Effects of Starch Content on Hydrogen and Methane Productions, Rumen Fermentation and Microbial Protein Synthesis During in Vitro Ruminal Culture

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Research

Keywords: Starch, Ruminal fermentation, Hydrogen, Methane, Microbial protein

Posted Date: August 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-808694/v1

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Abstract

**Background:** Starch has faster rate of rumen fermentation than fiber, and always causes a rapid increase in ruminal molecular hydrogen (H\(_2\)) partial pressure and microbial protein synthesis, which may promote other H\(_2\) sinks to compete H\(_2\) from methanogenesis. The study was designed to investigate the effects of increasing starch content on methane (CH\(_4\)), hydrogen gas (gH\(_2\)) production, rumen fermentation, metabolic hydrogen ([H]) production, microbial protein (MCP) synthesis through *in vitro* ruminal batch incubation.

**Methods:** Seven different treatments was prepared by replacing corn straw with corn grain, and starch content were 72, 185, 297, 410, 525, 634 and 747 g/kg DM.

**Results:** Elevating starch content increased DM degradation (*P*\(_{\text{linear}}\) < 0.001), and decreased the CH\(_4\) (*P*\(_{\text{linear}}\) and *P*\(_{\text{quadratic}}\) < 0.001) and gH\(_2\) (*P*\(_{\text{linear}}\) < 0.001) productions relative to DM degraded. Elevating starch content increased VFA concentration (*P*\(_{\text{linear}}\) < 0.001), propionate molar percentage (*P*\(_{\text{linear}}\) < 0.001; *P*\(_{\text{quadratic}}\) = 0.001) and MCP concentration (*P*\(_{\text{linear}}\) and *P*\(_{\text{quadratic}}\) < 0.001), and decreased acetate molar percentage (*P*\(_{\text{linear}}\) < 0.001), acetate to propionate ratio (*P*\(_{\text{linear}}\) < 0.001) and estimated net [H] production relative to DM degraded (*P*\(_{\text{linear}}\) < 0.001). Elevating starch content decreased molar percentage of [H] utilized for CH\(_4\) (*P*\(_{\text{quadratic}}\) = 0.003) and gH\(_2\) (*P*\(_{\text{linear}}\) < 0.001) production.

**Conclusion:** Increasing starch content alters rumen fermentation pathway from acetate to propionate production with reduction in efficiency of [H] production, promotes H\(_2\) utilization with enhanced MCP synthesis and leads to the reduction in efficiency of CH\(_4\) and gH\(_2\) production.

**Background**

Methane (CH\(_4\)) is an important greenhouse gas, which receives great attention worldwide for its impact on global climatic change [1]. Ruminant CH\(_4\) emissions accounts for approximately 17% of the global CH\(_4\) emissions [2]. Furthermore, CH\(_4\) emission is also an important energy loss which represents 2–12% of dietary gross energy and strongly associated with efficiency of ruminants production [3, 4]. Thus, CH\(_4\) mitigation is beneficial to the environment and animal performance.

Molecular hydrogen (H\(_2\)) is the precursor of ruminal methanogenesis and mainly produced during the fermentation of carbohydrate to volatile fatty acid (VFA) [5]. Comparing with forage fiber, starch has faster rate of rumen fermentation and ATP production [6] and is always accompanied with a rapid increase in ruminal H\(_2\) partial pressure [7]. Other H\(_2\) sinks, such as reductive acetogenesis, biohydrogenation, propionate production and microbial protein (MCP) synthesis [8], can be promoted and serve as H\(_2\) competitors in the rumen, when ruminal H\(_2\) partial pressure is increased [9]. Studies have investigated relationship between fermentation pathways and methanogenesis [7, 10]. However, few studies have conducted to investigate contribution of H\(_2\) utilization by methanogenesis on the metabolic hydrogen ([H]) generated by VFA production, when different types of carbohydrates was fermented.
We hypothesized that increasing starch content could decrease the contribution of H₂ disposal by methanogenesis, so that fermentation pathway might not be only cause of the decreased CH₄ production in starchy diet. *In vitro* ruminal batch culture was employed, as it is effective method to measure the actual net fermentation products. Increasing starch content was then achieved by replacing corn straw with corn grain. We measured the kinetics of total gas, CH₄ and H₂ gas (gH₂) production, fermentation end products, estimated net [H] production and MCP after 48-h *in vitro* ruminal fermentation.

