Effects of Flaxseed Cake Replacing Soybean Meal on Rumen Metabolism in Sheep: Postprint

Authors: Yu Shengchan, Hao Xiaoyan, Wu Xiaodong, Ding Na, Diao Xiaogao, Xiang Binwei, Wenjia Zhang, Zhang Jianxin

Date: 2018-12-24T00:00:00+00:00

Abstract

This study investigated the effects of replacing soybean meal with flaxseed cake on rumen metabolism in sheep by examining rumen fermentation parameters, digestive enzyme activities, and the expression levels of genes related to volatile fatty acid (VFA) absorption in rumen epithelial tissue. Twenty-four Dorper × Small-tailed Han crossbred F1 male lambs at 5 months of age with a body weight of (26.0$±$1.0) kg were selected and randomly divided into 4 groups [control (CK), , , and], with 6 lambs per group, and fed diets with flaxseed cake replacing soybean meal at inclusion levels of 0, 6%, 12%, and 18%, respectively. At the end of the experiment, the lambs were slaughtered and samples were collected, including rumen fluid and rumen tissue, to investigate rumen fermentation parameters, digestive enzyme activities, and the expression of genes related to VFA absorption in rumen epithelial tissue. The pre-experimental period was 10 d, and the formal experimental period was 60 d. The results showed that: 1) The average daily gain (ADG) of groups and showed no significant difference compared with the CK group (P>0.05), but was significantly higher than that of group (P<0.05); The feed to gain ratio (F/G) of group was significantly lower than that of the CK group (P<0.05). 2) Compared with the CK group, the activities of carboxymethyl cellulase, β-glucosidase, and protease in groups and were significantly increased (P<0.05), and the activities of xylanase and pectinase in group were significantly increased (P<0.05). 3) Compared with the CK group, replacing soybean meal with flaxseed cake had no significant effect on rumen fluid pH, propionate and lactate concentrations, or acetate to propionate ratio in sheep (P>0.05), but the concentrations of acetate, butyrate, and total volatile fatty acids (TVFA) in group were significantly increased (P<0.05); The concentrations of ammonia nitrogen (NH3-N) in groups , , and were significantly decreased (P<0.05), while the concentration of microbial crude protein (MCP) was significantly increased (P<0.05). 4) Compared with the CK group, the relative mRNA expression levels of monocarboxylate trans-

porter 1 (MCT1) and monocarboxylate transporter 4 (MCT4) in group were significantly increased (P<0.05), while the relative mRNA expression level of down-regulated in adenoma (DRA) was significantly decreased (P<0.05); The relative mRNA expression level of Na+/H+ exchanger 3 (NHE3) in group was significantly increased (P<0.05), while the relative mRNA expression levels of MCT1, MCT4, and DRA were significantly decreased (P<0.05); Replacing soybean meal with flaxseed cake had no significant effect on the mRNA expression level of anion exchanger 2 (AE2) (P>0.05). In conclusion, replacing soybean meal with flaxseed cake at a certain proportion in sheep diets can improve rumen metabolic status, and under the conditions of this experiment, the appropriate inclusion level of flaxseed cake replacing soybean meal in the diet was 12%.

Full Text

Effects of Replacement of Soybean Meal by Flaxseed Cake on Rumen Metabolism in Sheep

YU Shengchen[1], HAO Xiaoyan[1], WU Xiaodong[1], DING Na[1], DIAO Xiaogao[1], XIANG Binwei[2], ZHANG Wenjia[2], ZHANG Jianxin[1]*

[1]College of Animal Science and Technology, Shanxi Agricultural University, Taigu 030801, China
[2]Animal Husbandry Bureau of Youyu County, Youyu 037200, China

