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Hydrogen-rich water 400ppb as a potential strategy for improving ruminant nutrition and mitigating methane emissions

Kang Mao[1†], Guwei Lu[1†], Yitian Zang[1], Qinghua Qiu[1], Xianghui Zhao[1], Kehui Ouyang[1], Mingren Qu[1] and Yanjiao Li[1*]

Abstract

The objective of this study was to evaluate the effects of different concentrations of hydrogen-rich water (HRW) on in vitro rumen fermentation characteristics and the dynamics of bacterial communities. The experiment included four treatment groups: a control (CON) and hydrogen-rich water (HRW) at 200, 400, and 800 ppb. Each group was analyzed at 12-hour (h) and 48-hour (h) time points with five replicates, totaling 40 samples. The experimental results highlighted the HRW_{800ppb} group as the top production in terms of gas production and CH_4 content. In contrast, the HRW_{200ppb} group exhibited significantly lower methane levels at both 12 h and 48 h ($P<0.05$). Regarding rumen fermentation, the HRW_{400ppb} group significantly increased the levels of ammonia nitrogen (NH_3-N) and microbial crude protein (MCP) at 12 h fermentation, but reduced the dry matter degradation rate ($P<0.05$). After 48 h, the HRW_{400ppb} group had highest MCP content ($P<0.05$), but no significant differences in NH_3-N and dry matter degradation rate compared with the CON group ($P>0.05$). Although HRW did not significantly benefit the synthesis of total volatile fatty acids (TVFA) and individual VFA, the HRW_{800ppb} group significantly increased the ratio of acetate to propionate ($P<0.05$). Based on CH_4 emissions and MCP synthesis, we selected the HRW_{400ppb} group for subsequent bacterial community analysis. Bacterial community analysis showed that at 12 h, compared with the CON group, the Bacterial community analysis revealed that the HRW_{400ppb} group had significant increases in the Simpson index, Firmicutes, Streptococcus, Schwartzia, *Prevotellaceae_YAB2003_group*, and *Oribacterium*, and decreases in *Prevotella*, *Ruminobacter*, *Succinivibrio*, *unclassified_Succinivibrionaceae*, and *Prevotellaceae_UCG-003* ($P<0.05$). At 48 h, the *Prevotellaceae_YAB2003_group* and *Oribacterium* abundances continued to rise significantly, while *Rikenellaceae_RC9_gut_group* and *Succiniclasticum* abundances fell in the HRW_{400ppb} group ($P<0.05$). Correlation analysis indicated a negative link between CH_4 and *Streptococcus*, and a positive correlation between the abundance of *Rikenellaceae_RC9_gut_group* and CH_4. Collectively, these results indicate that HRW can modulate rumen fermentation and microbial community structure to reduce methane emissions without significantly affecting VFA synthesis, highlighting its potential as drinking water for enhancing ruminant nutrition and mitigating the environmental impact of livestock farming.

Keywords Drinking water, Hydrogen-rich water, Microbial diversity, *In vitro* ruminal fermentation, Methanogenesis

†Kang Mao and Guwei Lu contributed equally to this work.

*Correspondence:
Yanjiao Li
yanjiaoli221@163.com

[1]Jiangxi Province Key Laboratory of Animal Nutrition/Animal Nutrition and Feed Safety Innovation Team, College of Animal Science and Technology, Jiangxi Agricultural University, Nanchang, China

Background

Ruminants can convert fibrous plants into edible meat and milk products for human consumption. This process requires the participation of rumen microorganisms, including bacteria, archaea, fungi, and ciliated protozoa which can produce volatile fatty acids (VFA), microbial proteins (MCP), and vitamins for the host animals [1, 2]. VFA supplies 70–80% of the energy to ruminants [3] and MCP provides a high level of protein resources for host animals [4]. While this fermentation is vital for the nutritional enhancement of dietary intake, it also inevitably leads to methane production—a potent greenhouse gas. Research has found that efficient beef cattle produce 20% less methane than inefficient ones [5]. Therefore, exploring strategies to regulate the activity of rumen microbiota to reduce methane production while maintaining animal production efficiency is of great scientific and practical significance.

Hydrogen-rich water (HRW) is a form of potable water that has been super-saturated with molecular hydrogen gas (H_2) through pressurized dissolution [6]. The hydrogen molecules are extremely small, so they can easily penetrate water and stay dissolved for a while. In recent years, it has been widely used and applied in many fields such as medicine, agriculture, sports, and beauty [7]. The widespread adoption of HRW can be largely attributed to its beneficial properties, such as its antioxidant, anti-inflammatory, and anti-apoptotic effects, coupled with a proven high safety profile [8]. However, there are few studies on hydrogen-rich water in ruminants. Kuru [9] found that administering HRW to goats during the peripartum period may improve the health and survival of kids and reduce their mortality.

At present, the specific mechanism of HRW is still unclear in ruminants. Some studies speculated that intestinal microorganisms might be the main target organ of hydrogen molecules [10]. Hydrogen metabolism is related to many microorganisms in the intestinal microbiota [11]. HRW intake could increase the abundance of Lactobacillus, Ruminococcus, and Clostridium [12], strengthening intestinal structural integrity and increasing butyrate-producing bacteria, thereby improving clinical features associated with gut microbiota disturbance [10]. On the other hand, in ruminants, improving the metabolic efficiency of hydrogen can affect the proliferation of hydrogenotrophic bacteria, thereby reducing the production of ruminal methane [13]. However, there is a lack of in-depth research on the impact of HRW on the structure and function of the rumen microbiota in ruminant animals, as well as its mechanism of action on the rumen fermentation and methane production process.

This study aims to fill this research gap by using a comprehensive set of technical methods, including in vitro fermentation tests, microbial community analysis, and metabolite detection, to explore the potential impact of HRW on rumen microbiota. We hypothesize that HRW may alter the hydrogen metabolism pathways within the rumen by regulating the composition and metabolic activities of rumen microbiota, thereby exerting a regulatory effect on methane production. The expected results of this study will provide new insights into the understanding and regulation of rumen microbiota metabolic activities, and offer potential solutions for reducing methane emissions from ruminants.

Results

Total gas production and methane production

The production of total gas and methane at 12 h and 48 h fermentations are shown in Fig. 1. After 12 h of fermentation, the HRW_{800ppb} group demonstrated the highest production of total gas and methane gas, reaching 53.35 mL and 13.58 mL, respectively, with the total gas production significantly exceeding the other three groups ($P<0.05$). However, the production of methane gas showed no significant difference compared to the CON group ($P>0.05$). At 48 h of fermentation, $HRW800_{ppb}$ maintained the

