ($\times 10^4$ cells/mL)	23.22	18.56	4.18	0.28	0.51	0.87
Milk urea nitrogen (mg/dL)	16.70	16.31	1.47	0.80	0.15	0.32

[a,b] Different superscript letters within the same row indicate significant between-group differences ($p < 0.05$). FCM, fat-corrected milk; SEM, standard error of the mean.

3.2. Blood Indices of Antioxidant Parameters, Immunity, and Biochemical

The HMEC group exhibited significantly higher serum levels of SOD ($p < 0.05$) and GSH-Px ($p < 0.01$) compared with the SFC group (Table 3). Additionally, feed containing HMEC had a significant impact on the levels of IgG, IgA, and IgM in the immune index (all $p < 0.05$). In contrast, the SFC group exhibited notably elevated levels of CREA and CHOL compared with the HMEC group (both $p < 0.05$). Nevertheless, there were no significant distinctions between the two feeding groups in terms of MDA, T-AOC, CAT, urea, albumin, glucose, TG, or TNF-α concentration ($p > 0.05$).

Table 3. Serum biochemical, immune, and antioxidant indices in the SFC and HMEC groups.

Items	Treatment		SEM	*p*-Value		
	SFC	HMEC		Treatment	Time	Treatment × Time
SOD (U/mL)	103.09 [b]	111.69 [a]	3.58	0.03	0.02	0.07
MDA (nmol/mL)	4.75	4.51	0.42	0.58	0.05	0.36
T-AOC (U/mL)	9.38	9.39	0.43	0.98	0.64	0.22
CAT (U/mL)	7.30	7.24	0.44	0.90	0.14	0.32
GSH-Px (U/mL)	865.42 [b]	922.91 [a]	17.66	0.01	0.64	0.04
IgG (g/L)	10.55 [b]	11.78 [a]	0.44	0.01	0.30	0.05
IgA (g/L)	0.75 [b]	0.88 [a]	0.05	0.02	0.07	0.04
IgM (g/L)	2.70 [b]	3.27 [a]	0.57	0.03	0.02	0.53
UREA (mmol/L)	5.51	4.98	0.48	0.28	0.05	0.57
CREA (μmol/L)	5.24 [a]	3.56 [b]	2.11	0.02	0.45	0.89
ALB (g/L)	36.57	36.71	0.96	0.88	0.73	0.64
GLU (mmol/L)	3.51	3.58	0.12	0.20	0.31	0.05
TG (mmol/L)	0.16	0.16	0.02	0.97	0.17	0.46
CHOL (mmol/L)	6.67 [a]	5.62 [b]	0.47	0.04	0.13	0.86
TNF-α (pg/mL)	42.03	41.54	17.55	0.98	0.10	0.28

[a,b] Different superscripts within the same row indicate significant between-group differences ($p < 0.05$). ALB, albumin; CAT, catalase; CHOL, cholesterol; CREA, creatinine; GLU, glucose; GSH-Px, total glutathione peroxidase; IgM/G/A, immunoglobulins M/G/A; MDA, malondialdehyde; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TNF-α, tumor necrosis factor-α; TG, triglyceride; UREA, urea.

3.3. Ruminal Fermentation Parameters

The impact of replacing SFC with HMEC on ruminal fermentation parameters (pH, NH_3-N, VFAs) is presented in Table 4. Compared with the SFC group, the HMEC group showed significant increases in the concentrations of total VFAs (TVFAs) ($p < 0.05$) and acetate ($p < 0.05$). There was also a tendency towards an increase in the concentration of propionate ($p = 0.07$) and a decrease in the concentration of NH_3-N ($p = 0.061$) in the HMEC group. Nonetheless, we did not discern any significant impacts of either diet on pH, concentrations of isobutyrate, butyrate, isovalerate, or valerate, or the acetate:propionate ratio (A:P) ($p > 0.05$).

Table 4. Comparison of ruminal fermentation parameters in the SFC and HMEC groups.

Items	Treatment		SEM	*p*-Value
	SFC	HMEC		
pH	6.56	6.59	0.28	0.90
NH_3-N (mg/dL)	12.65	8.25	2.03	0.06
TVFA (mmol/L)	97.70 [b]	120.09 [a]	9.96	0.03
Acetate (mmol/L)	53.12 [b]	70.58 [a]	6.56	0.03
Propionate (mmol/L)	24.63	35.81	5.42	0.07
Isobutyrate (mmol/L)	0.95	0.95	0.24	1.00
Butyrate (mmol/L)	10.97	9.71	2.34	0.60
Isovalerate (mmol/L)	1.45	1.56	0.35	0.77
Valerate (mmol/L)	1.58	1.48	0.33	0.77
A:P [1]	2.31	2.04	0.40	0.51

[a,b] Different superscripts within the same row indicate significant between-group differences ($p < 0.05$). [1] A:P, acetate: propionate ratio; NH_3-N, ammonia nitrogen; TVFAs, total volatile fatty acids.