Abstract: This study investigated the effects of replacing soybean meal with flaxseed cake on rumen metabolism in sheep by examining rumen fermentation parameters, digestive enzyme activities, and expression of genes related to volatile fatty acid (VFA) absorption in rumen epithelial tissue. Twenty-four five-month-old Dorper × Small Tail Han sheep ram lambs with body weight of (26.0$±$1.0) kg were randomly assigned to four groups [control (CK), I, II, and III] of six animals each. The lambs were fed diets in which flaxseed cake replaced soybean meal at inclusion levels of 0, 6%, 12%, and 18%, respectively. At the end of the experiment, all lambs were slaughtered for collection of rumen fluid and tissue samples. The pre-trial period lasted 10 days, followed by a 60-day formal experimental period. The results showed: 1) Average daily gain (ADG) in groups I and II did not differ significantly from the CK group (P>0.05) but was significantly higher than in group III (P<0.05). The feed-to-gain ratio (F/G) in group II was significantly lower than in the CK group (P<0.05). 2) Compared with the CK group, activities of carboxymethyl cellulase, β-glucosidase, and protease were significantly increased in groups II and III (P<0.05), while xylanase and pectinase activities were significantly increased in group III (P<0.05). 3) Replacement of soybean meal with flaxseed cake had no significant effect on rumen pH, propionate concentration, or acetate-to-propionate ratio (P>0.05), but group II showed significantly increased concentrations of acetate, butyrate, and total VFA (TVFA) (P<0.05). Ammonia nitrogen (NH_3-N) concentration was significantly decreased (P<0.05) and microbial protein (MCP) concentration was significantly increased (P<0.05) in groups I, II, and III. 4) Compared

with the CK group, mRNA relative expression of monocarboxylate transporter 1 (MCT1) and monocarboxylate transporter 4 (MCT4) was significantly increased in group II ($P<0.05$), while down-regulated in adenoma (DRA) mRNA expression was significantly decreased ($P<0.05$). In group III, Na^+/H^+ exchanger 3 (NHE3) mRNA expression was significantly increased ($P<0.05$), but MCT1, MCT4, and DRA mRNA expression were significantly decreased ($P<0.05$). No significant effect was observed on anion exchanger 2 (AE2) mRNA expression ($P>0.05$). In conclusion, replacing soybean meal with flaxseed cake at an appropriate proportion can improve rumen metabolism in sheep, with the optimal inclusion level being 12% under the conditions of this study.

Keywords: flaxseed cake; lambs; rumen fermentation; enzyme activity; VFA-related gene expression

Introduction

Shanxi Province is one of the major flax-producing regions in China, with a total output of 63,000 tons. Flaxseed cake, the main by-product of flaxseed oil extraction, represents a valuable feed resource that can reduce waste when utilized effectively. Flaxseed cake is rich in polyunsaturated fatty acids (PUFA), particularly α-linolenic acid, which can influence rumen microbial hydrogenation pathways, regulate rumen fermentation patterns, and alter the formation of fermentation end-products. Additionally, PUFA can reduce rumen methane production by participating in fiber digestion and inhibiting the toxic effects of hydrogen-producing microorganisms. Previous research by Wu et al. demonstrated that flaxseed cake can replace soybean meal as a protein source for fattening sheep, improving meat quality and antioxidant capacity. Flaxseed cake also contains flaxseed gum, a dietary fiber with health benefits, and is relatively abundant in minerals, vitamins, and disease-resistant amino acids.

However, flaxseed cake also contains anti-nutritional factors such as linamarin, anti-vitamin B6 factors, and phytic acid. When animals consume flaxseed, linamarin can be degraded by β-glucosidase and α-hydroxynitrile lyase from damaged tissue cells, releasing toxic hydrogen cyanide (HCN), which adversely affects normal rumen metabolism and development. Therefore, investigating the effects of flaxseed cake on rumen metabolism and development is crucial for its rational utilization in ruminant production.

This study used Dorper × Small Tail Han sheep crossbred ram lambs to investigate the effects of replacing soybean meal with flaxseed cake on rumen metabolism by measuring rumen fermentation parameters, rumen fluid digestive enzyme activities, and expression of genes related to VFA absorption. The objective was to determine the optimal replacement proportion and provide a scientific basis for the rational use of flaxseed cake in ruminant production.

1.1 Experimental Animals and Design

Twenty-four healthy Dorper × Small Tail Han sheep crossbred ram lambs, aged five months with body weight of (26.0$±$1.0) kg, were selected for this study. A completely randomized design was employed, with the lambs divided into four groups [control (CK), I, II, and III] of six animals each. The lambs were fed diets in which flaxseed cake replaced soybean meal at inclusion levels of 0, 6%, 12%, and 18%, respectively.

