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Effects of replacing peanut vine hay with wheat husk on in vitro rumen fermentation, nutrient degradability, and microbial community dynamics of buffalo

Linke Huo
Guangxi Buffalo Research Institute
Mengwei Li
Guangxi Buffalo Research Institute
Tao Lyu
Guangxi Buffalo Research Institute
Haiyu Bai
Guangxi Buffalo Research Institute
Ziming Zeng
Guangxi Buffalo Research Institute
Pinfeng Liao
Guangxi Buffalo Research Institute
Huade Xie
Guangxi Buffalo Research Institute
Jingzhen Li
Guangxi Buffalo Research Institute
Xiaoqiang Guo
Guangxi Buffalo Research Institute
Chengjian Yang
ycj0746@sina.com
Guangxi Buffalo Research Institute

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Abstract

This study examined the effect of replacing peanut vine hay (PVH) with wheat husk (WH) on fermentation profiles, nutrient degradability and ruminal bacterial communities *in vitro* ruminal fermentation system in buffaloes. Five treatment groups (0% A, 25% B, 50% C, 75% D, 100% E), each with eight replicates, were established by replacing WH with PVH at equal graded levels, followed by anaerobic incubation at 39°C for 72 h.The results indicated that total gas production, dry matter (DM), and crude protein (CP) degradability decreased linearly with increasing WH inclusion ($P < 0.05$). Neutral detergent fibre (NDF) degradability and hydrogen production increased linearly with higher WH inclusion ($P < 0.05$). WH increased butyrate ($P < 0.05$). Alpha diversity was unchanged, while principal coordinate analysis revealed distinct clustering among treatments ($P < 0.05$). *Bacteroidota* (45.76–49.90%) and *Firmicutes* (30.91–33.72%) predominated. At the genus taxonomic level, *norank_Rikenellaceae* and *Xylanibacter* were the most abundant taxa across all treatments. Correlation analysis identified *Ruminobacter* and *Treponema* as key drivers of fermentation shifts, with strong associations with volatile fatty acid profiles and H_2 production ($P < 0.05$). Functional prediction indicated that membrane transport and signal transduction were the dominant pathways. In summary, WH is a viable alternative to PVH in buffalo diets. Despite decreasing protein degradability, it improves fibre utilisation and exerts favourable modulatory effects on the rumen microbial community, with no concomitant rise in CH_4 production.

1 Introduction

The shortage of conventional feed resources has become a major constraint on the sustainable development of ruminant livestock, particularly buffalo production in southern China[1]. The sustainable development of livestock is constrained by three major challenges: insufficient feed supply, rising production costs, and increasing methane emissions[2]. Enteric methane from ruminants accounts for approximately 6% of global anthropogenic greenhouse gas emissions and causes a 6–12% loss of dietary gross energy, which hinders low carbon and efficient production in modern animal husbandry[3]. As a key dietary component regulating ruminal microbiota and fermentation pathways, roughage directly determines feed utilization efficiency, animal performance, and enteric methane output[4]. Optimizing roughage utilization is therefore critical to balancing livestock productivity and methane reduction[5].

Peanut vine hay (PVH) serves as a widely available high quality roughage in tropical and subtropical regions[6]. It contains 10–14% crude protein, with 56.12% organic matter digestibility and 8.98 MJ/kg DM metabolizable energy, which fully meets the nutritional requirements of growing ruminants[7]. Even so, large-scale PVH application is limited by multiple practical constraints: unbalanced seasonal and regional supply, competition with other industries for raw materials, high spoilage risk during long-term storage, and unstable ruminal fermentation after feeding[8]. Faced with feed shortages and low-carbon production demands, developing stable, low cost and eco-friendly alternative roughages to replace PVH is essential to secure feed supply and cut breeding costs in intensive ruminant farms[9].

China generates massive quantities of crop by-products every year, yet most lignocellulosic residues lack effective utilization and possess great recycling potential[10]. Wheat husk (WH), a cheap and easily accessible wheat processing by-product, contains high levels of neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin and cannot be digested by monogastric animals[11]. In contrast, the complex microbial community in the ruminant rumen can effectively degrade recalcitrant lignocellulose[12]. Compared with beef cattle, buffaloes carry more active fibrolytic microbes and tolerate low-quality lignified roughages better, so they can efficiently utilize high-lignin raw materials such as WH[13].

Existing studies have verified that ruminal fermentation and methanogenesis are highly sensitive to roughage structural traits, lignification degree, and nutritional quality[14]. Fibre-rich substrates with high cellulose and lignin contents usually trigger acetate-dominated fermentation and hydrogen accumulation, thus promoting methane production[15]. In contrast, appropriate substitution with high-lignin roughages shifts fermentation toward propionate synthesis; propionate acts as a competitive hydrogen sink and suppresses methanogenic activity[16]. Previous research also confirms that dietary malate reduces ruminal methane yield, while excessive NDF or starch accumulation boosts the growth of methanogens[17].

WH and PVH differ substantially in fibre structure and lignification level. Replacing PVH with WH may therefore reshape ruminal volatile fatty acid profiles and change methanogenic metabolic pathways[18].

Although multiple studies have explored alternative roughages, few reports systematically clarify how graded WH substitution affects nutrient degradation, fermentation stability and methane mitigation. This research gap limits the precise application of WH as low carbon roughage substitutes for ruminants. For this reason, we conducted an *in vitro* rumen fermentation trial to explore the effects of different WH substitution ratios on gas production kinetics, nutrient degradability, fermentation characteristics and methane emissions. We suggested that moderate WH inclusion could optimise fibre fermentation, improve nutrient utilisation and lower methane production without damaging feed nutritive value. This study provides theoretical support for lignocellulosic roughage replacement and methane regulation, and offers practical data for WH recycling and low-cost, low carbon roughage feeding strategies for buffaloes.

2 Materials and Methods

2.1 Substrates and Treatments

Fermentation substrates including corn silage, WH, PVH, corn meal and soybean meal were sourced from the breeding farm of Guangxi Buffalo Research Institute. Raw materials were oven-dried (LBAO-250, STIK, China) at 65°C for 72 h, then ground with a cyclone mill (Pulverisette 15, Fritsch, Germany) fitted with a 100-mesh sieve.Nutritional profiles of the substrates are presented in Table 1.1.0 g of fermentation substrate was applied, at a dietary concentrate-to-forage ratio of 3:7.Five treatment

groups were designed, in which WH were used to equally substitute PVH at substitution ratios of 0% (A), 25% (B), 50% (C), 75% (D) and 100% (E), with 8 replicates per group (Table 2).

Table 1
Nutritional profiles of fermentation substrates

Items	Corn silage	Wheat husk	Peanut vine hay	Corn meal	Soybean meal
DM (%)	29.21	88.30	86.01	88.43	89.16
OM (% DM)	95.23	93.92	92.29	98.65	93.09
Ash (% DM)	4.80	6.08	7.71	1.35	6.91
CP (% DM)	12.03	8.65	8.63	8.64	46.91
NDF (% DM)	55.42	67.39	49.21	61.04	14.43
ADF (% DM)	29.96	30.81	34.35	29.93	9.67
NE (MJ/Kg DM)	6.23	6.08	5.46	2.49	6.31

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; NE, net energy.