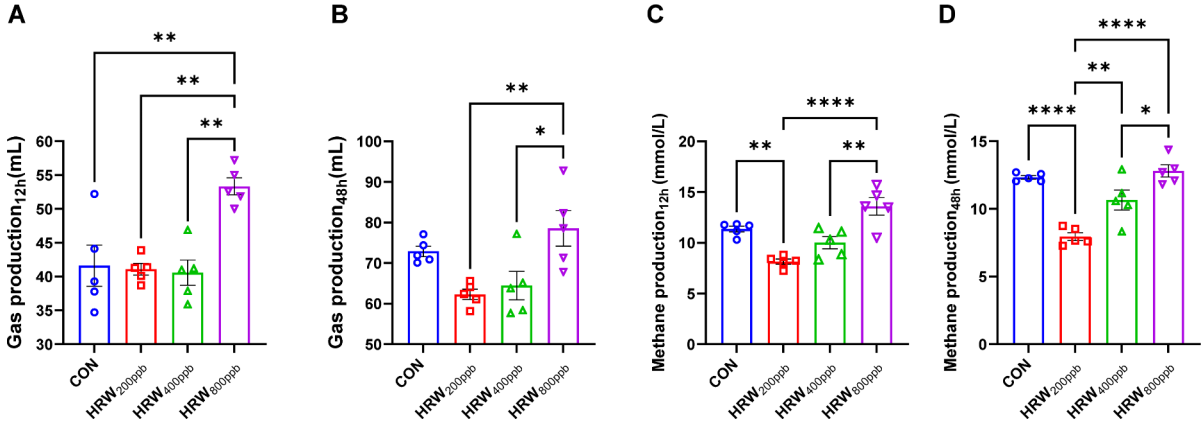

Fig. 1 Total gas production and methane production at 12 h and 48 h. **A** 12 h gas production; **B** 48 h gas production; **C** 12 h methane production; **D** 48 h methane production. CON = control, HRW = hydrogen-rich water; *$P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.001$

highest production of total gas and methane gas, with 78.56 mL and 12.80 mL, respectively, both of which were significantly higher than those in the HRW_{200ppb} and HRW_{400ppb} groups ($P<0.05$). Nevertheless, there were no significant differences compared to the CON group. The methane gas production of the HRW_{200ppb} group was significantly lower than the other three groups at both 12 and 48 h of fermentations.

Rumen characteristics

The results of rumen fermentation characteristics are shown in Table 2. Different concentration of HRW affected rumen fermentation parameters. After 12 h of fermentation, significant differences in pH values were observed among the four groups, with HRW_{800ppb} (pH=6.59) and HRW_{400ppb} (pH=7.03) showing significantly lower pH values compared to HRW_{200ppb} (pH=7.25, $P<0.001$) and the CON group (pH=6.43, $P<0.001$). In terms of MCP, the HRW_{400ppb} group exhibited the highest MCP content at 31.67 mg/dL, followed by the HRW_{200ppb} group at 27.45 mg/dL, while the HRW800ppb group had a significantly lower MCP content than that of the CON group (20.85 mg/dL vs. 28.38 mg/dL, $P<0.001$). At 48 h of fermentation, the trend in pH values was similar to that at 12 h, with HRW_{400ppb} and HRW_{800ppb} groups showing significantly higher pH values compared to the HRW_{200ppb} and CON groups ($P<0.001$). Regarding MCP, the HRW_{400ppb} group maintained the highest MCP content, followed by the HRW_{200ppb} group, and both were significantly higher compared to the CON group ($P=0.001$). After 12 h of fermentation, the HRW group of NH_3-N levels were significantly higher than those in the CON group ($P<0.001$). While, at 48 h of fermentation, the CON group exhibited the highest NH_3-N levels at 12.21 mg/dL, which were significantly different from those in the HRW groups ($P=0.018$). The dry matter degradation rate at 12 h was significantly higher in the CON and HRW_{800ppb} groups compared to the HRW_{200ppb} and HRW_{400ppb} groups ($P<0.001$), while there were no significant effects between the HRW groups and the CON group ($P=0.187$) at 48 h.

The results of VFAs are shown in Table 3. After 12 h of fermentation, the CON group demonstrated the highest levels of acetate, propionate, branched-chain amino acids, and TVFA, with concentrations of 32.39 mM, 16.16 mM, 0.56 mM, and 52.20 mM, respectively, which were significantly higher than those in the HRW_{200ppb} and HRW_{400ppb} groups ($P<0.05$). Notably, the HRW_{800ppb} group exhibited the lowest propionate content ($P<0.05$). The acetate-to-propionate ratio and the non-glucogenic-to-glucogenic acids ratio at 12 h of fermentation were significantly higher in the HRW_{800ppb} group compared to the other groups ($P<0.05$). After 48 h of fermentation, the HRW_{200ppb} group exhibited markedly reduced isobutyrate levels in comparison to the other groups ($P<0.05$). In contrast, the HRW_{800ppb} group displayed significantly elevated butyrate concentration and an increased acetate-to-propionate ratio among the four groups ($P<0.05$). The CON group had significantly higher valerate and isovalerate contents compared to the HRW_{200ppb} and HRW_{800ppb} groups ($P<0.05$). Additionally, the TVFA content in the CON group is also significantly higher than the other 3 groups ($P<0.05$), with a notable decrease observed in the HRW_{200ppb} group ($P<0.05$). The HRW_{800ppb} group had the highest non-glucogenic to glucogenic acids ratio, but a lowest fermentation efficiency compared to the CON and HRW_{200ppb} groups ($P<0.05$). At 48 h, the HRW_{200ppb} group had a significantly lower non-glucogenic to glucogenic acids ratio and a higher fermentation efficiency than the other groups ($P<0.05$).

Rumen Bacteria

From the 20 samples, a total of 909,772 clean reads were detected with an average of 45488.6 for each sample (Table. S1). The composition of bacteria across the 20 samples was dominated by 951 OTU, 16 phyla, and 224 genera (Table. S2). The alpha diversity at 12 h and 48 h of fermentation was estimated by the Simpson and Ace index (Fig. 2). Compared with the CON group, the HRW group significantly increased the Simpson index (Fig. 2A, C), whereas no significant differences were observed in Ace at 12 h and 48 h (Fig. 2B, D). To measure the extent of similarity between the microbial communities, beta diversity was calculated using a weighted normalized UniFrac, and the PCoA was performed. As shown in Fig. 2E, F. The microbial community profiles of the HRW were grouped to the right of the PCoA, and CON was grouped to the left of the PCoA. PERMANOVA analysis found that the two groups were significantly different at 12 h and 48 h ($R=0.988$, $P=0.004$; $R=0.660$, $P=0.004$).

The taxonomic analysis of the reads revealed that the dominant phyla were Firmicutes, Bacteroidota, and Proteobacteria at 12 h and 48 h of fermentation, accounting for >99% of total reads (Fig. 3A, B). Among the three phyla, supplementing with HRW could significantly increase the relative abundance of Firmicutes, and decrease the relative abundance of Bacteroidota and Proteobacteria at 12 h of fermentation (Fig. 4A, B, C, $P<0.05$), while no significant differences were observed at 48 h of fermentation (Fig. 5A, B, C, $P<0.05$). At the genus level, the predominance of the genera is depicted in Fig. 3C and D for the 12 h and 48 h fermentation stages, respectively. Difference analysis of TOP 12 genera (Fig. 4D-O) indicated that the abundance of *Streptococcus*, *Schwartzia*, *Prevotellaceae_YAB2003_group*, and *Oribacterium* were significantly higher, and *Prevotella*, *Succinivibrio*, *unclassified_f__Succinivibrionaceae*, and

