Molecular hydrogen produced by elemental magnesium inhibits rumen fermentation and enhances methanogenesis in dairy cows

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ABSTRACT

Hydrogen is a key metabolite that connects microbial fermentation and methanogenesis in the rumen. This study was to investigate the effects of elevated H₂ produced by elemental Mg on rumen fermentation and methanogenesis in dairy cows. Four nonlactating Chinese Holstein dairy cows were employed for this experiment in a replicated crossover design. The 2 dietary treatments included a basal diet supplemented with Mg(OH)₂ (14.5 g/kg of feed dry matter) or elemental Mg (6.00 g/kg of feed dry matter). When compared with Mg(OH)₂ treatment, cows fed diet with elemental Mg had similar rumen Mg²⁺ concentration, but higher rumen dissolved H₂ and methane concentrations at 2.5 h after morning feeding. Also, elemental Mg supplementation decreased feed digestibility, rumen volatile fatty acid concentration, and relative abundance of group *Ruminococcaceae_UCG-014*, genus *Bifidobacterium*, and group *Mollicutes_RF9*, increased acetate to propionate ratio, succinate concentration, and abundance of family *Christensenellaceae*. Elemental Mg supplementation increased enteric CH₄ emission, altered methanogen community with increased abundance of order *Methanomassiliicoccales*, 16S ribosomal RNA gene copies of methanogens, and order *Methanobacteriales*. In summary, the pulse of elevated dissolved H₂ after feeding produced by elemental Mg inhibited rumen fermentation and feed digestibility by decreasing the abundance of carbohydrate-degrading bacteria, promoted H₂ incorporation into succinate by increasing family *Christensenellaceae* and genus *Bacteroidales*.

BS11, and increased H₂ utilization for methanogenesis by favoring growth of methanogens.

Key words: elemental magnesium, dissolved hydrogen, feed digestibility, rumen fermentation, methanogenesis

INTRODUCTION

Molecular hydrogen is mainly produced by carbohydrate fermentation and then used by methanogens to produce CH₄ in the rumen (Hungate, 1967; Robinson and Tiedje, 1982). Starch in concentrate diets has greater and faster rumen degradability than fiber in forage and produces a large amount of H₂ (Wang et al., 2016b). Also, high dietary starch has been observed to cause a reduction in rumen fiber digestibility in vitro (Grant and Mertens, 1992) and in vivo (Yang et al., 2001). Methanogenesis is the largest H₂ sink in the rumen and maintains low H₂ partial pressures, which in turn promotes re-oxidization of NADH/NADPH and supports continuous fermentation processes (Robinson and Tiedje, 1982). When methanogenesis is inhibited, rumen H₂ accumulates followed by a reduction in rumen fermentation and feed degradation (Goel and Makkar, 2012; Ungerfeld, 2015). This shows that increased rumen H₂ is associated with the inhibition of rumen fermentation and feed degradation.

Rumen H₂ has been proposed to influence the fermentation pathways thermodynamically, and increased H₂ partial pressure can inhibit NADH/NADPH generation and decrease the acetate to propionate ratio (Ungerfeld and Kohn, 2006; Janssen, 2010). However, rumen H₂ may not always be associated with shifts in fermentation pathways. For example, Wenner et al. (2017) reported that increased passage rate does not alter rumen H₂, but shifts acetate toward propionate production. Wang et al. (2018) reported that increased rumen H₂ is not associated with changes of VFA profiles in goats

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fed high starch. Martinez-Fernandez et al. (2018) reported 3-NOP supplementation did not influence H₂ gas emissions but facilitated acetate production in beef steers. Further studies are still needed to investigate the relationship between rumen H₂, fermentation pathways and associated microbiota *in vivo*.

Rumen methanogenesis mainly involves 3 types of methanogenic metabolic pathways, which includes hydrogenotrophic (reduction of CO₂ coupled to the oxidation of H₂), methylotrophic (conversion of methyl-group containing compounds), and acetoclastic pathways (Lovley and Ferry, 1985; Rother and Krzycki, 2010). The hydrogenotrophic pathway accounts for the first dominant metabolic pathway of methanogenesis in the rumen (Hungate, 1967; Hook et al., 2010). Increasing rumen H₂ may enhance the hydrogenotrophic methanogenesis, and thus affect H₂ utilization and methanogen communities in the rumen.

