Effects of Different Rumen-Degradable Starch Levels on In Vitro Rumen Fermentation Under Low-Starch Diet Conditions (Postprint)

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Date: 2017-10-10T00:00:00+00:00

Abstract

This study aimed to investigate the effects of different levels of rumen-degradable starch (RDS) on in vitro rumen fermentation under low-starch diets with corn as the starch source. Using rumen fluid collected from three healthy Holstein cows fitted with permanent rumen fistulas as inoculum, different RDS level diets were used as fermentation substrates for each group, and gas production and rumen fermentation parameters at 48 h of incubation, as well as changes in rumen microbial flora at 24 h, were determined using the in vitro gas production method. The results showed that: 1) With increasing dietary RDS level, gas production, potential gas production fraction, and gas production rate at 48 h of in vitro incubation increased linearly ($P<0.05$), gas production from the rapidly fermentable fraction decreased linearly ($P<0.05$), and dry matter disappearance increased linearly ($P<0.05$); 2) With increasing dietary RDS level, microbial protein, acetate, propionate, butyrate, and total volatile fatty acid concentrations in the culture fluid at 48 h of in vitro incubation increased linearly ($P<0.05$), while pH and ammonia nitrogen concentrations showed no significant changes ($P>0.05$); 3) With increasing dietary RDS level, the relative abundance of Ruminococcus albus and Ruminobacter amylophilus in the culture fluid at 24 h of in vitro incubation increased linearly ($P<0.05$), while the relative abundance of Ruminococcus flavefaciens, Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Streptococcus bovis, and Succinimonas amylolytica showed no significant changes ($P>0.05$). Taken together, increasing RDS level under low-starch diet conditions is beneficial for rumen fermentation.

Full Text

Effects of Ruminally Degradable Starch Level in Low Starch Diets on in Vitro Ruminal Fermentation

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Abstract

This trial was conducted to investigate the effects of different ruminally degradable starch (RDS) levels in low-starch diets on in vitro ruminal fermentation, using corn as the starch source. Three healthy Holstein cows fitted with permanent rumen fistulas served as rumen fluid donors. Diets with varying RDS levels were used as fermentation substrates, and gas production, ruminal fermentation parameters at 48 h of incubation, and ruminal microbial flora changes at 24 h were measured using the in vitro gas production method. The results showed that: 1) With increasing dietary RDS level, gas production, potential gas production, and gas production rate increased linearly ($P<0.05$) after 48 h of in vitro incubation, while gas production from the rapidly fermentable fraction decreased linearly ($P<0.05$), and dry matter disappearance rate increased linearly ($P<0.05$). 2) With increasing dietary RDS level, microbial protein, acetate, propionate, butyrate, and total volatile fatty acid concentrations in the culture fluid increased linearly ($P<0.05$) after 48 h of incubation, while pH and ammonia nitrogen concentration remained unchanged ($P>0.05$). 3) With increasing dietary RDS level, the relative abundance of *Ruminococcus albus* and *Ruminobacter amylophilus* in the culture fluid increased linearly ($P<0.05$) after 24 h of incubation, while the relative abundance of *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Streptococcus bovis*, and *Succinimonas amylolytica* showed no significant changes ($P>0.05$). In conclusion, increasing RDS level in low-starch diets is beneficial for ruminal fermentation.

Keywords: ruminally degradable starch; gas production; rumen fermentation; microflora

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Starch is a crucial energy source in dairy cow diets, serving as a precursor for propionate production in the rumen and as a direct source of glucose absorbed in the small intestine. Starch constitutes 70%–80% of non-structural carbohydrates in grains and is typically rapidly degraded in the rumen, with nearly complete digestion (digestibility exceeding 90% for many cereal starches). Grains are widely used in dairy cattle to meet their starch and energy requirements. However, with increasing demand for grains in developing countries and biofuel markets, grain prices have risen continuously, affecting dairy farm profitability

and drawing attention to low-starch diets. Current recommendations for lactating dairy cows suggest dietary starch levels of 23%–30% on a dry matter basis [1]. Dann [2] reviewed that substituting byproduct feeds (such as soybean hulls and beet pulp) for corn in lactating cow diets, low-starch levels (18%–21%) did not negatively affect ruminal fermentation or lactation performance.