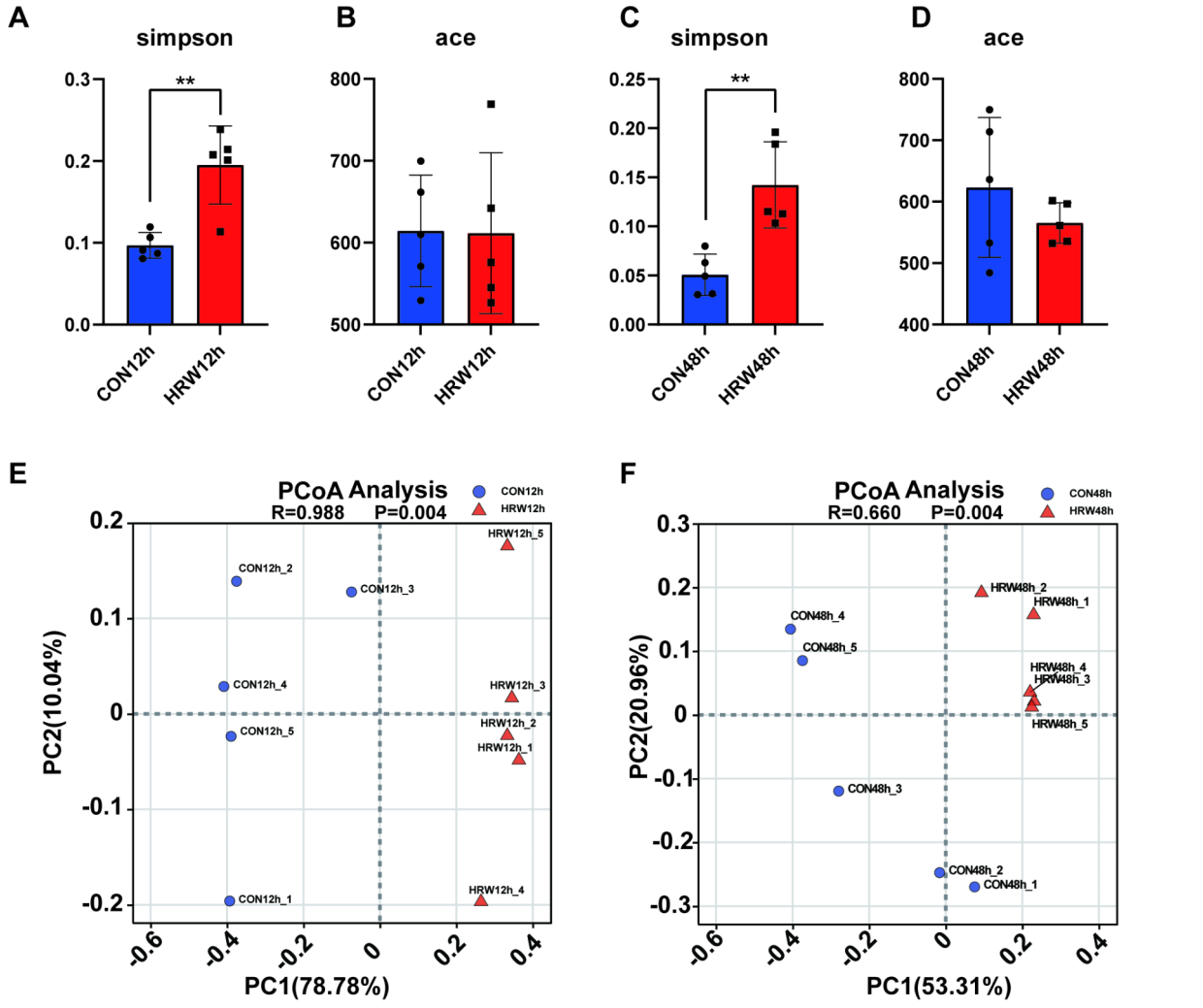

Fig. 2 Bacteria alpha diversity and principal-coordinate analysis (PCoA) based on OUT level. **A-D**: Bacteria alpha diversity analysis between HRW and CON group at 12 h and 48 h fermentation; **E-F**: Bacteria PCoA analysis between HRW and CON group at 12 h and 48 h fermentation. CON = control, HRW = hydrogen-rich water ** $P < 0.01$

Prevotellaceae_ UCG-003 were significantly lower in the HRW group compared with the CON group at 12 h of fermentation ($P < 0.05$). While, among the 5 differential genera at 48 h (Fig. 5D, G, H, M, N), the abundance of *Prevotellaceae_YAB200 3_group*, *Oribacterium*, *Streptococcus*, *and Ruminobacter* were significantly increased, and *Rikenellaceae_RC9_gut_group* and *Succiniclasticum* were significantly decreased in the HRW group compared with the CON group ($P < 0.05$).

Correlation analysis

Correlations analysis was conducted between rumen fermentation characteristics and main bacteria in genus level at 12 h and 48 h (Fig. 6). There were 9 significant correlations at 12 h of fermentation ($|R| > 0.5$, $P < 0.05$). The acetate and propionate were significantly positively related to *Prevotella*, *Ruminobacter*, *unclassified_f__Succinivibrionaceae*, and *Prevotellaceae_ UCG-003* ($P < 0.05$), while significantly negatively related to *Streptococcus*, *Schwartzia*, *Prevotellaceae_YAB2003_ group*, and *Oribacterium* ($P < 0.05$). The NH_3-N was significantly positively related to *Streptococcus*, *Schwartzia*, *Prevotellaceae_YAB2003_group*, and *Oribacterium* ($P < 0.05$), and significantly negatively related to *Prevotella*, *Ruminobacter*, *Succinivibrio*, *unclassified_f__Succinivibrionaceae*, and *Prevotellaceae_UCG-003* ($P < 0.05$). On the other hand, there were 7 significant correlations at 48 h of fermentation ($|R| > 0.5$, $P < 0.05$). The acetate and propionate were significantly positively related to *Rikenellaceae_RC9_gut_group* and *Succiniclasticum* ($P < 0.05$), and significantly negatively related to *Prevotellaceae_YAB2003_grou*, *Oribacterium*, and *Streptococcus* ($P < 0.05$).