**Materials And Methods**

The experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

**Experimental design**

The corn grain and corn straw (Table 1) were employed to generate different starch contents for the incubated substrates. Seven treatments were ratios of corn grain to corn straw being 6:0, 5:1, 4:2, 3:3, 2:4, 1:5 and 0:6, and grounded to pass 1-mm aperture sieve. The starch content of seven treatments were 72, 185, 297, 410, 525, 634 and 747 g/kg DM.

| Item                  | Corn grain | Corn straw |
|-----------------------|------------|------------|
| Organic matter        | 985        | 915        |
| Crude protein         | 76.4       | 38.7       |
| Neutral detergent fiber| 132       | 792        |
| Acid detergent fiber  | 34.5       | 482        |
| Starch                | 747        | 72.4       |
| Gross energy (MJ/kg DM)| 16.5      | 14.8       |

**In vitro ruminal batch incubation**

Rumen fluid was collected from two of three Xiangdong black goats with permanent rumen cannula before morning feeding. Goats were fed a total mixed diet containing corn straw and concentrated mixture (1:1) containing CP 137 g kg⁻¹ of DM and NDF 380 g kg⁻¹ of DM, respectively. The rumen fluid was filtered through six layers of cheesecloth into a pre-warmed insulated bottle and taken to the laboratory.

About 0.6 g of substrate was accurately weighed into a 135-ml fermentation bottle. Then buffered rumen fluid containing 12-ml rumen fluid and 48-ml McDougall’s buffer [11] were added into bottle under a stream of CO₂ at 39.5 °C. Bottles were immediately placed into the automatic incubation system described by Wang et al. [12], with venting pressure set at 10.0 kPa. As the incubation bottle was in line with gas chromatograph
(GC, Agilent 7890 A, Agilent Inc., Palo Alto, California, USA) via a computer-controlled three way solenoid valve, the released gas was automatically vented into a GC for measuring CH₄ and hydrogen gas concentrations. Gas production (GP), CH₄ and gH₂ accumulations were calculated using the equation equation described by Wang et al. [13].

In vitro ruminal fermentation was stopped at 48 h. About 2 mL of liquid without visible particles were collected from each bottle and centrifuged at 15,000 g for 10 min at 4°C. The supernatant (1.5 mL) was acidified using 0.15 mL of 25% (w/v) metaphosphoric acid, and stored at -20°C for analysis of VFA and ammonia. The pH was measured immediately with a portable pH meter (Starter 300; Ohaus Instruments Co. Ltd., Shanghai, China). About 8 ml of samples were collected for measuring microbial protein after intense shaking of the bottle to ensure that representative portions of liquid and particle fractions. Solid residues were filtered into pre-weighed Gooch filter crucibles, dried at 105°C to constant weight and weighed to determine degradation of incubated substrates.

Each run had four replicates for each treatment. Two bottles were used for measuring pH and DM degradation, and the other two bottles were used for obtaining samples for measuring fermentation end-product and microbial protein. Each run was repeated three times, each on different days, so that each treatment was conducted in triplicate.

Sample analyses

The dry matter (DM) content was determined by drying at 105°C for 24 hours in an oven, and the organic matter (OM) content was determined by ashing at 550°C for 12 hours in a muffle furnace. Gross energy (GE) was measured using an isothermal automatic calorimeter (5EAC8018; Changsha Kaiyuan Instruments Co. Ltd, Changsha, China). The contents of crude protein (CP) (N × 6.25) in feed samples were determined according to procedures of AOAC [14]. The contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) in feed samples were determined according to the methods described by Van Soest et al. [15] and expressed as inclusive of ash. Heat stable α-amylase was added to for NDF analysis.

Volatile fatty acid concentration was measured according to the procedure described by Wang et al. [16], using a GC (Agilent 7890 A, Agilent Inc., Palo Alto, California, USA). Ammonia concentration was measured colorimetrically according to Chaney and Marbach [17]. Rumen microorganisms was separated from feed particles according to Makkar et al. [18] with filtration (four layers of gauze), shaking (125 rpm/min for 1h) and centrifugation (150 × g for 10 min), and microbial nitrogen production was measured colorimetrically according to Bradford [19], Using a Coomassie brilliant blue kit (Build a biopharmaceutical research institute, Nanjing, China).