NE was a calculated value obtained from the Chinese Feed Composition and Nutritive Values Table; all other components were analytically determined.

Table 2
Composition of fermentation substrates (dry matter basis)

Groups	Corn silage (g)	Wheat husk (g)	Peanut vine hay (g)	Corn meal (g)	Soybean meal (g)
A	0.350	0.000	0.350	0.228	0.073
B	0.350	0.088	0.263	0.231	0.069
C	0.350	0.175	0.175	0.235	0.065
D	0.350	0.263	0.088	0.239	0.061
E	0.350	0.350	0.000	0.243	0.057

Groups A, B, C, D and E represented WH substitution levels of PVH at 0%, 25%, 50%, 75% and 100%, respectively.

2.2 In vitro ruminal incubation

Three ruminally cannulated buffaloes (514 ± 18 kg live weight) were selected as inoculum sources, with experimental protocols approved by the Animal Ethics Committee of Guangxi Buffalo Research Institute (GXBRI202260520). Prior to the morning meal, rumen liquor was aspirated from each animal, pooled,

and squeezed through four-layer gauze. The pooled filtrate was combined with CO_2-flushed artificial saliva at a volume ratio of 1:2. Artificial saliva was prepared under anaerobic conditions according to the method described by Theodorou et al.[19]. For the fermentation assay, 50 mL of this buffered mixture was dispensed into 180 mL serum bottles, each containing 1 g of experimental substrate (with or without TSO). To ensure strict anaerobiosis, the bottles were gassed with carbon dioxide before being hermetically sealed. Finally, all bottles were subjected to a 72-h incubation in an orbital water bath set at 39°C and 100 oscillations per minute.

2.3 Analysis of total gas, hydrogen and methane production

During the fermentation process, the dynamic gas production and the methane and hydrogen production were measured at 3, 6, 9, 12, 24, 36, 48, and 72 hours. At each time point, a graduated glass syringe balanced internal and external bottle pressure, and we recorded total gas volume. Gas samples collected in air bags were injected into a gas chromatograph (Model 7890A; Agilent Technologies, USA) to test methane and hydrogen concentrations.

2.4 Nutrient digestibility analysis

At the end of the 72 h fermentation, each serum bottle was uncapped and its entire contents poured into 100-mesh nylon bags (10 cm × 10 cm) for residue collection. The bags were washed with distilled water to remove loosely attached microorganisms and soluble materials, then dried at 65°C until a stable weight was achieved (72 h). The dried residues were used to determine the contents of DM, OM, NDF, ADF, and CP. The degradation rate of each nutrient was calculated as:

Nutrient degradability (%) = (1 − post-digestion residue weight / pre-fermentation substrate weight) × 100

2.5 Analysis of ruminal fermentation parameters

After 72 h of incubation, the serum bottles were swirled in an ice-water bath to terminate fermentation. Immediately after opening the bottles, pH was measured using a pH meter (HANNA HI8424, Italy). Multiple samples of the supernatant from fermentation bottles were collected separately, and volatile fatty acid (VFA) fractions were determined by gas chromatography with crotonic acid as the internal standard[20]. Microbial crude protein (MCP) content was determined via colorimetry using an ultraviolet-visible spectrophotometer (Model PE Lambda 35; Shanghai Pudi Biotechnology Co., Ltd.)[21]. The ammonia N (NH_3-N) content using the phenol-hypochlorite method.

2.6 Determination of rumen bacteria through 16S RNA gene sequencing

At the end of fermentation, total DNA was extracted as described previously[22]. The V3–V4 region of 16S rRNA was amplified with barcoded primers 341F/805R and sequenced on the Illumina MiSeq PE300 platform. Raw sequencing data underwent quality control according to our previous analysis pipeline. We conducted species annotation and relative abundance analysis to describe bacterial community

composition across groups, and performed alpha diversity calculation and correlation analysis with environmental factors to identify inter-group microbial differences.

2.7 Statistical Analysis

All data were processed with SPSS 26.0 software. One-way ANOVA was applied to test treatment effects, and Duncan's multiple range test separated mean values. Differences were considered statistically significant at $P < 0.05$. All results are presented as mean ± standard error of the mean. Pearson correlation coefficients quantified linear relationships between bacterial genera and fermentation parameters. Principal coordinate analysis (PCoA) and permutational multivariate analysis of variance compared overall community structure among groups. The Kruskal–Wallis test screened differentially abundant taxa. We predicted microbial functional profiles via Tax4Fun software based on 16S rRNA sequencing data.

3 Results

3.1. *In Vitro* Total Gas, Methane and Hydrogen(H_2) Production

As presented in Table 3, wheat husk replacing peanut vines hay highly significantly reduced total gas production ($P < 0.01$), significantly altered methane yield ($P < 0.05$) and increased H_2 production ($P < 0.05$).

Table 3
Effects of wheat hull substitution on in vitro gas production parameters

Items	A	B	C	D	E	SEM	*P*-value
Gas production(ml/g DM)							
Total gas	219.50 ± 9.26^{a}	214.13 ± 8.51^{ab}	205.75 ± 5.92^{bc}	197.00 ± 12.67^{c}	183.63 ± 6.80^{d}	3.273	<0.001
H_2	0.46 ± 0.46^{b}	0.95 ± 0.54^{b}	1.11 ± 0.14^{b}	1.18 ± 0.56^{b}	2.48 ± 2.26^{a}	0.071	0.011
CH_4	19.35 ± 2.32^{ab}	15.65 ± 3.41^{c}	20.11 ± 2.20^{a}	17.11 ± 3.89^{abc}	16.52 ± 2.01^{bc}	0.801	0.016

Values within the same row with different superscript letters differ significantly ($P < 0.05$). The same as below.Groups A, B, C, D and E represented WH substitution levels of PVH at 0%, 25%, 50%, 75% and 100%, respectively.SEM, standard error of the mean.

3.2 *In vitro* substrate degradation

Table 4 shows nutrient degradability results across treatments. As WH substitution ratios increased, DM and CP declined gradually and extremely significantly; A had the highest values while E recorded the lowest ($P < 0.01$). ADF and OM showed no significant inter-group differences ($P > 0.05$). NDF in D and E was significantly higher than values of other treatment groups ($P < 0.01$).

Table 4
Effects of WH substitution for PVH on *in vitro* nutrient degradability

Items	A	B	C	D	E	SEM	*P*-value
DM (%)	72.26^{a}	71.57^{b}	70.61^{c}	70.43^{cd}	70.23^{d}	0.157	<0.001
NDF (%)	56.18^{b}	57.25^{b}	56.01^{b}	58.98^{a}	59.19^{a}	0.004	0.001
ADF (%)	37.54^{b}	42.93^{a}	41.16^{ab}	40.78^{ab}	38.19^{b}	0.012	0.055
OM (%)	59.47	57.04	56.84	55.39	55.05	0.012	0.231
CP (%)	43.25^{a}	37.06^{b}	44.97^{a}	31.30^{c}	25.77^{d}	13.14	< 0.001

Values within the same row with different superscript letters differ significantly ($P < 0.05$).Groups A, B, C, D and E represented WH substitution levels of PVH at 0%, 25%, 50%, 75% and 100%, respectively.DM, dry matter;NDF, neutral detergent fibre; ADF, acid detergent fibre; OM, organic matter; CP, crude protein.SEM, standard error of the mean.