The rate and extent of dietary starch degradation in the rumen are determined by several factors, including starch source, diet composition, single meal intake, mechanical processing (grain processing and chewing), chemical effects (gelatinization degree), and rumen microbial adaptation [3]. NRC (2001) [4] provided minimum recommendations for neutral detergent fiber (NDF) and maximum recommendations for non-fiber carbohydrates (NFC) in total mixed rations for lactating cows. However, variations in RDS level affect rumen pH [5], ruminal VFA composition [6-7], dry matter intake (DMI) [5,8-9], digestibility [8,10], milk fat percentage [8], and nitrogen utilization efficiency [7,11]. Therefore, appropriate RDS levels are essential for optimal dairy cow performance, as they influence the suitable amounts of NDF and NFC in the diet. In China, corn is the primary source of starch in dairy diets, with steam-flaked corn and ground corn being commonly used processing and feeding forms. Steam-flaked corn increases ruminal starch degradability compared to ground corn (80.3% vs. 67.9%) [12]. This experiment designed three diets with different RDS levels by altering the ratio of ground corn to steam-flaked corn in the diet. Using the in vitro gas production method, we investigated the effects of different RDS levels on in vitro ruminal gas production parameters, fermentation parameters, and microbial flora under low-starch conditions [20% starch (dry matter basis)], providing a theoretical basis for the effective application of low-starch diets and starch in dairy production.

1.1 Feed Sample Preparation and Composition Analysis

Three experimental diets with different RDS levels were formulated according to the *Feeding Standards of Dairy Cattle* [13] (NY/T 34—2004): low RDS diet (61.07% RDS, L-RDS group), medium RDS diet (67.82% RDS, M-RDS group), and high RDS diet (73.74% RDS, H-RDS group). The composition and nutrient levels of the experimental diets are shown in Table 1 .

Dietary RDS levels were determined using the semi-in situ nylon bag method [14]. Three healthy Chinese Holstein cows fitted with permanent rumen fistulas [body weight: (678±27) kg; milk yield: (20.6±2.5) kg; days in milk: (276±19) d] were used. The cows were fed a diet consisting of 28.9% corn silage, 24.2% Chinese wild rye hay, and 46.9% concentrate (all on a dry matter basis), with two daily feedings and milkings, ad libitum access to feed and water, and individual tie-stall housing. Diets were ground to pass through a 3 mm sieve, and 5 g samples were accurately weighed into labeled nylon bags (10 cm×20 cm; 50 m pore size; provided by Ankom) and incubated in the rumen for 72, 48, 24, 12, 8, 4, 2, and 0 h. Nylon bag incubation and post-incubation procedures followed Yu et al. [21]. Samples were dried at 65°C for 48 h, equilibrated to constant

weight, and ground through a 1 mm sieve for determination of ruminal starch degradation parameters, which are presented in Table 2 .

1.2 Rumen Fluid Collection and Processing

In June 2016, three Holstein cows fitted with permanent rumen fistulas (approximately 600 kg body weight) were selected from the Nestlé Dairy Training Center in Shuangcheng City, Heilongjiang Province, as rumen fluid donors. Rumen fluid was collected 2 h before morning feeding through the rumen fistula, mixed, and transported to the laboratory in a preheated thermos. The collected rumen fluid was thoroughly mixed and filtered through four layers of cheesecloth (while continuously flushing with CO) for later use. All operations were performed in a 39°C water bath.

1.3 In Vitro Fermentation

The in vitro fermentation system consisted of an automatic gas production recording device (model: Cerabar T PMP131, Memograph M RSG40) and software system (provided by Alltech) manufactured by Endress+Hauser, Germany. Experimental samples were ground to pass through a 1 mm sieve. Accurately weighed 0.5 g samples were placed into 150 mL anaerobic fermentation bottles, with six replicates per treatment and three blanks. During inoculation, 50 mL of pre-warmed buffer solution (at 39°C) and 25 mL of fresh rumen fluid were added while stirring. The liquid medium was prepared according to Menke et al. [15]. After flushing with CO for 5 s, the sensor of each gas production device was connected to the data logger, and fermentation bottles were continuously incubated at 39.2°C for 24 and 48 h.

