Evaluation of the Combined Effects of Mulberry Leaves and Leymus chinensis Using In Vitro Rumen Fermentation: Postprint

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Abstract

This experiment aimed to investigate the associative effects between different ratios of mulberry leaves and leymus grass using in vitro rumen fermentation method, thereby screening for the optimal combination ratio between the two. Mulberry leaves and leymus grass were mixed at ratios of 0:100 (T0 group), 20:80 (T20 group), 40:60 (T40 group), 60:40 (T60 group), 80:20 (T80 group), and 100:0 (T100 group) as substrates for continuous 72-h in vitro gas production incubation and in vitro batch culture experiments. The results showed that: 1) With increasing proportion of mulberry leaves in the substrate, theoretical gas production, cumulative gas production, microbial crude protein (MCP) concentration in the culture fluid, and in vitro organic matter digestibility (IVDOM) all exhibited an increasing trend; 2) At 72 h, the theoretical maximum gas production, cumulative gas production, and IVDOM of the T100 group were significantly higher than those of other groups ($P<0.05$), and the MCP concentration in culture fluid of T60 and T100 groups was significantly higher than that of other groups ($P<0.05$); 3) At 48 and 72 h, the culture fluid pH of T60, T80, and T100 groups was significantly lower than that of other groups ($P<0.05$); 4) At 72 h, there were no significant differences in total volatile fatty acids (TVFA) and acetate concentrations in culture fluid among T60, T80, and T100 groups ($P>0.05$), but all were significantly higher than those of other groups ($P<0.05$); 5) At 6, 12, and 72 h, the ammonia nitrogen (NH_3-N) concentration in culture fluid of T60, T80, and T100 groups was significantly higher than that of other groups ($P<0.05$); 6) The multiple factor associative effect index (MFAEI) of the T60 group was higher than that of other groups. In conclusion, the combination of mulberry leaves and leymus grass can improve in vitro rumen fermentation characteristics, indicating positive associative effects, with the optimal ratio of mulberry leaves to leymus grass being 60:40.

Full Text

Evaluation of Associative Effects of Mulberry Leaves and *Leymus chinensis* by *In Vitro* Ruminal Fermentation Method

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Abstract: This experiment aimed to investigate the associative effects of different proportions of mulberry leaves (ML) and *Leymus chinensis* (LC) using the *in vitro* ruminal fermentation method, and to identify the optimal combination ratio. ML and LC were mixed at ratios of 0:100 (T0 group), 20:80 (T20 group), 40:60 (T40 group), 60:40 (T60 group), 80:20 (T80 group), and 100:0 (T100 group) and used as fermentation substrates for continuous 72-hour *in vitro* gas production and batch culture experiments. The results showed: 1) Theoretical gas production, accumulated gas production, microbial crude protein (MCP) concentration in culture fluid, and *in vitro* digestibility of organic matter (IVDOM) all exhibited increasing trends as the proportion of ML in the substrate increased. 2) At 72 h, the theoretical maximum gas production, accumulated gas production, and IVDOM in the T100 group were significantly higher than those in all other groups ($P<0.05$), while the MCP concentration in culture fluid in the T60 and T100 groups was significantly higher than in other groups ($P<0.05$). 3) At 48 and 72 h, culture fluid pH in the T60, T80, and T100 groups was significantly lower than in the T0, T20, and T40 groups ($P<0.05$). 4) At 72 h, total volatile fatty acid (TVFA) and acetate concentrations in the T60, T80, and T100 groups showed no significant differences among themselves ($P>0.05$), but were all significantly higher than those in other groups ($P<0.05$). 5) At 6, 12, and 72 h, NH -N concentration in culture fluid in the T60, T80, and T100 groups was significantly higher than in other groups ($P<0.05$). 6) The multiple-factor associative effect index (MFAEI) was highest in the T60 group. In conclusion, the combination of ML and LC can improve *in vitro* ruminal fermentation characteristics, demonstrating positive associative effects, with the optimal ratio being 60:40.