3.3 *In vitro* fermentation parameters

Table 5 lists all ruminal fermentation indicators. Treatment exerted no significant influence on fermentation pH ($P > 0.05$). NH_3-N and MCP concentrations rose significantly as WH inclusion increased, with E reaching the maximum values ($P < 0.01$). Total VFA concentrations in C and D were significantly higher than those in A, B and E ($P < 0.01$). Acetic acid, propionic acid, butyric acid, isovaleric acid and valeric acid concentrations differed significantly across groups ($P < 0.05$): acetic acid and Total VFA peaked in C, and butyric acid concentrations in C, D and E were significantly higher than A. Acetate/propionate ratio and isobutyric acid concentration remained unchanged among all treatments ($P > 0.05$).

Table 5
Effects of wheat husk substitution on *in vitro* fermentation parameters

Items	A	B	C	D	E	SEM	*P*-value
pH	6.02 ± 0.04	6.03 ± 0.08	6.01 ± 0.08	5.97 ± 0.03	6.01 ± 0.03	0.022	0.091
NH_3-N (mg/mL)	5.38 ± 0.30^{c}	5.43 ± 0.364^{c}	5.77 ± 0.541^{bc}	6.82 ± 0.556^{ab}	743 ± 0.421^{a}	0.424	0.005
MCP (μg/mL)	0.057 ± 0.004^{bc}	0.053 ± 0.003^{c}	0.053 ± 0.005^{c}	0.063 ± 0.006^{ab}	0.075 ± 0.005^{a}	0.004	0.004
Acetate	66.38 ± 14.52^{b}	67.51 ± 13.87^{b}	78.48 ± 7.21^{a}	73.70 ± 10.38^{ab}	65.78 ± 9.25^{b}	5.490	0.004
Propionate	19.96 ± 3.65^{ab}	20.32 ± 3.05^{ab}	22.30 ± 2.67^{a}	21.84 ± 2.79^{a}	19.65 ± 2.16^{b}	1.380	0.038
Acetate/Propionate	3.24 ± 0.43	3.47 ± 0.12	3.54 ± 0.08	3.47 ± 0.09	3.25 ± 0.34	0.160	0.088
Isobutyrate	0.97 ± 0.22^{a}	1.03 ± 0.13^{a}	1.13 ± 0.20^{a}	1.09 ± 0.17^{a}	1.15 ± 0.16^{a}	0.081	0.067
Butyrate	11.32 ± 3.03^{b}	13.37 ± 2.21^{ab}	14.88 ± 2.81^{a}	14.40 ± 2.36^{a}	14.43 ± 1.73^{a}	1.144	0.008
Isovalerate	1.42 ± 0.39^{b}	1.57 ± 0.25^{ab}	1.77 ± 0.23^{a}	1.63 ± 0.23^{ab}	1.62 ± 0.15^{ab}	0.152	0.050
Valerate	1.25 ± 0.31^{b}	1.34 ± 0.28^{ab}	1.64 ± 0.21^{a}	1.59 ± 0.26^{a}	1.66 ± 0.16^{a}	0.126	0.017
Total VFA (mmol/L)	101.30 ± 17.71^{b}	105.14 ± 16.07^{b}	120.20 ± 11.41^{a}	114.25 ± 10.82^{a}	104.28 ± 9.42^{b}	6.691	<0.001

Values within the same row with different superscript letters differ significantly ($P < 0.05$).Groups A, B, C, D and E represented WH substitution levels of PVH at 0%, 25%, 50%, 75% and 100%, respectively.SEM, standard error of the mean.

3.4 Rumen microbial community diversity and structure

Rarefaction curves flattened as sequencing reads increased, confirming sufficient sequencing depth to cover most ASVs in all treatment groups (Fig. 1). Bacterial alpha diversity indices differed significantly across treatments ($P < 0.05$). A had higher OTU number, ACE and Chao1 richness indices and Shannon diversity index than D and E, while the Simpson index was lower in A. These data indicated A possessed the highest microbial richness and diversity, and diversity declined in high substitution D and E (Table 6). We applied PCoA based on Bray-Curtis distance to assess inter-group community dissimilarity; PC1 and

PC2 explained 15.27% and 11.68% of total community variation respectively (Fig. 2). All samples split into two distinct clusters: A, B and C clustered on the left axis, while D and E distributed on the right axis with obvious spatial separation between clusters.

Table 6
Alpha diversity parameters of rumen bacteria among all treatments

Items	A	B	C	D	E	SEM	*P*-value
OTUs	2907.88^{a}	2839.38^{ab}	2817.00^{b}	2678.00^{c}	2661.50^{c}	34.080	<0.001
ACE	3369.51^{a}	3307.87^{a}	3299.07^{a}	3163.80^{b}	3180.14^{b}	2.861	<0.001
Chao1	3327.43^{a}	3276.21^{a}	3278.94^{a}	3132.34^{b}	3169.24^{b}	33.391	<0.001
Shannon	6.16^{a}	6.11^{a}	6.12^{a}	5.96^{b}	5.96^{b}	0.029	<0.001
Simpson	0.012^{b}	0.012^{b}	0.011^{b}	0.013^{a}	0.013^{ab}	0.001	0.003

Values within the same row with different superscript letters differ significantly ($P < 0.05$).Groups A, B, C, D and E represented WH substitution levels of PVH at 0%, 25%, 50%, 75% and 100%, respectively.SEM, standard error of the mean.

3.5 Compositional profiles of the rumen microbiota and taxonomic differences

The relative abundance of bacterial phyla and genera are presented in Fig. 3. The relative abundance of bacterial phyla showed that *Bacteroidota* (49.90-45.76%) and *Bacillota* (30.91–33.72%) were the most dominant phyla detected in all treatments. At the genus level, *norank_Rikenellaceae* and *Xylanibacter* were dominant, accounting for at least 9.73% and 7.52% of the total rumen microbiota, respectively, regardless of the treatment.

The Kruskal-Wallis test was used to identify differentially abundant genera among treatment groups (Fig. 4). At the genus level, the *g__norank_F082* and *f__F082* were more enriched in A. *f__norank_Oscillospirales*, *g__norank_Oscillospirales* were enriched in B. *norank_f_Prevotellaceae* showed more abundance in C. *Ruminobacter*, *Candidatus_Saccharimonas* and *Pseudobutyrivibrio* were more abundant in E.