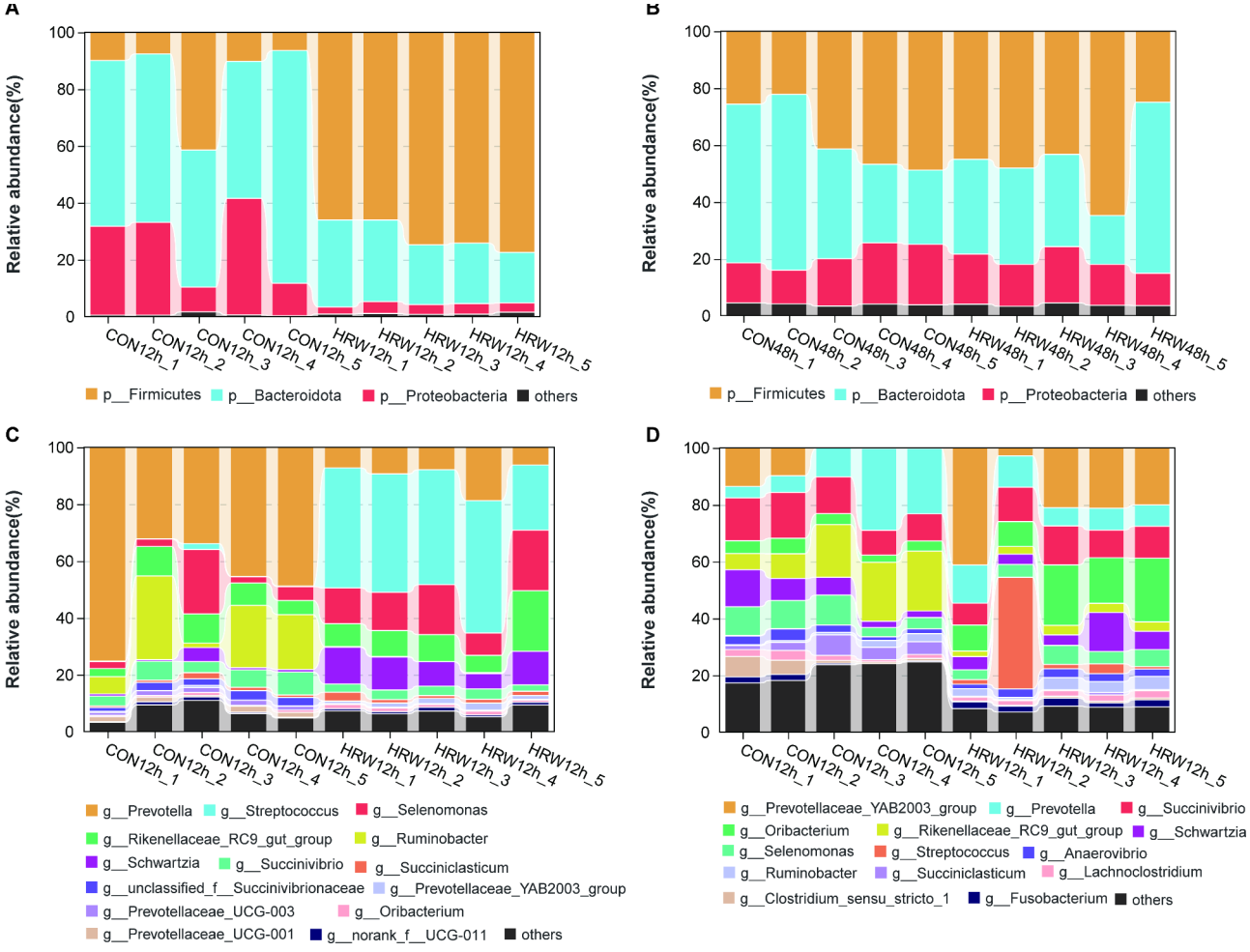

Fig. 3 Microbial compositional profiles of phylum and genus. **A-B**: Microbial compositional profiles of phylum between CON and HRW group at 12 h and 48 h fermentation. **C-D**: Microbial compositional profiles of genus between CON and HRW group at 12 h and 48 h fermentation. CON = control, HRW = hydrogen-rich water

Discussion

HRW, which is derived through a unique technological process that integrates hydrogen gas into water, boasts numerous beneficial effects on human health [7]. Nevertheless, the realm of research pertaining to its utilization in ruminants remains relatively unexplored, with the majority of studies predominantly focused on monogastric animals. Under normal growth conditions, HRW treatment did not affect the feed intake and growth performance in the piglets and broiler chickens [14, 15], which might be related to nutrient digestibility. In general, the improvement in nutrient digestibility is accompanied by an elevation in growth performance [16]. Therefore, we speculate that HRW has no significant effect on nutrient digestibility. In our study, there was no significant difference in dry matter degradability between the HRW group and CON group at the 48 h of fermentation, which was consistent with our hypothesis. Fermentation gas is derived from the digestion of carbohydrates during the fermentation process and is associated with rumen degradability of the organic matter [17]. It has been reported that there exists a close correlation between the dry matter degradation rate and gas production [18], where a higher dry matter degradation rate typically coincides with an elevated gas production. While, our result that HRW_{800ppb} has the highest dry matter degradability and gas production at 12 h and 48 h, but only gas production at 12 h fermentation was statistically significant compared to the CON group. This could imply that the initial effect of the treatment diminishes over time. CH_4, a potent greenhouse gas, is predominantly generated through microbial fermentation in the rumen ecosystem. In this process, methanogenic archaea play a pivotal role by engaging in methanogenesis, a metabolic pathway that assimilates hydrogen and carbon dioxide, thereby converting them into methane [19]. HRW_{200ppb} significantly decreased the content of CH_4 at 12 h and 48 h fermentation. But interestingly, as the content of

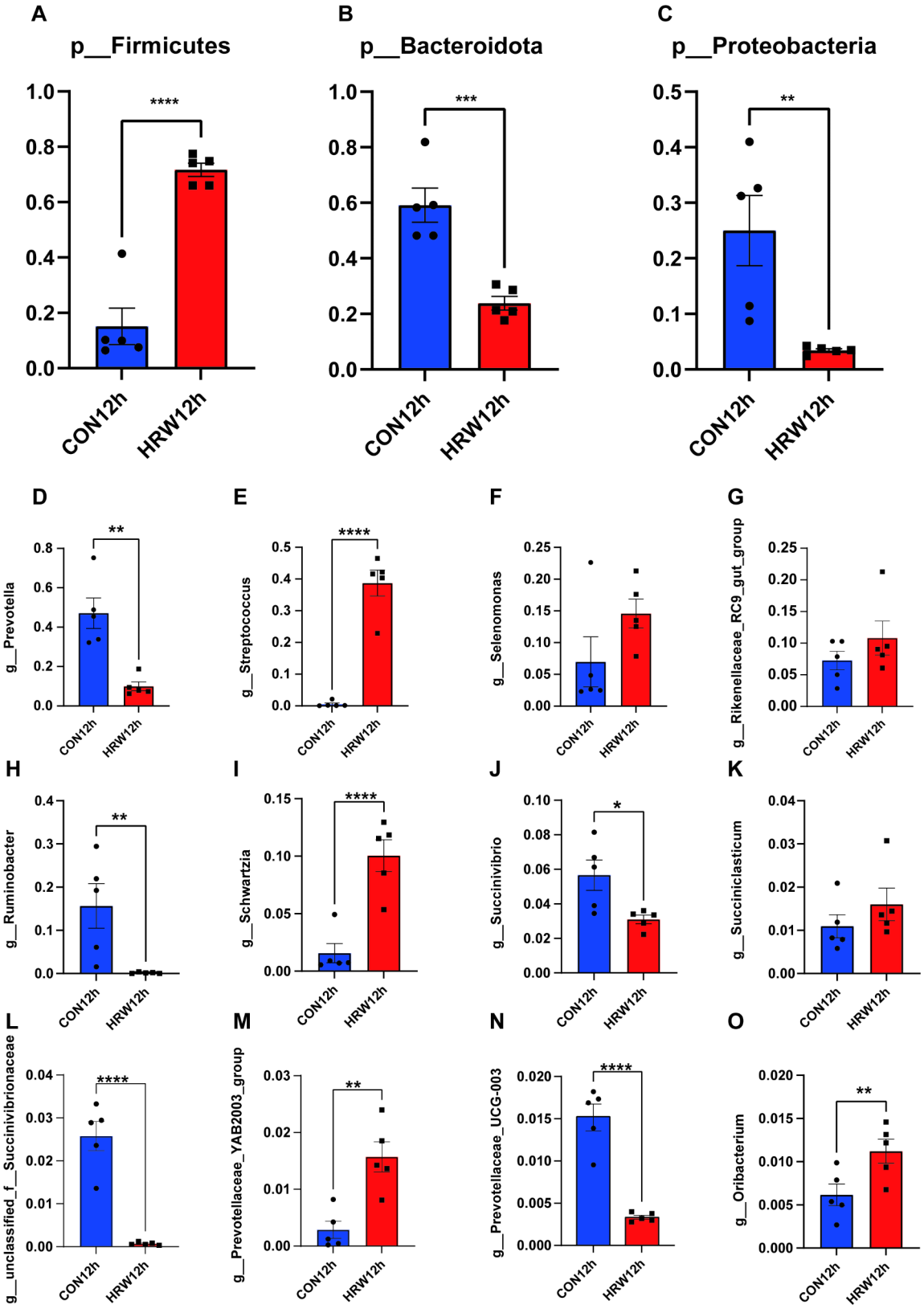

Fig. 4 Differential rumen bacteria phylum and genus at 12 h fermentation. **A-C**: Analysis of differences in abundance among the top 3 phylum level bacteria between CON and HRW group; **D-O**: Analysis of differences in abundance among the top 12 phylum level bacteria between CON and HRW group. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$