1.4 Sample Collection and Pretreatment

After 24 and 48 h of in vitro incubation, three fermentation bottles were removed from each treatment group. At 24 h, 10 mL of culture fluid was quickly transferred and stored at -80°C for total microbial DNA extraction. At 48 h, fermentation was terminated, pH was measured immediately, and 50 mL of fermentation fluid was collected into centrifuge tubes. A 25% metaphosphoric acid solution was added at a 1:5 ratio to the fermentation fluid, mixed, and stored at -20°C for determination of microbial protein (MCP), ammonia nitrogen (NH -N), and volatile fatty acid (VFA) concentrations. The 48 h fermentation fluid was filtered through nylon bags (pore size: 40 m; specification: 45 mm×55 mm). The residue and nylon bags were rinsed under running water until clear, dried at 65°C to constant weight, and used to calculate in vitro dry matter disappearance (DMD).

1.5 Measurement Indicators and Methods

Dry matter, crude ash, crude protein, crude fat, and inorganic matter contents were determined according to AOAC (1990) [16] methods. pH was measured

using a Sartorius Basic pH Meter PB-20 (Sartorius Scientific Instruments Beijing Co., Ltd.). Starch content was determined using the amyloglucosidase/ -amylase method with a kit purchased from Megazyme, Ireland. Acid detergent fiber (ADF) and NDF contents were determined according to Van Soest et al. [17] using an Ankom 220 Fiber Analyzer (ANKOM, USA).

NH -N concentration was determined by the indophenol colorimetric method described by Broderick et al. [18] using a UV-2000 spectrophotometer (Shanghai Unico Instrument Co., Ltd.). MCP concentration was determined by the purine method described by Makkar et al. [19]. The specific procedure was: a standard curve was prepared using yeast RNA; 8 mL of fermentation fluid was centrifuged at 13,200 r/min for 20 min, pretreated, and measured at 260 nm using a UV-2000 spectrophotometer. The RNA measurement value was calculated based on absorbance and the standard curve. The MCP concentration was calculated as follows:

Microbial protein nitrogen concentration (mg/mL) = RNA measurement value (mg/mL) × RNA nitrogen content (17.83%) / bacterial RNA nitrogen content (10%) × dilution factor;
MCP concentration (mg/mL) = microbial protein nitrogen concentration (mg/mL) × 6.25.

VFA concentration was determined using a Shimadzu GC-200 gas chromatograph [20] under the following conditions: injector parameters: carrier gas nitrogen (N), split ratio 40:1, injection volume 0.4 L, temperature 220°C; column parameters: HP-INNOwax capillary column in constant flow mode, flow rate 2.0 mL/min, average linear velocity 38 cm/s; oven parameters: programmed temperature 120°C (3 min) → 10°C/min → 180°C (1 min); detector parameters: hydrogen (H) flow 40 mL/min, air flow 450 mL/min, column flow + tail gas flow 45 mL/min, flame ionization detector (FID) temperature 250°C.

Microbial flora determination: Total rumen microbial DNA was extracted using the bead-beating cetyltrimethylammonium bromide (CTAB) method [21]. After extraction, the concentration and purity of the extracted total DNA were measured using a UV-Vis spectrophotometer, ensuring the optical density (OD) ratio at 260 nm to 280 nm was between 1.6 and 1.8. Real-time quantitative PCR (RT-qPCR) was used to detect microbial relative abundance using an ABI7500 PCR instrument. RT-qPCR reaction conditions were established according to SYBR Premix Ex Taq™ reagent in a 20 L reaction system. Bacterial 16S rDNA was amplified from total rumen microbial DNA. The PCR reaction system consisted of: 2 L 10× buffer, 0.4 L 10 mmol/L dNTP, 0.8 L each of 10 mol/L forward and reverse primers, 1.6 L 25 mmol/L magnesium chloride (MgCl), 0.4 L template (total rumen DNA), 2 L Taq DNA polymerase, and 13.6 L double-distilled water (ddH O), for a total volume of 20 L. PCR parameters: denaturation at 95°C for 7 min, 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 3 min, and final extension at 72°C for 7 min. Primer sequences and references are shown in Table 3 , and primers were synthesized by Shanghai Sangon Biotech Co., Ltd.