Rumen H₂ exists in 2 forms: the gaseous H₂ and the dissolved H₂ (dH₂), and only the dH₂ is biologically relevant for rumen microorganisms (Wang et al., 2016a). Elemental Mg supplementation previously increased rumen dH₂ concentration and influenced rumen fermentation and CH₄ emission in goats (Wang et al., 2017). Interestingly, elemental Mg tends to react with H⁺ in rumen fluid to produce extra H₂ according to the reaction $\text{Mg} + 2\text{H}^+ \rightarrow \text{Mg}^{2+} + \text{H}_2$ to produce H₂. Studies are needed to comprehensively investigate the biological function of dH₂ in dairy cows. In this experiment, Mg was added to TMR to increase dH₂, whereas Mg(OH)₂ was included as the control group, because Mg(OH)₂ can also react with ruminal H⁺ according to the reaction $\text{Mg(OH)}_2 + 2\text{H}^+ \rightarrow \text{Mg}^{2+} + 2\text{H}_2\text{O}$. Therefore, we hypothesized that elemental Mg, compared with Mg(OH)₂ supplementation, could increase dH₂ concentration in the liquid phase of the rumen, which would influence feed digestibility, rumen fermentation, methanogenesis, and microbiota in dairy cows.

MATERIALS AND METHODS

All animal procedures followed our institutional guidelines for the care and use of animals and were approved by the Animal Care Committee (approval number ISA-W-201702), Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Experimental Design and Animals

Four nonlactating Chinese Holstein rumen-fistulated dairy cows of about 7 yr of age (BW = 503.3 ± 83.21 kg, DMI = 7.0 ± 1.12 kg, daily CH₄ emission = 175.4

Table 1. Ingredients and chemical compositions of basal diet

Item	Value
Ingredient, g/kg	
Corn silage	500
Corn	142
Soybean meal	115
Wheat middlings	100
Wheat bran	100
Palm fat powder	10
Limestone ¹	8
Calcium hydrophosphate	5
Urea	5
Sodium chloride	5
Vitamin/trace mineral premix ²	10
Chemical composition, g/kg	
CP	145
NDF	435
ADF	211
Starch	216
Ash	155
DE, ³ MJ/kg of DM	14.1

¹The limestone included 38.4% Ca and 0.8% Mg.

²Premix provided per kilogram of concentrate: 1,000,000 IU of vitamin A, 200,000 IU of vitamin D, 1,250 IU of vitamin E, 8,000 mg of Zn, 80 mg of Se, 120 mg of I, 2,000 mg of Fe, 40 mg of Co, 2,500 mg of Mn, and 2,000 mg of Cu.

³DE = digestible energy, calculated according to NRC (2001).

± 12.07 g; mean ± SD) were used for this experiment. The experiment was a replicated crossover design with 2 dietary treatments, which were a basal diet (Table 1) supplemented with Mg(OH)₂ (14.5 g/kg of DM) or elemental Mg (6.00 g/kg of DM).

Cows were housed in a tiestall barn and fed individually at 0630 and 1630 h. All cows had free access to fresh water. The TMR provided allowed less than 1% of feed refusal to ensure the similar DMI [7.06 ± 1.30 kg/d for Mg(OH)₂ treatment; 7.08 ± 0.96 kg/d for elemental Mg treatment; mean ± SD] of 2 groups. In each experimental period, 30 d in total, cows passed through 15 d of diet adaptation, 5 d of feces and diet refusal collection, 8 d for measuring CH₄ emissions (2 d in a respiration chamber for 4 cows), and 2 d of rumen content collection.

Nutrient Digestibility

Total feces and feed refusals were collected for 5 consecutive days and processed according to the procedure described by Wang et al. (2016b). The DM (method 934.01), ash (method 942.05), and CP (method 990.03) were determined according to AOAC (1990). The NDF and ADF were measured following the procedures of Van Soest et al. (1991) by using a Fibertherm analyzer (C. Gerhardt Ins., Königswinter, Germany) and expressed inclusive of residual ash. The α-amylase and

sodium sulfite (Sigma, Shanghai, China) were added to remove starch and protein within NDF analysis. The starch content was determined after pre-extraction with 80% ethanol (vol/vol), and glucose released from starch by enzyme hydrolysis was measured using amyloglucosidase (Sigma) according to Karthner and Theurer (1981). Gross energy was measured with an automatic calorimeter (5E-AC8018, Changsha Kaiyuan Instruments Co. Ltd., Changsha, China).

Methane Emissions

Four cows of each experimental period were sequenced individually through the respiration chamber by 2 blocks. Each block of 2 cows contained 1 cow from 2 treatments, and emissions from each cow were measured for 2 d. Thus, 4 cows were passed through the chamber within 8 d.