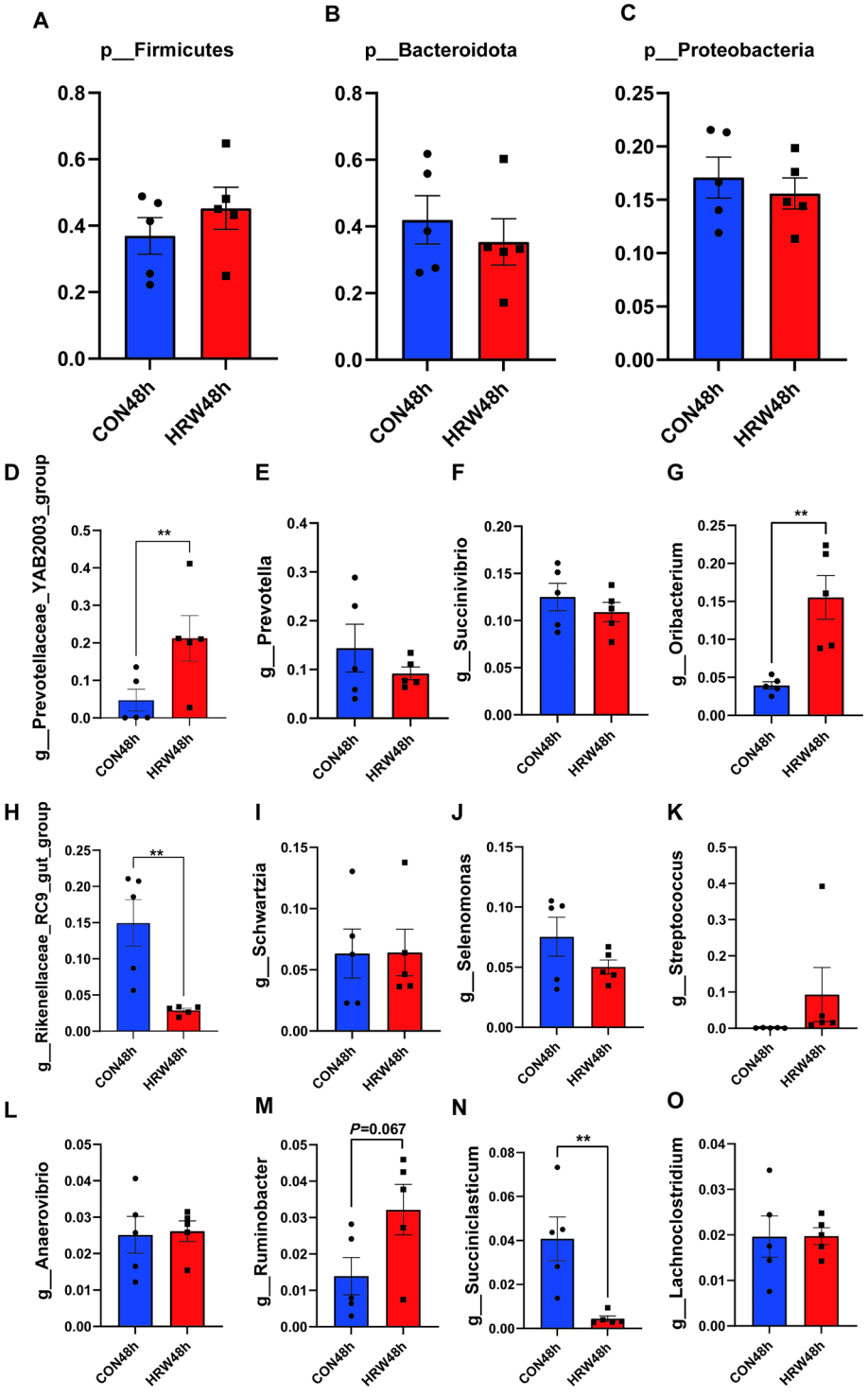

Fig. 5 Differential rumen bacteria phylum and genus at 48 h fermentation. **A-C**: Analysis of differences in abundance among the top 3 phylum level bacteria between CON and HRW group; **D-O**: Analysis of differences in abundance among the top 12 phylum level bacteria between CON and HRW group. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$

HRW increases, CH_4 production also increases. It might be that low doses of hydrogen can alter the fermentation pathways of rumen microorganisms, while high content of hydrogen provides a substrate for methane production [20]. The rumen pH, NH_3-N, MCP, and VFA are important indicators for evaluating rumen function. The rumen pH fluctuates from 6.0 to 7.2, which is conducive to rumen microorganisms and the normal function of

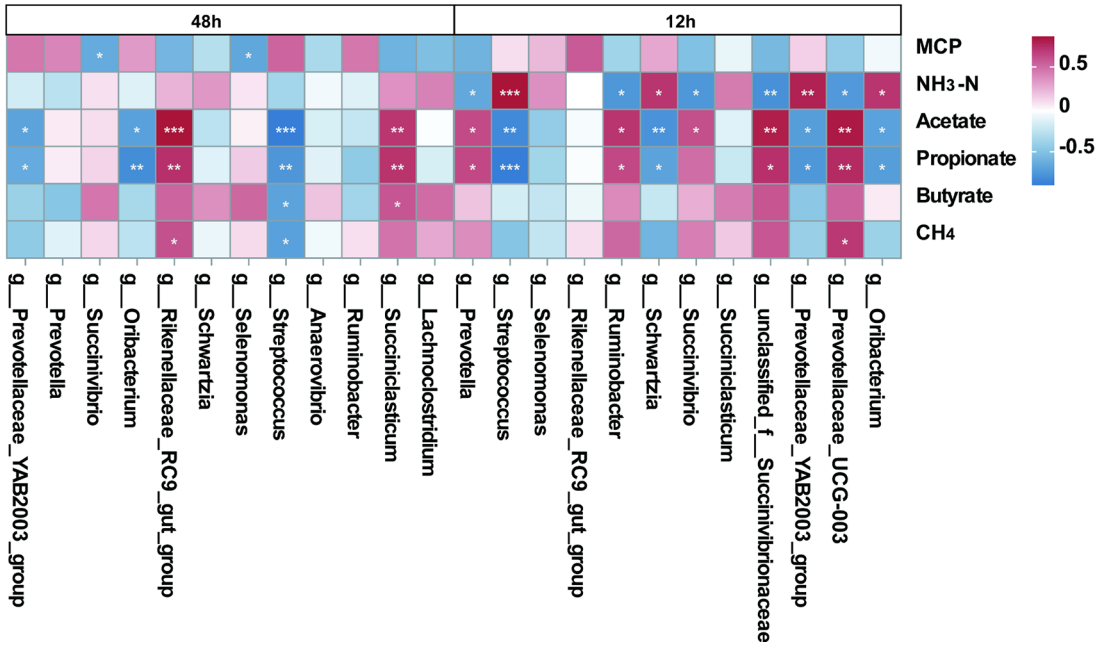

Fig. 6 Sperman correlations between rumen fermentation characteristics and TOP 12 rumen bacterial community in genus level at 12 h and 48 h fermentation. |R| > 0.5 and $P < 0.05$ indicate significant correlation