3.6 Microbial correlations and functional predictions

The genus level correlation heatmap revealed linear associations between core ruminal genera and key fermentation parameters (Fig. 5). *Ruminobacter* showed an extremely significant positive correlation with valerate ($P < 0.01$). *Treponema* correlated positively with butyrate ($P < 0.05$), and highly positively with valerate and hydrogen yield ($P < 0.01$). *norank_f_F082* exhibited significant negative correlations with

butyrate and valerate ($P < 0.05$), while *norank_c_Clostridia* correlated negatively with valerate ($P < 0.05$) and highly negatively with hydrogen yield ($P < 0.01$). *Ruminococcus* and *Saccharofermentans* were highly positively correlated with butyrate, valerate and hydrogen yield ($P < 0.01$). *Xylanibacter* showed a significant positive correlation with valerate ($P < 0.05$) and an extremely significant positive correlation with hydrogen yield ($P < 0.01$). *norank_f_Rikenellaceae* had significant negative correlations with acetate, butyrate, TAFA and valerate ($P < 0.05$). *Succiniclasticum* only correlated negatively with hydrogen yield ($P < 0.05$), whereas *Succinivibrio* correlated positively with valerate ($P < 0.05$) and highly positively with hydrogen yield ($P < 0.01$). *norank_f_Christensenellaceae* and n*orank_o_Oscillospirales* correlated negatively with valerate ($P < 0.05$) and highly negatively with hydrogen yield ($P < 0.01$). No significant linear correlations existed between propionate, isovalerate and any detected bacterial genera. Remaining genera showed no significant correlation with all measured fermentation indicators ($P > 0.05$).

Tax4Fun predicted KEGG Level 2 functional pathways based on 16S rRNA sequencing data. Membrane transport occupied the highest relative abundance (0.0846–0.0869), followed by signal transduction (0.0237–0.0250). Other functional categories recorded lower relative abundance, ranked from high to low as global and overview maps, carbohydrate metabolism, energy metabolism, nucleotide metabolism and replication and repair, with abundance values ranging from 0.0016 to 0.0084. The relative abundance variation of each pathway across groups was less than 0.003, indicating minor differences in core microbial functions among fermentation systems. Overall, membrane transport represented the dominant functional pathway in all groups, while other core metabolic pathways maintained stable low-level expression. Coordinated operation of these functional modules sustained stable metabolic homeostasis of ruminal microbes under different substrate conditions.

Discussion

4.1 Gas Production Dynamics and Hydrogen Metabolism

Total gas yield declined linearly as WH substitution ratios rose, consistent with the downward trend of DM. This observation indicated that WH had lower fermentability than PVH. Previous research confirmed in vitro gas production positively correlates with dietary organic matter and crude protein content, and negatively correlates with NDF and ADF concentrations[23]. Zhang et al.[24] reported similar results in an in vitro fermentation study using donkey caecal and colonic fluid, where dry matter disappearance of fibrous feeds was positively correlated with organic matter and crude protein, and negatively correlated with NDF and ADF. Therefore, the higher NDF content and lower crude protein content of WH in this study may have jointly limited gas production potential.

Methane yield fluctuated among treatments, being lowest in B and highest in C. Lee et al.[25] found that methane production from feedstuffs decreased as crude fibre and crude protein increased, but increased with higher non-fibrous carbohydrate content. In the present study, the high fibre content of WH may have partly suppressed methane production. Hydrogen yield in Group E was approximately 5.4 times higher than in A, while methane yield did not increase correspondingly, indicating a clear diversion

of metabolic hydrogen flow. Under normal rumen conditions, most metabolic hydrogen is used by methanogenic archaea to reduce CO_2 to CH_4[26]. The uncoupling between increased H_2 and stable CH_4 in this study suggests that alternative hydrogen sinks may have been activated.

Wang et al.[27]demonstrated that partial replacement of forage fibre with non-forage fibre sources (wheat bran and bean hulls) increased H_2 production by 8.45% and decreased CH_4 production by 14.06%, accompanied by a significant increase in propionate concentration. These changes indicated that hydrogen metabolism was partly shifted from methanogenesis to propionate formation. The authors further attributed this shift to the replacement of Firmicutes by bacteroidetes, and the replacement of Methanobrevibacter by Methanomassiliicoccus among methanogens. The substantial increase in H_2 yield with relatively stable CH_4 in Group E may reflect a similar mechanism, in which hydrogen was directed towards alternative pathways such as butyrate synthesis or reductive acetogenesis rather than methanogenesis[28]. Additionally, hydrogen metabolism in in vitro systems is influenced by both substrate fibre structure and inoculum source. Witzig[29] pointed out that dietary differences among donor animals could affect rumen microbial communities and consequently alter *in vitro* fermentation parameters.

4.2 Nutrient Degradation Characteristics

Replacing PVH with WH noticeably changed the degradation pattern of nutrients in the *in vitro* rumen system. DM decreased significantly and linearly with increasing replacement, which agreed with the results of Ma et al.[30], who reported that dry matter disappearance of fibrous feeds was positively correlated with organic matter and crude protein, and negatively correlated with NDF and ADF. Although OM showed a decreasing trend, the difference was not significant, indicating that WH replacement did not severely impair overall organic matter utilisation.

NDF was significantly higher in D and E than in other groups, suggesting that a high proportion of WH replacement promoted the degradation of structural carbohydrates. Bradford et al.[31] found that partial replacement of forage fibre with non-forage fibre sources significantly improved DM, NDF and ADF. Chaucheyras-Durand et al.[32]reported that fibrolytic microorganisms such as *Butyrivibrio fibrisolvens* could colonise fibrous substrates and enhance NDF degradation. They also found that live yeast supplementation increased the abundance of *Butyrivibrio fibrisolvens* on fibrous materials and improved NDF degradation of bean hulls, indicating that the degradation efficiency of fibrous substrates depends heavily on microbial colonisation. Zontini et al.[33] reported that NDF of non-forage fibrous feeds varied considerably with substrate source and fermentation time. In this study, the high NDF content of WH may have provided sufficient substrate for fibrolytic microbes, possibly stimulating the secretion of extracellular fibrolytic enzymes such as cellulase and hemicellulase.

Acid detergent ADF was highest in B, but overall differences were only marginally significant, suggesting that the effect of WH on ADF degradation may be dose-dependent. CP decreased extremely significantly with increasing replacement. This was probably due to the low crude protein content of WH and the presence of protein-fibre complexes, which may have reduced protease accessibility[34]. Okon et al.[35]

also confirmed that crude protein content was positively correlated with dry matter disappearance. These results suggest that WH replacement improved fibre degradation while moderately reducing protein degradation.

4.3 Rumen Fermentation Characteristics

There was no significant difference in pH among treatments, with values ranging from 5.97 to 6.03. This indicates that WH replacement did not increase the risk of acidosis and that the rumen environment remained relatively stable (Sadarman et al., 2023). It has been reported that methanogenesis may be completely inhibited when pH drops below 6.0[14]. In this study, pH remained close to 6.0 across all groups, which may have provided suitable conditions for methanogens and partly explained why methane yield did not change dramatically.