Keywords: mulberry leaves; *Leymus chinensis*; *in vitro* ruminal fermentation method; associative effect

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Mulberry leaves (ML) are the leaves of *Morus alba* L., a traditional Chinese plant used for both medicinal and food purposes. ML can serve as a high-quality livestock feed due to their high crude protein content (approximately 20% of dry matter), good palatability, high digestibility, and rich content of trace elements, vitamins, and appropriately proportioned amino acids. Additionally, ML contain bioactive substances such as flavonoids, polysaccharides, phytosterols, and -aminobutyric acid, as well as functional nutrients like triterpenoids and coumarin that benefit rumen microbial growth, maintain a healthy and stable rumen environment, and enable more efficient degradation of dietary components. Mulberry trees are widely cultivated across China, but many mulberry plantations in southern regions have been abandoned due to rising labor costs and the decline of the sericulture industry, resulting in wasted ML resources. *Leymus chinensis* (LC), also known as alkaline grass, is an excellent gramineous forage that produces dark green, aromatic, and nutrient-rich hay when dried. The associative effect of dietary components refers to the overall interaction among nutritional, non-nutritional, and anti-nutritional substances from different feed sources. Enhancing positive associative effects between feeds can improve feed utilization efficiency and reduce production costs. Menke et al. from the University of Hohenheim first developed the *in vitro* syringe fermentation gas production method to evaluate feed nutritional value, which has been widely applied due to its simplicity, economy, and rapidity. Current research on ML has primarily focused on their use as protein feed substitutes and extraction of active components, with limited studies investigating their direct utilization as roughage or potential associative effects with LC. Therefore, this study employed artificial rumen technology to mix ML and LC at different ratios as fermentation substrates, measuring gas production parameters and fermentation indices to evaluate their associative effects and determine the optimal combination ratio for use as ruminant roughage.

1.1 Experimental Materials

The ML and LC used in this experiment were provided by the Sericulture Research Institute of the Chinese Academy of Agricultural Sciences and the Experimental Farm of Yangzhou University, respectively. After collection, the samples were dried at 65°C to produce air-dried samples, which were then ground and passed through a 40-mesh sieve for storage. Dry matter (DM) and crude ash content were determined according to the methods described in *Feed Analysis and Feed Quality Detection Technology* edited by Zhang Liying. Crude ether extract (EE) content was measured following GB/T 6433-2006 *Determination of Crude Fat in Feeds*. Crude protein (CP) content was determined using the Kjeldahl method, while neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed using the Van Soest method.

1.2 Experimental Animals and Rumen Fluid Collection

Four healthy Saanen goats with similar body weight and permanent rumen fistulas were selected as rumen fluid donors. The goats were fed oat hay supplemented with concentrate (corn:soybean meal = 6:4) twice daily (08:00 and 17:00) with free access to water. On the experimental day, before morning feeding, rumen fluid was collected from three fistulated goats using a self-made vacuum negative pressure device. The fluid was immediately mixed, filtered through four layers of cheesecloth, and placed in a pre-warmed (39°C) thermos flask filled with CO . The flask was transported to the laboratory and kept in a 39°C water bath with continuous CO infusion until inoculation.

1.3 Artificial Saliva Preparation

Artificial saliva was prepared according to the method of Cone et al. The solution contained 8.75 g NaHCO , 1.00 g NH HCO , 1.43 g Na HPO , 1.55 g KH PO , 0.15 g MgSO · 7H O, 0.52 g Na S, 0.015 g MnCl · 4H O, 0.002 g CoCl · 6H O, 0.012 g FeCl · 6H O, 0.017 g CaCl · 2H O, and 1.25 mg resazurin dissolved in 1 L distilled water. Before inoculation, the solution was preheated in a 39°C water bath and slowly infused with high-purity CO until pH reached 6.8.

1.4 *In Vitro* Fermentation Experiments

1.4.1 *In Vitro* Gas Production Test ML and LC were mixed at ratios of 0:100 (T0), 20:80 (T20), 40:60 (T40), 60:40 (T60), 80:20 (T80), and 100:0 (T100) as fermentation substrates (nutrient levels shown in Table 1). Approximately 0.5 g of each mixed substrate was weighed into separate 100 mL fermentation bottles with seven replicates per group. Fifty milliliters of artificial saliva and 25 mL of rumen fluid were rapidly injected into each bottle, followed by CO infusion for approximately 5 seconds before immediately sealing with rubber stoppers. The bottles were connected to a 64-channel AGRS-III automatic gas production recording system and incubated at 39°C for 72 hours.