ruminants [21]. In this experiment, the pH of the rumen in each group fluctuated within the normal range of 6.43 to 7.2, indicating that HRW supplementation did not disrupt the balance of the acid-base environment. The fluctuation of NH_3-N concentration in the rumen reflects the degradation of dietary N and the utilization of NH_3-N by rumen microorganisms [22]. The content of NH_3-N in the HRW_{400ppb} group has no significant difference compared with the CON group at 48 h of fermentation, which might be that HRW_{400ppb} did not promote the dry matter degradability. The result that lacking of significant effect on nitrogenous at 48 h fermentation was consistent with other studies. For instance, Choi et al. [23] found that the use of HRW had no significant effect on the quality of duck manure in Beijing ducks, including pH, total nitrogen, and ammonia nitrogen. Although the HRW_{400ppb} does not affect the rumen ammonia nitrogen content, it increases the content of MCP, which can be explained by the higher bacterial diversity. The increasing diversity of rumen microorganisms may be improving the utilization efficiency of available nitrogen. VFA is known as the main end product of carbohydrates, which can provide 70–80% of ruminant energy needs [24]. Structural carbohydrates and nonstructural carbohydrates continue to degrade as the fermentation process advances, producing acetate and propionate, respectively [25]. In this study, at 48 h of fermentation, although the levels of TVFA, individual VFAs, and BCVFA were higher, the contents of TVFA, acetate, and propionate were lower in the HRW group compared to the CON group. To date, despite the absence of direct studies exploring the effect of HRW on ruminal microorganisms, research findings have pointed towards HRW's capacity to modulate the gut microbiota in humans [10]. In light of this, we employed 16 S rRNA sequencing technology to delve into the potential effects of HRW on the structure of the ruminal microbiota, and to subsequently dissect the intricate relationship between these alterations and the production of VFA. Utilizing this approach, we aim to gain a clearer understanding of the mechanisms by which HRW modulates ruminal microbial activity and VFA production, thereby providing a scientific basis for optimizing the feeding management of ruminants. Based on rumen fermentation indicators and methane gas production, HRW_{400ppb} (abbreviated as HRW hereafter) was selected as the subsequent treatment group for analysis. Bacterial alpha diversity includes species richness and diversity, which are primarily described by Ace and Simpson indexes, respectively. In this study, differences were found in diversity between the HRW and CON groups at 12 h and 48 h of fermentation. Currently, the effects of HRW on gut microbial diversity are inconsistent. Under normal physiological conditions, HRW has been observed to exert no significant influence on the α-diversity of gut bacteria in mice [12]. However, conversely, research has demonstrated a marked enhancement in the α-diversity of gut bacteria among female athletes [26]. This discrepancy suggests that the impact of HRW on gut microbiota may vary among different populations and distinct physiological states, necessitating in-depth research to unravel the underlying mechanisms and explore potential applications. Bacteroidetes, Firmicutes, and Proteobacteria

were regarded as the three phyla with the most abundance in ruminal bacteria [27], which was consistent with our results. Firmicutes are capable of breaking down cellulose into VFA, thereby supplying energy to the host, and Bacteroidetes contribute to the enhancement of the host's nutrient utilization by degrading carbohydrates and proteins [28]. Firmicutes degrade dietary fiber to produce acetate and butyrate, while Bacteroidota mainly produces propionate through nonfibrous substance degradation. In this study, at 12 h of fermentation, we observed a correlation between Bacteroidota and propionate, with their trends moving in tandem. In contrast, an increase in Firmicutes abundance was associated with a decrease in both acetate and butyrate contents. This discrepancy may be attributed to the enriched HRW, which potentially enhanced the capacity of Firmicutes to synthesize MCP. Consistent with our findings, previous studies have reported that the Firmicutes phylum accelerates the utilization of ruminal ammonia-N and the synthesis of MCP [29], thereby underscoring its pivotal role in the metabolic transformations within the rumen ecosystem. At the same time, the abundance of Proteobacteria decreased in the HRW group at 12 h. The phylum Proteobacteria plays an essential role in the rumen microbiome, particularly in the degradation of carbohydrates, where they are primarily responsible for the breakdown of cellulose and hemicellulose. Consequently, a decrease in the abundance of Proteobacteria may directly lead to a reduced efficiency of fibrous material degradation in the rumen [30]. This change not only affects the metabolic activities of the rumen microbiota but also subsequently impacts the production of VFA. In the present study, it is evident that the variation in the abundance of Proteobacteria significantly influences the efficiency of rumen fermentation, and its decline could be a key factor contributing to the decrease in dry matter degradation rate and VFA production. But at 48 h of fermentation, the abundance of Bacteroidota, Firmicutes, and Proteobacteria had no significant difference between the HRW and CON groups. It might be that, as fermentation time passes, the hydrogen in the HRW gradually gets consumed, thereby leading to the normalization of its fermentation pattern. Subsequently, a deeper analysis was conducted on the differential bacteria genera at 12 h of fermentation, the results showed that the relative abundance of *Prevotella* and *Prevotellaceae_UCG-003* were significantly decreased in the HRW group. *Prevotella* and *Prevotellaceae_UCG-003*, belonging to the Bacteroidetes phylum, both possess a potent capacity to degrade nonstructural carbohydrates and proteins. Additionally, they are capable of fermenting sugars via the acrylic and succinic acid pathways, leading to the production of propionate [31]. Meanwhile, both of these also had a positive correlation with propionate. Thus, the decrease in propionate levels in the HRW group is directly associated with the reduced abundance of *Prevotella*. Additionally, *Prevotella*, *Ruminobacter*, and *Succinivibrio* are all hydrogen-producing bacteria [32], and the supplementation of HRW may have suppressed their activity. On the other hand, in the HRW group, there was a significant increase in the abundance of the *Streptococcus* genus. Although no studies have directly investigated the correlation between the increased abundance of *Streptococcus* and the utilization of ruminal nitrogen, the findings of Jin et al. [33] suggest that the *Streptococcus* genus possesses unique advantages in the utilization of nitrogen within the rumen. Based on this, we hypothesize that the increased MCP synthesis may be associated with the rise of *Streptococcus* abundance. However, in this study, after 48 h of fermentation, the types of bacteria changed varied. Previous research has reported that *Rikenellaceae_RC9_gut_group* plays a crucial role in the degradation of carbohydrates within the gut microbiota [34], while *Succinivibrionaceae* exhibits a significant positive correlation with the production of total VFA as well as the contents of acetate and propionate [35]. The reduction in the abundance of these two bacterial families in our experiment is consistent with the observed trends in VFAs. However, the precise biological mechanisms underlying their influence necessitate further research for clarification. Additionally, correlation analysis revealed a significant positive relationship between *Rikenellaceae_RC9_gut_group* and CH_4 content, which may suggest a role for this family in the decline of methane levels during this period. This finding provides a novel perspective for further exploration of the potential role of *Rikenellaceae_RC9_gut_group* in regulating methane production. In addition, a significant negative correlation was observed between the presence of Streptococcus and CH_4 production. This phenomenon suggests that bacteriocins produced by Streptococcus may play a role in inhibiting the activity of methanogenic archaea or facilitate the redirection of H_2 towards other reductive microorganisms that do not generate CH4 [36]. Consequently, the reduction in methane levels we observed is likely associated with an increase in Streptococcus abundance, which may be attributed to the antimicrobial effects of bacteriocins or the metabolic redirection they induce. Particular attention was given to the genus *Oribacterium*, which was significantly increased in the HRW group at 12 and 48 h of fermentation. *Oribacterium* has been identified as one of the primary bacteria in the rumen of cows fed with forage [37, 38]. However, current research on this bacterium is still limited, with existing studies merely speculating a relationship between ruminal *Oribacterium* and the production of alanine [39]. Therefore, the mechanism by which

HRW affects *Oribacterium* requires further investigation.