NH_3-N concentration gradually increased with increasing WH replacement, which was opposite to the trend observed for CP. This inconsistency may be related to a higher proportion of non-protein nitrogen in WH, slow release of fibre-bound protein, or changes in microbial nitrogen utilisation efficiency[36]. Yan et al.[37]suggested that ammonia nitrogen concentration in fibrous substrates is regulated by both protein degradation rate and microbial nitrogen assimilation. MCP concentration increased significantly with higher replacement, implying that WH substitution may have improved microbial growth efficiency through better synchronisation of energy and nitrogen supply, or through stimulation of fibre-associated microbes[38].

Total VFA concentration was significantly higher in C and D than in other groups, showing a parabolic response that first increased and then decreased. Chen et al.[39] reported that *in vitro* VFA production from fibrous substrates was closely related to substrate nutrient composition. Zhao et al.[40] found that dietary fibre source affected the fermentation characteristics of fibre fractions in pigs, and that VFA concentration was positively correlated with insoluble dietary fibre digestibility. In the present study, moderate WH replacement may have provided a more balanced ratio of fibre and fermentable carbohydrates, thereby promoting synergistic microbial fermentation. Acetate concentration was highest in C, propionate concentrations were higher in C and D, and butyrate concentrations in C, D and E were significantly higher than in A. Butyrate is an important energy source for rumen epithelial cells and plays a role in maintaining rumen barrier function[41].

Wang et al.[27] found that replacement with non-forage fibre sources significantly increased propionate concentration, but did not affect acetate concentration or the acetate-to-propionate ratio. Amelchanka et al.[42] reported that buck WH extract increased acetate and total short-chain fatty acid production, and tended to increase butyrate production. In this study, the acetate/propionate ratio did not differ significantly among treatments, indicating that acetate remained the main fermentation product and that the overall energy metabolism pathway was not fundamentally altered[43]. These results suggest that moderate WH replacement may optimise VFA production and increase butyrate proportion, which could be beneficial for rumen health.

4.4 Overall Differences in Rumen Microbial Community

Rarefaction curves tended to plateau in all groups, indicating that sequencing depth was sufficient to cover most microbial diversity in the samples. Alpha diversity analysis showed that A had significantly higher OTU number, Chao1, ACE and Shannon indices than D and E, while the Simpson index was higher in D and E. These results suggest that high levels of WH replacement (≥ 75%) reduced microbial richness and evenness, possibly because the high lignin content of WH limited the growth of non-fibrolytic bacteria[44].

PCoA analysis showed that PC1 and PC2 explained 15.27% and 11.88% of the total variation, respectively. A, B and C were clearly separated from D and E along the PC1 axis, forming two distinct clusters. This indicates that microbial community structure changed substantially when the replacement ratio reached 75% or higher, and that these changes were consistent with the observed differences in nutrient degradation and fermentation parameters. Thoetkiattikul et al.[45] also found that replacing forage fibre with non-forage fibre sources significantly altered bacterial community structure, with Firmicutes decreasing and Bacteroidetes tending to increase at the phylum level. They also reported that Methanomassiliicoccus increased significantly, while Methanobrevibacter tended to decrease among methanogens[46]. In the present study, low- to medium-replacement groups (A, B, C) clustered closely, suggesting that replacement ratios ≤ 50% had limited effects on community structure. However, high-replacement groups (D, E) were clearly separated from low-replacement groups, indicating that substantial microbial community rearrangement had occurred.

It is worth noting that Wang et al.[27] also found that most high-abundance bacteria were relatively insensitive to changes in dietary fibre source, and that only some amylolytic and fibrolytic genera responded significantly. This is consistent with the present study, in which the dominant phyla *Bacteroidota* and *Bacillota* remained relatively stable, while differential genera were selectively enriched at the genus level. These findings support the view that rumen microbial communities respond to dietary changes in a manner characterised by overall stability but localised restructuring.

4.5 Effects of WH Replacement on Rumen Microbial Community Composition

At the phylum level, Bacteroidota and Bacillota were dominant in all groups, which is consistent with the typical rumen microbial structure reported in ruminants[47]. Thoetkiattikul et al.[45] found that non-forage fibre replacement significantly decreased the relative abundance of Firmicutes and tended to increase Bacteroidetes. In this study, the relative abundance of these two phyla fluctuated slightly but did not change significantly, which may be related to differences in replacement levels and substrate types.

At the genus level, *Ruminobacter*, *Candidatus_Saccharimonas* and *Pseudobutyrivibrio* were significantly enriched in Group E. *Pseudobutyrivibrio* is a known butyrate-producing bacterium, and its enrichment may partly explain the significantly higher butyrate concentration in Group E. Chaucheyras-Durand et al.[32] showed that the colonisation of fibrolytic bacteria such as *Butyrivibrio fibrisolvens* on fibrous substrates was closely related to NDF degradation efficiency. Metabolomic analysis by Hua et al.[48] further showed that a lipogenic diet significantly increased the relative abundance of cellulolytic genera

such as *Ruminococcus*, *Butyrivibrio*, *Eubacterium*, and *Lachnospira*, while glucogenic diets favoured amylolytic bacteria such as *Ruminobacter* and *Succinivibrionaceae_UCG-002*. The enrichment of Ruminobacter in the full-replacement group of this study is consistent with these findings, suggesting that the fibrous structure of WH may selectively promote the growth of certain fibrolytic microbes.

The enrichment of *Candidatus_Saccharimonas* in E also implies a potential role in fibre degradation or hydrogen metabolism. Li et al.[49] observed that WH extract increased the relative abundance of unclassified *Bacteroidales*, *Clostridiales* and *Ruminococcaceae*, and that *Butyrivibrio* tended to increase. These findings support the idea that fibrous substrates such as WH can selectively enrich microbes involved in fibre degradation and butyrate synthesis. The genus level changes observed in this study suggest that high-proportion WH replacement may reshape rumen fermentation function by enriching specific fibrolytic and butyrate-producing populations.

4.6 Microbial Correlation and Functional Pathways

Genus level correlation analysis revealed significant linear relationships between key genera and fermentation parameters. *Ruminobacter* showed an extremely significant positive correlation with valerate. *Treponema* was positively correlated with butyrate, valerate and H_2 yield, which supports its potential role as a fibrolytic bacterium involved in hydrogen transfer and VFA production[50]. R*uminococcus* and *Saccharofermentans* were highly significantly positively correlated with butyrate, valerate and H_2 ($P < 0.01$), indicating their importance in fibre degradation and hydrogen-producing fermentation. Xue et al.[51] also reported a significant relationship between VFA production and fibre digestibility.

Xylanibacter was positively correlated with valerate and H_2, suggesting that it may play an important role in xylan degradation and hydrogen metabolism. *norank_f_Rikenellaceae* showed significant negative correlations with acetate, butyrate, Total VFA and valerate, indicating that this genus may be involved in competitive metabolic pathways. *Succinivibrio* was positively correlated with valerate and H_2, whereas *Succiniclasticum* was negatively correlated with H_2 only, suggesting that different succinate-pathway bacteria may have distinct effects on hydrogen metabolism. Ravelo et al.[52] found that succinate-pathway bacteria such as *Ruminobacter* and *Succinivibrionaceae_UCG-002* were more abundant in glucose rich diets, which was associated with higher propionate production. The enrichment of Ruminobacter and the increase in H_2 yield observed in this study may reflect similar regulatory mechanisms.