Calculations and statistical analysis

The logistic-exponential model [20] was employed to analyze the kinetics of total gas and CH₄ production by using the Nonlinear Regression Analysis Program (NLREG, version 5.4) [21], and was expressed as follows:
\[ GP_t = VF \frac{1 - \exp(-kt)}{1 + \exp(b-kt)} \]

Where \( GP_t \) is the accumulated gas or CH\(_4\) production at time \( t \) (ml/g); \( VF \) is the final asymptotic gas or CH\(_4\) production (ml/g); \( k \) is the fractional rate of gas or CH\(_4\) production (/h); \( b \) is the shape parameter.

The kinetics of gH\(_2\) production \( (V_{H2}) \) was analyzed using the equations provided by Wang et al. [22], which was expressed as follows:

\[ V_{H2t} = \frac{VF_{H2}\left\{1 - \exp\left[ -k_{H2}\left(t - lag_{H2}\right) \right]\right\} \left\{1 + c_{H2}\exp\left[ -\mu_{H2}\left(t - lag_{H2}\right) \right]\right\}}{1 + \exp\left[ b_{H2} - k_{H2}\left(t - lag_{H2}\right) \right]} \]

Where \( V_{H2t} \) is the accumulated H\(_2\) gas production at time \( t \) (ml/g); \( VF_{H2} \) is the final asymptotic H\(_2\) gas volume (mL/g), \( b_{H2} \) and \( c_{H2} \) are shape parameters of H\(_2\) gas curve without dimension, \( k_{H2} \) is the fractional rate of H\(_2\) gas formation (/h), \( \mu_{H2} \) is the fractional rate of H\(_2\) gas re-resolution (/h), and \( lag_{H2} \) is discrete lag time (h).

The stoichiometric equations developed by Wang et al. [16] was used to calculate the net [H] production \( (P_{NH2}, \text{mM}) \) and estimated [H] production relative to the amount of total VFA produced \( (R_{NH2}, \text{mol}/100 \text{ mol of VFA}) \), which was expressed as follows:

\[ P_{NH2} = 2(\text{Ace} + \text{But} + \text{Isobut}) - (\text{Pro} + \text{Val} + \text{Isoval}) \]

\[ R_{NH2} = 100 \frac{P_{NH2}}{\text{Ace} + \text{But} + \text{Isobut} + \text{Pro} + \text{Val} + \text{Isoval}} \]

where ace, but, pro, val, isobut and isoval were concentration (mM) of acetate, propionate, valerate, isobutyrate, and isovalerate respectively.

The data were analyzed using general linear model (GLM) with SPSS 26.0 (Chicago, IL, USA), and are presented as mean and SEM. The analytic model included treatment \( (n = 7) \) as fixed effect and run \( (n = 3) \) as random effect, and were analyzed for linear or quadratic responses to starch content using orthogonal contrasts. Statistical significance was considered at \( P \leq 0.05 \) with \( 0.05 < P \leq 0.10 \) considered as a tendency.

**Results**

Elevating starch content increased DMD \( (P_{linear} < 0.001) \) and altered kinetic of gas production, with increase in 48-h gas production \( (P_{linear} \text{ and } P_{quadratic} < 0.001) \), final asymptotic gas production \( (P_{linear} < 0.001; P_{quadratic} = 0.007) \) and fractional rate of gas production \( (P_{linear} < 0.001; P_{quadratic} = 0.005) \) (Fig. 1A and Table 2). Elevating starch content altered kinetics of CH\(_4\) accumulation, with increase in 48-h CH\(_4\) production \( (P_{linear} \text{ and } P_{quadratic} < 0.001) \), final asymptotic CH\(_4\) production \( (P_{quadratic} < 0.001) \), fractional rate of CH\(_4\) production \( (P_{quadratic} = 0.007) \), and reduction in 48-h CH\(_4\) production relative to DM degraded \( (P_{linear} < 0.001; P_{quadratic} = 0.007) \) (Fig. 1B and Table 2). Elevating starch content altered kinetics of gH\(_2\)
accumulation, with reduction in 48-h gH₂ production ($P_{\text{linear}} = 0.011; P_{\text{quadratic}} = 0.003$), final asymptotic gH₂ production ($P_{\text{linear}} = 0.03; P_{\text{quadratic}} = 0.002$), and 48-h gH₂ production relative to DM degraded ($P_{\text{linear}} < 0.001$), and increase in fractional rate of gH₂ resolution ($P_{\text{linear}} = 0.035$) (Fig. 1C and Table 4).