3.4. Rumen Microbial Richness and Diversity

The impact of HMEC feeding on alpha-diversity indices of the ruminal microbiome is presented in Table 5. The replacement of SFC with HMEC in the rations given to our dairy cows had no significant effects on Simpson, Shannon, Chao 1, or ACE indices (all $p > 0.05$), indicating that there were no noteworthy alterations in the alpha diversity of the ruminal microbial community. Differences in the beta diversity of this community between the two

feeding groups were assessed through NMDS and PCoA analyses. The findings clearly demonstrated overlapping clustering of microbiota between the two treatment groups, as depicted in Figure 1A,B. The beta-diversity analysis of the ruminal fluid showed similar patterns within the microbial communities of the HMEC and SFC groups, suggesting no significant variations.

Table 5. Comparison of microbial alpha-diversity indices in the SFC and HMEC groups.

Items	Treatment		SEM	*p*-Value
	SFC	HMEC		
ACE	1287	1221.8	48.13	0.19
Chao 1	1304.5	1224.9	52.38	0.14
Shannon	5.33	5.21	0.11	0.32
Simpson	0.014	0.020	<0.01	0.23

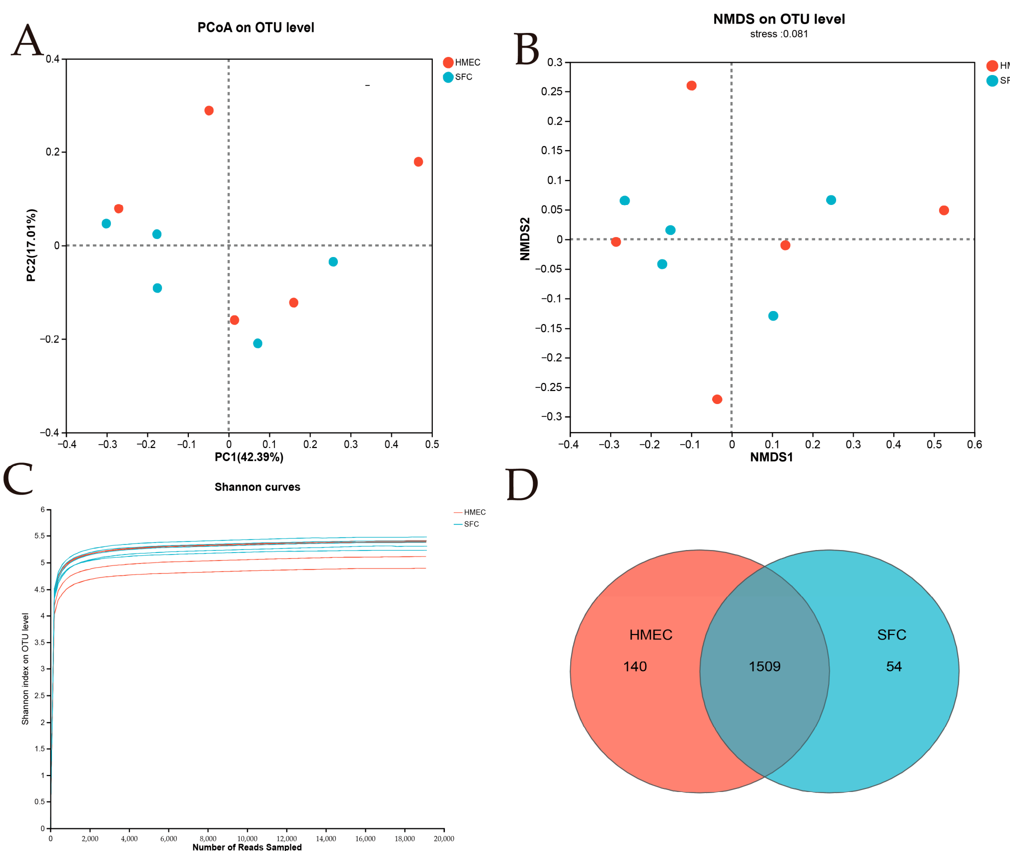

Figure 1. The impact of replacing dietary SFC with HMEC on the composition of the ruminal microbiome in two groups of dairy cows. (**A**) Principal coordinate analysis (PCoA) of beta diversity. (**B**) Non-metric multidimensional scaling analysis (NMDS) of beta diversity. (**C**) Rarefaction curve. (**D**) Venn diagram. OTU, operational taxonomic unit.

