British Journal of Nutrition (2017), **118**, 401–410
doi:10.1017/S0007114517002161

Molecular hydrogen generated by elemental magnesium supplementation alters rumen fermentation and microbiota in goats

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(Submitted 8 May 2017 – Final revision received 29 June 2017 – Accepted 24 July 2017 – First published online 20 September 2017)

Abstract

We tested the hypotheses that supplementation of a diet with elemental Mg increases ruminal dissolved H_2 (dH_2) in rumen fluid, which in turn alters rumen fermentation and microbial community in goats. In a randomised block design, twenty growing goats were allocated to two treatments fed the same basal diet with 1·45 % $Mg(OH)_2$ or 0·6 % elemental Mg. After 28 d of adaptation, we collected total faeces to measure total tract digestibility, rumen contents to analyse fermentation end products and microbial groups, and measured methane (CH_4) emission using respiration chambers. Ruminal Mg^{2+} concentration was similar in both treatments. Elemental Mg supplementation increased dH_2 at 2·5 h post morning feeding (+180 %, $P<0·001$). Elemental Mg supplementation decreased total volatile fatty acid concentration (−8·6 %, $P<0·001$), the acetate:propionate ratio (−11·8 %, $P<0·03$) and fungal copy numbers (−63·6 %, $P=0·006$), and increased propionate molar percentage (+11·6 %, $P<0·001$), methanogen copy numbers (+47·9 %, $P<0·001$), dissolved CH_4 (+35·6 %, $P<0·001$) and CH_4 emissions (+11·7 %, $P=0·03$), compared with $Mg(OH)_2$ supplementation. The bacterial community composition in both treatments was overall similar. Ruminal dH_2 was negatively correlated with acetate molar percentage and fungal copy numbers ($P<0·05$), and positively correlated with propionate molar percentage and methanogen copy numbers ($P<0·05$). In summary, elemental Mg supplementation increased ruminal dH_2 concentration, which inhibited rumen fermentation, enhanced methanogenesis and seemed to shift fermentation pathways from acetate to propionate, and altered microbiota by decreasing fungi and increasing methanogens.

Key words: Elemental magnesium: Fermentation pathways: Dissolved hydrogen: Methane: Rumen microbiota

Volatile fatty acids (VFA), carbon dioxide (CO_2) and molecular H (H_2) are produced during carbohydrate fermentation by bacteria, protozoa and fungi in the rumen. VFA serve as a major energy source for the host animal[(1)], with acetate and propionate being the main precursors of fat and glucose, respectively. The main consumers of H_2 are methanogenic Archaea, producing methane (CH_4) as an end product of fermentation, which keeps a low H_2 partial pressure in the rumen[(2)]. It is understood that the accumulation of H_2, which typically occurs when methanogenesis is inhibited, could hinder the re-oxidation of reduced electron carriers and adversely affect fermentation and fibre digestion[(3)].

Besides being used as a substrate for methanogenesis, H_2 is also involved in VFA production[(2,4)]. This is because different numbers of moles of H_2 (or of reducing equivalent pairs in reduced co-factors), are released or incorporated in the production pathway of each VFA. For example, fermentation of 1 mol of glucose to acetate releases 4 mol of electron pairs, most of which are transferred to protons to form H_2. In contrast, fermentation of glucose to propionate involves H_2 or metabolic H incorporation[(2)]. When animals are switched from fibrous to starchy diets, or rumen methanogenesis is inhibited, dissolved H_2 (dH_2) in the rumen elevates, and, as expected from the stoichiometry of acetate and propionate production, fermentation shifts from acetate to propionate[(5–7)].

However, when the effect of direct addition of H_2 as gas (gaseous H_2, gH_2) has been studied in *in vitro* batch cultures[(8–10)] and *in vivo*[(11)], the results observed in acetate and

Abbreviations: CH_4, methane; CO_2, carbon dioxide; dCH_4, dissolved CH_4; dCO_2, dissolved carbon dioxide; dH_2, dissolved H_2; gCO_2, gaseous CO2; gH_2, gaseous H_2; H_2, molecular hydrogen; OUT, operational taxonomic units; VFA, volatile fatty acid.

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propionate do not fully agree with that rationale. In batch cultures, Broudiscou *et al.*[12] found that the effect of an initial 0·5 or 1 kPa gH_2 headspace on acetate and propionate production depended on the inoculum used, and when using an inoculum adapted to a fibrous substrate, gH_2 addition unexpectedly increased acetate and decreased propionate. Similarly, Patra & Yu[8] found that including gH_2 in the initial culture headspace unexpectedly resulted in lower propionate molar percentage. Similarly, Qiao *et al.*[10] found that increasing gH_2 in the headspace of batch cultures actually increased acetate molar percentage and the acetate:propionate ratio, and decreased propionate molar percentage. Infusion of gH_2 in the rumen of dairy cows did not result in changes in total VFA concentration or VFA profile[11]. The effect of gH_2 on dH_2 concentration was not reported in those studies. Given that dH_2 and gH_2 have been shown not to be at equilibrium *in vitro*[13] and *in vivo*[14], and that the form of H_2 available to microbes is dH_2 rather than gH_2, we propose that the role of H_2 on fermentation needs further examination by studying the effect of dH_2 addition.

Our first hypothesis for this study using goats as the experimental ruminant model was that dH_2 could be increased through supplementing elemental Mg to the diet, as elemental Mg would react with water in rumen fluid releasing H_2:$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$[15]. Our second hypothesis was that increased dH_2 would alter rumen fermentation and the microbial community composition.

Methods

All animal procedures were approved by the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences (CAS), Changsha, China.