Table 1 Chemical compositions of corn grain and corn straw. Components are expressed in g kg⁻¹ of dry matter (DM).

| Item                        | Corn grain | Corn straw |
|-----------------------------|------------|------------|
| Organic matter              | 985        | 915        |
| Crude protein               | 76.4       | 38.7       |
| Neutral detergent fiber     | 132        | 792        |
| Acid detergent fiber        | 34.5       | 482        |
| Starch                      | 747        | 72.4       |
| Gross energy (MJ/kg DM)     | 16.5       | 14.8       |

Table 2 Effects of starch content on DMD, FRD₀ and the kinetic parameters of total gas production (GP) after 48-h of in vitro ruminal incubation.

| Items¹                      | Starch content (g/kg) | SEM | $P$-value |
|-----------------------------|-----------------------|-----|-----------|
|                             |                      |     |           |
|                             | 72        | 185 | 297       | 410       | 522       | 634       | 747       | Linear | Quadratic |
| DMD (g/kg)                  | 511       | 579 | 645       | 706       | 777       | 854       | 916       | 8.4    | < 0.001  | 0.592 |
| GP (ml/g DM)                | 270       | 305 | 360       | 384       | 412       | 428       | 444       | 5.8    | < 0.001  | < 0.001|
| VF₆₀ (ml/g DM)              | 307       | 338 | 384       | 397       | 411       | 418       | 432       | 9.4    | < 0.001  | 0.007 |
| $k_{GP}$ (10⁻²/h)           | 5.43      | 5.22 | 5.84       | 6.97       | 9.65       | 12.81       | 16.08       | 1.059   | < 0.001  | 0.005 |

¹DMD = dry matter degradation; VF₆₀ = the final asymptotic volume of total gas production; $k_{GP}$ = the fractional rate of gas production.
Table 3
Effects of starch content on the kinetic parameters of methane (CH$_4$) production after 48-h of *in vitro* ruminal incubation.

| Items$^1$ | Starch content (g/kg) | SEM | P-value |
|-----------|-----------------------|-----|---------|
|           | 72 185 297 410 522 634 747 |     |         |
| CH$_4$    |                       |     |         |
| ml/g DM   | 44.8 49.4 53.4 57.7 58.9 57.7 52.5 | 0.88 | < 0.001 |
| ml/g of DDM | 88.0 85.6 82.9 81.7 75.8 67.6 57.3 | 1.71 | < 0.001 |
| VF$_{CH4}$ (ml/g DM) | 46.1 53.4 55.2 56.2 56.0 51.9 46.8 | 1.18 | 0.970 < 0.001 |
| k$_{CH4}$ (10$^{-2}$/h) | 8.40 6.70 7.21 8.48 9.81 12.76 16.54 | 1.320 | < 0.001 0.007 |

$^1$DDM = degraded dry matter; VF$_{CH4}$ = the final asymptotic volume of CH$_4$ production; k$_{CH4}$ = the fractional rate of CH$_4$ production.

Table 4
Effects of starch content on the kinetic parameters of hydrogen gas (gH$_2$) production after 48-h of *in vitro* ruminal incubation.

| Items$^1$ | Starch content (g/kg) | SEM | P-value |
|-----------|-----------------------|-----|---------|
|           | 72 185 297 410 522 634 747 |     |         |
| gH$_2$    |                       |     |         |
| ml/g DM   | 0.097 0.110 0.102 0.108 0.102 0.098 0.076 | 0.005 | 0.011 0.003 |
| ml/g of DDM | 0.19 0.19 0.16 0.15 0.13 0.12 0.08 | 0.012 | < 0.001 0.344 |
| VF$_{H2}$ (ml/g DM) | 0.095 0.110 0.103 0.110 0.104 0.100 0.076 | 0.006 | 0.030 0.002 |
| k$_{H2}$ (10$^{-2}$/h) | 34.0 43.3 30.3 26.7 27.1 28.5 33.9 | 5.52 | 0.281 0.252 |
| µ$_{H2}$ (h) | 4.16 3.89 5.56 5.96 6.68 6.54 8.00 | 1.429 | 0.035 0.986 |

