Postprint: Evaluation of Associative Effects of Corn Stover, Rice Straw, Corn Stover Silage and Concentrate Using the In Vitro Gas Production Method

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Abstract

The present study aimed to investigate the associative effects among corn stover, rice straw, corn stover silage, and concentrate using the in vitro gas production method. A single-factor experimental design was adopted, and three combination screening experiments were conducted: First, a corn stover and rice straw combination experiment was performed to screen for the optimal ratio of corn stover to rice straw (corn stover-rice straw); then, a corn stover-rice straw and corn stover silage combination experiment was conducted to screen for the optimal ratio of corn stover-rice straw to corn stover silage (corn stover-rice straw-corn stover silage); finally, a corn stover-rice straw-corn stover silage and concentrate combination experiment was carried out to screen for the optimal ratio of corn stover-rice straw-corn stover silage to concentrate. All combinations were subjected to in vitro fermentation experiments at ratios of 100.0:0, 80.0:20.0, 60.0:40.0, 50.0:50.0, 40.0:60.0, 20.0:80.0, and 0:100.0, with three replicates per combination. The in vitro gas production method was used to analyze the effects of different feed combinations on 48 h gas production, dry matter disappearance rate (DMD), pH, and concentrations of microbial crude protein (MCP), ammonia nitrogen (NH3-N), and volatile fatty acids (VFA). The single-factor associative effect index (SFAEI) and multi-factor associative effect index (MFAEI) were calculated for each combination. The results showed that: 1) Different proportional combinations of feeds had significant or extremely significant effects on gas production ($P<0.05$ or $P<0.01$), and the SFAEI for gas production of corn stover:rice straw, corn stover-rice straw:corn stover silage, and corn stover-rice straw-corn stover silage:concentrate reached maximum values at ratios of 60.0:40.0, 40.0:60.0, and 20.0:80.0, respectively; 2) Different proportional combinations of feeds also had significant or extremely significant effects on

DMD ($P<0.05$ or $P<0.01$), and the SFAEI for DMD of corn stover:rice straw, corn stover-rice straw:corn stover silage, and corn stover-rice straw-corn stover silage:concentrate reached maximum values at ratios of 50.0:50.0, 40.0:60.0, and 20.0:80.0, respectively; 3) Different ratios of corn stover-rice straw-corn stover silage:concentrate had an extremely significant effect on pH ($P<0.01$); 4) Different proportional combinations of feeds also had significant or extremely significant effects on MCP concentration ($P<0.05$ or $P<0.01$), and the SFAEI for MCP concentration of corn stover:rice straw, corn stover-rice straw:corn stover silage, and corn stover-rice straw-corn stover silage:concentrate reached maximum values at ratios of 80.0:20.0, 40.0:60.0, and 20.0:80.0, respectively; 5) Different proportional combinations of feeds also had significant or extremely significant effects on NH3-N concentration ($P<0.05$ or $P<0.01$), with a range of 20.20~31.59 mg/dL; 6) The acetate/propionate ratio of corn stover and rice straw combinations and the butyrate concentration of corn stover-rice straw-corn stover silage and concentrate combinations showed no significant differences among ratios ($P>0.05$), while different proportional combinations of feeds had significant or extremely significant effects on the remaining VFA and TVFA concentrations ($P<0.05$ or $P<0.01$). Based on MFAEI evaluation, the optimal ratios of each feed combination were as follows: corn stover to rice straw was 60.0:40.0; corn stover, rice straw, and corn stover silage was 24.0:16.0:60.0; and corn stover, rice straw, corn stover silage, and concentrate was 9.6:6.4:24.0:60.0.

Full Text

Associative Effects of Corn Stalk, Rice Straw, Corn Stalk Silage and Concentrate Evaluated by Gas Production Technique In Vitro