1.4.2 *In Vitro* Batch Culture Test Artificial saliva and substrates were prepared as described above, with an additional blank group containing only rumen fluid and artificial saliva. Each group had 18 replicates and was incubated in a SHA-A thermostatic oscillating water bath for 72 hours. Samples were collected at 0, 3, 6, 12, 24, 48, and 72 hours, with three fermentation bottles removed from each group at each time point. The entire contents were filtered through filter bags to collect residues and culture fluid. The culture fluid was aliquoted into two 10 mL centrifuge tubes and three 2 mL centrifuge tubes, then stored at -80°C for subsequent analysis of volatile fatty acids (VFA), ammonia nitrogen (NH -N), and microbial crude protein (MCP) concentrations. The residues were washed, dried, and used for IVDOM determination.

1.5 Measurement Methods

1.5.1 Gas Production Calculation Gas production data were fitted using the nonlinear regression model proposed by Ørskov et al.:

$$GP_t = a + b \times (1 - e^{-ct})$$

where GP_t is the accumulated gas production at time t (mL/g DM), a is the rapidly fermentable fraction (gas production at initial time, mL/g DM), b is the slowly fermentable fraction (theoretical maximum gas production, mL/g DM), c is the gas production rate constant (h ^{1}), and $a + b$ represents the potential gas production (mL/g DM).

1.5.2 VFA Concentration Determination VFA concentrations were measured by gas chromatography according to Khorasani et al. Culture fluid was centrifuged at 10,000×g for 10 minutes, and 1 mL of supernatant was mixed with 0.2 mL of 20% metaphosphoric acid containing 60 mmol/L crotonic acid. After mixing and centrifugation at 10,000×g, 0.4 L of supernatant was injected into a gas chromatograph (GC-9A, Shimadzu, Japan) for analysis. Total VFA (TVFA) concentration was calculated as the sum of acetate, propionate, and butyrate concentrations.

1.5.3 NH -N Concentration Determination NH -N concentration was determined using a colorimetric method. Four milliliters of culture fluid were centrifuged at 400×g for 10 minutes, and 50 L of supernatant was transferred to a 10 mL test tube. Phenol reagent (prepared by dissolving 9.9757 g phenol and 50.65 mg sodium nitroprusside in water and diluting to 1000 mL) and sodium hypochlorite reagent (prepared by dissolving 5 g NaOH in a small amount of distilled water, cooling, adding 20 mL sodium hypochlorite, mixing, and diluting to 1000 mL) were added sequentially (3 mL each). After mixing, the solution was heated in a 60°C water bath for 10 minutes, immediately cooled in cold water, and the absorbance at 546 nm (OD) was measured using a 756 UV-Vis spectrophotometer.

1.5.4 MCP Concentration Determination MCP concentration in culture fluid was determined according to the method of Su Haiya. Standard curve preparation: 1) Weigh 5, 15, 25, 35, 45, and 55 mg of yeast RNA into 10 mL centrifuge tubes, add 2 mL of 0.6 mol/L perchloric acid (HClO), and heat in a 90-95°C water bath for 1 hour, then cool; 2) Add 6 mL of 28.5 mmol/L ammonium dihydrogen phosphate (NH H PO) solution to each tube, heat at 90-95°C for 15 minutes, cool, and centrifuge at 3,000×g for 10 minutes; 3) Transfer 1.6 mL of supernatant, add 6 mL of 0.2 mol/L NH H PO solution, and adjust pH to 2-3 with 85% phosphoric acid; 4) Take 3.8 mL of the pH-adjusted solution, add 0.2 mL of 0.4 mol/L silver nitrate (AgNO) solution, mix, and store overnight at 5°C in the dark; 5) After overnight storage, centrifuge at 3,000×g for 10 minutes, discard supernatant, wash precipitate with 4.5 mL distilled water (pH 2), centrifuge again at 3,000×g for 10 minutes, and discard supernatant; 6) Add 5 mL of 0.5 mol/L HCl to the precipitate, mix, and heat in a 90-95°C water bath for 30 minutes, then centrifuge at 3,000×g for 10 minutes; 7) Dilute the supernatant 40-fold with 0.5 mol/L HCl, use 0.5 mol/L HCl as reference, measure absorbance at 260 nm, and prepare the standard curve based on absorbance values.