1.6 Calculation Formulas

Dietary RDS level was calculated using a rumen kinetic mathematical exponential model [27], and data were processed using SAS 9.2 nonlinear regression least squares method.

The dynamic fermentation model GP=a+b(1-e) [28] was used to determine a, b, and c values based on nonlinear least squares principle, where a represents gas production from the rapidly fermentable fraction (mL), b represents gas production from the slowly fermentable fraction (mL), c represents the gas production rate constant for fraction b (%/h), a+b represents potential gas production (mL), and GP represents gas production at time t (mL).

Sample dry matter disappearance (%) = [(sample weight - residue weight) / sample weight] × 100.

Rumen microbial relative abundance was expressed as a percentage relative to total bacterial 16S rDNA using the following formula:

Target bacteria relative abundance (%) = 2 Ct Ct × 100.

Where: Ct is the Ct value measured with target bacteria primers, and Ct is the Ct value obtained with total bacteria primers.

1.7 Statistical Analysis

Experimental data were processed using Excel 2010 software and analyzed statistically using the MIXED model in SAS 9.1 software. Orthogonal polynomial contrasts were performed to test linear and quadratic effects of dietary RDS level. Differences were considered significant at $P<0.05$, and trends were identified at $0.05 P<0.10$.

2.1 Effects of Different RDS Level Diets on Gas Production, Gas Parameters, and Dry Matter Disappearance After 48 h In Vitro Incubation

As shown in Table 4 , with increasing dietary RDS level, gas production, potential gas production, gas production rate, and dry matter disappearance increased linearly ($P<0.05$) after 48 h of in vitro incubation, while gas production from the rapidly fermentable fraction decreased linearly ($P<0.05$). No significant differences were observed between the L-RDS and M-RDS groups for any parameter ($P>0.05$). Except for gas production from the rapidly fermentable fraction, which was significantly lower than in the L-RDS group ($P<0.05$), all other parameters in the H-RDS group were significantly higher than in the L-RDS group ($P<0.05$). Gas production and potential gas production in the H-RDS group were significantly higher than in the M-RDS group ($P<0.05$), with no significant differences in other parameters between these two groups ($P>0.05$).

2.2 Effects of Different RDS Level Diets on pH and MCP, NH -N, and VFA Concentrations After 48 h In Vitro Incubation

As shown in Table 5 , dietary RDS level had no significant effect on culture fluid pH, NH -N concentration, or acetate/propionate ratio (P>0.05). With increasing dietary RDS level, MCP, acetate, propionate, butyrate, and total volatile fatty acid (TVFA) concentrations increased linearly (P<0.05). The M-RDS group showed significantly lower MCP concentration compared to the H-RDS group (P<0.05), with no significant differences in other parameters between these two groups (P>0.05). The H-RDS group had significantly higher MCP, acetate, propionate, butyrate, and TVFA concentrations than the L-RDS group (P<0.05). The L-RDS group showed no significant differences in MCP, propionate, and butyrate concentrations compared to the M-RDS group (P>0.05), but had significantly lower acetate and TVFA concentrations (P<0.05).

2.3 Effects of Different RDS Level Diets on Microbial Flora After 24 h In Vitro Incubation

As shown in Table 6 , with increasing dietary RDS level, the relative abundance of *Ruminococcus albus* and *Ruminobacter amylophilus* in the culture fluid increased linearly (P<0.05). The H-RDS group had significantly higher relative abundance of *R. albus* and *R. amylophilus* than both the L-RDS and M-RDS groups (P<0.05), with no significant differences between the L-RDS and M-RDS groups (P>0.05). Dietary RDS level had no significant effect on the relative abundance of *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Streptococcus bovis*, and *Succinimonas amylolytica* (P>0.05).