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Abstract: This experiment was conducted to investigate the associative effects among corn stalk (CS), rice straw (RS), corn stalk silage (CSS) and concentrate (CC) using an in vitro gas production technique. A single-factor experimental design was employed across three sequential screening trials: first, CS was combined with RS to screen for the optimal CS-RS ratio; second, the optimal CS-RS combination was recombined with CSS to screen for the optimal CS-RS-CSS ratio; and third, the optimal CS-RS-CSS combination was recombined with CC to screen for the optimal CS-RS-CSS:CC ratio. Each combination was tested in vitro at proportions of 100.0:0, 80.0:20.0, 60.0:40.0, 50.0:50.0, 40.0:60.0, 20.0:80.0, and 0:100.0, with three replicates per combination. The in vitro gas production method was used to analyze the effects of different feed combinations on 48-hour gas production, dry matter disappearance rate (DMD), pH, and concentrations of microbial crude protein (MCP), ammonia nitrogen (NH_3-N), and volatile fatty acids (VFA). Single factor associative effects index (SFAEI) and multiple factors associative effects index (MFAEI) were calculated for each combination. The results showed that: (1) Different combination ratios of feeds had significant or extremely significant effects on gas production ($P<0.05$ or $P<0.01$), with SFAEI for gas production reaching maximum values at CS:RS, CS-RS:CSS, and CS-RS-CSS:CC ratios of 60.0:40.0, 40.0:60.0, and 20.0:80.0, respectively; (2) Different combination ratios also had significant or extremely significant effects on DMD ($P<0.05$ or $P<0.01$), with optimal SFAEI values observed at CS:RS, CS-RS:CSS, and CS-RS-CSS:CC ratios of 50.0:50.0, 40.0:60.0, and 20.0:80.0, respectively; (3) Different CS-RS-CSS:CC ratios had extremely significant effects on pH ($P<0.01$); (4) Different combination ratios had significant or extremely significant effects on MCP concentration ($P<0.05$ or $P<0.01$), with optimal SFAEI values at CS:RS, CS-RS:CSS, and CS-RS-CSS:CC ratios of 80.0:20.0, 40.0:60.0, and 20.0:80.0, respectively; (5) Different combination ratios had significant or extremely significant effects on NH_3-N concentration ($P<0.05$ or $P<0.01$), ranging from 20.20 to 31.59 mg/dL; (6) No significant differences were observed in acetic acid/propionic acid ratio among different CS:RS combinations or in butyric acid concentration among different CS-RS-CSS:CC combinations ($P>0.05$), though other VFA and TVFA concentrations were significantly or extremely significantly affected by different combination ratios ($P<0.05$ or $P<0.01$). Based on MFAEI evaluation, the optimal ratios were determined to be: CS:RS = 60.0:40.0; CS:RS:CSS = 24.0:16.0:60.0; and CS:RS:CSS:CC = 9.6:6.4:24.0:60.0.

Key words: corn stalk; rice straw; corn stalk silage; concentrate; associative effects

China is rich in crop straw resources, with annual production reaching 820 million tons, yet the utilization rate remains low, resulting in significant resource waste. Crop straw is poorly utilized by ruminants due to its low digestibility, insufficient fermentable nitrogen sources, low bypass protein content, low glucogenic substances, and mineral nutrition imbalances. Therefore, improving the feeding value of straw has attracted considerable attention from researchers and producers. Studies on physical, chemical, and biological treatments of straw have revealed that feed nutritional value is related to feed combinations. When the interaction between feeds results in nutrient utilization or intake higher than the weighted average of individual feeds, a positive associative effect is demonstrated. Utilizing associative effects among feeds to promote rumen fermentation represents an important measure for improving the utilization of straw-based feeds such as rice straw.

Previous research has investigated pairwise associative effects among rice straw with cornstarch, corn silage, and alfalfa; among soybean straw, peanut vine, and corn silage; and among treated wheat straw, corn silage, and concentrate, identifying combinations with positive associative effects. However, no studies have reported on the associative effects among four feed types: corn stalk, rice straw, corn stalk silage, and concentrate. This study aimed to investigate the effects of different combination ratios of these feeds on in vitro gas production and rumen fermentation characteristics, determine appropriate mixing proportions, and provide a scientific basis for ruminant diet formulation and improved straw utilization.

1.1 Experimental Materials

Corn stalk, rice straw, corn stalk silage, and concentrate were collected from dairy farms in Baoding and Chengde, Hebei Province. Samples were dried at 65°C to produce air-dried samples, ground to pass through a 20-mesh sieve, and stored sealed for later use. The nutrient levels of the three straw feeds are shown in Table 1, and the composition and nutrient levels of the concentrate are shown in Table 2.

Table 1 Nutrient levels of corn stalk, rice straw and corn stalk silage (DM basis) %

Table 2 Composition and nutrient levels of concentrate (DM basis)

Note: NEmf was a calculated value, while the other nutrient levels were measured values. The same applies to Table 3.