Methane recovery was valid before and after measurement, and a correction factor was then developed by comparing the amount of pure CH₄ released into the empty chamber with measured flux of CH₄ from the chamber. Methane emissions from each cow were measured in a respiration chamber using the protocol by Wang et al. (2015). Airflow was maintained under negative chamber pressure (flow rate = 190 ± 2 m³/h), controlled by an air pump. The outlet gas from the chamber and ambient gas were connected to a Multiport Inlet Unit of the Gas Analyzer (GGA-30p, Los Gatos Research, CA) using 1/8 inch (0.32 cm) polyurethane tubing. Methane concentration in the chamber and ambient gas was recorded every 30 min. The amount of daily CH₄ emissions was then calculated by multiplying correction factor and measured flux of CH₄ from chamber. Staff entered each chamber twice daily at 0530 and 1530 h for approximately 20 min for feeding. Within the chamber, cows were restrained in the bin with free access to drinking water.

Rumen Sampling and Analysis

Rumen contents were sampled from the middle of the ventral sac for 2 d after CH₄ measurement through a rumen cannula at 0, 2.5, and 6 h after the morning feeding. One subsample (100 mL) was immediately frozen in liquid nitrogen and stored at -80°C for subsequent DNA extraction and quantification of microbial groups. Rumen fluids were immediately separated from rumen contents by squeezing through cheesecloths (350 μm , Tang Rong Medical Supplies Co. Ltd., Yangzhou, China). Because the mesh size of cheesecloth we used was higher than the 250 μm reported in the literature (Maekawa et al., 2002), 6 layers rather than the typical 4 layers of cheesecloths were conducted to filter rumen

fluids. Two other subsamples (35 mL each) of rumen fluid were immediately transferred into 50-mL plastic syringes for the measurement of dH₂ and dissolved methane (dCH₄) concentration. About 20 mL of rumen fluids were used for immediate measurement of rumen pH by using a portable pH meter (Starter 300, Ohaus Instruments Co. Ltd., Shanghai, China), calibrated before each sampling using standard buffers (pH 4.0 and 7.0). Samples of 2 mL of rumen fluid were centrifuged at $12,000 \times g$ for 10 min at 4°C , and aliquots of the supernatants (1.5 mL) were transferred into plastic tubes each containing 0.15 mL of 25% (wt/vol) metaphosphoric acid and stored at -20°C overnight for subsequent measurement of VFA and ammonia. The remaining samples (50 mL) were stored at -20°C for measuring Mg²⁺, formate, and succinate.

Dissolved CH₄ and dH₂ in rumen fluid were extracted according to the protocol of Wang et al. (2014). In brief, an approximately 35-mL subsample of rumen fluid was quickly transferred into a 50-mL plastic syringe, which was connected with a 20-mL syringe filled with 10 mL of N₂. Nitrogen gas was then injected into the 50-mL syringe via polyurethane tubing, and the gases dissolved in the rumen fluid were extracted into the N₂ gas phase by shaking in an orbital shaker (Wuhan Huichen Biotech Co., Wuhan, China) at a speed of 200 rpm for 5 min. Extracted gas samples were collected in evacuated tubes, and concentrations of CH₄ and H₂ were measured by using GLC (Agilent 7890A, Agilent Inc., Palo Alto, CA). The standard gas concentration (L/L) grades were 0.1, 0.01, and 0.001% for H₂ and 10, 5, 1, and 0.5% for CH₄. All standard gases were prepared and qualified by Aerospace Ruida Standard Material Technology (Beijing) Co. Ltd. Concentrations of dH₂ and dCH₄ in the original liquid fraction were calculated according to equations described in Wang et al. (2016a).

The concentration of Mg²⁺ was determined by Inductively Coupled Plasma-Optical Emission Spectrometers using Varian 720-ES (Agilent Inc.) as described by Wang et al. (2017). Individual VFA concentrations were analyzed using gas-liquid chromatography (Agilent 7890A, Agilent Inc.) according to the procedure of Wang et al. (2016a). Ammonia concentration was measured using the procedure of Shahinian and Reinhold (1971). The formate and succinate were measured using HPLC (Agilent 1260, Agilent Inc.) according to the procedure described by Gagen et al. (2014).