The dilution curve of the Shannon index of ruminal fluid samples (Figure 1C) exhibited a moderating trend, indicating that the sequencing depth approached saturation. This suggested that the sequencing depth was reliable, capturing a substantial portion of microorganisms in the samples, which enabled accurate analysis of their composition and ensured that there was sufficient coverage for the flora diversity analysis. Based on the optimized sequences, a total of 1711 OTUs were obtained, with 1509 OTUs being common to all samples (Figure 1D).

3.5. Taxonomic Analysis of Ruminal Microbial Communities

Bacteria are the dominant microorganisms in the rumen of dairy cows, comprising a wide array of phyla and genera. In this study, 19 microbial phyla were identified, with Firmicutes (50.60 ± 17.64%), Bacteroidetes (43.32 ± 17.20%), and Actinobacteria (3.13 ± 2.35%) dominating across all categories (Figure 2A). At the family level, Prevotellaceae (34.27 ± 16.05), Lachnospiraceae (18.43 ± 6.42%), and Oscillospiraceae (8.28 ± 5.50%) were the three most abundant families (Figure 2B). At the genus level, we identified a collective of 240 microbial groups in the samples. The most prominent genera were Prevotella (31.26 ± 16.16%), NK4A214-group (6.79 ± 5.36%), and Succiniclasticum (5.90 ± 4.87%) (Figure 2C). However, the relative abundances of the ruminal microbes were not significantly affected by the dietary inclusion of HMEC at any of these taxonomic levels ($p > 0.05$).

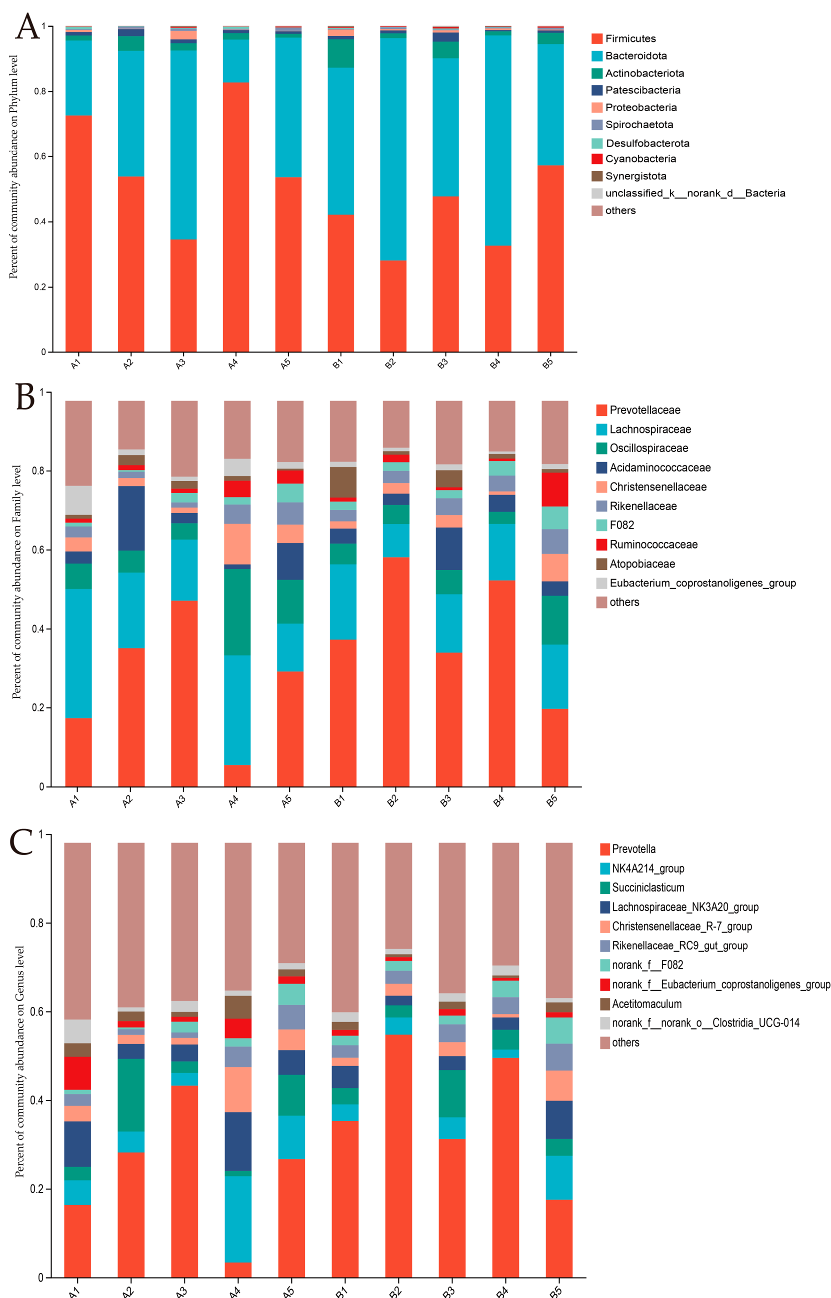

Figure 2. Relative abundances of ruminal microbes in the two feeding groups at three taxonomic levels: phylum (**A**), family (**B**), and genus (**C**). The HMEC group consists of A1–A5, and the SFC group consists of B1–B5.