1.2 Rumen Fluid Donor Animals

Three healthy castrated steers weighing approximately 550 kg and fitted with permanent rumen fistulas were selected as rumen fluid donors. The steers were fed total mixed ration (TMR) twice daily with free access to water. The diet composition and nutrient levels of the fistulated steers are shown in Table 3.

Table 3 Composition and nutrient levels of the diet of fistulated steers (DM basis) %

Note: The premix provided the following per kg of diet: VA 4300 IU, VD_3 *650 IU, VE 25 IU, Cu (as copper sulfate) 8 mg, Fe (as ferrous sulfate) 70 mg, Mn (as manganese sulfate) 40 mg, Zn (as zinc sulfate) 60 mg, I (as potassium iodide) 0.5 mg, Se (as sodium selenite) 0.1 mg, Co (as cobalt chloride) 0.4 mg.*

1.3 Experimental Design

A single-factor seven-level experimental design was adopted. Initially, CS was combined with RS to screen for the optimal combination, which was then recombined with CSS to screen for the optimal roughage combination, and finally recombined with CC. Each combination had three replicates. The different feed combinations and proportions are shown in Table 4.

Table 4 Different combinations and proportions of feeds %

1.4.1 Preparation of In Vitro Rumen System

The ANKOM RFS gas measurement system (ANKOM Technology Corporation, USA) was used, consisting of pressure sensor modules, 250 mL gas production bottles, an incubator, and computer software. The software interface comprises three sections: setup, real-time monitoring, and data recording. The monitoring section displays current module status and updates according to response time, while the recording section displays system data including pressure, absolute pressure, and battery capacity. The system wirelessly connects multiple vessels, with pressure information recorded in computer spreadsheets through an interactive interface.

Artificial rumen buffer solution was prepared according to Goering and Van Soest. A mixture was prepared containing 520.2 mL distilled water, 0.1 mL trace element solution (A), 208.1 mL buffer solution (B), 208.1 mL macro element solution (C), and 1.0 mL resazurin solution (D) in a glass bottle with a stopper. CO_2 gas was continuously infused while the solution was preheated to 39°C in a water bath. Immediately before use, 62.4 mL reducing agent solution (E) was added and CO_2 infusion continued until the buffer changed from light blue to nearly colorless.

The preparation methods for solutions A, B, C, D, and E were as follows:

Solution A (Trace elements): 13.2 g $CaCl_2 \cdot 2H_2O$, 10.0 g $MnCl_2 \cdot 4H_2O$, 1.0 g $CoCl_2 \cdot 6H_2O$, and 8.0 g $FeCl_3 \cdot 6H_2O$ were dissolved in distilled water and brought to 1000 mL.

Solution B (Buffer): 4.0 g NH_4HCO_3 and 35.0 g $NaHCO_3$ were dissolved in distilled water and brought to 1000 mL.

Solution C (Macro elements): 5.7 g Na_2HPO_4, 6.2 g KH_2PO_4, and 6 g $MgSO_4 \cdot 7H_2O$ were dissolved in distilled water and brought to 1000 mL.

Solution D (Resazurin): 0.1% (m/V) solution prepared by dissolving 100.0 mg resazurin in distilled water and bringing to 100 mL.

Solution E (Reducing agent, prepared fresh): 625.0 mg cysteine hydrochloride was dissolved in 95 mL distilled water, then 4 mL of 1 mol/L NaOH solution and 625.0 mg $Na_2S \cdot 9H_2O$ were added and the volume was brought to 100 mL with distilled water.

On the day of each trial, 1000 mL of rumen fluid was collected from donor steers before morning feeding, placed in a pre-warmed thermos bottle flushed with CO_2, immediately sealed, and rapidly transported to the laboratory. The rumen fluid was mixed thoroughly, filtered through four layers of cheesecloth into a receiving bottle, and maintained at 39°C in a water bath with continuous CO_2 infusion to ensure anaerobic conditions.

Exactly 1 g of fermentation substrate was weighed into each 250 mL gas production bottle, which was preheated in a 39°C incubator for 30-60 minutes. The filtered rumen fluid was then mixed with the prepared artificial rumen buffer solution at a 1:4 volume ratio, and 150 mL of this mixture was accurately measured into each preheated gas production bottle (with continuous CO_2 infusion during the operation). Each bottle was flushed with CO_2 for an additional 2 minutes to ensure anaerobic conditions before immediately sealing with the corresponding sensor module. All bottles were incubated at (39.0$±$0.5)°C in a shaking water bath for 48 hours of in vitro fermentation, with blank bottles included as controls.