Goats and diets

A randomised block design with two treatments was used to investigate the effects of H_2 generated by the reaction of elemental Mg with water in the rumen fluid of goats. In all, twenty growing Xiangdong black male goats (mean initial body weight = 20·3 (SD 3·42) kg) were allocated to ten blocks according to body weight and CH_4 emission (g/kg DM intake) measured in a preliminary experiment (unpublished results). Two treatments were formulated with equal dietary Mg content: basal diet plus 1·45 % (DM basis) $Mg(OH)_2$ powder (99 % purity; Beijing Taizejiaye Technology Development Co., Ltd) for the control group and basal diet with 0·60 % (DM basis) elemental Mg powder (99 % purity; Beijing Taizejiaye Technology Development Co., Ltd) for the hypothesised elevated dH_2 group. In a preliminary experiment (unpublished results), these treatments had been shown to be harmless to the health of goats. Each block of animals contained two goats and each goat within a block was randomly assigned to one of the two dietary treatments.

Goats were kept in individual pens and had free access to fresh water. The diet was formulated to meet 1·4 times maintenance requirements of goats. The composition of the basal diet is shown in Table 1. Dietary forage and concentrate were not mixed, and both forage and concentrate were offered separately, each divided into two equal portions at 08.00 and 17.00 hours. Diets were offered for a 28-d adaptation period before conducting measurements. The elemental Mg and Mg $(OH)_2$ supplements were mixed with the concentrate fraction immediately before feeding to avoid the reaction with water in the environment, and the concentrate fraction containing the elemental Mg and $Mg(OH)_2$ supplements was eaten completely within approximately 1 h. During the initial 10 d of adaptation to diets, feed was offered *ad libitum* targeting 5 % refusals. The amount of feed allocated daily during the next 18 d of adaptation was adjusted to 100 % of the DM intake previously measured in order to minimise feed selection. The refusals, when present, were collected and analysed to determine the actual nutrient intakes.

Table 1. Ingredients and chemical composition of the basal diet offered to goats (g/kg DM)

Items	Diet
Dietary ingredient (g/kg DM)	
Forage	
Rice straw	300
Concentrate	
Soyabeans	54
Maize	304
Wheat bran	290
$CaCO_3$	5
Fat	11
NaCl	5
Urea	11
Premix*	20
Chemical composition (g/kg DM)	
DM	960
Organic matter	912
Ash	88·3
Crude protein	150
Starch	262
Neutral detergent fibre	312
Acid detergent fibre	186
Diethyl ether extract	35·0
Gross energy (MJ/kg)	18·2

* Premix was formulated to provide the following (per kg of premix): 400 g of $NaHCO_3$, 2 g of Fe, 1 g of Cu, 0·01 g of Co, 0·05 g of I, 6·6 g of Mn, 4·4 g of Zn, 0·003 g of Se, 333 mg of retinol, 5 mg of cholecalciferol, 838 mg of α-tocopherol.

Nutrient digestibility

Nutrient digestibility was determined over a 5-d period from days 29 to 33. Total faeces and urine were collected twice daily, weighed and mixed daily. A subsample (approximately 1 %) was frozen immediately at −20°C, and another subsample (approximately 1 %) was acidified using 10 % (w/w) H_2SO_4 to prevent N loss and then frozen immediately at −20°C. The faeces samples were later dried at 60°C for 48 h in a forced-air oven, and ground through a 1-mm screen. The acidified oven-dried samples were used for total N analysis, whereas non-acidified, oven-dried samples were used for other chemical analyses.

Rumen sampling

Collection of rumen contents was performed at 0, +2·5 and +6 h relative to the commencement of the morning feeding on days

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34 and 35. Rumen contents (300 ml) were collected by oral stomach tubing as described by Wang *et al.*[16], with the initial 100 ml discarded to avoid saliva contamination. The pH of rumen contents was measured immediately after sampling using a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd). Two subsamples of 35 ml each were immediately transferred into 50-ml plastic syringes for measuring dH_2 and dissolved CH_4 (dCH_4) concentration, as described by Wang *et al.*[13]. Two other 35-ml subsamples were immediately frozen at −80°C in liquid N_2 for DNA extraction and subsequent microbial analyses. In addition, 2-ml samples of rumen contents were collected and centrifuged at 15 000 ***g*** for 10 min at 4°C, and 1·5 ml of supernatant was acidified using 0·15 ml of 25 % (w/v) metaphosphoric acid, and stored at −20°C for subsequent measurement of VFA concentration. The remaining sample of rumen contents was stored at −20°C for the measurements of ammonium (NH_4^+), Mg^{2+} and glucose concentration.

Methane and carbon dioxide emissions

CH_4 and CO_2 emissions were measured in three plexiglass respiration chambers that permitted the goats to see each other, thereby minimising stress. CH_4 and CO_2 emissions were measured individually for each goat for 48 h using the protocol of Wang *et al.*[17] slightly modified. In brief, during seven periods of 2 d each, each block of animals containing two goats assigned to different treatments was randomly assigned to a chamber. One goat from each block was then randomly assigned to a measurement period, and the second goat from that block was placed in the same chamber in the subsequent period. Within the chamber, the goats were restrained with free access to a feed bin and drinking water. Airflow was maintained under negative pressure (flow rate = 35 m^3/h). Outlet gas from the chamber and ambient gas were connected to a multiport inlet unit of a gas analyser (GGA-30p; Los Gatos Research) for measuring CH_4 and CO_2 concentration. The cycling time to measure CH_4 and CO_2 concentration produced in the chamber was 30 min, with 8 min for analysing gases from each of the three chambers and 6 min for analysing background environmental concentrations of CH_4 and CO_2 in incoming air. Daily CH_4 and CO_2 emissions were calculated using net CH_4 and CO_2 concentrations and flow rate of air through each chamber at 30-min intervals, and differences between chambers were corrected using the methodology described by McGinn *et al.*[18]. The chambers were opened twice a day at 08.00 and 18.00 hours to deliver the diets. The chamber cleaning, such as swapping faeces and urine trays, took place during the morning before feeding.