Pyrosequencing of 16S rRNA Gene Amplicons

Microbial DNA was extracted according to the protocol described by Yu and Morrison (2004), with the exception that DNA was purified by phenol/chlo-

roform/isopentyl alcohol (25:24:1 vol/vol/vol, Solarbio Co., Shanghai, China) as described by Minas et al. (2011), then the vacuum-dried DNA pellets were dissolved in Tris EDTA buffer (Tris 10 mM, EDTA 1 mM, pH = 8.0) after being washed with 70% ethanol alcohol twice. The concentration and purity of DNA were measured using an ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The 16 S rRNA gene amplicons were produced as described in our previous work (Ma et al., 2018) and were sequenced with the Illumina MiSeq platform by Majorbio BioPharm Technology, Shanghai, China. Primers for bacterial and methanogens amplicons are shown in Table 2. Raw reads were submitted to the NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) database under accession number SRP149140. Quality control of the sequence reads was performed using the MOTHUR v.1.40 (Schloss et al., 2009) and followed the protocol described by Kozich et al. (2013). Reads were clustered into operational taxonomic units at 97% similarity, and the data were normalized according to the samples with the smallest number of reads. Taxonomic analysis was carried out using the RDP classifier v.11.1 (Wang et al., 2007) with a minimum support threshold of 80% against the SILVA.nr.132 database including 188,247 bacteria, 4,626 archaea, and 20,246 eukarya sequences (Quast et al., 2013). A principal coordinate analysis was performed based on Bray-Curtis similarity distances (Bray and Curtis, 1957).

Quantitative Real-Time PCR Analyses

The absolute quantification of total methanogens, order *Methanobacteriales*, and genus *Methanobrevibacter* was measured by quantitative real-time PCR (qPCR) with primers validated in our laboratory (Table 2). Quantitative PCR assays were performed according to the procedures described by Ma et al. (2018) by using a LightCycler 480 (Roche Molecular Systems Inc., Pleasanton, CA) and qPCR SuperMix (Transgen, Bei-

jing, China). Final absolute amounts of target groups were estimated by relating the cycle threshold value to standard curves and expressed as copies per milliliter of rumen fluid ($\log_{10}$ transformed).

Statistical Analyses

Statistical analyses were performed using a linear mixed model of SPSS 17.0 software (SPSS Inc., Chicago, IL). The first analytic model included treatment as a fixed effect, and animal, period, and block as random effects. When sampling time was considered, the second analytic model was used to include treatment, an interaction of treatment and sampling time as the fixed effect, sampling time as a repeated measurement, and animal, period, and block as the random effect. When significant interaction of treatment and sampling time was observed, statistical analysis was re-performed for each sampling time point by using the first model. Statistical significance of effects was declared at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.10$.

RESULTS

Elemental Mg supplementation decreased ($P = 0.018$; Table 3) total-tract OM digestibility in dairy cows. Compared with cows fed diet with $\text{Mg}(\text{OH})_2$, cows supplemented with elemental Mg had greater enteric CH_4 emission in terms of grams per day (+37.2%, $P = 0.09$), g/kg of DMI (+33.8%, $P = 0.003$), g/kg of OM digested (+48.7%, $P = 0.002$), and g/kg of NDF digested (+48.0%, $P = 0.005$).

Concentrations of Mg^{2+} and ammonia, and pH in the rumen of cows fed diets with elemental Mg did not differ significantly ($P \geq 0.41$; Table 4) to cows fed diet with $\text{Mg}(\text{OH})_2$. Interactions between treatment and time were observed for rumen dH_2 ($P = 0.005$) and dCH_4 ($P = 0.003$) concentrations. Elemental Mg supplementation greatly increased dH_2 (+129.6%, $P = 0.007$) and dCH_4 (+110.8%, $P = 0.024$) in rumen fluid

Table 2. Primers for amplicons and quantitative real-time PCR (qPCR) of microbial groups

Microbial group	Primer	Sequence (5'→3')	Reference
Amplicons			
Bacteria	Forward	ACTCCTACGGGAGGCAGCA	Amato et al. (2013)
	Reverse	GGACTACHVGGGTWTCTAAT	
Methanogens	Forward	TGYCAGCCGCCGCGTAA	Pires et al. (2012)
	Reverse	YCCGGCGTTGAVTCCAATT	
qPCR			
Total methanogens	Forward	GGATTAGATACCCSGGTAGT	Denman and McSweeney (2006)
	Reverse	GTTGARTCCAATTAAACCGCA	
<i>Methanobacteriales</i>	Forward	CGWAGGGAAGCTGTAAAGT	Yu et al. (2005)
	Reverse	TACCGTCGTCCACTCCTT	
<i>Methanobrevibacter</i>	Forward	CCTCCGCAATGTGAGAAATCGC	Huang et al. (2016)
	Reverse	TCWCCAGCAATTCCACAGTT	