1.4.2 Determination of Feed Nutrient Levels

Nutrient levels were determined according to Zhang Liying’ s “Feed Analysis and Feed Quality Detection Technology.” Crude protein (CP) content was measured by the Kjeldahl method using a FOSS Kjeltec analyzer (Denmark). Ether extract (EE) was determined by Soxhlet extraction using a Soxhlet apparatus. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed by the Van Soest method using an ANKOM A2000i automated fiber analyzer (USA). Calcium content was determined by potassium permanganate titration using an electric furnace, muffle furnace, and crucible. Phosphorus content was measured by molybdenum yellow colorimetry using a UV-2102 PCS UV-Vis spectrophotometer.

1.4.3 Determination of Gas Production

Gas production was measured using the ANKOM RFS gas measurement system (USA), which automatically records pressure generated in fermentation bottles and converts pressure to gas volume. Based on the ideal gas law, gas production was calculated as:

$$V_x = V_j \times P_{psi} \times 0.068004084$$

where V_x is the gas volume at 39°C (mL), V_j is the headspace volume of the gas production bottle (mL), and P_{psi} is the pressure automatically recorded by the gas measurement system.

Gas volumes were recorded at 2, 4, 8, 12, 24, 36, and 48 hours of incubation. The recorded values were corrected by subtracting the gas production of blank fermentation bottles.

1.4.4 Determination of Dry Matter Disappearance Rate (DMD)

After 48 hours of in vitro fermentation, bottles were immediately placed in crushed ice to terminate fermentation. The contents were filtered through preweighed nylon cloth (50 m), and the bottles were rinsed several times with distilled water to ensure no residue remained. The filtered rumen fluid was collected in a receiving bottle, and the nylon cloth was carefully transferred to an oven and dried at 65°C for 48 hours to constant weight.

$$DMD(\%) = \frac{\text{Initial DM weight} - \text{Residual DM weight}}{\text{Initial DM weight}} \times 100$$

1.4.5 pH Determination

pH was measured using a UB-7 pH meter.

1.4.6 Determination of Ammonia Nitrogen (NH_3-N) Concentration

NH_3-N concentration was determined by the colorimetric method of Feng Zongci et al. Ten milliliters of post-fermentation rumen fluid were centrifuged at 3500-4000 r/min for 10 minutes. Two milliliters of supernatant were transferred to a 15 mL test tube, mixed with 8 mL of 0.2 mol/L HCl, and measured using a UV-2102 PCS UV-Vis spectrophotometer.

1.4.7 Determination of Microbial Crude Protein (MCP) Concentration

MCP was isolated by differential centrifugation according to Cotta and Russell. Post-fermentation rumen fluid was filtered through 40-60 m nylon cloth, and 25 mL was centrifuged at 150×g for 15 minutes at 39°C to remove protozoa and large feed particles. Twenty milliliters of supernatant were then centrifuged at 16,000×g for 20 minutes at 4°C to separate bacteria. The supernatant was discarded, and the pellet was washed twice with 15 mL of 0.85% saline solution. The final bacterial pellet was carefully transferred to a digestion tube, and MCP concentration was determined by the Kjeldahl method using a FOSS Kjeltec analyzer.

1.4.8 Determination of Volatile Fatty Acid (VFA) Concentration

VFA concentration was determined by gas chromatography according to Wang Jiaqi. Five milliliters of post-fermentation rumen fluid were centrifuged at 10,000×g for 10 minutes. One and a half milliliters of supernatant were transferred to a centrifuge tube, mixed with 0.15 mL of 25% metaphosphoric acid, vortexed, allowed to stand for 30 minutes, and centrifuged again at 10,000×g for 15 minutes. The supernatant was analyzed using an Agilent 7890A gas chromatograph (USA).

1.4.9 Calculation of Associative Effects Indices

Single factor associative effects index (SFAEI) and multiple factors associative effects index (MFAEI) were calculated according to the method used by Wang Xu:

$$\text{Weighted estimate} = (\text{Actual measured value of feed A} \times \text{proportion}) + (\text{Actual measured value of feed B} \times \text{prop}$$

$$SFAEI = \frac{\text{Measured value after combination} - \text{Weighted estimate}}{\text{Weighted estimate}}$$

$$MFAEI = \sum \text{Single factor associative effects values}$$

1.5 Data Processing and Analysis

Experimental data were initially processed using Excel 2007 and then analyzed using the General Linear Model procedure in SPSS 19.0 software.