Sample analysis

All samples of feeds, refusals and faeces were dried and ground to pass through a 1-mm sieve. Contents of DM (method 945.15), organic matter (OM) (method 942.05), crude protein (method 954.01) and diethyl ether extract (method 920.39) were determined according to published methodologies[19]. Gross energy was determined using an isothermal automatic calorimeter (5E-AC8018; Changsha Kaiyuan Instruments Co. Ltd). Contents of neutral detergent fibre (NDF) and acid detergent fibre were determined and expressed inclusive of residual ash[20], and NDF was assayed with the addition of a heat-stable amylase, but without sodium sulphite. The starch content was determined after pre-extraction with ethanol (80 %), and glucose released from starch by enzyme hydrolysis was measured using amyloglucosidase[21].

Frozen acidified rumen samples were thawed and centrifuged at 15 000 ***g*** for 10 min at 4°C, and individual VFA concentrations in the supernatant were measured using GC (Agilent 7890A; Agilent Inc.), according to the method described by Wang *et al.*[13]. The estimated net H_2 production relative to the amount of total VFA produced (R_{NH2}) was estimated according to the stoichiometric equation developed by Wang *et al.*[16], under the assumption of equal fractional rates of individual VFA absorption. Ammonia and glucose in the supernatant were determined colorimetrically according to the methods of Weatherburn[22] and Nelson[23], respectively. The concentration of Mg^{2+} in the supernatant was determined by Inductively Coupled Plasma-Optical Emission Spectrometers using Varian 720-ES series (Agilent Inc.).

Dissolved gases were also measured immediately after sampling using the procedure described by Wang *et al.*[16] with a slight modification. In brief, a 20-ml syringe containing 10 ml of N_2 gas was connected to a 50-ml plastic syringe containing 35-ml rumen content samples via polyurethane tubing. The N_2 gas was then injected into the 50-ml syringe, and the gases dissolved in the rumen fluid were extracted into the N_2 gas phase by vigorously hand shaking for 5 min. Gaseous H_2 and CH_4 (gCH_4) concentrations in the gas phase were measured by GC (Agilent 7890A). The dH_2 and dCH_4 concentrations in the original rumen fluid were calculated using equations described by Wang *et al.*[13]. Gaseous CO_2 (gCO_2) was calculated as the total dissolved gas extracted minus dCH_4 extracted, assuming that rumen gases would be composed of CO_2 and CH_4. Total dissolved CO_2 (dCO_2) concentration in the original rumen fluid (C_{TdCO_2}, mol/l) was calculated by combining equations from Wang *et al.*[16] and Hille *et al.*[24], and expressed as follows:

$$C_{TdCO_2} = C_{eTdCO_2} + V_g C_{gCO_2}/(22 \cdot 4 V_l)$$

$$C_{eTdCO_2} = \alpha_{CO_2} C_{gCO_2} 10^{(pH - pK_{CO_2})} + \alpha_{CO_2} C_{gCO_2}$$

$$C_{gCO_2} = (V_g - V_N - V_g C_{gCH_4})/V_g$$

$$\alpha_{CO_2} = \frac{100}{22 \cdot 4 \exp(-6 \cdot 8346 + 1 \cdot 2817(10^4/T) - 3 \cdot 7668(10^6/T^2) + 2 \cdot 997(10^8/T^3))}$$

where C_{eTdCO_2} is the total dCO_2 concentration (mol/l) in the rumen fluid at equilibrium after extraction; C_{gCO_2} the gCO_2 concentration (litres/l) measured in the gas phase of the 20-ml syringe at equilibrium after extraction of dissolved gases; C_{gCH_4} the gCH_4 concentration (litres/l) measured in the gas phase of the 20-ml syringe at equilibrium after extraction of dissolved gases; V_g the gas volume (ml) at equilibrium after extraction of dissolved gases; V_l the volume of liquid (ml); V_N the injected N_2 gas volume (10 ml); pK_{CO_2} the dissociation constant of bicarbonate and set to be 6·11[24]; α_{CO_2} the Bunsen absorption coefficient (mol/l·atm) for CO_2[25]; and T the temperature in K (273 + temperature in °C).

Estimation of Gibbs free-energy changes

Gibbs energy changes (ΔG) of fermentation reactions (online Supplementary Table S1) were estimated using measured concentrations of metabolites at 0, +2·5 and +6 h relative to the commencement of the morning feeding. Gibbs energy changes of reactions at standard conditions of 298 K were calculated from standard Gibbs energy of formation of reactants and products, and then adjusted to a rumen temperature of 312 K (39°C) using the van't Hoff equation[26]. Gibbs energy changes estimated for actual rumen conditions were subsequently adjusted using measured concentrations of soluble metabolites (10^{-pH}, glucose, acetate, propionate and butyrate) and dissolved gases (dH_2, dCO_2 and dCH_4)[14].

Quantitative real-time PCR analyses

Rumen samples (2 ml) at each of the three sampling time points were pooled and freeze-dried, and then physically disrupted using a bead beater for 1 min. Genomic DNA was extracted using the QIAamp DNA Stool Mini kit (Qiagen) according to the manufacturer's instructions. The quantity of DNA was measured based on absorbance at 260 and 280 nm using a NanoDrop ND 100 (Nano Drop Technologies). The absolute quantification of total bacteria, protozoa, fungi, methanogens and select bacterial species was measured by quantitative real-time PCR (qPCR) using primers validated in our laboratory (online Supplementary Table S2)[27]. Quantitative PCR was performed according to the procedures described by Jiao *et al.*[27]. Final absolute amounts of target groups or species were estimated by relating the C_T value to the standard curves and expressed as $\log_{10}$ copies/DM rumen contents. The abundances of six select rumen bacterial species (*Ruminococcus albus, R. flavefaciens, Fibrobacter succinogenes, Selenomonas ruminantium*, *Ruminobacter amylophilus* and *Prevotella ruminicola*) and of genus *Prevotella* spp. were measured using qPCR and species-specific 16S rRNA gene-targeted primers (online Supplementary Table S2) and expressed relative to the total bacterial DNA. The abundances of each bacterial species and of genus *Prevotella* spp. were determined using the ΔC_T method[28].