Table 3. Feed intake and digestibility and methane emission in dairy cows fed a diet with Mg(OH)₂ or elemental Mg (n = 4)

Item	Treatment		SEM	P-value
	Mg(OH) ₂	Mg		
DMI, kg	7.08	7.06	—	—
Apparent total-tract digestibility, g/kg				
DM	713	690	3.0	0.063
OM	716	690	1.8	0.018
CP	800	780	2.2	0.097
NDF	637	586	5.5	0.066
ADF	598	536	3.1	0.014
Starch	991	996	0.17	0.29
Gross energy, %	74.1	72.0	0.22	0.044
Methane emissions				
g/d	148	203	8.9	0.09
g/kg of DMI	21.6	28.9	0.21	0.003
g/kg of OM digested	35.1	49.7	0.29	0.002
g/kg of NDF digested	77.1	114.1	1.26	0.005

collected at 2.5 h after morning feeding (Figure 1). Elemental Mg supplementation decreased total VFA concentration (−11.1%, $P = 0.029$), and increased acetate to propionate ratio (+15.3%, $P = 0.011$) and succinate concentration (+265%, $P = 0.054$) in the rumen (Table 4).

Bacteria and methanogens community were further analyzed by high-throughput sequencing. The principal coordinate analysis result of bacteria showed that cow 1 was distinctly different from the other 3 cows (Figure 2A). A total of 194 bacteria genera were annotated, and 22 genera with abundances larger than 1% accounted for 76.7% of total sequences (Table 5). Elemental Mg supplementation decreased the relative abundance of the *Ruminococcaceae_UCG-014* group (−51.8%, $P =$

0.040), genus *Bifidobacterium* (−61.4%, $P = 0.051$), and the *norank_o_Mollicutes_RF9* group (−33.3%, $P = 0.024$), but increased the relative abundance of the *Ruminococcaceae_UCG-010* group (+60.0%, $P = 0.005$), *norank_f_Bacteroidales_BS11* group (+71.2%, $P = 0.032$), and family *Christensenellaceae* (+57.0%, $P = 0.034$) in rumen. Cow 1 had distinctly different methanogen communities from cow 3 and 4, although we did not find a separation in methanogen communities between the 2 treatments (Figure 2B). Cows fed the diet with elemental Mg had lower abundance of order *Methanobacteriales* ($P = 0.005$), and greater abundance of order *Methanomassiliicoccales* ($P = 0.007$) in the rumen (Table 6). The qPCR results showed that elemental Mg supplementation increased ($P \leq 0.005$)

Table 4. Dissolved gases and fermentation end products in the rumen of dairy cows fed a diet with Mg(OH)₂ or elemental Mg (n = 4)

Item	Treatment			P-value		
	Mg(OH) ₂	Mg	SEM	Treatment	Time ¹	Treatment × time
Mg ²⁺ , mM	13.1	13.1	1.15	0.99	0.004	0.84
pH	6.65	6.49	0.102	0.38	0.075	0.67
Ammonia, mM	12.7	12.2	1.96	0.63	<0.001	0.41
Dissolved gas						
Hydrogen, μM	1.76	2.68	0.435	0.035	<0.001	0.005
Methane, mM	0.41	0.50	0.038	0.024	0.14	0.003
Total VFA, mM	89.6	79.7	4.06	0.029	0.013	0.18
Molar concentration of individual VFA, mM						
Acetate	47.3	43.3	1.92	0.056	0.046	0.16
Propionate	21.4	16.9	1.29	0.003	0.007	0.35
Butyrate	14.4	13.3	0.89	0.25	0.012	0.26
Valerate	2.04	1.75	0.123	0.035	0.002	0.31
Iso-butyrate	1.26	1.29	0.041	0.49	0.035	0.30
Iso-valerate	3.18	3.14	0.128	0.78	0.13	0.06
Acetate to propionate ratio	2.29	2.64	0.12	0.011	0.013	0.99
Formate, mM	0.38	0.31	0.070	0.32	0.12	0.68
Succinate, mM	0.452	1.65	0.408	0.054	0.59	0.64

¹Relative time after morning feeding.

Figure 1. Dissolved hydrogen (dH₂) and methane (dCH₄) in the rumen fluids collected at 0, 2.5, and 6 h after morning feeding in dairy cows fed diets with Mg(OH)₂ or elemental Mg (n = 4). Error bars show SD.

16S rRNA gene copies of methanogens and abundance of order *Methanobacteriales* (Table 6).