$^1$DDM = degraded dry matter; VF$_{H2}$ = the final asymptotic volume of hydrogen gas production; k$_{H2}$ = the fractional rate of hydrogen gas production; µ$_{H2}$ = the fractional rate of hydrogen gas utilization.
Elevating starch content decreased pH ($P_{\text{linear}} < 0.001$) and increased total VFA concentration ($P_{\text{linear}} < 0.001$). Elevating starch content decreased acetate ($P_{\text{linear}} < 0.001$), butyrate ($P_{\text{quadratic}} = 0.002$) and isobutyrate ($P_{\text{quadratic}} = 0.02$) molar percentage and acetate to propionate ratio ($P_{\text{linear}} < 0.001$), and increased propionate ($P_{\text{linear}} < 0.001$; $P_{\text{quadratic}} = 0.001$), valerate ($P_{\text{linear}} < 0.001$; $P_{\text{quadratic}} = 0.009$) and isovalerate ($P_{\text{linear}} < 0.001$ and $P_{\text{quadratic}} < 0.001$) molar percentage (Table 5). Elevating starch content increased estimated net [H]$^+$ production ($P_{\text{linear}} < 0.001$; $P_{\text{quadratic}} = 0.005$), and decreased the estimated net [H]$^+$ production relative to DM degraded ($P_{\text{linear}} < 0.001$) and estimated net [H]$^+$ production relative to total VFA produced ($P_{\text{linear}} < 0.001$; $P_{\text{quadratic}} = 0.004$). Elevating starch content decreased molar percentage of [H]$^+$ utilized for CH$_4$ ($P_{\text{quadratic}} = 0.003$) and gH$_2$ ($P_{\text{linear}} < 0.001$) production. Elevating starch content increased MCP ($P_{\text{linear}}$ and $P_{\text{quadratic}} < 0.001$) and ammonia concentration ($P_{\text{quadratic}} = 0.004$) (Table 6).

### Table 5
Effects of starch content on ruminal pH and the profile of volatile fatty acids (VFA) after 48-h of *in vitro* ruminal incubation.

| Items                  | Starch content (g/kg) | SEM | $P$-value |
|------------------------|-----------------------|-----|-----------|
|                        | 72       | 185 | 297      | 410   | 522   | 634   | 747   |
| pH                     |          |     |          |       |       |       |       |
|                        | 6.58     | 6.53| 6.49     | 6.44  | 6.40  | 6.34  | 6.27  |
| Total VFA (mM)         |          |     |          |       |       |       |       |
|                        | 56.8     | 64.4| 66.3     | 72.0  | 79.0  | 84.9  | 86.0  |
| Molar percentage of individual VFA (mol/100 mol) | | | | | | | |
| Acetate                | 71.0     | 69.0| 68.1     | 66.3  | 64.4  | 62.4  | 60.4  |
| Propionate             | 19.8     | 20.9| 21.5     | 22.9  | 24.6  | 27.0  | 29.6  |
| Butyrate               | 5.89     | 6.49| 6.52     | 6.67  | 6.61  | 6.30  | 5.82  |
| Isobutyrate            | 1.04     | 1.09| 1.10     | 1.12  | 1.13  | 1.07  | 0.99  |
| Valerate               | 0.86     | 0.97| 1.04     | 1.12  | 1.20  | 1.22  | 1.26  |
| Isovalerate            | 1.37     | 1.60| 1.75     | 1.92  | 2.04  | 2.02  | 1.93  |
| Acetate to propionate ratio | 3.61     | 3.30| 3.17     | 2.89  | 2.62  | 2.32  | 2.04  |
|                        | $0.010$  |     |           |       |       |       |       |
|                        | < 0.001  |     |           |       |       |       |       |

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Table 6
Effects of starch content on $P_{NH2}$, $R_{NH2}$, molar percentage of metabolic hydrogen ([H]) utilized, ammonia concentration, and MCP after 48-h of in vitro ruminal incubation.