3.1 Effects of Different RDS Level Diets on Rumen Gas Production, Gas Parameters, and Dry Matter Disappearance After 48 h In Vitro Incubation

In vitro gas production is an important indicator for evaluating feed fermentability; stronger feed fermentability corresponds to higher microbial activity in the rumen and greater gas production, and vice versa. In this experiment, 48 h gas production increased with dietary RDS level, consistent with results reported by Palizdar et al. [29] and Zhang et al. [30]. This occurs because replacing ground corn with steam-flaked corn in the diet makes starch more readily available to microorganisms and enzymes [31], and starch degradation generates substantial energy that promotes microbial growth and proliferation, thereby increasing fermentation gas production. The results also showed that high RDS diets increased potential gas production and gas production rate, consistent with the findings of Palizdar et al. [29]. Rapid starch degradation promotes microbial utilization of nutrients and enhances fermentation, providing partial explanation for the increased dry matter disappearance rate. The decreasing trend in gas production from the rapidly fermentable fraction with increasing dietary RDS level may be attributed to the reduced soluble fraction of dietary starch during rumen incubation.

3.2 Effects of Different RDS Level Diets on pH and MCP, NH -N, and VFA Concentrations After 48 h In Vitro Incubation

pH is a crucial indicator of rumen internal environment changes, influenced by diet composition, saliva secretion, and organic acid accumulation. The prevailing view suggests that increasing fermentable carbohydrate levels enhances short-chain fatty acid production and the risk of rumen acidosis, leading to decreased rumen pH [32]. However, this experiment showed that dietary RDS level had no significant effect on culture fluid pH after 48 h of in vitro incubation, consistent with the in vitro results of Zhang et al. [30]. This may be because as incubation time extends, starch degradation rates among treatments become similar, and pH is highly correlated with starch degradation [33], resulting in non-significant pH differences among groups at 48 h. Rumen NH -N concentration is an important indicator of rumen nitrogen metabolism, reflecting both protein degradation extent and microbial ammonia utilization capacity. Murphy et al. [34] reported that the optimal NH -N concentration for microbial fermentation ranges from 6.3 to 27.5 mg/dL, and all treatment groups in this experiment fell within this suitable range. The results also indicated that increasing dietary RDS level had no significant effect on NH -N concentration. Zhang et al. [30] reported that substituting steam-flaked corn for ground corn in diets had no significant effect on fermentation fluid NH -N concentration in vitro, which aligns with our findings. However, these results differ from in vivo reports by Zhong et al. [8] and Aldrich et al. [35], who found that increasing RDS level through steam-flaked corn decreased rumen NH -N concentration. The discrepancy may be explained by two factors: First, grain processing increases in vitro starch degradation rate during the 4–8 h period [36], when synchronized energy and nitrogen release can promote microbial growth and MCP synthesis, enhancing microbial NH -N utilization, while extended incubation makes starch degradation rates similar among groups, leading to consistent microbial NH -N utilization capacity. Second, steam-flaking technology disrupts corn protein spatial structure, increasing ruminal protein degradability [37] and consequently protein breakdown and NH -N production.

This experiment demonstrated that increasing dietary RDS level enhanced MCP synthesis, consistent with previous reports by Plascencia et al. [6] and Theurer et al. [38]. MCP synthesis depends on the availability of rumen-degradable energy and nitrogen, as well as fermentation rate synchronization. The higher relative abundance of *R. albus* and *R. amylophilus* under high RDS diets indicates that high RDS levels promote the growth of relevant microorganisms, likely because high RDS levels provide greater rumen-degradable organic matter [39] that supplies energy for microbial growth and proliferation. Additionally, increased ruminal degradability of steam-flaked corn protein may be another reason for elevated MCP concentration. Some reports [11] have shown that increasing dietary RDS level had no significant effect on MCP concentration, and this inconsistency may be due to rapidly degradable starch reducing ruminal fiber digestibility, negatively affecting rumen fermentation, decreasing rumen-