Functional prediction indicated that carbohydrate metabolism and membrane transport were the most dominant pathways, which is consistent with the upregulation of transporters and glycosidases under high-fibre substrates[51]. This may reflect an adaptive response of the microbial community to the fibrous composition of WH. Amino acid metabolism pathways remained relatively stable, which was consistent with the decrease in CP and the increase in MCP. Metabolomic analysis by Lock et al.[53] showed that lipid-rich diets improved lipid digestion, particularly fatty acid metabolism, but reduced amino acid digestion, which is similar to the CP response observed in this study. Overall, the correlation

analysis and functional prediction support the conclusion that WH substitution selectively enriched fibrolytic and butyrate-synthetic genera. While maintaining basic energy metabolism, this substitution appeared to optimise hydrogen flow and VFA production patterns, providing a possible microbial mechanism for the rumen adaptation to WH as an alternative roughage source.

Conclusions

Replacing peanut vine hay with wheat husk in buffalo diets improved nutrient digestibility, reduced fermentation gas production, and modified the composition of ruminal fermentative microorganisms. Under the experimental conditions of this study, the maximum recommended replacement ratio of wheat husk was 50%. This level maintained stable fermentation without increased methane emissions, enhanced fiber degradation and butyrate proportion, and optimized the ruminal microecosystem.

Abbreviations

PVH
peanut vine hay
WH
wheat husk
DM
dry matter
CP
crude protein
NDF
neutral detergent fibre
VFA
volatile fatty acid
H_2
hydrogen
CH_4
methane.

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Animal Ethics Committee of Guangxi Buffalo Research Institute (Approval No. GXBRI202260520). All animal sampling procedures complied with the institutional guidelines for the care and use of experimental animals.

Consent for publication

Not applicable.

Availability of data and materials

Raw 16S rRNA gene sequencing data have been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1529365 (SUB16484034). The data will be released upon publication of this manuscript.

Competing Interests

The authors declare no conflicts of interest.

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Authors' contributions

Conceptualization—M.L and L.H; Methodology—M.L; Validation—L.H, T.L and Z.Z; Formal analysis—L.H; Resources—M.L and P.L; Data curation—J.L; Writing-original draft—L.H; Writing-review and editing—L.H and H.X; Supervision—X.G; Project administration—L.H; Funding acquisition—C.Y; All authors have read and agreed to the published version of the manuscript.