3.6. Correlation of Ruminal Microbes with Ruminal Fermentation Indices and Serum Indicators

Spearman correlation analysis revealed significant associations between microbial genera found in the rumen, serum indicators, and ruminal fermentation indices (Figure 3). Treponema exhibited positive correlations with acetate and propionate, but negative correlations with isobutyrate, butyrate, and valerate. In contrast, Norank-f-UCG-001 displayed positive correlations with all of these acids. The A:P ratio and IgM levels were found to be positively correlated with Syntrophococcus.

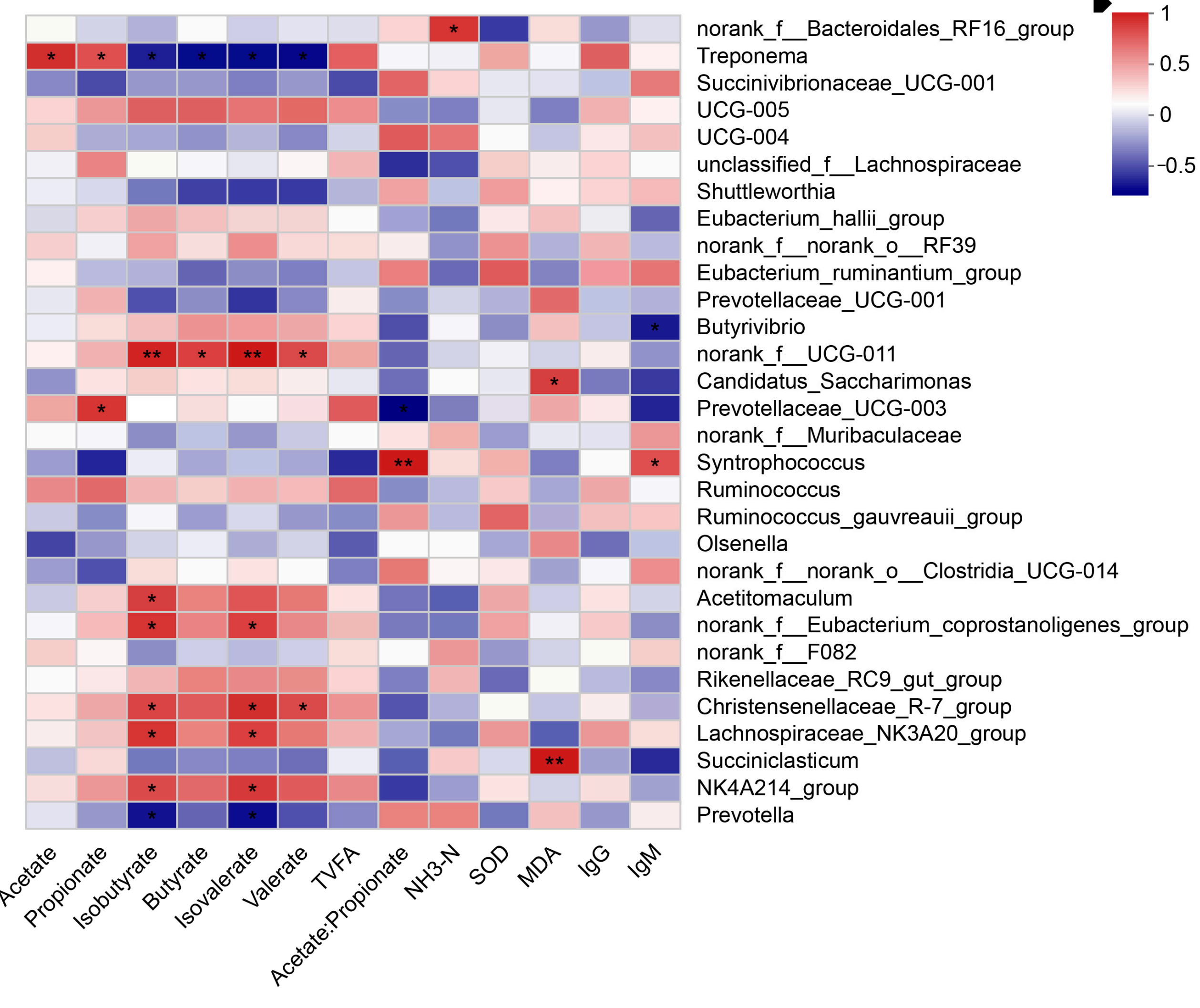

Figure 3. Spearman's correlation plot of ruminal fermentation parameters, blood biochemical indicators, and ruminal microbial populations. * indicates $p < 0.05$, ** indicates $p < 0.01$.