degradable organic matter levels, and impairing MCP synthesis. VFAs are the primary energy source for ruminant energy metabolism, with acetate, propionate, and butyrate accounting for 80% of major energy-yielding compounds. This study found that increasing dietary RDS level increased acetate, propionate, and butyrate concentrations to varying degrees. Zhang et al. [30] reported that substituting steam-flaked corn for regular corn in diets tended to increase acetate concentration and significantly increased propionate and butyrate concentrations in vitro, similar to our results. However, related in vivo studies showed that increasing dietary RDS level decreased acetate concentration [7], increased propionate concentration [10], and had no significant effect on butyrate concentration [7,10]. Starch in steam-flaked corn is more readily utilized by microorganisms and enzymes than starch in ground corn, and rapid starch degradation leads to increased propionate concentration. The increased acetate and butyrate concentrations may be due to higher rumen-degradable organic matter levels [39] under high RDS diets, which provide energy for microbial growth and promote microbial decomposition of nutrients, thereby increasing acetate and butyrate production. This experiment showed that dietary RDS level had no significant effect on the acetate/propionate ratio, consistent with Zhang et al. [30] but inconsistent with Shen et al. [7], possibly because different dietary conditions alter the effects of RDS on rumen fermentation, leading to variations in acetate and propionate concentrations.

3.3 Effects of Different RDS Level Diets on Microbial Flora After 24 h In Vitro Incubation

The major fiber-degrading bacteria in the rumen include *R. flavefaciens*, *R. albus*, and *F. succinogenes*. This experiment showed that the relative abundance of *R. albus* in culture fluid increased with dietary RDS level after 24 h of in vitro incubation, while no significant differences were observed in *R. flavefaciens* and *F. succinogenes* among groups. Li [40] reported that substituting wheat for corn at different ratios in dairy goat diets resulted in linearly decreased relative abundance of *R. flavefaciens* and quadratic trends in *F. succinogenes* relative abundance, with no significant effect on *R. albus*. Hu [41] found that increasing dietary corn proportion in dairy goat diets gradually decreased the relative abundance of three fiber-degrading bacteria (*R. flavefaciens*, *F. succinogenes*, and *R. albus*). These inconsistent results may be caused by differences in rumen pH, as increasing fermentable carbohydrate levels enhances short-chain fatty acid production and rumen acidosis risk, decreasing rumen pH [32,42]. Fiber-degrading bacteria are generally sensitive to rumen pH, and growth of major fiber-degrading bacteria such as *Ruminococcus* spp. and *F. succinogenes* is inhibited when pH falls below 6.0 [43]. In this experiment, culture fluid pH remained within the normal range across all groups, and NH -N concentration was within the suitable range for microbial fermentation. The increased relative abundance of *R. albus* may be because high RDS levels provide more rumen-degradable organic matter [39], thereby supplying energy for microbial growth and proliferation. Different strains of *B. fibrisolvens* show considerable varia-

tion, with some exhibiting strong starch-degrading activity and others strong fiber-degrading activity. The non-significant differences in *B. fibrisolvens* relative abundance among groups in this experiment are consistent with the results of Shen [44]. *S. bovis*, *R. amylophilus*, and *S. amylolytica* are major starch-degrading bacteria. Shen [44] reported that substituting steam-flaked corn for ground corn in dairy cow diets increased *S. bovis* relative abundance, decreased *R. amylophilus* relative abundance, and had no significant effect on *S. amylolytica*. Li [40] found that substituting steam-flaked corn for ground corn increased *S. amylolytica* relative abundance with no significant effects on *S. bovis* and *R. amylophilus*. These inconsistent results may be related to differences in dietary RDS levels and starch degradation rates, and the underlying reasons require further investigation.

In conclusion, increasing dietary RDS level in low-starch diets increased 48 h gas production, potential gas production, and gas production rate, elevated MCP, acetate, propionate, butyrate, and TVFA concentrations after 48 h of in vitro incubation, had no significant effect on pH and ammonia nitrogen concentration, and increased the relative abundance of *Ruminococcus albus* and *Ruminobacter amylophilus* after 24 h of incubation. Therefore, increasing dietary RDS level under low-starch conditions is beneficial for ruminal fermentation.

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Note: Figure translations are in progress. See original paper for figures.