| Items$^1$ | Starch content (g/kg) | SEM | $P$-value |
|-----------|-----------------------|-----|-----------|
|           | 72  | 185 | 297 | 410 | 522 | 634 | 747 |
| $P_{NH2}$ |     |     |     |     |     |     |     |
| mM        | 76.1 | 83.5 | 84.2 | 87.9 | 92.0 | 92.9 | 87.4 | 1.98 | < 0.001 | 0.005 |
| mM/g of DDM | 149.7 | 144.9 | 130.8 | 124.3 | 118.3 | 108.8 | 95.4 | 4.26 | < 0.001 | 0.642 |
| $R_{NH2}$ | 134 | 130 | 127 | 122 | 116 | 109 | 102 | 1.3 | < 0.001 | 0.004 |
| Molar percentage of [H] utilized (mol/100 mol $P_{NH2}$) | | | | | | | | | |
| CH$_4$   | 49.9 | 49.5 | 52.5 | 53.8 | 51.7 | 49.5 | 47.2 | 1.28 | 0.227 | 0.003 |
| gH$_2$  | 0.053 | 0.055 | 0.050 | 0.050 | 0.045 | 0.042 | 0.034 | 0.003 | < 0.001 | 0.076 |
| Others  | 50.1 | 50.4 | 47.4 | 46.1 | 48.2 | 50.4 | 52.8 | 1.28 | 0.222 | 0.003 |
| Ammonia (mM) | 17.2 | 19.0 | 19.0 | 21.1 | 21.0 | 20.0 | 17.8 | 0.90 | 0.262 | 0.004 |
| MCP (mg/ml) | 0.75 | 0.73 | 0.78 | 0.81 | 0.84 | 0.91 | 1.08 | 0.022 | < 0.001 | < 0.001 |

$^1$ $P_{NH2}$ = estimated net [H] production; DDM = degraded dry matter; $R_{NH2}$ = estimated net [H] production relative to the amount of total VFA produced; gH$_2$ = hydrogen gas; MCP = microbial protein.

Discussion

It's well-known that corn grain and corn straw have different types of carbohydrate components and are rich in starch and fiber respectively. Ruminal degradation rate of carbohydrates depends on their monosaccharide and bond composition, molecular size, sugar arrangement at molecular level and physical morphology [23, 24]. Starch is mainly formed by $\alpha$-1,4 glycosidic bond and easily hydrolyzed by enzyme [6, 25], while fiber is the major component of cell wall, formed by $\beta$-1,4 glycosidic bond, and contains "crystals" which formed by the connection of cellulose macromolecules through hydrogen bonds and resistant to acid and enzyme hydrolysis [26]. Comparing with starch, cellulose and hemicellulose are less susceptible to microbial degradation in rumen [5]. In our study, increasing starch content linearly increased substrate degradation, gas production and fractional rate of gas production during in vitro 48-h batch incubation. These results were consistent with previous studies [27, 28], which report that starch has greater and faster rumen degradability in comparison with fiber.
Methane is the end-product during ruminal carbohydrate fermentation [3]. It is not surprising that elevated starch content linearly increased fractional rate of CH₄ production, as starch has faster rate of fermentation than straw fiber. The amount of CH₄ produced is related to the degree of substrate degradation and efficiency of CH₄ produced (i.e. CH₄ produced per unit of substrate degraded) [5]. Starch and fiber fermentation have different efficiency of CH₄ produced. Readily fermentable feed high in starch leads to lower CH₄ production, while slowly fermentable feed in cellulose and hemicellulose causes higher CH₄ production [29–31]. In the present study, the elevated starch content quadratically increased the 48-h and final asymptotic volume of CH₄ production, but linearly decreased amount of CH₄ produced per unit of DM degraded. Although starch had greater fermentation rate than straw fiber, reduced CH₄ production in starchy treatment could be caused by the reduction in efficiency of CH₄ produced.

Hydrogen is produced during carbohydrate fermentation to VFA and mainly consumed by methanogens to produce CH₄ as the end product [32]. The unused H₂ will be evolved from liquid to gas phase in the headspace and vent to air. Normally, the ruminal H₂ partial pressure is very low to facilitate the rumen fermentation [5]. Rooke et al. [33] report that H₂ emitted just accounts for less than 2% of the estimated total H₂ production from fermentation in beef cattle. In our study, both 48-h and final asymptotic gH₂ production was less than 1 ml/g DM, indicating that most of H₂ produced were utilized by methanogens to produce CH₄. Furthermore, starch and fiber had different profile of gH₂ production. For example, elevated starch content increased fractional rate of gH₂ consumption, although fractional rate of gH₂ production was not altered. Elevated starch content also quadratically decreased the 48-h and final asymptotic volume of gH₂ production and linearly decreased amount of gH₂ produced per unit of DM degraded. We proposed that the starch exhibited lower efficiency of H₂ production than straw fiber, which can be likely to be related to their different pathways of rumen fermentation.