MCP concentration in culture fluid: Transfer 8 mL of culture fluid to three 10 mL centrifuge tubes, centrifuge at 20,000×g for 20 minutes, discard supernatant, add 2.104 mL of 0.6 mol/L HClO , heat in a 90-95°C water bath for 1 hour, then cool. Follow steps 2-6 of the standard curve preparation. Use 0.5 mol/L HCl as reference, measure absorbance at 260 nm, determine RNA content from the standard curve, and calculate MCP concentration using the following formula:

$$\text{MCP concentration (mg/mL)} = \frac{\text{RNA measured (mg/mL)} \times \text{RNA N content (17.83\%)} \times \text{dilution factor} \times 6.25}{\text{Bacterial RNA N content (10\%)}}$$

1.5.5 IVDOM and Culture Fluid pH Determination IVDOM was determined according to the method in *Feed Analysis and Feed Quality Detection Technology* edited by Zhang Liying, calculated as:

$$\text{IVDOM (\%)} = 100 \times \frac{[m_1 - (m_2 - m_3)]}{m_1}$$

where m_1 is the organic matter content of the substrate (g), m_2 is the organic matter content of the residue (g), and m_3 is the organic matter content of the blank group residue (g). Culture fluid pH was measured using a pHS-3C pH meter.

1.5.6 Associative Effect Evaluation Formulas Weighted estimate = (T0 group measured value × LC proportion %) + (T100 group measured value × ML proportion %) [13];

Single-factor associative effect index (SFAEI, %) = 100 × (measured value - weighted estimate) / weighted estimate [13];

Multiple-factor associative effect index (MFAEI, %) = ΣSFAEI = SFAEI_{accumulated gas production} + SFAEI_{IVDOM} + SFAEI_{MCP} + SFAEI_{NH_3-N} + SFAEI_{TVFA} [14].

1.6 Statistical Analysis

All experimental data were organized using Excel 2016 and analyzed using one-way ANOVA in SPSS 22.0 software. Multiple comparisons were performed using the LSD method, with $P<0.05$ considered statistically significant.

2.1 Effects of Different ML and LC Ratios on 72 h *In Vitro* Fermentation Gas Production Parameters

As shown in Table 2 , theoretical maximum gas production, potential gas production, and accumulated gas production showed increasing trends with increasing ML proportion. The T100 group exhibited significantly higher theoretical maximum gas production, potential gas production, and accumulated gas production compared to all other groups (P<0.05), while no significant differences were observed among the T0, T20, and T40 groups for these three parameters (P>0.05).

2.2 Effects of Different ML and LC Ratios on 72 h *In Vitro* Culture Fluid pH

Table 3 shows that culture fluid pH decreased over time. No significant differences in pH were observed among groups at 3, 6, and 12 h (P>0.05). At 24 h, the T100 group had significantly lower pH than the T0, T20, and T40 groups (P<0.05). At 48 and 72 h, the T60, T80, and T100 groups all showed significantly lower pH compared to the T0, T20, and T40 groups (P<0.05).

2.3 Effects of Different ML and LC Ratios on 72 h *In Vitro* Culture Fluid VFA Concentrations

Table 4 indicates that acetate and propionate concentrations generally increased initially then varied over time. At 12 and 48 h, the T80 group had significantly higher acetate and TVFA concentrations than all other groups (P<0.05). At 72 h, no significant differences in acetate and TVFA concentrations were found among the T60, T80, and T100 groups (P>0.05), but all were significantly higher than other groups (P<0.05). At 12 and 48 h, propionate concentrations in the T80 and T100 groups were significantly higher than in other groups (P<0.05). At 12 and 72 h, the acetate/propionate ratio in the T60 and T80 groups was significantly higher than in other groups (P<0.05).