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Figures

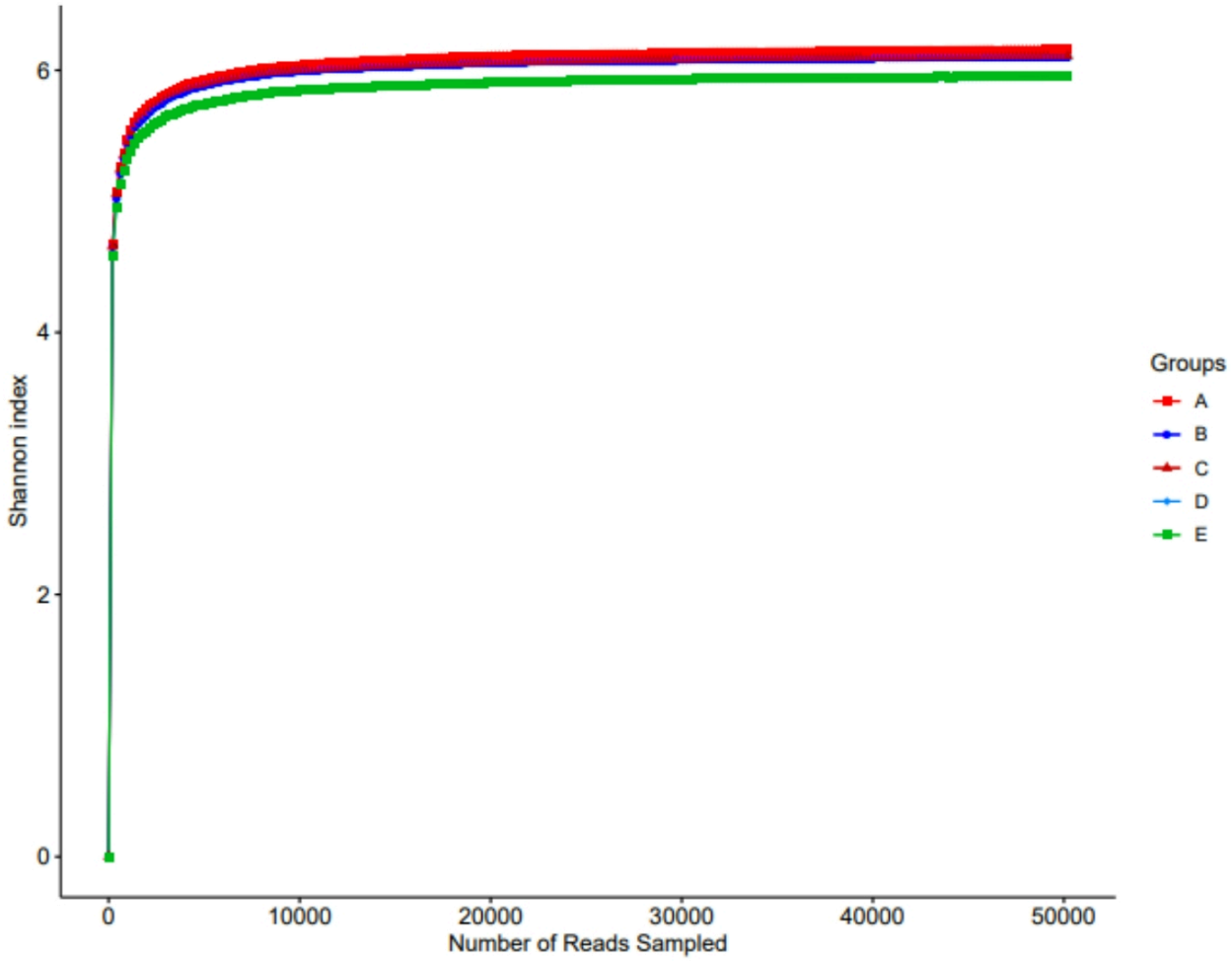


Figure 1

Rarefaction curves of amplicon sequence variants

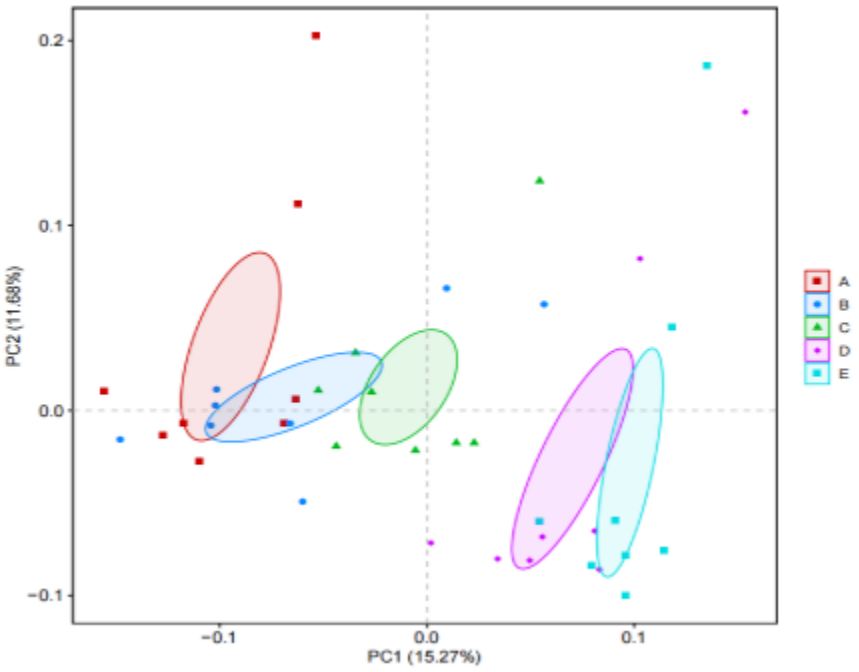


Figure 2

Principal component analysis (PCoA) of the bacterial community

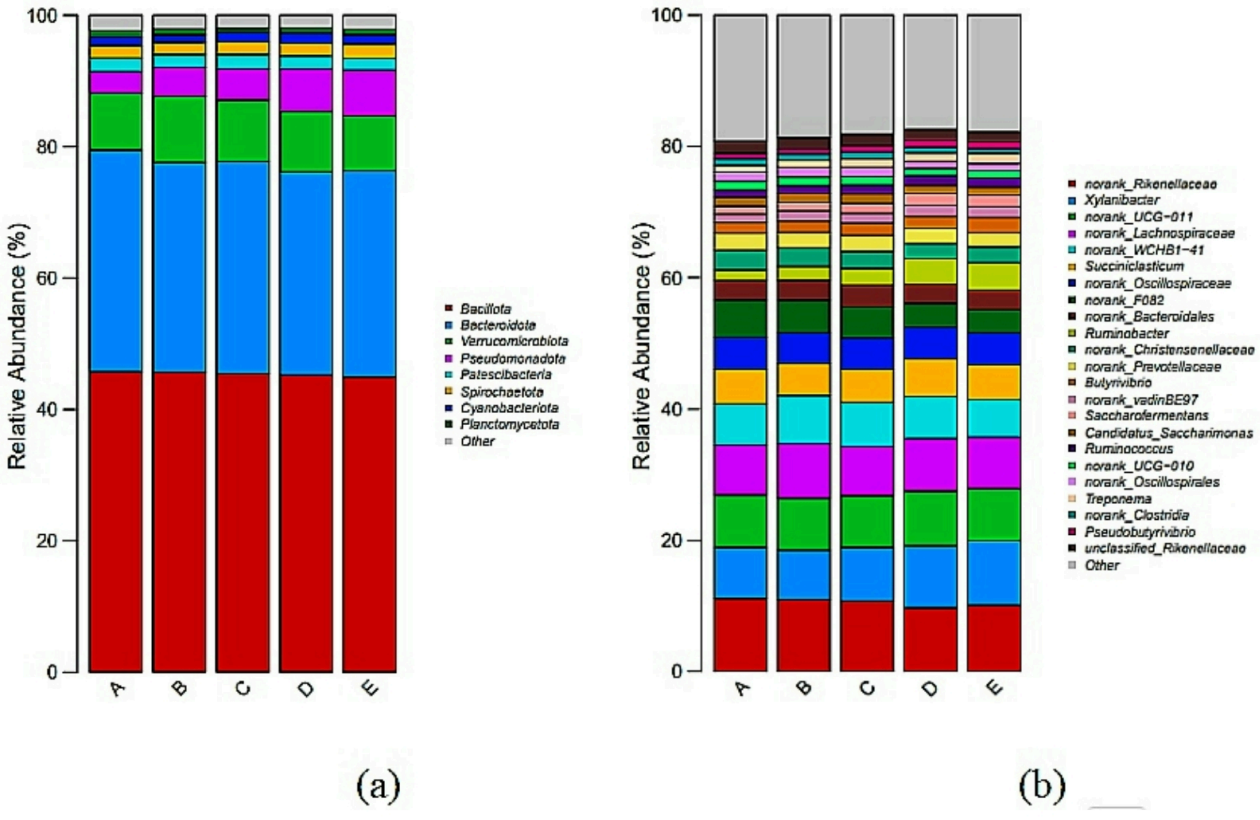


Figure 3

Relative abundance of rumen microbial communities in buffalo phylum level (a) and genus level (b).

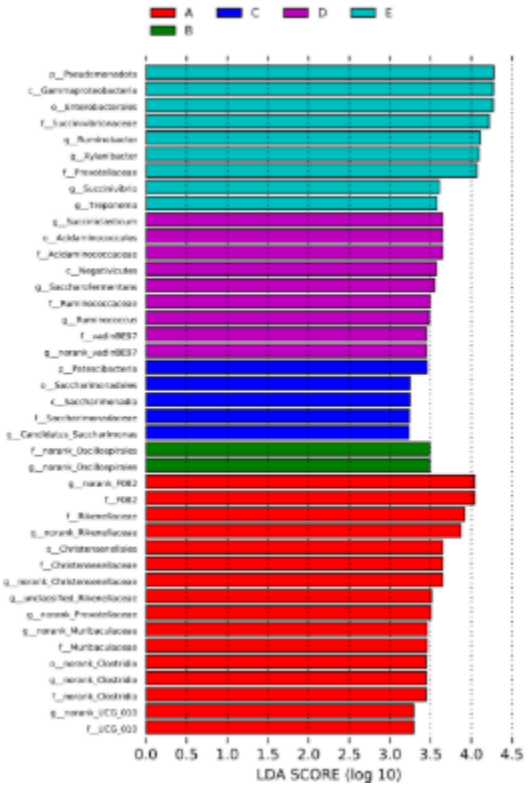


Figure 4

Differentially abundant genera were identified using the Kruskal-Wallis test.