High-throughput sequencing and analysis

Extracted purified DNA (50 ng) from each rumen sample was subjected to PCR amplification of the V3–V4 region of 16S rRNA gene using universal bacterial primers 338 F (5'-ACTCCTACG GGAGGCAGCA-3') and 806 R (5'-GGACTACHVGGGTWT CTAAT-3')[29]. PCR was performed using a GeneAmp® 9700 thermal cycler (Applied Biosystems). The PCR products were purified using the AxyPrep™ DNA Gel Extraction Kit (Axygen Biosciences) according to the manufacturer's instructions and quantified using the QuantiFluor™-ST system (Promega). Purified PCR products were high-throughput-sequenced using an IlluminaMiSeq PE300 instrument at Majorbio Bio-Pharm Technology Co., Ltd, using protocols recommended by procedures of Miseq reagent kits v3 (Illumina). Sequences were quality filtered and demultiplexed using exact matches to the supplied DNA barcodes.

Bacterial phylotypes were identified using uclust[30] and assigned to operational taxonomic units (OTU) at 97 % sequence identity. Taxonomic identity of each phylotype was determined using the Greengenes database[31]. The resulting OTU were combined into an OTU table that represented abundance of each OTU in each microbial sample. Alpha diversity of bacterial communities was obtained using Mothur version 1.30.1. Similarity between bacterial communities was assessed using the Bray–Curtis distance metric and visualised using principal coordinates (PCoA) analysis.

Statistical analyses

The statistical model used included dietary treatment and block as fixed effects and sampling day as random effect. When sampling time was included, the model included dietary treatment and block as fixed effects, sampling time as a repeated-measure variable, and the interaction between dietary treatment and sampling time and sampling day as random effects. The best linear or log linear regression was derived between dH_2 and concentration of other rumen metabolites using ordinary least squares. Associations between response variables were studied through calculating their Pearson's correlation coefficient (r) and statistical significance. $P \leq 0{\cdot}05$ was considered significant, and $0{\cdot}05 < P \leq 0{\cdot}1$ was accepted as a tendency to significance. The SPSS 12.0 software was used for the statistical analyses.

Results

Although no differences were observed for feed intake and digestibility between both treatments, elemental Mg supplementation increased CH_4 emissions expressed as g/d (+11·7 %, $P = 0{\cdot}03$) and as g/kg OM intake (+9·87 %, $P = 0{\cdot}03$) (Table 2). No differences were observed in ruminal Mg^{2+} concentration and pH between the two treatments (Table 3). The pH was negatively correlated ($r - 0{\cdot}75$, $P < 0{\cdot}001$) with ruminal Mg^{2+} concentration (online Supplementary Fig. S1).

Elemental Mg supplementation increased the average dH_2 (+95·1 %, $P = 0{\cdot}02$) and dCH_4 concentrations (+39·0 %, $P < 0{\cdot}001$) (Table 3). Interactions between treatment and time were observed for dH_2 ($P = 0{\cdot}02$) and dCH_4 ($P < 0{\cdot}001$). Elemental Mg supplementation increased dH_2 only at +2·5 h (+180 %, $P < 0{\cdot}001$) but not at 0 or +6 h relative to the commencement of the morning feeding, and increased dCH_4 at +2·5 (+63·4 %, $P < 0{\cdot}001$) and +6 h (+50·5 %, $P < 0{\cdot}001$) but not at 0 h relative to the commencement of the morning feeding (Fig. 1). At +2·5 h, ruminal dH_2 was positively correlated with dCH_4 (r 0·50, $P = 0{\cdot}02$) (online Supplementary Fig. S2). Furthermore, ruminal dH_2 concentration varied widely among the 10 goats treated with elemental Mg supplementation, the highest dH_2 concentration being 17 mmol/l. When this highest dH_2 point was removed, the Pearson's correlation coefficient between dH_2 and dCH_4 was 0·486 ($P = 0{\cdot}03$, data not shown).

Elemental Mg supplementation decreased total VFA molar concentration (−11·6 %, $P < 0{\cdot}001$), acetate molar percentage (−3·80 %, $P < 0{\cdot}001$), the acetate to propionate ratio (−11·8 %, $P < 0{\cdot}03$) and R_{NH2} (−42·1 %, $P < 0{\cdot}03$), and increased propionate molar percentage (+11·6 %, $P < 0{\cdot}001$) (Table 3). Elemental

Mg supplementation slightly increased ΔG of glucose fermentation to acetate ($P=0{\cdot}03$) and to butyrate ($P=0{\cdot}04$), did not alter ΔG of glucose to 2/3 acetate + 4/3 propionate ($P=0{\cdot}36$) and tended ($P=0{\cdot}009$) to decrease ΔG of glucose + $2H_2$ to propionate at +2·5 h relative to the commencement of the morning feeding (Table 4).