2.4 Effects of Different ML and LC Ratios on 72 h *In Vitro* Culture Fluid NH -N Concentration

Table 5 shows that NH -N concentration increased over time in all groups. At 3 h, the T40, T60, T80, and T100 groups had significantly higher NH -N concentrations than the T0 group (P<0.05). At 6, 12, and 72 h, the T60, T80, and T100 groups exhibited significantly higher NH -N concentrations than the T0, T20, and T40 groups (P<0.05). At 24 h, the T60 group showed significantly higher NH -N concentration than all other groups (P<0.05). At 48 h, the T80 group had significantly higher NH -N concentration than the T0, T20, T40, and T60 groups (P<0.05).

2.5 Effects of Different ML and LC Ratios on 72 h *In Vitro* Culture Fluid MCP Concentration

Table 6 reveals that at 12 and 48 h, no significant differences in MCP concentration were observed among the T60, T80, and T100 groups (P>0.05). At 72 h, the T60 and T100 groups showed no significant difference in MCP concentration between them (P>0.05), but both were significantly higher than other groups (P<0.05). At 6 and 24 h, the T100 group had significantly higher MCP concentration than all other groups (P<0.05).

2.6 Effects of Different ML and LC Ratios on 72 h *In Vitro* IVDOM

Table 7 demonstrates that IVDOM increased over time and with increasing ML proportion. At 6, 24, 48, and 72 h, the T100 group exhibited significantly higher IVDOM than all other groups (P<0.05).

2.7 Associative Effect Indexes of Different ML and LC Ratios

Table 8 shows that negative associative effects occurred for accumulated gas production, TVFA, MCP, and NH -N SFAEI and MFAEI in the T40 group, as well as for accumulated gas production in the T20 and T80 groups and NH - N SFAEI in the T20 group. All other indexes across groups showed positive associative effects or no effect. The T60 group had higher SFAEI for MCP, TVFA, and NH -N than other groups, while SFAEI for IVDOM decreased with increasing ML proportion. The MFAEI was highest in the T60 group.

3.1 Effects of ML and LC Combination on Gas Production Parameters

Gas production reflects the fermentability of feed, determined jointly by rumen microbial degradation capacity and substrate characteristics. Crude protein in substrates is relatively easy to ferment, while NDF is not easily fermented. Nsahlai et al. found that *in vitro* accumulated gas production positively correlates with substrate CP content and negatively correlates with NDF content. In this experiment, ML had higher CP and lower NDF content than LC. As ML proportion increased, substrate CP content increased and NDF content decreased, leading to increased accumulated gas production. Liu et al. reported similar results when mixing rice straw with ML at different ratios for *in vitro* fermentation, where accumulated gas production increased with ML proportion.

3.2 Effects of ML and LC Combination on Culture Fluid pH

pH reflects rumen fermentation status to some extent, with the optimal range for rumen microbial growth being 6-7. In this experiment, VFA accumulation from *in vitro* fermentation caused culture fluid pH to decrease over time, but all values remained within the suitable range and would not affect rumen microbial activity.

3.3 Effects of ML and LC Combination on Culture Fluid VFA Concentrations

Monosaccharides derived from dietary polysaccharide degradation are rapidly converted to VFAs by rumen microbes, serving as the primary energy source for ruminants. VFA proportions and concentrations in rumen fluid relate to dietary composition; when dietary fiber content increases, TVFA concentration decreases while acetate proportion increases. Allen and Han et al. both found a strong positive correlation between acetate concentration and dietary NDF content. In this experiment, as ML proportion increased and substrate NDF content decreased, acetate and propionate concentrations first increased then decreased, with the T60 and T80 groups showing higher concentrations than other groups. This may be because ML are more fermentable than LC and can be more easily degraded by microbes, rapidly producing VFAs like acetate and propionate. The associative effect between ML and LC resulted in higher acetate and propionate concentrations in the T60 and T80 groups compared to the T0 and T100 groups, consistent with Sun Lisha' s findings on associative effects between silkworm excrement and rice straw.