Conclusion

In conclusion, our findings indicate that HRW at 400ppb significantly enhances rumen fermentation, thereby improving the overall efficiency of the rumen ecosystem. Contrary to initial hypotheses, HRW does not directly contribute to the synthesis of ruminal VFA. Nonetheless, HRW_{400ppb} exhibits a notable capacity to mitigate methane emissions, which correlates with *Streptococcus* and *Rikenellaceae_RC9_gut_group*, offering a critical environmental benefit. Additionally, HRW's influence on the rumen microbiota's composition indirectly facilitates the synthesis of MCP, which is essential for ruminant nutrition. These results underscore the potential of HRW as a sustainable feed additive, offering dual advantages in enhancing ruminant nutrition and reducing the environmental footprint of livestock farming. This research thus contributes pivotal insights into the strategic integration of HRW in ruminant diets for improved animal health and environmental stewardship.

Methods

Preparation of hydrogen-rich water

Preparation of 800 ppb HRW: Distilled water (2 L) was added to a negative ion water generator (Model V8, Mrs. Li's Electrical Appliance Co., Ltd., Zhongshan City) using a measuring cylinder. The apparatus was powered for 0.5 h to produce alkaline hydrogen-rich electrolyzed water. The resulting alkaline electrolyzed water had a pH of 8.69, an ORP of -554 mV, and a hydrogen gas concentration of 0.81 mg/L.

Table 1 Composition and nutrient levels of experimental diet (air-dry basis, %)

Ingredients	Content	Nutritional composition	g/kg of DM
Wheat Straw	50.71	Metabolic energy (ME), MJ/kg	9.59
Corn	20.00	Crude protein (CP)	141.2
wheat bran	3.49	MP to CP ratio, MJ/g	0.068
Soybean meal	19.80	Neutral detergent fiber	428.0
Calcium bicarbonate	0.50	Acid detergent fiber	266.4
Calcium hydrophosphate	0.50		
Premix[a]	4.00		
Limestone	0.50		
Salt	0.50		
Tatol	100		

[1]The premix (per kg of diet) is: 1400 mg of Fe, 1200 mg of Zn, 250 mg of Cu, 900 mg of Mn, 100,000 IU of vitamin A, 27,000 IU of vitamin D3, and 800 IU of vitamin E

Preparation of 400 ppb: 1 L of HRW at a concentration of 800 ppb was mixed with 1 L of distilled water to obtain 2 L of HRW at a concentration of 400 ppb. The resulting alkaline electrolyzed water had a pH of 7.64, an ORP of -72 mV, and a hydrogen gas concentration of 0.44 mg/L.

Preparation of 200 ppb HRW: 1 L of HRW at a concentration of 400 ppb was mixed with 1 L of distilled water to obtain 2 L of HRW at a concentration of 200 ppb. The resulting alkaline electrolyzed water had a pH of 7.52, an ORP of -14 mV, and a hydrogen gas concentration of 0.22 mg/L.

Rumen fluid collection

Three Jinjiang cattle with permanent ruminal fistula installed (weight = 365.2 ± 27.4 kg) were taken as the rumen fluid donors for rumen content collection. The rumen content was obtained 1 h before morning feeding and then was filtered by four layers of gauze. All three collections from bulls were evenly mixed into a sterile bottle, which was finally used as the rumen fluid (culture medium) for the in *vitro* test. The rumen fluid pH of three cattle, measured immediately with a Rex PHBJ-260 m upon arrival at the laboratory using a Rex PHBJ-260 pH meter (Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China), averaged 6.82. The fermentation substrate was the total mixed ration for Jinjiang cattle, the ingredients and nutrient composition of the diet are listed in Table 1.

In vitro cultivation medium and experimental design

Mixing the following reagents in volume as cultivation medium: 520.2 mL of distilled water (treatment group using 200 ppb, 400ppb, and 800ppb HRW), 208.1 mL of buffer solution (4.0 g NH_4HCO_3 + 35 g $NaHCO_3$ dissolved in distilled water and made up to 1000 mL), 208.1 mL of constant element solution (9.45 g $Na_2HPO_4 \cdot 12H_2O$ + 6.2 g anhydrous KH_2PO_4 + 0.6 g $MgSO_4 \cdot 7H_2O$ dissolved in distilled water and made up to 1000 mL), 0.1 mL of trace element solution (13.2 g $CaCl_2 \cdot 2H_2O$ + 10.0 g $MnCl_2 \cdot 4H_2O$ + 1.0 g $CoCl_2 \cdot 6H_2O$ + 8.0 g $FeCl_3 \cdot 6H_2O$ dissolved in distilled water and made up to 1000 mL), and 62.4 mL of reducing solution (4.0 mL of 1 mol/L NaOH + 625 mg $Na_2S \cdot 9H_2O$ + 625 mg cysteine hydrochloride + 95 mL distilled water), which was bubbled with CO_2 until the solution turned colorless from light blue.

The prepared cultivation medium was warmed at 39 ℃. Proportionally prepared fermentation substrate (0.50 g) was placed in a glass bottle with a total volume of 100 mL, and then 40 mL of pre-warmed cultivation medium and 20 mL of rumen fluid were added to the above bottle and CO_2 was injected to get rid of oxygen. The bottle was incubated in SHA-B oscillators (Guohua Enterprise, Changzhou, Jiangsu, China) for in vitro gastric fermentation experiments.

Table 2 Rumen fermentation characteristics in vitro rumen fermentation

Item	CON	HRW_{200ppb}	HRW_{400ppb}	HRW_{800ppb}	SEM	*P*-value
pH value						
12 h	6.43^{d}	7.20^{a}	7.03^{b}	6.59^{c}	0.025	< 0.001
48 h	6.60^{b}	6.98^{a}	7.08^{a}	6.50^{b}	0.027	< 0.001
Microbial crude protein, mg/dL						
12 h	28.38^{ab}	27.45^{b}	31.67^{a}	20.85^{c}	0.588	< 0.001
48 h	36.08^{b}	45.23^{a}	45.97^{a}	34.34^{b}	0.986	0.001
Ammonia nitrogen, mg/dL						
12 h	4.64^{c}	7.66^{a}	6.62^{ab}	5.99^{b}	0.194	< 0.001
48 h	12.21^{a}	9.76^{bc}	11.95^{ab}	8.86^{c}	0.387	0.018
Dry matter degradability, %						
12 h	45.07^{a}	30.16^{c}	40.66^{b}	48.53^{a}	0.725	< 0.001
48 h	65.35^{ab}	62.71^{b}	66.13^{ab}	68.62^{a}	0.907	0.187

$^{a, b}$ Means within a row with no common superscript differ significantly ($P < 0.05$). CON = control; HRW = hydrogen-rich water. SEM = stand error of mean

Table 3 Rumen fermentation total volatile fatty acids (VFA) and individual VFAs in vitro rumen fermentation