DISCUSSION

The 6 g/kg of dietary Mg supplementation was relatively high but within the safe dose (≤ 7 g/kg) reported by Quillian et al. (1980). No diarrhea occurred in any of the animals used. Both elemental Mg and Mg(OH)₂ can be converted to Mg²⁺ in the liquid phase of the rumen, producing H₂ and H₂O as end products, respectively. Compared with cows fed the diet with Mg(OH)₂, cows fed the diet with elemental Mg had similar Mg²⁺ concentration and greater dH₂ concentration. Elemental Mg supplementation also increases ruminal dH₂ concentration in goats (Wang et al., 2017). The elemental Mg supplementation only increased dH₂ concentration at 2.5 h after morning feeding, whereas dH₂ concentrations of the Mg treatment returned to the same level as the Mg(OH)₂ treatment at 6 h after morning feeding. The rumen dH₂ concentration can be affected by many factors, such as exhausting H₂ production during the reaction of elemental Mg with H⁺ and the consumption of elevated H₂ by increased H₂ users.

The increase in partial pressure of H₂ can prevent the oxidation of NADH to NAD⁺, and thus inhibits rumen fermentation (Miller and Wolin, 1995). In addition, increased H₂ is suggested to be linked with reduction in rumen fermentation and feed digestibility (Goel and Makkar, 2012; Ungerfeld, 2015). Elemental Mg supplementation decreased OM digestibility and total VFA concentration, which could also be caused

by the extra H₂ produced by elemental Mg. Inhibition of rumen fermentation can be associated with a shift in the bacterial community (Weimer, 1998). Compared with the control group, elemental Mg supplementation decreased the relative abundance of the *Prevotellaceae_UCG-001* group, *Ruminococcaceae_UCG-014* group, *Mollicutes_RF9* group, and genus *Bifidobacterium*. The *Prevotellaceae_UCG-001* group is a hemicellulose degrader (Thoetkiattikul et al., 2013; He et al., 2017). Members of the *Ruminococcaceae_UCG-014* group can also be essential fiber degraders, as its abundance is positively associated with dietary NDF intake (Zhang and Wang, 2018). The metagenome-assembled genomes of the *Mollicutes_RF9* group have the CAZme associated with degrading starch (Svartstrom et al., 2017). Although genus *Bifidobacterium* cannot degrade polysaccharide, it synergizes with starch degraders, as genus *Bifidobacterium* is increased by feeding starchy diet (Trovatelli and Matteuzzi, 1976). It seems that elevated dH₂ caused by elemental Mg supplementation inhibits some groups of carbohydrate-degrading bacteria. However, elemental Mg supplementation increased the relative abundance of the *Ruminococcaceae_UCG-010* group, which also belongs to the family *Ruminococcaceae*. Unfortunately, group *Ruminococcaceae_UCG-010* is an uncultured bacterium, and its biological function and ecological role in rumen need further investigation.

Fermentation of glucose to acetate and propionate involves net H₂ production and incorporation, respectively (Janssen, 2010). When H₂ is accumulated in the rumen, fermentation theoretically favors propionate production other than acetate production (Ungerfeld,

2013). A negative correlation was observed between dH_2 and propionate molar proportion (Wang et al., 2016b, 2017). However, our study indicated that elevated dH_2 caused by elemental Mg supplementation increased acetate to propionate ratio in dairy cows. We suspect that other H_2 sinks may be promoted, as the complex rumen microbial ecosystem can cope with elevated H_2 in the rumen (Ungerfeld, 2015; Wang et al., 2017).

Figure 2. Principal coordinate (PC) analysis plot of the bacteria (A) and methanogen (B) community at the operational taxonomic unit level based on Bray-Curtis distances. Hollow and solid symbols indicate $\text{Mg}(\text{OH})_2$ and elemental Mg treatment, respectively; circle (○), triangle (Δ), and square (□) represent samples collected at 0, 2.5, and 6 h after morning feeding, respectively; red, black, green, and blue represent dairy cow 1, 2, 3, and 4, respectively; and solid circles indicate separation in bacteria and methanogen community of cows from each other.