2.1.1 Effects of Different Combination Ratios on Gas Production

As shown in Table 5, gas production increased with incubation time for all CS and RS combinations, and at each time point, gas production decreased as the proportion of CS decreased. At all time points, the 100.0:0 CS:RS ratio produced significantly or extremely significantly higher gas production than other ratios ($P<0.05$ or $P<0.01$). At 48 hours of fermentation, gas production decreased significantly as the RS proportion increased ($P<0.01$).

Table 5 Effects of different proportions of CS and RS on gas production at different in vitro fermentation times

Note: Values in the same column with different lowercase superscripts differ significantly (P<0.05), while different uppercase superscripts differ extremely significantly (P<0.01). The same or no superscripts indicate no significant difference (P>0.05). The same applies to subsequent tables.

2.1.2 Effects of Different Combination Ratios on Fermentation Parameters

As shown in Table 6, DMD and concentrations of MCP and NH_3-N all increased with increasing CS proportion in the combinations. DMD differed significantly or extremely significantly among all combinations ($P<0.05$ or $P<0.01$). MCP and NH_3-N concentrations did not differ significantly between the 100.0:0 and 80.0:20.0 ratios ($P>0.05$), but differed significantly or extremely significantly among other combinations ($P<0.05$ or $P<0.01$). pH values did not differ significantly among combinations ($P>0.05$), ranging from 6.72 to 6.85.

Table 6 Effects of different proportions of CS and RS on DMD, pH and concentrations of MCP and NH_3-N after 48 h in vitro fermentation

As shown in Table 7, acetic acid, propionic acid, and total volatile fatty acid (TVFA) concentrations showed a trend of initial increase followed by decrease with increasing RS proportion, reaching maximum values of 55.30, 38.97, and 104.90 mmol/L, respectively, at the 60.0:40.0 ratio. TVFA concentration was significantly or extremely significantly higher than other combinations ($P<0.05$ or $P<0.01$). Butyric acid concentration generally increased with RS proportion, reaching a maximum of 11.94 mmol/L at the 50.0:50.0 ratio. The acetic acid/propionic acid ratio did not differ significantly among combinations ($P>0.05$).

Table 7 Effects of different proportions of CS and RS on VFA concentrations after 48 h in vitro fermentation

2.1.3 Associative Effects of CS and RS

As shown in Table 8, SFAEI evaluation revealed negative associative effects for gas production, DMD, MCP, and NH_3-N at CS:RS ratios of 40.0:60.0 and 20.0:80.0, with positive effects observed at other ratios. Conversely, pH showed positive associative effects at 40.0:60.0 and 20.0:80.0, with a maximum value of 0.0037. Except for butyric acid showing a negative effect at 80.0:20.0, acetic acid, propionic acid, and butyric acid all showed positive associative effects across combinations. MFAEI evaluation showed that the effect value initially increased then decreased with decreasing CS proportion, reaching a maximum at the 60.0:40.0 ratio. Therefore, the optimal CS:RS ratio was determined to be 60.0:40.0.

Table 8 Associative effects of CS and RS

2.2.1 Effects of Different Combination Ratios on Gas Production

As shown in Table 9, gas production increased with incubation time. Different CS-RS:CSS ratios had extremely significant effects on gas production at all time points ($P<0.01$). As the CSS proportion increased, gas production initially decreased then slightly increased.

Table 9 Effects of different proportions of CS-RS and CSS on gas production at different in vitro fermentation times

Note: The proportion in CS-RS was 60.0:40.0. The same applies to Tables 10, 11, and 12.

2.2.2 Effects of Different Combination Ratios on Fermentation Parameters

As shown in Table 10, different CS-RS:CSS ratios had extremely significant effects on DMD and concentrations of MCP and NH_3-N ($P<0.01$). DMD decreased with increasing CSS proportion, while MCP concentration increased. NH_3-N concentration showed a decreasing then increasing trend, ranging from 19.88 to 24.95 mg/dL. pH values did not differ significantly among combinations ($P>0.05$).