Table 2. Feed intake and digestibility, methane (CH_4) and carbon dioxide (CO_2) emissions in growing goats fed diets with 1·45 % $Mg(OH)_2$ or 0·6 % elemental magnesium after 28 d of adaption (n 10)
(Mean values with their standard errors)

	$Mg(OH)_2$		Elemental Mg		
Items	Mean	SE	Mean	SE	P
Intake (g/d)					
DM	577	17·4	573	17·4	0·46
OM	524	16·2	522	15·9	0·73
CP	86·4	2·60	87·3	2·49	0·25
NDF	251	7·91	247	8·21	0·21
ADF	106	3·5	103	3·9	0·10
Starch	175	5·2	178	5·0	0·11
GE	7·67	0·229	7·81	0·219	0·10
Total tract apparent digestibility (g/kg)					
OM	683	15·3	689	9·2	0·78
CP	779	16·1	797	9·7	0·47
NDF	572	26·7	591	16·9	0·57
Starch	980	5·8	981	5·3	0·65
CH_4 emissions					
g/d	8·50	0·481	9·50	0·584	0·03
g/kg OM intake	16·2	0·85	17·8	0·71	0·03
g/kg OM digested	23·9	1·42	25·9	0·83	0·10
CO_2 emissions					
g/d	363	15·0	388	18·6	0·25

CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; GE, gross energy.

Because the hypothesised effect on dH_2 was observed only at +2·5 h relative to the commencement of the morning feeding, we focused on this time point for studying the effects of elemental Mg supplementation on the associations between concentration of dH_2 and other rumen metabolites. Ruminal dH_2 was negatively correlated with total VFA concentration (r −0·60, $P=0{\cdot}005$), acetate molar percentage (r −0·76, $P<0{\cdot}001$), acetate:propionate ratio (r −0·60, $P=0{\cdot}005$) and R_{NH2} (r −0·40, $P=0{\cdot}08$), and positively correlated with propionate molar percentage (r 0·47, $P=0{\cdot}03$) at +2·5 h relative to the commencement of the morning feeding (online Supplementary Fig. S3). When the greatest dH_2 data point was removed, only a trend (r −0·39, $P=0{\cdot}09$, data not shown) to a correlation between dH_2 and acetate molar percentage at +2·5 h was observed.

Elemental Mg supplementation decreased the copy numbers of fungi (−63·6 %, $P=0{\cdot}006$) and the relative abundance of *S. ruminantium* (−39·7 %, $P=0{\cdot}008$), and increased the copy numbers of methanogens (+47·9 %, $P<0{\cdot}001$) and relative abundance of *R. flavefaciens* (+71 %, $P=0{\cdot}02$) (online Supplementary Table S3). Ruminal +2·5 h dH_2 was negatively correlated with copy numbers of fungi (r −0·53, $P=0{\cdot}02$) and the relative abundance of *S. Ruminantium* (r −0·43, $P=0{\cdot}05$), and positively correlated with copy numbers of methanogens (r 0·54, $P=0{\cdot}01$) (Fig. 2). When the greatest dH_2 data point was removed, a significant correlation (r −0·54, $P=0{\cdot}01$, data not shown) between +2·5 h dH_2 and copy numbers of fungi was still observed.

Pyrosequencing of the 16S rRNA gene resulted in 38 730 (SD 3761) sequences per sample (range of 30 525–44 554 sequences per sample). Supplementation with elemental Mg increased community Ace ($P=0{\cdot}04$) and bias-corrected Chao 1 ($P=0{\cdot}04$) richness, but did not alter bacterial diversity of

Table 3. Concentration of rumen metabolites in growing goats fed diets with 1·45 % $Mg(OH)_2$ or 0·6 % elemental magnesium after 28 d of adaption (n 10)
(Mean values with their standard errors)

	$Mg(OH)_2$		Elemental Mg		P		
Items	Mean	SE	Mean	SE	Treatment	Time	Treatment × Time
Mg^{2+} (mmol/l)	5·71	0·332	5·29	0·314	0·16	<0·001	0·39
pH	6·66	0·027	6·64	0·034	0·31	<0·001	0·003
Glucose (mmol/l)	4·64	0·389	5·28	0·352	0·18	0·254	0·92
Dissolved gases							
dH_2 (μmol/l)	1·02	0·121	1·99	0·415	0·02	<0·001	0·02
dCH_4 (mmol/l)	1·05	0·045	1·46	0·058	<0·001	<0·001	<0·001
dCO_2 (mmol/l)	57·4	5·82	58·3	4·75	0·82	0·002	0·48
Rumen fermentation							
Ammonia (mmol/l)	7·54	0·829	8·23	0·994	0·17	0·02	0·13
Total VFA (mmol/l)	98·5	2·59	90·0	2·29	<0·001	<0·001	0·85
Acetate:Propionate	3·38	0·060	2·98	0·087	<0·001	0·003	0·97
Individual VFA (mol/100 mol total VFA)							
Acetate	63·1	0·62	60·7	0·77	<0·001	0·19	0·99
Propionate	18·9	0·25	21·1	0·43	<0·001	<0·001	0·81
Butyrate	11·9	0·61	11·8	0·62	0·91	0·69	0·99
Valerate	1·84	0·088	1·92	0·116	0·57	<0·001	0·65
Iso-butyrate	1·45	0·067	1·56	0·078	0·10	<0·001	0·29
Iso-valerate	2·88	0·119	3·00	0·157	0·39	<0·001	0·68
Estimated net H_2 production relative to the amount of total VFA produced (mol/mol)							
R_{NH2}	1·29	0·008	1·22	0·012	<0·001	<0·001	0·88

dH_2, dissolved H_2; dCH_4, dissolved methane; dCO_2, dissolved carbon dioxide; VFA, volatile fatty acids.