3.7. LEfSe Analysis of Microbial Community-Specific Bacteria in Rumen Samples

Figure 4A,B displays the ruminal microbial taxa with significant differences between the two groups. In the HMEC group, six bacterial taxa showed significantly higher abundance (LDA > 2) compared with the SFC group: Peptostreptococcales-Tisssierellales, Anaerovoracaceae, Mogibacterium, Eubacterium-nodatum-group, norank-f-Lachnospiraceae, and Eubacterium-brachy-group ($p < 0.05$). Conversely, Aneurinibacillales, Aneurinibacillaceae, Aneurinibacillus, Denitrobacterium, and Colidextribacter were significantly less abundant in the HMEC group than in the SFC group ($p < 0.05$).

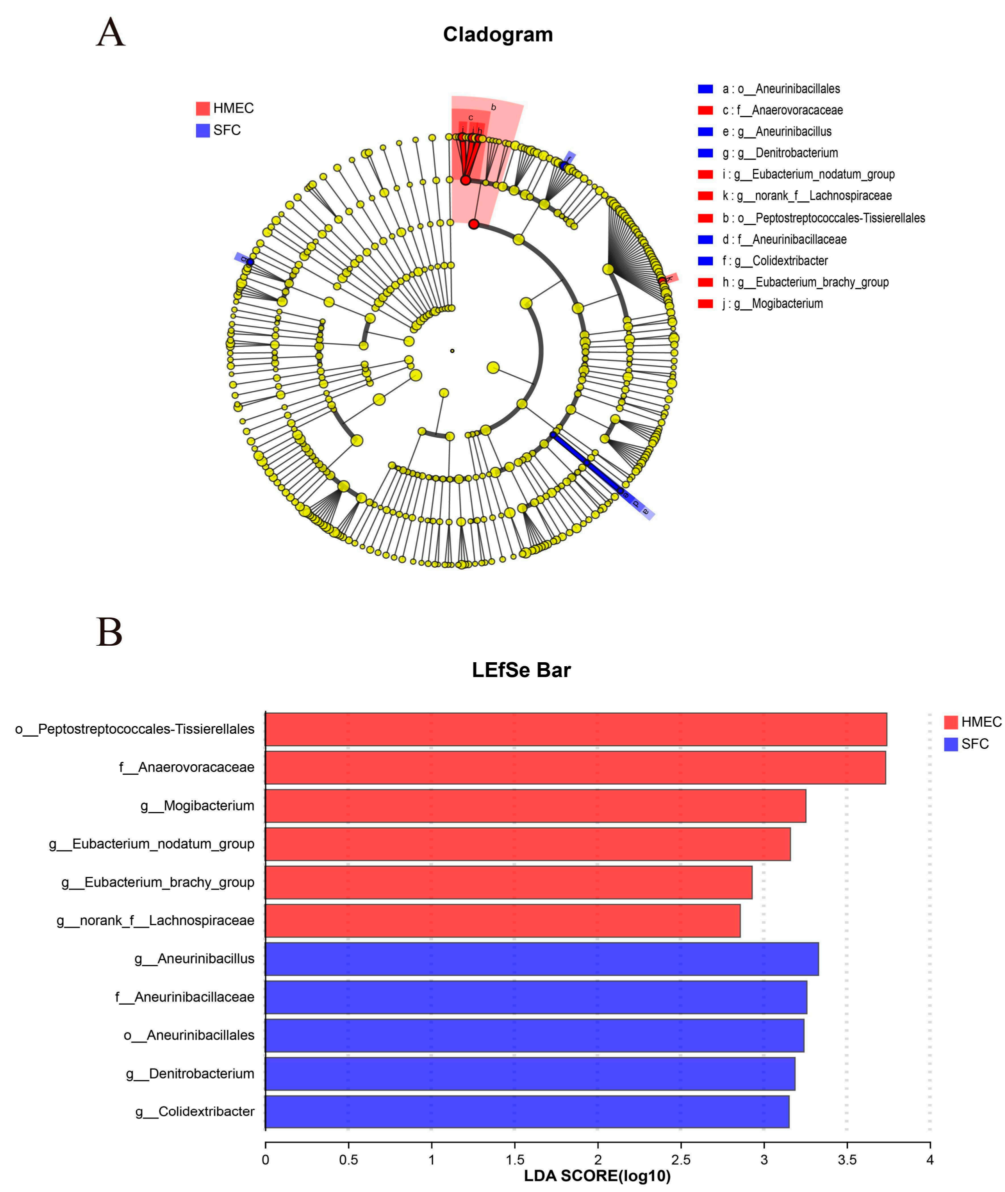

Figure 4. Linear discriminant analysis effect size (LEfSe) analysis of the microbial communities in cows fed with HMEC or SFC. (**A**) Evolutionary branching diagram. (**B**) Bar graph of linear discriminant analysis (LDA) scores in the two groups.