Succinate is an essential precursor of propionate and can be an H_2 sink (Blackburn and Hungate, 1963). Formation of succinate from pyruvate involves 2 steps of metabolic hydrogen incorporation, which are oxaloacetate to malate (Kyoto Encyclopedia of Genes and Genomes reaction: R00342, www.kegg.jp) and fumarate to succinate (Kyoto Encyclopedia of Genes and Genomes reaction: R00402). Elemental Mg supplementation increased succinate concentration and decreased propionate concentration, indicating an enhancement of succinate formation or inhibition of succinate decarboxylation to propionate. The *Bacteroidales_BS11* group belongs to succinate producers because it produces succinate dehydrogenase, an enzyme catalyzing the reaction of fumarate to succinate (Solden et al., 2017). Furthermore, fumarate plus malate supplementation facilitate the growth of the family *Christensenellaceae* (De Nardi et al., 2016), which can also be related to succinate formation according to the fermentation pathways of fumarate or malate degrading to propionate. Elemental Mg supplementation increased the relative abundance of the *Bacteroidales_BS11* group and family *Christensenellaceae*, which may contribute to the accumulation of succinate.

Formate formation and methanogenesis are other important H_2 sinks in the rumen. Elemental Mg supplementation did not alter formate concentration, indicating that elevated dH_2 concentration did not change ruminal formate metabolism. However, elemental Mg supplementation increased the dCH_4 concentration and CH_4 emissions, suggesting that elevated dH_2 enhances rumen methanogenesis. Similarly, previous studies also reported that H_2 gas infusion promotes CH_4 production in vitro (Hungate, 1967; Qiao et al., 2015) and in vivo (Olijhoek et al., 2016; Wang et al., 2017). In our study, 2 mol of Mg supplemented theoretically produces 0.5 mol of CH_4 , which accounted for about 15% of extra CH_4 production, so the extent of increase in CH_4 emissions observed (3.4 mol) was beyond our expectations. Elemental Mg supplementation increased population of methanogens, which could use more H_2 and compete more successfully with propionate producers for the H_2 . Increased methanogens may also use H_2 coming from other unknown sources, which needs further verification.

The methanogen community can be divided into 3 categories: hydrogenotrophic, methylotrophic, and acetoclastic methanogens. Genus *Methanobrevibacter* belongs to hydrogenotrophic methanogens and can reduce CO_2 coupled to the oxidation of H_2 to produce CH_4 . Kim (2012) reported that *Methanobrevibacter* are more competitively successful than other genera under high H_2 partial pressure in the rumen. This is not the case in our study. Although elevated dH_2 produced by

Table 5. Abundances (% assessed by amplicon sequences of 16S rRNA gene) of major bacteria in rumen fluid of dairy cows fed diets with Mg(OH)₂ or elemental Mg (n = 4)

Bacteria group ¹	Treatment			P-value		
	Mg(OH) ₂	Mg	SEM	Treatment	Time ²	Treatment × time
f_Prevotellaceae	27.1	24.9	4.37	0.63	0.42	0.96
g_Prevotella_1	23.2	22.2	3.71	0.79	0.43	0.99
g_Prevotellaceae_UCG-001	1.51	0.98	0.302	0.10	0.055	0.50
f_Ruminococcaceae	22.0	22.6	2.14	0.76	0.23	0.79
g_Ruminococcaceae	6.23	6.98	0.890	0.41	0.16	0.58
g_Ruminococcus_2	3.08	3.39	0.891	0.71	0.016	0.46
g_Ruminococcaceae_UCG-014	2.72	1.31	0.621	0.040	0.98	0.87
g_Saccharofermentans	2.16	1.92	0.397	0.56	0.30	0.43
g_Eubacterium	1.51	1.62	0.261	0.69	0.25	0.52
g_Ruminococcaceae_UCG-011	1.47	1.88	0.272	0.15	0.52	0.28
g_Ruminococcaceae_UCG-010	1.00	1.60	0.130	0.005	0.07	0.21
f_Lachnospiraceae	10.1	10.2	1.80	0.93	0.086	0.61
g_Butyrvibrio_2	1.86	1.73	0.538	0.82	0.46	0.09
g_Lachnospiraceae	1.85	1.64	0.428	0.62	0.06	0.26
f_Bacteroidales	6.80	7.20	2.102	0.84	0.13	0.41
g_Norank_f_Bacteroidales	6.80	7.20	2.102	0.84	0.13	0.41
f_Unknown_p_Saccharibacteria	3.83	2.95	0.705	0.23	0.46	0.11
g_Candidatus_Saccharimonas	3.83	2.95	0.705	0.23	0.46	0.11
f_Rikenellaceae	3.86	4.80	0.637	0.24	0.19	0.11
g_Rikenellaceae	3.23	4.40	0.560	0.16	0.22	0.12
f_Bacteroidales_BS11	2.89	4.95	0.420	0.032	0.14	0.12
g_Norank_f_Bacteroidales_BS11	2.89	4.95	0.420	0.032	0.14	0.12
f_Christensenellaceae	2.89	4.54	0.484	0.034	0.86	0.76
g_Christensenellaceae	2.89	4.54	0.484	0.034	0.86	0.76
f_Norank_c_WCHB1-41	2.68	1.97	0.461	0.15	0.86	0.057
g_Norank_c_WCHB1-41	2.68	1.97	0.461	0.15	0.86	0.057
f_Bifidobacteriaceae	2.28	0.88	0.419	0.051	0.053	0.75
g_Bifidobacterium	2.28	0.88	0.419	0.051	0.053	0.75
f_Acidaminococcaceae	1.68	2.00	0.796	0.68	0.85	0.55
g_Succiniclasticum	1.68	2.00	0.796	0.68	0.85	0.55
f_Norank_o_Mollicutes_RF9	1.47	0.98	0.186	0.024	0.49	0.15
g_Norank_o_Mollicutes_RF9	1.47	0.98	0.186	0.024	0.49	0.15
f_Bacteroidales	1.19	1.10	0.285	0.76	0.30	0.53
g_Norank_f_Bacteroidales	1.19	1.10	0.285	0.76	0.30	0.53
f_Fibrobacteraceae	1.43	1.05	0.316	0.25	0.91	0.79
g_Fibrobacter	1.43	1.05	0.316	0.25	0.91	0.79