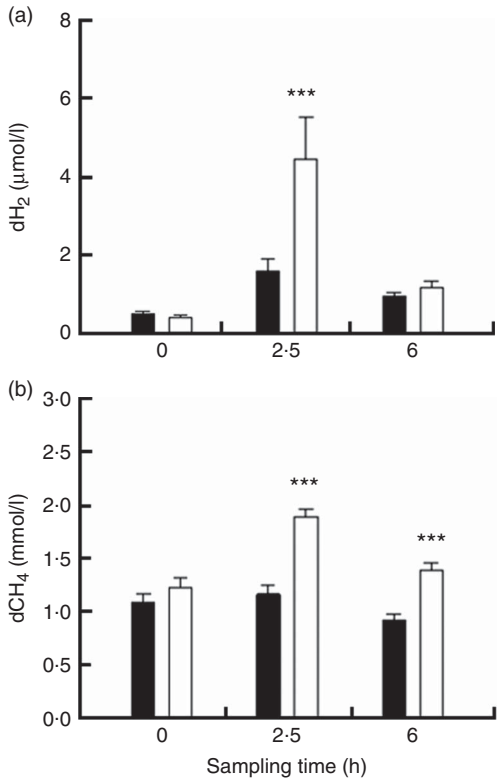

Fig. 1. Dissolved hydrogen (dH_2, a) and methane (dCH_4, b) in rumen contents at 0, 2·5 and 6 h after the commencement of the morning feeding in goats fed diets with 1·45 % $Mg(OH)_2$ (■) or 0·6 % elemental magnesium (□) after 28 d of adaption. Values are means (n 10), with their standard errors represented by vertical bars. *** $P < 0·001$.

Shannon and Simpson index (online Supplementary Table S4). The PC1 and PC2 explained 15·2 and 10·1 % of variation in bacterial OTU, respectively, and the score plot did not show a distinct clustering of goats supplemented with $Mg(OH)_2$ or elemental Mg (online Supplementary Fig. S4). Bacteroidetes and Firmicutes were the most abundant ruminal bacterial phyla, and did not differ between treatments (Table 5). Few differences were observed in the abundance of genera with >1 % abundance, which are shown in Table 5. Elemental Mg supplementation decreased the abundance of Bacteroidaceae ($P = 0·03$) and increased the abundance of Veillonellaceae ($P = 0·06$) and *Succiniclasticum* ($P = 0·009$) (Table 5).

Discussion

H_2 is produced during the reaction of elemental Mg with water: $Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2$. The $Mg(OH)_2$ so produced would be further hydrolysed in the rumen environment, in a reaction favoured by low pH: $Mg(OH)_2 + 2H^+ \rightarrow Mg^{2+} + 2H_2O$. Ruminal Mg^{2+} concentration was thus expected to be pH dependent, which agreed with the negative association observed between Mg^{2+} concentration and pH. At all time points, ruminal Mg^{2+} concentration was similar for both treatments of elemental Mg and $Mg(OH)_2$ supplementation, which suggests that rumen fluid passage rates to the lower tract were similar for both treatments. The reaction of elemental Mg with water resulting in H_2 formation might have been short-lived, or perhaps incomplete because of rumen outflow of elemental Mg, because dH_2 increased at +2·5 h, but not at +6 h, relative to the commencement of the morning feeding.

Table 4. Estimated Gibbs energy changes (kJ/reaction) of seven reaction pathways in the rumen of goats fed diets 1·45 % $Mg(OH)_2$ or 0·6 % elemental magnesium after 28 d of adaption (n 10)*
(Mean values with their standard errors)

	$Mg(OH)_2$		Elemental Mg		
Items	Mean	SE	Mean	SE	P
0 h relative to the commencement of morning feeding					
Glucose → 2 acetate + 4 H_2	−337	1·32	−341	2·14	0·10
Glucose → acetate + 1/2 butyrate + 3 H_2	−331	1·13	−334	1·5	0·11
Glucose → butyrate + 2 H_2	−324	1·05	−327	0·97	0·15
Glucose → acetate + propionate + H_2	−407	0·92	−410	1·41	0·02
Glucose → 2/3 acetate + 4/3 propionate	−338	1·00	−339	0·94	0·10
Glucose + 2 H_2 → 2 propionate	−350	1·68	−350	1·25	0·99
CO_2 + 4 H_2 → CH_4	−30·3	1·49	−27·7	1·79	0·24
+2·5 h relative to the commencement of morning feeding					
Glucose → 2 acetate + 4 H_2	−324	2·56	−316	3·96	0·03
Glucose → acetate + 1/2 butyrate + 3 H_2	−321	2·23	−314	3·24	0·03
Glucose → butyrate + 2 H_2	−319	1·93	−315	2·56	0·04
Glucose → acetate + propionate + H_2	−397	1·52	−393	2·29	0·07
Glucose → 2/3 acetate + 4/3 propionate	−334	0·67	−333	0·77	0·36
Glucose + 2 H_2 → 2 propionate	−351	1·24	−354	1·81	0·09
CO_2 + 4 H_2 → CH_4	−39·8	2·14	−46·4	3·89	0·05
+6 h relative to the commencement of morning feeding					
Glucose → 2 acetate + 4 H_2	−328	1·13	−329	2·25	0·67
Glucose → acetate + 1/2 butyrate + 3 H_2	−324	1·15	−325	1·93	0·67
Glucose → butyrate + 2 H_2	−321	1·22	−322	1·67	0·68
Glucose → acetate + propionate + H_2	−401	0·79	−402	1·18	0·50
Glucose → 2/3 acetate + 4/3 propionate	−335	0·76	−336	0·51	0·52
Glucose + 2 H_2 → 2 propionate	−350	1·23	−350	1·29	0·96
CO_2 + 4 H_2 → CH_4	−37·0	1·24	−36·0	2·27	0·70

* Water is omitted for simplicity.

