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Short communication

Carbon-13 stable isotope analysis reveals the existence but insignificance of ruminal methanogenic pathway from acetate in a batch culture system

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ABSTRACT

A series of *in vitro* batch culture incubations were conducted to investigate the extent of carboxyl group and methyl group in acetate converted to CH_4 using a stable isotopic tracer method. Ground lucerne hay was incubated as a substrate with buffered ruminal fluid, and ^{13}C labeled $CH_3{}^{13}COONa$ or $^{13}CH_3COONa$ was supplied into the incubation bottles at a dose of 0, 15, 35 or 55 mM. Increasing $CH_3{}^{13}COONa$ or $^{13}CH_3COONa$ supplementation linearly increased ($P < 0.05$) absolute $^{13}CH_4$ and $^{13}CO_2$ production (mL/bottle) at all the time points measured. Yet, the conversion rates (CR) of $^{13}CH_4$ and $^{13}CO_2$ produced from labeled $CH_3{}^{13}COONa$ or $^{13}CH_3COONa$ were very low. For instance, the relative $^{13}CH_4$ CR (%) was ranged from 0.0028-0.14% with incubation time extended to 48 h, and the relative $^{13}CO_2$ CR (%) was ranged from 0.054 to 1.3%. Moreover, acetate supplementation linearly increased ($P < 0.01$) the concentration of butyrate, 16S rRNA gene copies of protozoa, total methanogens, *Methanosphaera stadtmanae*, *Methanobrevibacter smithii* and *Methanosarcina barkeri*. In summary, our study indicates existence of methanogenesis from acetate, but such pathway of methanogenesis makes little contribution to the total CH_4 production through *in vitro* ruminal fermentation.

1. Introduction

Methane is a potent greenhouse gas linked with ruminant production (Henry and Eckard, 2009). Methane produced by ruminants accounts for a quarter of all anthropogenic CH_4 emissions (Patra, 2014), contributing significantly to anthropogenic greenhouse gas emissions in agricultural section. In addition, enteric CH_4 production also represents the energy not used by the host animal, and accounts 2–12% of gross dietary energy through eructation (Johnson and Ward, 1996). Gaining more insight into the methanogenesis in the rumen can be critical for developing strategies to mitigate enteric CH_4 emissions and increase dietary energy use efficiency of host animals (Llonch et al., 2017).

Abbreviations: CR, conversion rate; -CH_3, methyl group; -COOH, carboxyl group; *M. barkeri*, *Methanosarcina barkeri*; *M. stadtmanae*, *Methanosphaera stadtmanae*; *M. ruminantium*, *Methanobrevibacter ruminantium*; *M. smithii*, *Methanobrevibacter smithii*; VFA, volatile fatty acids

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Acetic acid is a major ruminal volatile fatty acid and accounts for more than 50% of the total volatile fatty acids (VFA) produced in the rumen. Acetate is recognized as an important substrate for methanogens, which refers to as acetoclastic methanogenesis (Weiland, 2010). Acetate contains two carbons, which are one from carboxyl group ($-COOH$) and the other one from methyl group ($-CH_3$). Oppermann et al. (1961) have reported that carboxyl and methyl group of acetate make a little contribution to ruminal methanogenesis *in vivo* by using 14C labeled sodium acetate. However, ruminal methanogenesis from acetate may be underestimated *in vivo*, because a portion of the added 14C sodium acetate can be absorbed by ruminal epithelium and flow to lower tract through ruminal passage.

The advantage of *in vitro* technique is that rumen fermentation will not be affected by ruminal absorption and passage rate and very useful to estimate the total production in comparison to *in vivo* technique. The objectives of this study were to investigate the extents of carboxyl and methyl group in acetate being converted to CH_4 by rumen microorganisms in batch culture system using a stable isotopic tracer method and to characterize the changes of *in vitro* gas production, fermentation parameters and ruminal methanogenic populations in response to increasing acetate supplementation.