3.4 Effects of ML and LC Combination on Culture Fluid NH -N Concentration

NH -N in the rumen is the primary nitrogen source for rumen microbes. Appropriate NH -N concentration promotes microbial growth, while excessive concentration wastes nitrogen and insufficient concentration limits microbial activity. At 6, 12, and 72 h, the T60, T80, and T100 groups had significantly higher NH -N concentrations than other groups. Since culture fluid NH -N mainly originates from microbial degradation of substrate nitrogenous substances, and these three groups had higher protein content in their substrates, this likely caused the elevated NH -N concentrations.

3.5 Effects of ML and LC Combination on Culture Fluid MCP Concentration

Microbial crude protein synthesis is significantly influenced by diet type. Stern et al. found that dietary carbohydrate and protein content and their synchronous degradation determine rumen microbial growth, thereby altering MCP synthesis efficiency. In this experiment, as ML proportion increased, high-quality protein content in substrates increased, leading to an upward trend in MCP concentration, consistent with Li Yan et al.' s research findings.

3.6 Effects of ML and LC Combination on IVDOM

Higher dietary crude fiber content results in lower digestibility, while higher CP content increases digestibility. Liu et al. found that IVDOM significantly correlates with dietary CP and NDF content. Menke et al. reported a strong positive correlation between IVDOM and accumulated gas production. In this experiment, as ML proportion increased, CP content increased while NDF content decreased, resulting in increased accumulated gas production and IVDOM, consistent with previous research.

3.7 Associative Effects of ML and LC

Lu Dexun proposed using MFAEI to comprehensively quantify multiple indicators from *in vitro* fermentation across multiple time points for evaluating dietary associative effects. This experiment comprehensively analyzed accumulated gas production, VFA, NH -N, MCP, and IVDOM, finding that the T60 group showed the greatest associative effect at 72 h, while the T40 group exhibited negative associative effects. This may be because MFAEI was only calculated for the 72 h time point, introducing some temporal bias and temporary negative effects that did not affect overall results. In practical production, goats receive concentrate supplementation. Since concentrate is more easily degraded by rumen microbes, it rapidly ferments into short-chain fatty acids shortly after entering the rumen, with minimal roughage degradation occurring simultaneously. When concentrate is nearly depleted, rumen microbes then utilize the more difficult-to-degrade roughage. Therefore, the optimal ML:LC ratio identified *in vitro* has practical relevance for production.

4 Conclusion

The combination of mulberry leaves and *Leymus chinensis* can improve *in vitro* ruminal fermentation characteristics, demonstrating positive associative effects, with the optimal ratio being 60:40.

References

[1] HOU Qirui, ZHAO Weiguo, WANG Mengzhi, et al. Effects of mulberry leaf powder addition on lipid metabolism and liver antioxidant capacity in rex rabbits[J]. Journal of Jiangsu University of Science and Technology (Natural Science Edition), 2017, 31(4): 550–554.

[2] ZHAO He. Study on the nutritional value of *Leymus chinensis* for dairy cows and its dietary associative effects[D]. Master's thesis. Daqing: Heilongjiang Bayi Agricultural University, 2008.

[3] ZHANG Ziyi. Chinese Feed Science[M]. Beijing: China Agriculture Press, 2000: 289–290.

[4] MENKE K H, RAAB L, SALEWSKI A, et al. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*[J]. The Journal of Agricultural Science, 1979, 93(1): 217–222.

[5] HUYEN N T, WANAPAT M, NAVANUKRAW C. Effect of mulberry leaf pellet (MUP) supplementation on rumen fermentation and nutrient digestibility in beef cattle fed on rice straw-based diets[J]. Animal Feed Science and Technology, 2012, 175(1): 8–15.

[6] JESZKA-SKOWRON M, FLACZYK E, JESZKA J, et al. Mulberry leaf extract intake reduces hyperglycaemia in streptozotocin (STZ)-induced diabetic

rats fed high-fat diet[J]. Journal of Functional Foods, 2014, 8: 9–17.

[7] ZHANG Liying. Feed Analysis and Feed Quality Detection Technology[M]. 2nd ed. Beijing: China Agricultural University Press, 2003.