Table 10 Effects of different proportions of CS-RS and CSS on DMD, pH and concentrations of MCP and NH_3-N after 48 h in vitro fermentation

As shown in Table 11, different CS-RS:CSS ratios had significant or extremely significant effects on TVFA, individual VFA concentrations, and acetic acid/propionic acid ratio ($P<0.05$ or $P<0.01$). Acetic acid concentration reached a maximum of 39.30 mmol/L at the 0:100.0 ratio, which was extremely significantly higher than at 50.0:50.0 ($P<0.01$). Propionic acid and TVFA concentrations peaked at the 60.0:40.0 ratio (34.05 and 82.96 mmol/L, respectively). Butyric acid concentration and acetic acid/propionic acid ratio ranged from 9.67 to 12.09 mmol/L and 1.14 to 1.98, respectively.

Table 11 Effects of different proportions of CS-RS and CSS on VFA concentrations after 48 h in vitro fermentation

2.2.3 Associative Effects of CS-RS and CSS

As shown in Table 12, SFAEI evaluation revealed positive associative effects for gas production and NH_3-N at CS-RS:CSS ratios of 40.0:60.0 and 20.0:80.0, with negative effects at other ratios. DMD, pH, MCP, and propionic acid showed positive associative effects across all combinations, while butyric acid showed negative effects throughout. Acetic acid showed positive effects at 80.0:20.0 and 60.0:40.0, with negative effects at other ratios. MFAEI evaluation identified a maximum value at the 40.0:60.0 ratio. Therefore, the optimal CS-RS:CSS ratio was determined to be 40.0:60.0.

Table 12 Associative effects of CS-RS and CSS

2.3.1 Effects of Different Combination Ratios on Gas Production

As shown in Table 13, gas production increased with incubation time. For different combinations, gas production at each time point increased with increasing CC proportion, with extremely significant differences among combinations ($P<0.01$).

Table 13 Effects of different proportions of CS-RS-CSS and CC on gas production at different in vitro fermentation times

Note: The proportion in CS-RS-CSS was 24.0:16.0:60.0. The same applies to Tables 14, 15, and 16.

2.3.2 Effects of Different Combination Ratios on Fermentation Parameters

As shown in Table 14, different CS-RS-CSS:CC ratios had extremely significant effects on DMD, pH, and concentrations of MCP and NH_3-N ($P<0.01$). DMD and concentrations of MCP and NH_3-N all increased with increasing CC proportion, ranging from 62.78% to 82.08%, 30.42 to 43.55 mg/dL, and 21.78 to 27.48 mg/dL, respectively. pH decreased with increasing CC proportion, ranging from 6.57 to 6.80.

Table 14 Effects of different proportions of CS-RS-CSS and CC on DMD, pH and concentrations of MCP and NH_3-N after 48 h in vitro fermentation

As shown in Table 15, different CS-RS-CSS:CC ratios had significant or extremely significant effects on TVFA, individual VFA concentrations, and acetic acid/propionic acid ratio ($P<0.05$ or $P<0.01$). Acetic acid concentration and acetic acid/propionic acid ratio both decreased with increasing CC proportion. Propionic acid concentration increased with CC proportion, reaching a maximum of 29.82 mmol/L. Butyric acid concentration did not differ significantly among combinations ($P>0.05$). TVFA concentration decreased with increasing CC proportion, with the 100.0:0 ratio showing 9.08% higher TVFA than the 0:100.0 ratio ($P>0.05$).

Table 15 Effects of different proportions of CS-RS-CSS and CC on VFA concentrations after 48 h in vitro fermentation

2.3.3 Associative Effects of CS-RS-CSS and CC

As shown in Table 16, SFAEI evaluation revealed positive associative effects for gas production and MCP at CS-RS-CSS:CC ratios of 40.0:60.0 and 20.0:80.0. pH, propionic acid, and butyric acid showed positive associative effects across all combinations. DMD showed negative effects at 80.0:20.0 and 60.0:40.0, while NH_3-N showed negative effects only at 80.0:20.0, with positive effects at other ratios. SFAEI for acetic acid ranged from -0.0815 to 0.0237. MFAEI evaluation identified a maximum value of 0.1116 at the 40.0:60.0 ratio. Therefore, the optimal CS-RS-CSS:CC ratio was determined to be 40.0:60.0.