Fermentation of feed rich in starch produces more propionate and butyrate, and less acetate than feed rich in cellulose and hemicellulose [5, 34]. In our study, elevated starch content linearly increased propionate molar percentage and reduced acetate molar percentage and acetate to propionate ratio. Formation of acetate and butyrate from carbohydrates is the net [H] production, while formation of propionate from pyruvate causes the net [H] utilization [35]. Elevated starch content linearly decreased estimated net [H] production per units of DM degraded. Increased estimated net [H] production could be caused by the greater extent of degradation, when starch content was increased. Furthermore, less than 55% of estimated net [H] produced was used for CH₄ and gH₂ synthesis. This suggests that a huge amount of [H] were redirected into other fermentation products other than CH₄ and gH₂. Elevated starch content quadratically decreased proportion of [H] produced for CH₄ production, indicating that elevated starch content may enhance [H] incorporation into other H₂ sinks. Rapid starch fermentation causes a fast increase in H₂ partial pressure [7, 36], which can energetically promote other H₂ utilization pathways, such as microbial protein synthesis, biohydrogenation of unsaturated fatty acid, acetogenesis and etc [9].

Microbial protein can be an alternative ruminal H₂ sink, and synthesized by utilizing ammonia or amino acids in the rumen [37]. Microbial growth and protein synthesis requires utilization of ATP generated during rumen fermentation [38].
fermentation [6, 38]. Non-fiber carbohydrates is the major energy substrate for ruminal microorganism, and thus could promote the incorporation of N into MCP synthesis [37, 39]. In our study, elevated starch content linearly increased MCP concentration, leading to a quadratical change in ammonia concentration. Ammonia concentration is determined by the balance between its production and utilization, and thus related to the substrate degradation rate and MCP synthesis. Thus, increasing starch content can result in a more rapidly available energy source for microorganisms, leading to enhanced utilization of ammoina for MCP synthesis [27, 40]. Other studies also proposes that more [H] can be incorporated into microbial biomass when available H₂ is increased [8]. Therefore, in comparison with straw fiber, starch fermentation is beneficial to MCP synthesis, which also contributed to the reduction in methanogenesis in starchy treatment.

**Conclusion**

In summary, comparing with fiber, starch has faster and greater rumen degradability. Increasing starch content alters rumen fermentation pathway from acetate to propionate production with reduction in efficiency of [H] production, increases MCP synthesis and decreases efficiency of CH₄ and gH₂ production. Such enhanced MCP synthesis is accompanied with increased molar percentage of [H] not for CH₄ and gH₂ production, and thus contributes to reduction in methanogenesis in starchy treatment.

**Abbreviations**

CH₄, methane; H₂, molecular hydrogen; [H], metabolic hydrogen; gH₂, hydrogen gas; MCP, microbial protein; DMD, dry matter degradation; VF₆GP, the final asymptotic volume of total gas production; b, shape parameter of gas production; k₆GP, the fractional rate of gas production; VF₆CH₄, the final asymptotic volume of CH₄ production; k₆CH₄, the fractional rate of CH₄ production; DDM, degraded dry matter; VF₆H₂, the final asymptotic volume of hydrogen gas production; b₆H₂, shape parameter of hydrogen gas; k₆H₂, the fractional rate of hydrogen gas production; μ₆H₂, the fractional rate of hydrogen gas utilization. P₆NH₂, estimated net [H] production; R₆NH₂, estimated net [H] production relative to the amount of total VFA produced.

**Declarations**

**Acknowledgements**

The authors would like to thank Dr. Qiushuang Li's kind help for Graphic drawing.

**Authors’ contributions**

SYY, XMZ designed the study; SYY, XZC, JWZ and CG performed the research; SYY analyzed data and wrote the paper; XMZ, MW, CXZ, BL and ZLT contributed to revision of the manuscript. The authors read and approved the final manuscript.

**Funding**
This study was supported by the National Natural Science Foundation of China (Grant No. 31922080, 32002204) and Strategic Priority Research Program (Grant No. XDA26040203).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on request.

Ethics approval and consent to participate

The experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that no competing interests exist. The manuscript has not been published previously.

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**Figures**

**Figure 1**

Effect of starch content on total gas (A), methane (CH4, B) and hydrogen gas (gH2, C) production through 48-h in vitro ruminal fermentation. Values are normalized by incubated dry matter (DM) weight.