[8] CONE J W, VAN GELDER A H, VISSCHER G J W, et al. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time-related gas production apparatus[J]. Animal Feed Science and Technology, 1996, 61(1-4): 113–128.

[9] YANG H J, TAMMINGA S, WILLIAMS B A, et al. *In vitro* gas and volatile fatty acids production profiles of barley and maize and their soluble and washout fractions after feed processing[J]. Animal Feed Science and Technology, 2005, 120(1-2): 125–140.

[10] ØRSKOV E R, MCDONALD I. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage[J]. The Journal of Agricultural Science, 1979, 92(2): 499–503.

[11] KHORASANI G R, OKINE E K, KENNELLY J J. Forage source alters nutrient supply to the intestine without influencing milk yield[J]. Journal of Dairy Science, 1996, 79(5): 862–872.

[12] SU Haiya. Study on associative effects between mulberry leaves and oilseed meals in ruminant diets[D]. Master' s thesis. Hangzhou: Zhejiang University, 2002.

[13] YAN Weijie. Study on associative effects between oilseed protein and *Leymus chinensis* NDF/corn starch mixtures[D]. Master' s thesis. Hangzhou: Zhejiang University, 2005.

[14] WANG Xu. Study on scientific roughage matching using GI technology and systematic optimization of sheep diet formulation[D]. Master' s thesis. Hohhot: Inner Mongolia Agricultural University, 2003.

[15] YANG Hongjian, LI Dahong, XIE Chunyuan, et al. Effects of ferulic acid esterase treatment on *in vitro* ruminal fermentation characteristics of *Leymus chinensis*, corn stover, rice straw, and wheat straw[J]. Chinese Journal of Animal Nutrition, 2010, 22(1): 207–211.

[16] NSAHLAI I V, SIAW D, OSUJI P O. The relationships between gas production and chemical composition of 23 browses of the genus *Sesbania*[J]. Journal of the Science of Food and Agriculture, 1994, 65(1): 13–20.

[17] LIU J X, SUSENBETH A, SÜDEKUM K H. *In vitro* gas production measurements to evaluate interactions between untreated and chemically treated rice straws, grass hay, and mulberry leaves[J]. Journal of Animal Science, 2002, 80(2): 517–524.

[18] FENG Yanglian. Ruminant Nutrition[M]. Beijing: Science Press, 2004: 340–360.

[19] ALLEN M S. Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber[J]. Journal of Dairy Science, 1997, 80(7): 1447-1462.

[20] HAN Jifu, FENG Yanglian, ZHANG Xiaoming, et al. Effects of diet type and *Leymus chinensis* particle size on volatile fatty acid proportion and energy conversion efficiency in beef cattle[J]. Acta Veterinaria et Zootechnica Sinica, 1998, 28(2): 97-104.

[21] SUN Lisha. Evaluation of silkworm excrement feed value and its utilization in mutton sheep feeding[D]. Master' s thesis. Yangzhou: Yangzhou University, 2015.

[22] STERN M D, VARGA G A, CLARK J H, et al. Evaluation of chemical and physical properties of feeds that affect protein metabolism in the rumen[J]. Journal of Dairy Science, 1994, 77(9): 2762-2786.

[23] LI Yan, HAN Xiaomin, LI Jianguo, et al. Evaluation of associative effects of corn stover, millet straw, and corn stover silage using *in vitro* method[J]. Acta Prataculturae Sinica, 2017, 26(5): 213-223.

[24] LIU Jie, DIAO Qiyu, TU Yan, et al. Study on prediction model of organic matter digestibility *in vitro* for mutton sheep feed[C]//Proceedings of the 2012 National Conference on Sheep Production and Academic Research of the Chinese Association of Animal Science and Veterinary Medicine Sheep Branch. Hengshan: Chinese Association of Animal Science and Veterinary Medicine, 2012: 299-302.

[25] MENKE K H, STEINGASS H. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid[J]. Animal Research and Development, 1988, 28: 7-55.

[26] LU Dexun. Introduction to Systematic Animal Nutrition[M]. Beijing: China Agriculture Press, 2004.

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