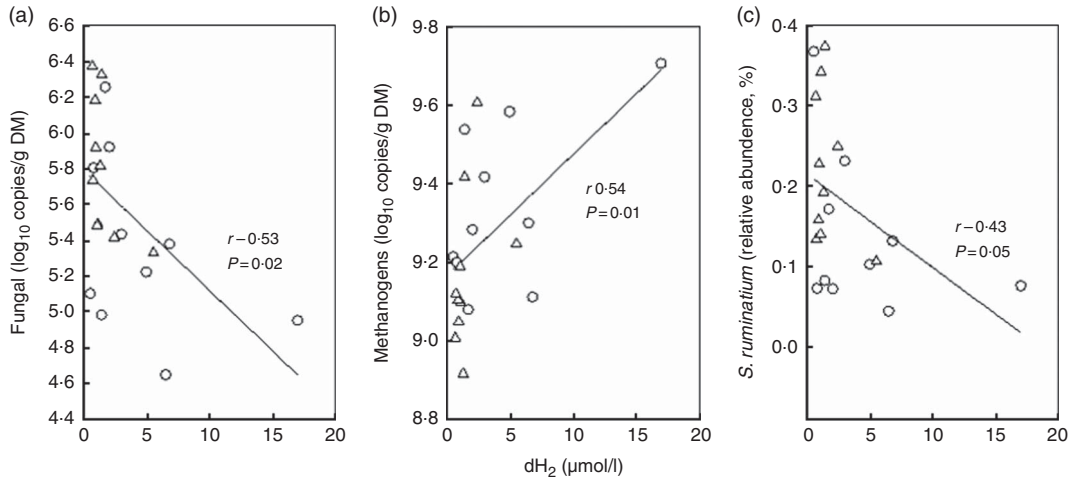

Fig. 2. Relationships between 2·5 h post-feeding dissolved hydrogen (dH_2) and select microorganisms (a, copy number of fungi; b, copy number of methanogens; c, relative abundance of *Selenomonas ruminantium*), as determined by RT-PCR, in goats (*n* 10) fed diets with 1·45 %$Mg(OH)_2$ or 0·6 % elemental Mg after 28 d of adaption. Each point represents one goat, with a total of 20 data points. △, Mg $(OH)_2$ treatment; ○, elemental magnesium treatment.

Table 5. Select bacterial phyla and genera, as determined by 16S rRNA pyrosequencing, in the rumen contents of growing goats fed diets with 1·45 % $Mg(OH)_2$ or 0·6 % elemental magnesium after 28 d of adaption (*n* 10)*
(Mean values with their standard errors)

		% of sequences				
		$Mg(OH)_2$		Elemental Mg		
Phyla	Genera	Mean	SE	Mean	SE	*P*
Bacteroidetes		63·8	2·96	62·1	2·09	0·61
	Bacteroidaceae†	0·871	0·139	0·532	0·109	0·03
Firmicutes		26·2	2·54	28·3	2·49	0·57
	Veillonellaceae‡	0·486	0·142	0·883	0·198	0·06
	Succiniclasticum	0·987	0·217	2·88	0·556	0·009

* Only the most abundant phyla and the genera that differed ($P < 0·10$) between treatments are shown.
† Unknown genera within family Bacteroidaceae.
‡ Unknown genera within family Veillonellaceae.

It is understood that ruminal H_2 accumulation hampers the oxidation of reduced electron carriers, such as NADH, to form H_2, leading to impaired glycolysis, decreased microbial ATP generation and fibre degradation in the rumen[32]. However, the rumen microbial ecosystem might exhibit the capability to cope with elevated H_2. Previous *in vivo* studies indicated that H_2 accumulation under methanogenesis inhibition did not affect feed intake, total tract digestibility and meat or milk production in ruminants[7,33,34]. In the current study, we also did not find differences in feed intake and digestibility in goats fed diets supplemented with elemental Mg and $Mg(OH)_2$, but elevated dH_2 resulting from elemental Mg supplementation decreased total VFA concentration in the rumen, which suggests that fermentation might have been impaired by elevated rumen dH_2. At first sight, it is difficult to reconcile the lack of effect of elevated dH_2 on digestibility with its negative effects on total VFA concentration. However, both measurements are proxies of digestion and fermentation events. We measured apparent overall tract digestibility, but any changes in microbial biomass in faeces could have masked differences in true overall digestibility. Similarly, we measured VFA concentration in the rumen, but we did not measure actual fluxes of VFA production. A more complete understanding of the effects of elevated dH_2 would require a more in-depth study of the dynamics of digestion, microbial biomass production and fluxes of VFA production in the rumen.

The rumen VFA profile varies widely depending on the types of carbohydrates fermented. Diets rich in readily degraded starch generally stimulate propionate production, whereas on the other hand cellulose is fermented preferentially to acetate[35]. These shifts of fermentation pathways are associated with the concentration of ruminal dH_2[5]. Lower dH_2 facilitates H_2 generation through acetate production, whereas greater dH_2 facilitates the disposition of electrons derived from fermentation into propionate production[2]. In this study, we confirmed rumen dH_2 as a direct effector of the shift of glucose fermentation pathways, as elevation of dH_2 caused by elemental Mg supplementation decreased acetate molar percentage and increased propionate molar percentage. This study allowed isolating the effects of elevated dH_2 on fermentation from other effects caused by changes in the diet.

Elevated dH_2 facilitated electron-incorporating glucose fermentation pathways, which agreed with the negative correlation between dH_2 and R_{NH2}. The shifts in fermentation pathways caused by elevated dH_2 resulting from elemental Mg

supplementation also agreed with the effects of elemental Mg supplementation on ΔG of the main fermentation pathways. The ΔG of glucose fermentation to acetate and H_2 was increased, and to propionate was decreased, by elemental Mg supplementation, along with the observed elevated dH_2. These results were in agreement with our previous study in dairy cows fed four types of carbohydrates[5]. The magnitude of the effects of elemental Mg supplementation on ΔG of most rumen pathways at +2·5 h relative to the commencement of the morning feeding were rather small, which suggested that stimulation of pathways incorporating H_2 might have taken place through more favourable kinetics of H_2 uptake rather than through thermodynamic changes.