4. Discussion

In this study, DMI, milk yield and 4% FCM were higher in cows fed HMEC than in those fed SFC, which is consistent with reports by Clark et al. and Wu et al. [20,21], who found cows had higher DMI, milk yield, and 4% FCM when fed with high-moisture shelled corn. These results may be due to the fact that high moisture corn is a fermented product with a relatively high percentage of lactic acid and a unique flavor that stimulates the appetite of cows, explaining the positive effect on DMI [22]. It is crucial to highlight that HMEC, being a "wet" feed, facilitates faster bacterial attachment, leading to more extensive digestion. In contrast, drier SFC requires "wetting" by rumen fluid before bacteria can attach and cultivate the SFC surfaces. Additionally, the fermented starch and soluble protein in HMEC may benefit microbial growth in the rumen, thus enhancing the production of microbial proteins and milk [5]. There was a tendency for the HMEC group to produce more milk protein than the SFC group, which is also consistent with Clark et al. [20], and may be attributable to the change in nutrient level utilization efficiency of the diet. In this study, no significant differences were observed in the content of milk fat, lactose, total solids,

and milk urea nitrogen, or the somatic cell count, between the two treatment groups. These results were also aligned with the study by Clark et al. [20], indicating that substituting HMEC for SFC had no adverse impact on the health of dairy cows within the particular conditions of this investigation. Additionally, in terms of economic considerations, HMEC can increase the biomass yield while the production cost is low. These aspects support the possibility of considering HMEC as a substitute for SFC in dairy cow diets.

Serum metabolite indicators serve as reflective markers of an animal's metabolic and health status. Within the oxidative stress response system, SOD, a crucial antioxidant enzyme, plays a pivotal role in scavenging superoxide radicals in the body. GSH-Px, by contrast, facilitates the decomposition of hydrogen peroxide into harmless byproducts, namely water and molecular oxygen, effectively shielding cells from potential oxidative stress-induced damage [23]. In this study, significantly higher levels of serum GSH-Px and SOD were observed in the HMEC group than in the SFC group, suggesting that the administration of HMEC increased the activity of serum antioxidant enzymes, thereby enhancing the antioxidant capacity of the cows. By contrast, Albornoz et al. [24] reported that feeding HMC had no discernible impact on the antioxidant status of cows; however, this discrepancy may be attributable to the differing compositions of HMEC and HMC. Wu et al. [4] revealed that, following fermentation, dietary HMEC led to a substantial proliferation of lactobacilli. These lactobacilli possess formidable antioxidant powers, including the ability to combat oxidative stress by excreting alcohols (e.g., glutathione), as well as antioxidative enzymes (e.g., SOD) [25].

The levels of IgA, IgG, and IgM can serve as indicators of the immune function in the body, with increasing concentrations enhancing the immune competence of lactating cows [26]. In a study by Wang et al. [27], HMC supplementation of weaned piglets led to a notable reduction in coliform bacteria within the intestinal tract, consequently enhancing the immune response of the piglets. The current study revealed that dairy cows fed HMEC exhibited significantly elevated serum levels of IgA, IgG, and IgM compared with those fed SFC. This finding indicated that the inclusion of HMEC resulted in an enhancement of dairy cow immunity. This effect may be attributable to the abundance of organic acids, including acetic acid and lactic acid, as well as β-carotene in HMEC [5,28]. Lactic and acetic acids would exert inhibitory effects on the activities of detrimental microbes while promoting the proliferation of beneficial bacteria in substantial quantities. This phenomenon ultimately leads to an augmentation of the immune response [29]. We found that Pickworth, et al. [5] reported that there is more β-carotene in HMEC that SFC. While β-carotene will be transformed to vitamin A, which has the immune enhancement function [30], we speculate, therefore, that the improvement in immune function may be related to β-carotene.