Item	DW	HRW_{200ppb}	HRW_{400ppb}	HRW_{800ppb}	SEM	*P*-value
Acetate, mM						
12 h	32.39^{a}	21.58^{c}	26.54^{b}	32.59^{a}	0.455	< 0.001
48 h	35.44^{a}	23.10^{c}	30.14^{b}	30.88^{b}	0.503	< 0.001
Propionate, mM						
12 h	16.16^{a}	11.17^{b}	11.47^{b}	7.63^{c}	0.270	< 0.001
48 h	19.76^{a}	14.91^{b}	15.88^{b}	14.73^{b}	0.235	< 0.001
Isobutyrate, mM						
12 h	0.04	0.04	0.05	0.05	0.002	0.282
48 h	0.13^{a}	0.08^{b}	0.14^{a}	0.17^{a}	0.006	0.002
Butyrate, mM						
12 h	3.19	3.77	3.09	4.33	0.301	0.457
48 h	4.55^{b}	4.15^{bc}	3.64^{c}	7.40^{a}	0.142	< 0.001
Isovalerate, mM						
12 h	0.14^{ab}	0.12^{b}	0.14^{ab}	0.15^{a}	0.003	0.011
48 h	0.36^{a}	0.21^{c}	0.32^{ab}	0.27^{bc}	0.015	0.017
Valerate, mM						
12 h	0.38^{a}	0.28^{b}	0.29^{b}	0.31^{b}	0.005	< 0.001
48 h	0.39^{a}	0.27^{b}	0.43^{a}	0.29^{b}	0.013	0.001
Branched-chain volatile fatty acids, mM						
12 h	0.56^{a}	0.45^{c}	0.48^{bc}	0.51^{ab}	0.009	0.003
48 h	0.87^{a}	0.57^{b}	0.90^{a}	0.73^{a}	0.031	0.006
Total volatile fatty acids, mM						
12 h	52.20^{a}	36.97^{b}	41.59^{b}	43.49^{b}	0.921	< 0.001
48 h	60.62^{a}	42.73^{c}	50.56^{b}	53.74^{b}	0.711	< 0.001
Acetate to propionate ratio						
12 h	2.00^{b}	1.95^{b}	2.32^{b}	4.21^{a}	0.100	< 0.001
48 h	1.79^{b}	1.55^{c}	1.90^{b}	2.10^{a}	0.032	< 0.001
Non-glucogenic to glucogenic acids ratio						
12 h	2.36^{c}	2.59^{bc}	2.80^{b}	5.10^{a}	0.069	< 0.001
48 h	2.23^{a}	2.09^{b}	2.32^{a}	3.07^{a}	0.043	< 0.001
Fermentation efficiency						
12 h	0.78^{a}	0.78^{a}	0.77^{a}	0.72^{b}	0.003	< 0.001
48 h	0.79^{b}	0.80^{a}	0.78^{bc}	0.77^{c}	0.002	< 0.001

$^{a, b}$ Means within a row with no common superscript differ significantly ($P < 0.05$). CON = control; HRW = hydrogen-rich water; Branched-chain volatile fatty acids are the sum of isobutyrate, valerate, and isovalerate. SEM = stand error of mean

The experiment comprised 4 groups, including CON (control), 200 ppb, 400 ppb, and 800ppb HRW, with 10 replicates for each group (5 replicates were stopped at 12 h (hours) and the other 5 at 48 h), and the indicators were strictly measured according to the experimental steps and requirements. Rumen fermentation characteristics were determined at the incubation time of 12 h and 48 h.

Rumen fermentation parameter determination

After 12 h and 48 h incubation, fermented contents were filtered with four layers of gauze to obtain supernatant samples. The pH value was measured by a pH meter (Testo 206-pH1, Desto Instrument Co., LTD, Shenzhen, China). These supernatant samples were stored at -80℃ to a determination of VFA, ammonia nitrogen (NH_3–N), MCP, and rumen microorganisms. The NH_3-N concentration was determined using the method of phenol-hypochlorite reaction as described in Broderick and Kang [40]. The Folin phenol method based on Lowry's assay was taken to determine the concentration of microbial crude protein (MCP), as described by Makkar et al. [41]. The VFA measurements were determined according to the method of Qiu et al. [42]: using a gas chromatograph (GC-2014Shimadzu Corporation, Kyoto, Japan) equipped with a 30 m capillary column (Rtx-Wax, 0.25 mm ID × 0.25 μm film, Restek, Evry, France) to determine the contents of acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid. The sum of the six VFAs was defined as total VFA (TVFA), and the sum of iso-butyric acid and iso-valeric acid was defined as branched-chain VFA. The peak area method was used for identification and content conversion of each VFA based on relative retention time. The standard curve was prepared under the same conditions using the same method. The non-glucogenic to glucogenic acids ratio (NGR) and fermentation efficiency (FE) were calculated as follows:

$$\mathrm{NGR} = (C_2 + 2 \times C_4 + C_5) / (C_3 + C_5)$$

$$\mathrm{FE} = (0.622 \times C_2 + 1.092 \times C_3 + 1.56 \times C_4) / (C_2 + C_3 + 2 \times C_4)$$

The culture medium was filtered through gauze, and the filter cake was transferred without damage into a nylon bag, which was then placed in a 65 °C drying oven to determine the solids content and calculate the degradation rate. The 12 h and 48 h solids were dried by reference to the method in GB/T6435-2006.

The in *vitro* solids degradation rate (V) = (W2-W3) / W1.

W1 = The weight of fermentation substrate (g).

W2 = The total weight of fermentation substrate and nylon bag (g).

W3 = The total weight of fermentation substrate and nylon bag after in *vitro* fermentation (g).

Net gas production rate and gas production parameters

The gas production was measured after incubating the culture for 3, 6, 9, 12, 18, 24, 27, 30, 36, and 48 h. The culture tubes were quickly removed from incubation and the piston displacement (mL) was immediately recorded. The net gas production for each period was calculated as:

Net gas production (mL) = Gas production at a time point (mL) - Gas production of blank at the same time point (mL).

Methane production (The CH_4 production was estimated using the equation described by Moss et al. [43].

CH_4 (mmol/L) = $0.45 \times C_2 - 0.275 \times C_3 + 0.40 \times C_4$.

Note: C_2 = Concentration of acetate (mmol/L), C_3 = Concentration of propionate (mmol/L), C_4 = Concentration of butyrate (mmol/L).

Bacterial community analysis

A total of twenty microbial community genomic DNA using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) were transported to the Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) or PCR amplification and MiSeq sequencing. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The amplification reaction system and program were the same as Mao et al. [27] report. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega, USA). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1174395).

The raw 16 S rRNA gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the

truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cut-off were clustered using UPARSE (version 7.1, http://drive5.com/uparse/), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by the RDP Classifier (http://rdp.cme.msu.edu/) against the 16 S rRNA database (e.g. Silva v138) using a confidence threshold of 0.7. Correlations between rumen fermentation characteristics and rumen bacterial community were presented with a heat map, which was performed using SPSS (version 17.0, IBM, Armonk, NY, USA) and Origin (version 2018, Origin Software, Inc., Northampton, Massachusetts, USA).

Statistical analysis

The data analyses were statistically analyzed by one-way ANOVA with SPSS statistical software (Version 17.0, IBM, Armonk, NY, USA). The results are shown as the mean and standard error mean (SEM). Differences among means were determined using Tukey's multiple range test was done when the interaction was significant. The level of statistical significance was set at $P<0.05$.

Abbreviations

HRW	hydrogen-rich water
CON	Control
MCP	Microbial crude protein
NH_3-N	Ammonia nitrogen
VFA	Volatile fatty acids
OTU	Operational taxonomic units
CH_4	Methane

Supplementary Information

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Supplementary Material 1

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Author contributions

KM and GL were responsible for the conception and design of the study. KM and GL were responsible for data extraction and interpretation of the results, and GL and YZ carried out the statistical analysis. YZ, GL, QQ, and KO supervised the research activity. KM and GL were mainly responsible for drafting the manuscript. YL, KM, and MQ were involved in revising the draft. All authors read and approved the final manuscript.

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Data availability

The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1174395).

Declaration

Ethics approval and consent to participate

This experiment was approved by the Committee for the Care and Use of Experimental Animals at Jiangxi Agricultural University (JXAULL-2021-10).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Ethics approval

Animal care and experimental procedures were approved by the Animal Care Committee of Jiangxi Agricultural University (Nanchang, China), and were under the university's guidelines for animal research.

Clinical trial number

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