¹f_ = family; g_ = genus.²Relative time after morning feeding.**Table 6.** Abundance (% assessed by amplicon sequences) and copies [log₁₀ transformed, assessed by quantitative real-time PCR (qPCR)] of 16S rRNA gene of major methanogen groups in the rumen fluids of dairy cows fed diets with Mg(OH)₂ or elemental Mg (n = 4)

Methanogen group ¹	Treatment			P-value		
	Mg(OH) ₂	Mg	SEM	Treatment	Time ²	Treatment × time
Relative abundance assessed by amplicon sequences, %						
o_Methanobacteriales	98.1	94.3	0.72	0.005	0.52	0.29
g_Methanobrevibacter	97.2	92.8	1.41	0.021	0.59	0.48
o_Methanomassiliicoccales	1.75	5.05	0.737	0.007	0.75	0.37
g_Candidatus_Methanomethylophilus	0.67	1.40	0.408	0.11	0.26	0.66
Copy numbers of 16S rRNA estimated by qPCR, log ₁₀ (copy/mL)						
Total methanogens	11.0	11.5	0.08	0.005	0.21	0.90
o_Methanobacteriales	11.0	11.5	0.06	0.001	<0.001	0.38
g_Methanobrevibacter	10.1	10.3	0.11	0.26	0.44	0.40

¹o_ = order; g_ = genus.²Relative time after morning feeding.

elemental Mg increased 16S rRNA gene copies of *Methanobrevibacter* by using qPCR analysis, it decreased the abundance of order *Methanobacteriales* (−3.48%). Furthermore, elemental Mg supplementation increased the abundance of order *Methanomassiliicoccales* (+188.6%), although the host animal was the major factor to separate methanogen communities. Members of the order *Methanomassiliicoccales* are methylotrophic methanogens and do not have the hydrogenotrophic parts of the methanogenesis pathway (Poulsen et al., 2013; Borrel et al., 2014; Li et al., 2016). It is surprising that elevated dH₂ caused by Mg supplementation favors the growth of order *Methanomassiliicoccales* when compared with *Methanobacteriales*. The metabolism of methyl groups needs the co-factor tetrahydrofolate, which is activated by metabolic hydrogen (Maden, 2000). We speculate that more folate might be activated by elevated dH₂ to carry methyl groups and generate more trimethylamine or methanol for the *Methanomassiliicoccales*. An increase in the abundance of *Methanomassiliicoccales* might explain the observed increase in CH₄ emissions.

In summary, elemental Mg supplementation causes a pulse of elevated dH₂ at 2.5 h after feeding, increases succinate concentration and CH₄ emission, decreases feed digestibility and VFA concentration, and alters the methanogen community in dairy cows. The pulse of dH₂ caused by elemental Mg inhibits rumen fermentation and feed digestion by decreasing carbohydrate-degrading bacteria, such as *Prevotellaceae_UCG-001*, *Ruminococcaceae_UCG-014*, and *Mollicutes_RF9* groups and genus *Bifidobacterium*. On the other hand, the pulse of dH₂ caused by elemental Mg promotes H₂ incorporation into succinate by increasing the abundance of family *Christensenellaceae* and *Bacteroidales_BS11* group enhances H₂ utilization for methanogenesis by favoring growth of methanogens.

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