Biochemical indicators in the blood encompass a range of enzymes and proteins, constituting the fundamental components essential for the body's vital functions. Furthermore, their content and change patterns directly reflect the growth and metabolism of animals [31]. For example, TGs serve as a biomarker for the absorption, metabolism, and utilization of lipids within the body. Serum TG levels are intricately regulated to maintain a dynamic equilibrium. The composition of the diet and feeding practices have a significant influence on TG levels, with inadequate energy intake resulting in a notable reduction in the blood TG concentration. In this study, feeding HMEC did not result in any significant alterations in serum TG levels, implying the absence of adverse effects on lipid metabolism in dairy cows. Creatinine and urea serve as indicators of renal function [32]. Creatinine is primarily eliminated through glomerular filtration, and its levels predominantly reflect the glomerular filtration capacity [33]. The findings of this study revealed that HMEC-fed cows exhibited significantly lower levels of creatinine compared with SFC-fed cows. This discovery indicated that HMEC feeding may lead to an enhancement in the kidney function of dairy cows. The predominant pathway for cholesterol metabolism in the body involves its conversion to bile acids in the liver. Numerous diseases have been closely linked to elevated serum levels of cholesterol [34]. In this study, we found that the HMEC group had

lower levels of cholesterol than the SFC group, indicating that HMEC has the potential to prevent cholesterol accumulation and promote its excretion from the body. This effect could be ascribed to the existence of β-carotene, a compound that inhibits the activity of the key enzyme responsible for cholesterol synthesis, within HMEC. This mechanism ultimately leads to a reduction in cholesterol synthesis [35].

Ruminal pH, NH_3-N, and VFA concentrations serve as crucial internal environmental markers for investigating fermentation dynamics in the rumen [36]. The composition and nutritional profile of the diet significantly influence the processes of ruminal fermentation, thereby assuming a critical role in molding both the ruminal microbial ecosystem and its metabolic functions [37]. Ruminal pH is a critical parameter for the assessment of ruminal fermentation and its normalcy. The typical pH range for ruminal fermentation is between 6 and 7, indicating a healthy microbial environment. Low ruminal fluid pH (<5.8) that is prolonged for more than 3 h is indicative of ruminal acidosis [38]. In our study, both groups of cows demonstrated ruminal pH values within the normal range, suggesting that there were no detrimental effects of either diet on ruminal fermentation. NH_3-N plays a critical role as a precursor for microbial protein synthesis in the rumen. The concentration of NH_3-N provides a comprehensive picture of the degradation process of nitrogenous compounds in the rumen and the subsequent utilization of NH_3-N by ruminal microbes. The optimal range of ruminal NH_3-N content in ruminants is 5–30 mg/dL [39]. In this study, the NH_3-N concentrations fell within the expected range in both groups; however, feeding HMEC led to a reduction in NH_3-N levels. This finding suggested that the inclusion of HMEC in the diet enhanced the utilization of NH_3-N, thereby reducing nitrogen loss and improving the fermentation environment of the rumen [40]. VFAs are the final metabolic products of the microbial degradation of feed within the rumen, serving as a crucial energy source and significantly impacting the digestion, absorption, and utilization of nutrients in ruminant animals. In this research, the TVFA concentration ranged from 97.70 to 120.09 mmol/L, consistent with the findings reported by Holmes et al. [41]; this is probably because of the good balance between nitrogen and energy of the diet. [42]. This study indicated that feeding HMEC resulted in increased concentrations of ruminal acetic acid as well as TVFA, consistent with the discoveries reported by Allen et al. [43]. Production of acetic acid is intricately linked to the degradation of fiber [38]. The rise in acetic acid concentration could be attributed, in part, to the decrease in non-structural carbohydrate content and the concurrent increase in fiber content observed in the HMEC group. Simultaneously, the inclusion of HMEC in the feed resulted in an augmentation of digestible carbohydrates, thereby leading to a substantial enhancement in TVFA production. Propionic acid, a crucial precursor for glucose synthesis, serves as an additional source of energy for cows [38]. Our study revealed a discernible upward trend in propionic acid levels in the HMEC group, indicating that the incorporation of HMEC in the diet may offer improved energy supplementation for dairy cows.

The taxonomic richness and community structure of the ruminal microbiome are key factors influencing ruminal function, with the composition of the microbial community largely determined by the composition of the diet. In this study, the incorporation of dietary HMEC did not have noteworthy impacts on the diversity and composition of microorganisms found in the ruminal fluid, consistent with the findings of Bach et al. [44]. This lack of effect could be attributable to the fact that HMEC fundamentally retains corn kernels as its main component; i.e., the inclusion of corn cob matter did not exert a substantial influence on microbial diversity or community dynamics.