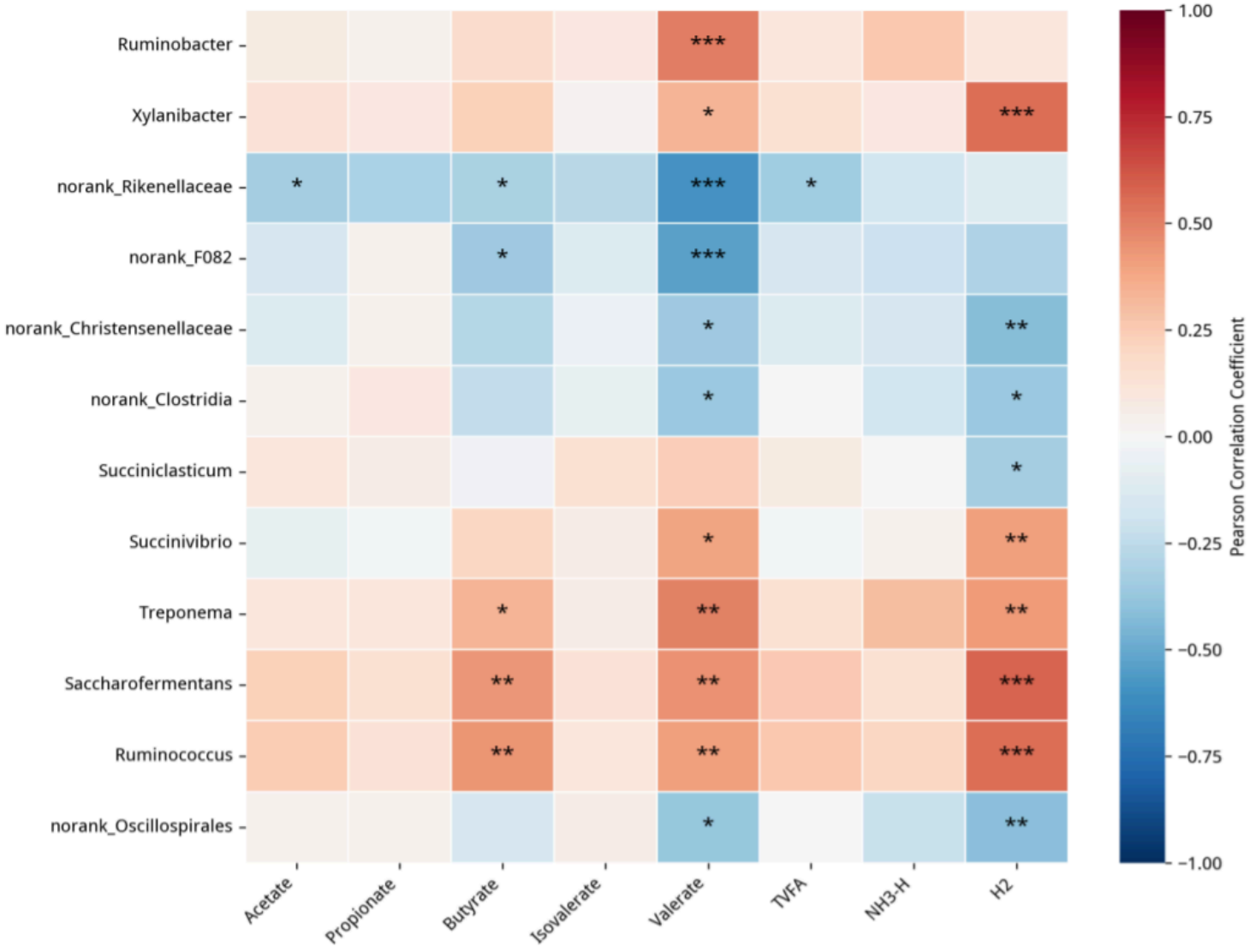


Figure 5

The correlation between microbial genera and rumen metabolism.

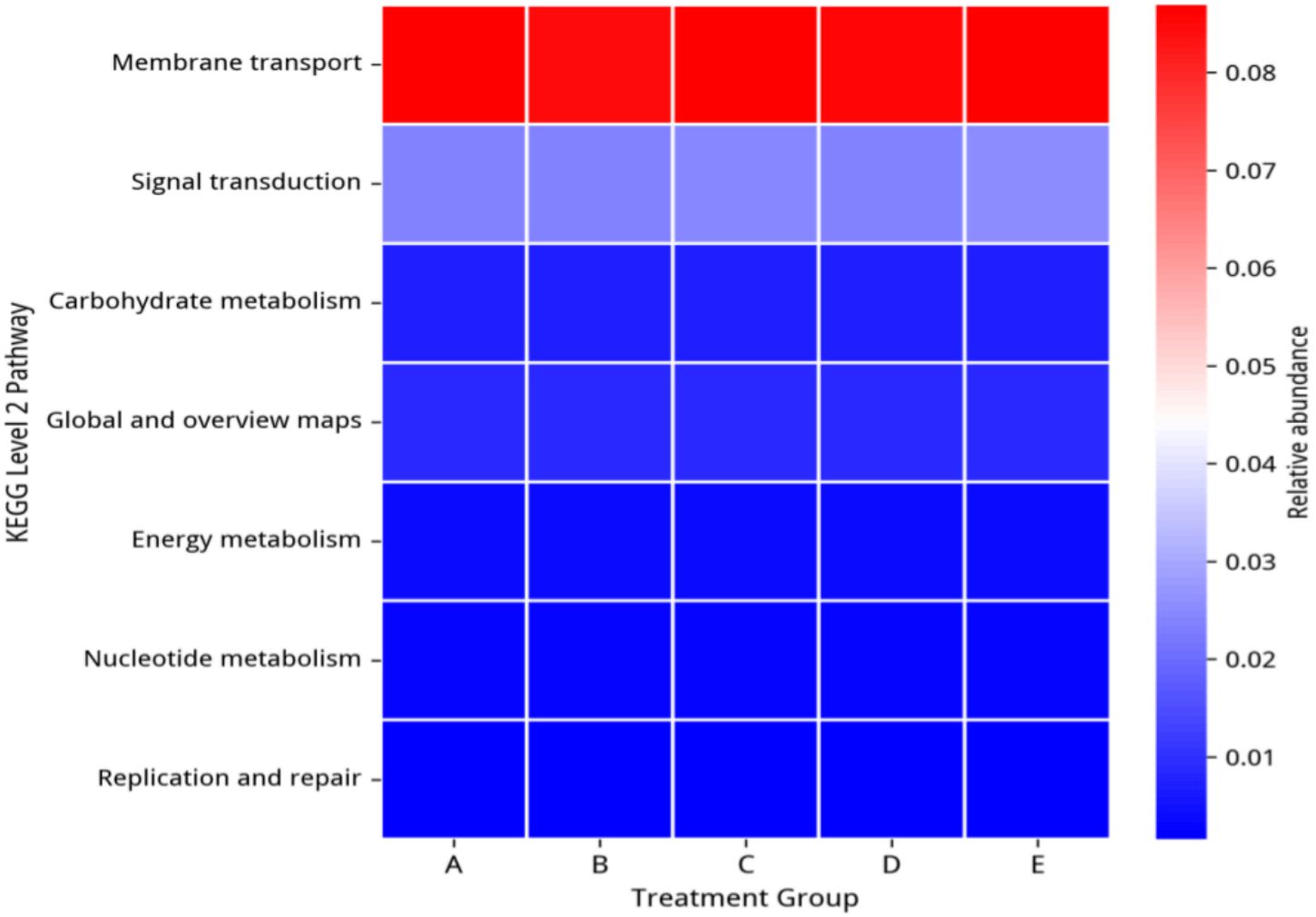


Figure 6

Prediction of functional metabolic pathways of microbial communities.