Changes in the composition of the rumen microbial community when gH_2 accumulates as a consequence of methanogenesis inhibition have been demonstrated[6,7,36]. In dairy cows fed four types of carbohydrates, greater dH_2 was associated with less acetate and lower number of H_2 producers such as anaerobic fungi[5]. In that study, however, the concentration of dH_2 was a consequence of the type of carbohydrate supplemented, and therefore changes in variables other than dH_2 resulting from the dietary changes could have also influenced the abundance of microbial groups. To our knowledge, the present study was the first investigation on how elevated dH_2 resulting from a non-fermentative source affected rumen microbiota, and we found that elevated dH_2 generated by elemental Mg supplementation greatly decreased (–63·6 %) the copy numbers of fungi. Decreased fungal numbers did not adversely affect fibre degradation, which might indicate that other fibrolytic organisms, such as *R. flavefaciens*, might have compensated for the negative effects of elemental Mg on fungi. *R. flavefaciens* produces H_2, but also succinate as a major product[37,38], and it would be possible that *R. Flavefaciens* shifted fermentation towards succinate production under elevated H_2 resulting from elemental Mg supplementation. That said, the relative abundance of *R. flavefaciens* was small, which casts doubt on the influence it might have had on fibre digestion.

The impact of increased dH_2 generated by supplementation with elemental Mg on the rumen bacterial community composition was further analysed using high-throughput sequencing. Supplementation with elemental Mg increased species richness in terms of Ace and Chao 1 diversity, although total copy numbers of bacteria were similar for both treatments. However, the PCoA score plot indicated that ruminal bacterial community was not distinctly different between both treatments. In a previous study, elevated H_2 was associated with increased Bacteroidetes: Firmicutes ratio when methanogenesis was inhibited by chloroform–cyclodextrin[39]. However, in the present study, elemental Mg supplementation decreased the abundance of family Bacteroidaceae, but did not alter the abundance of Bacteroidetes and Firmicutes. An interesting result was that genus *Succiniclasticum*, which belongs to family Firmicutes and is involved in converting succinate to propionate[40], was 2-fold more abundant in goats that received elemental Mg supplementation. This result was in agreement with the increased propionate molar percentage in the rumen when dH_2 concentration was increased by elemental Mg supplementation. In contrast, an unexpected observation was that elemental Mg supplementation and elevated dH_2 decreased the abundance of *S. ruminantium*, an important propionate-producing bacterium in the rumen[41]. The important increase in *Succiniclasticum* abundance might have compensated for the decreased abundance of *S. ruminantium* to produce propionate in this study, if in goats supplemented elemental Mg succinate conversion to propionate limited propionate production.

H_2 and CO_2 are the main substrates for methanogenesis in the rumen, and the majority of H_2 produced from carbohydrate fermentation is used in CH_4 production[42]. In our study, we found that elemental Mg supplementation increased dCH_4 concentration, the copy number of methanogens and CH_4 emissions, indicating that increased dH_2 generated by elemental Mg supplementation promoted CH_4 production in the rumen of goats. Olijhoek *et al.*[11] also reported that short-term infusion of H_2 into the rumen increased CH_4 production. Furthermore, we estimated that elemental Mg supplementation decreased ΔG of methanogenesis at +2·5 h after the commencement of the morning feeding, indicating that elevated dH_2 would have thermodynamically favoured growth and metabolism of methanogens at that time point. Dissolved H_2 concentration was positively correlated with copy numbers of methanogens, but not with CH_4 emissions. This suggests that ruminal dH_2 might have exceeded the capacity of methanogens to take up H_2 in the treatment with elemental Mg supplementation, so that methanogenic population and activity might have been limiting for CH_4 generation in the rumen.

In summary, goats adapted to elevated ruminal dH_2 generated by elemental Mg supplementation had lower rumen total VFA concentration, copy number of fungi and greater propionate molar percentage and CH_4 emissions when compared with goats fed the control diet supplemented with $Mg(OH)_2$. Elevation of ruminal dH_2 concentration seemed to inhibit rumen fermentation and altered fermentation pathways and some microbial groups, without affecting total tract digestibility. Effects of extra H_2 obtained by supplementing elemental Mg were short-lived, because elevated dH_2 had returned to control concentration by +6 h relative to the commencement of the morning feeding. Furthermore, the extent of elevated ruminal dH_2 concentration caused by elemental Mg supplementation varied widely among the ten goats, and the goat with the greatest dH_2 concentration (17 mmol/l) also had the greatest methanogen population and the lowest VFA concentration, acetate molar percentage, acetate:propionate ratio and copy number of fungi. This study provides insights on the effects of elevated dH_2 on fermentation in the rumen and methanogenesis, although high dietary Mg concentration in both treatments might have altered the availability of other minerals and exerted some postabsorptive effects in goats. More studies with sustained and consistent elevated dH_2 are needed to further elucidate how elevated dH_2 concentration affects the rumen ecosystem, fermentation and digestion.

Acknowledgements

This work was supported by National Natural Science Foundation of China (grant nos 31561143009, 31320103917 and 31472133), National key research and development program of China (grant no. 2016YFD0500504), Youth Innovation Promotion Association

CAS (2016327), China Agriculture Research System (grant no. CARS-37) and Hunan province science and technology plan (grant no. 2015WK3043).

M. W., R. W., H. X. M., D. L. L. and X. M. Z. conducted the research; M. W., R. W. and E. M. U. analysed the data; and M. W., E. M. U., K. A. B and Z. L. T. wrote the paper. M. W. and Z. L. T. designed the research and had primary responsibility for the final content. All authors read and approved the final manuscript.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114517002161

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