Sequencing of 16S rRNA amplicons in ruminal fluid revealed that the three most dominant clades in the rumen were Bacteroidetes, Firmicutes, and Actinobacteria, which is consistent with the findings of Yi et al. [45]. Crucial roles in ruminant digestion are played by Firmicutes and Bacteroidetes [46]. The Bacteroidetes phylum plays a crucial role in the degradation of non-fibers and the breakdown of plant polysaccharides, whereas the Firmicutes phylum primarily contributes to the breakdown of dietary fibers [47]. Consequently, both Bacteroidetes and Firmicutes assist their host organism in the digestion

and absorption of carbohydrates, proteins, and dietary fibers. In this study, at the phylum level, Firmicutes and Bacteroidetes emerged as the most abundant ruminal microbes, collectively constituting more than 90%, further validating the findings of previous studies. However, there were no significant differences between the groups, likely due to the lack of a substantial change in protein content resulting from the substitution of HMEC for SFC. Consequently, there were no significant differences in the abundances of the two microbial phyla detected between the groups [48].

At the genus level, *Prevotella*, *NK4A214-group*, and *Succiniclasticum* dominated in this study. *Prevotella* is the most widely distributed and abundant genus in the rumen [49]. *Prevotella* significantly contributes to lignocellulose degradation through its production of xylanase and carboxymethylcellulase. Additionally, *Prevotella* actively participates in the metabolism of proteins, starch, hemicellulose, and the production of VFAs. Within the rumen and gastrointestinal tract of ruminants, *Prevotella* spp. act as protein-degrading bacteria, facilitating the breakdown of pectins and non-fibrous polysaccharides [50]. *Succiniclasticum* is an integral part of the core ruminal microbial community, playing a significant role in the fermentation process through its conversion of succinate into propionic acid [51]. Within the *Ruminococcus* family exists the *NK4A214-group*, whose capacity to degrade resistant starch may be linked to variations in its abundance [52]. Furthermore, Spearman correlation analysis revealed positive correlations between *Treponema* and acetic acid, as well as propionic acid. These correlations may explain the observed increases in acetic acid and propionic acid in the HMEC group, which align with findings from previous studies [53]. Moreover, a significant positive correlation was evident between IgM levels and the presence of *Syntrophococcus*, which is known to participate in the utilization of non-fiber carbohydrates, thereby aiding in enhanced digestion, development, and immune function of the gastrointestinal tract [54].

Mogibacterium, *Eubacterium nodatum group*, and *norank-f-Lachnospiraceae* flora were significantly more abundant in the ruminal fluid of the HMEC group compared with that of the SFC group. *Mogibacterium* and *Prevotella* have similar roles in the rumen, and their abundance rates are significantly correlated. *Mogibacterium* is a glycolytic bacterial genus known for its involvement in ammonia assimilation. Ammonia utilization enables the synthesis of phenylacetic acid, which serves as a crucial precursor for the synthesis of phenylalanine. This metabolic pathway contributes to enhanced nitrogen digestion in dairy cows [55]. In the current study, a substantial elevation in the abundance of *Mogibacterium* was noted in the HMEC group. This discovery offers a potential explanation for the observed reduction in NH_3-N levels in this group; i.e., the enhanced presence of *Mogibacterium* may contribute to more efficient utilization of ammonia, resulting in a reduction of NH_3-N in the rumen. Within the Firmicutes phylum, the *Eubacterium nodatum group* has a vital role in the degradation of carbohydrates into acetic acid and butyric acid. This metabolic activity of the *Eubacterium nodatum group* may explain the observed increase in acetic acid levels in the HMEC group [56]. *Lachnospiraceae* plays a critical role in preserving intestinal health in ruminants [57]. The host's growth traits are modulated by this bacterial family through the production of short-chain fatty acids, which, thereby, facilitate the conversion of primary bile acids into secondary bile acids. Secondary bile acids support the host's resistance to colonization of intestinal pathogens, as expounded by Sorbara et al. [58]. The inclusion of HMEC in the diet also led to reduced populations of *Aneurinibacillus* and *Colidextribacter*, both of which are part of the *Bacillus* class of bacteria [59]. *Colidextribacter* has been linked to the promotion of tumor formation, as characterized by elevations in the expression of oncogenes *bcl-xl* and *bcl-2* and the inhibition of apoptosis in colon cells [60].

5. Conclusions

The replacement of 2 kg of SFC with HMEC on an equal dry mass basis increased DMI, milk production, and 4% FCM, while enhancing antioxidant capacity and immunity in dairy cows. HMEC feeding led to increased concentrations of acetate and TVFAs in ruminal fluid,

decreased NH_3-N content, improved the ruminal fermentation environment, and regulated the composition of the ruminal microbiome. HMEC has a lower cost compared to SFC, and it can also be fed to increase cow production in the future. This study demonstrates clear advantages of using HMEC as a replacement for SFC, and provides insights into the regulatory mechanism of HMEC on the ruminal microbiome.

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