Table 16 Associative effects of CS-RS-CSS and CC

3.1 Effects of Different Roughage and CC Combinations on Gas Production

Higher gas production indicates greater rumen microbial activity and more complete substrate fermentation, while lower gas production results from insufficient fermentable substrates for microbial growth. In this study, gas production from all three feed combinations increased with fermentation time, consistent with the findings of Zerbini et al. Furthermore, gas production increased with increasing CS proportion in CS-RS combinations and with increasing CC proportion in CS-RS-CSS-CC combinations. Except at 2 and 4 hours, gas production in CS-RS-CSS combinations increased with CSS proportion. This may be because CS, CSS, and CC contain readily fermentable substrates and relatively high crude protein content, whereas RS has low crude protein content insufficient to supply microbial nitrogen requirements, leading to decreased gas production with increasing RS proportion. These results are similar to those of Zhang et al., who reported increased gas production due to relatively high crude protein content in fermentation substrates providing adequate fermentable substrates.

3.2 Effects of Different Roughage and CC Combinations on In Vitro Fermentation Parameters

DMD is an indicator of the ability of ruminants to digest and utilize organic matter in diets, with dietary fiber content being a key factor affecting feed degradability. In this study, DMD of different feed combinations showed different patterns according to substrate composition, decreasing with decreasing CS proportion, decreasing with increasing CSS proportion, and increasing with increasing CC proportion. This may be because CS contains fiber levels more suitable for rumen microbial growth than RS, and CS-RS combinations are more favorable for microbial fermentation than CSS alone. In roughage-CC combinations, increasing CC proportion improved rumen microbial digestibility of feed dry matter, indicating enhanced microbial growth.

pH is a critical factor for normal rumen fermentation, with the optimal range being 6-7. In this study, pH values of all feed combinations fell within this suitable range, similar to the results of Chen et al. on feed associative effects.

NH_3-N concentration is a primary limiting factor for MCP synthesis. With adequate carbon sources, appropriate NH_3-N concentration ensures normal microbial growth and reproduction. Excessively high or low NH_3-N concentrations are detrimental—high concentrations waste nitrogen sources, while low concentrations reduce microbial activity and MCP synthesis. In this study, NH_3-N concentrations ranged from 19.88 to 31.59 mg/dL, within the range reported in the literature, indicating concentrations suitable for normal microbial growth, consistent with the findings of Deli et al.

MCP synthesis depends critically on energy and protein supply, with MCP concentration reflecting rumen microbial population size and indicating microbial growth rate and activity. In this study, MCP concentrations increased with increasing proportions of CS, CSS, and CC, likely because these feeds have higher nutrient content compared to others, providing relatively abundant nutrient sources for rumen microbes.

VFAs are essential components of rumen metabolism, providing 70%-80% of total energy requirements and reflecting microbial activity as a key indicator of rumen fermentation. Acetic acid is the primary precursor for milk fat synthesis, while propionic acid is a glucose precursor, making increased propionic acid concentration significant for supplying energy and improving weight gain efficiency in ruminants. VFA composition mainly depends on dietary carbohydrate proportions, with amounts and types affecting rumen fermentation environment and consequently VFA concentrations. In this study, acetic acid concentrations were higher than propionic acid across combinations, possibly because ruminants absorb VFAs in the order of butyric acid > propionic acid > acetic acid, and rumen VFA concentrations are not affected by dietary type, consistent with Copani et al. Additionally, the optimal roughage-CC combination showed decreasing acetic acid and increasing propionic acid concentrations with increasing CC proportion, resulting in decreased acetic acid/propionic acid ratio, consistent with Sun et al. This may be because high-CC diets alter rumen microbial flora and fermentation patterns of culture substrates.

3.3 Effects of Different Roughage and CC Combinations on Associative Effects Indices

SFAEI evaluates feed associative effects based on a single indicator, making comprehensive assessment difficult, whereas MFAEI integrates multiple indicators for more representative evaluation. In this study, MFAEI for CS-RS combinations showed positive associative effects across all ratios, with maximum positive effect at 60.0:40.0. For the optimal CS-RS-CSS combination, MFAEI peaked at 40.0:60.0. For the optimal roughage-CC combination, MFAEI also peaked at 40.0:60.0. These positive associative effects likely result from appropriate proportions of different feeds interacting to improve overall substrate fermentation and maximize feed digestibility and utilization, similar to the findings of Yu et al.

Conclusions: 1. The optimal combination ratio of CS and RS is 60.0:40.0. 2. The optimal combination ratio of CS, RS, and CSS is 24.0:16.0:60.0. 3. The suitable combination ratio of CS, RS, CSS, and CC is 9.6:6.4:24.0:60.0.

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