2. Materials and methods

2.1. In vitro batch culture

Carboxyl labeled sodium acetate ($CH_3{}^{13}COONa$, Catalog 279285, Sigma-Aldrich Shanghai Trading Co Ltd., Shanghai, China) and methyl labeled $^{13}CH_3COONa$ (Catalog 279315, Sigma-Aldrich Shanghai Trading Co Ltd.) were employed for *in vitro* ruminal fermentation. Four ruminally-fistulated Liuyang black male goats (19.8 ± 3.0 kg) fed a mixed ration (containing 650 g maize stover and 350 g concentrates per kilogram, dry matter basis) were used for collecting ruminal fluid. The procedures of *in vitro* batch culture were followed as described in study of He et al. (2018). Approximately 0.4 g of ground lucerne hay (908 g organic matter, 165 g crude protein, 433 g neutral detergent fiber and 290 g acid detergent fiber per kg; dry matter basis) was weighed into a 150 mL bottle on the day before incubation. Ruminal fluids were collected from various locations within the rumen before morning feeding (0800 h). After filtration through a four-layer cheesecloth, the rumen inoculum was mixed (1 : 9) with buffer solutions (292 mg K_2HPO_4, 240 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4{\cdot}7H_2O$, 64 mg $CaCl_2{\cdot}2H_2O$, 4 mg Na_2CO_3 and 600 mg cysteine hydrochloride per liter) in an anaerobic environment filled with N_2. Buffered ruminal fluid (40 mL) was then added to each bottle under N_2 flushing, then 1-^{13}C (carboxyl) labeled $CH_3{}^{13}COONa$ or 2-^{13}C (methyl) labeled $^{13}CH_3COONa$ was added into bottle to achieve a dose of 0, 15, 35 or 55 mM CH_3COONa in the buffered rumen fluid. The treatment without supplementing 13C labeled CH_3COONa (*i.e.*, dose 0) was used to as a control to correct the natural 13C abundance (Hao et al., 2015). After completion of dispensing the treatments and fluid, each bottle was then capped with a rubber stopper, and placed in an incubator at 39 °C for 6, 12, 24 or 48 h. Each run contained triplicates for each treatment and was conducted with pooled rumen fluids from 2 of 4 donor goats. The incubation runs were repeated 3 times with each on different days.

2.2. Sample collection

After incubation for 6, 12, 24 and 48 h, triplicates of gas samples (10 mL each) collected in graduated syringes were transferred to 10 mL pre-evacuated vials to measure gas composition, ^{13}C isotopic abundance in total CO_2 and ^{13}C isotopic abundance in total CH_4. Two milliliter of filtered fluids were collected from each bottle and centrifuged at 15,000 *g* for 10 min at 4 °C, and then 1.5 mL of supernatants were transferred to 2-mL tubes and acidified with 0.15 mL of 25% (w/v) HPO_3. The acidified samples were stored at −20 °C for measuring VFA concentration. Another 5 ml of fermented fluid samples were frozen in liquid N_2 and stored at −80 °C for measuring populations of microbial markers.

2.3. Gas calculation and analyses

After incubation for 6, 12, 24 and 48 h, headspace gas pressures were measured by a pressure transducer (CYG130-12, Kunshan Shuangqiao Sensor Measurement Controlling Co., Ltd., Kunshan, Jiangsu, China) through the stopper. Gas composition of the collected gas samples was determined using a gas chromatograph (Agilent 7890 A; Agilent Technologies, Palo Alto, CA, USA) with a packed column in nickel tubing (2.4 m × 0.2 mm i.d., Hayesep Q packing, 80–100 mesh, Agilent Technologies). Gas was flushed through a sampling loop of the gas chromatograph, which was fitted with a Flame Ionisation Detector (column temperature = 60 °C, injector and detector temperature = 280 °C). The carrier gas (N_2) flow rate was 21 mL/min. Volumes of CH_4 and CO_2 were calculated according to the equation proposed by Wang et al. (2013).

The values of ^{13}C isotopic abundance (expressed as mol^{13}C/[mol^{13}C + mol ^{12}C]) in total CO_2 were determined by a continuous flow technique with a Finnigan™MAT-253 stable isotope ratio mass spectrometry (IRMS; Thermo Scientific, Ringoes, NJ, USA). The gas was directly transferred to a gas chromatography with a Pora plot column (25 m × 0.32 mm i.d.) at 25 °C under 2×10^5 Pa to separate CO_2. The separated CO_2 was eventually eluted into an IRMS for ^{13}C isotopic abundance determination. For the values of ^{13}C isotopic abundance in total CH_4, the gas samples were firstly treated with a gas chromatography attached automated interface (PreCon, ThermoFinnigan, Ringoes, NJ, USA) for pre-gas chromatography concentration. The pre-gas chromatography concentration including a chemical trap filled with Mg $(ClO_4)_2$ and NaOH was primarily used to remove the CO_2 from total gas (Zhang et al., 2012). Then, the concentrated CH_4 was oxidized into CO_2 in a combustion furnace at 1000 °C and transferred to a gas chromatography for CO_2 separation. Lastly, the ^{13}C isotopic abundance in the separated CO_2 was determined by a Finnigan™MAT-253 IRMS. The $^{13}CH_4$