1.2 Experimental Diets

Flaxseed cake from a company in Youyu was selected, air-dried naturally, and stored for later use. Its nutrient composition is shown in Table 1 . The basal diet was formulated according to the NRC (2007) nutrient requirements for ram lambs weighing 25 kg with a daily gain of 300 g. All experimental diets were total mixed ration (TMR) pellets, with composition and nutrient levels presented in Table 2 .

Table 1 Nutrient composition of flaxseed cake (DM basis)
Table 2 Composition and nutrient levels of experimental diets (DM basis)

1.3 Animal Management

The experiment was conducted from May to August 2016 at the Hongyu Sheep Breeding Farm in Youyu County, Shanxi Province. The pre-trial period lasted 10 days, followed by a 60-day formal experimental period. All lambs were fed twice daily at 08:00 and 16:00, housed individually in separate pens, with free access to feed and water.

1.4 Sample Collection and Analysis

Determination of Dietary Nutrient Levels

Dry matter (DM), crude ash, ether extract (EE), and crude protein (CP) contents of flaxseed cake and experimental diets were determined using the methods described by Zhang. Acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were measured using the method of Van Soest et al. combined with ANKOM filter bag technology and an ANKOM A200i semi-automatic analyzer. Calcium (Ca) content was determined by atomic absorption spectrophotometry (EWAI, AA-7020), and phosphorus (P) content was measured by UV spectrophotometry (Mapada, UV-1800PC). Digestible energy (DE) was calculated using the formula:

DE = 19.509 - 0.170 × NDF - 0.006 × (DM - Ash) - 0.097 × CP ($R^2 = 0.973$, $P < 0.001$).

Growth Performance Measurement

Average daily feed intake (ADFI) was recorded daily by weighing feed offered and refusals for each lamb (on an as-fed basis). Average daily gain (ADG) was

determined by weighing all lambs at the start of the formal period and every 30 days thereafter after overnight fasting. Feed-to-gain ratio (F/G) was calculated as:

F/G = ADFI / ADG.

Sample Collection

At the end of the experimental period, all lambs were fasted for 12 hours starting at 20:00 and slaughtered at 08:00 the following day. After slaughter, the rumen was removed and opened along the coronary groove with scissors. Rumen fluid was immediately collected from different regions using a sterile beaker, filtered through four layers of sterile gauze, and pH was measured in real-time using a PHS-3G pH meter. Ten milliliters of rumen fluid were placed in 15 mL centrifuge tubes, snap-frozen in liquid nitrogen, and stored at -20°C. After emptying the rumen contents and rinsing the rumen wall with physiological saline, a 1 cm^2 sample from the left dorsal sac was collected, snap-frozen in liquid nitrogen, and stored at -80°C.

Determination of Rumen Fermentation Parameters

A deproteinizing solution was prepared by dissolving 25 g of metaphosphoric acid and 0.6464 g of crotonic acid in a 100 mL volumetric flask. Rumen fluid (1.5 mL) was centrifuged at 10,621×g for 10 min, then 1 mL of supernatant was mixed with 0.2 mL deproteinizing solution. After 30 min in an ice bath, the mixture was centrifuged at 15,294×g for 5 min, and VFA concentrations were determined by gas chromatography (Thermo Fisher, Trace GC). Ammonia nitrogen (NH_3-N) concentration was measured by the sodium nitroprusside-sodium hypochlorite colorimetric method using a UV spectrophotometer. Lactate concentration was determined using a lactate assay kit (Nanjing Jiancheng Bioengineering Institute). Microbial protein concentration was measured by the colorimetric method of Makkar et al. after centrifuging 1 mL of rumen fluid at 430×g for 5 min to remove protozoa and feed particles.

Rumen fluid digestive enzyme activities were determined according to the methods of Agarwal et al. for carboxymethyl cellulase, β-glucosidase, xylanase, and pectinase. α-Amylase and protease activities were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute).

Determination of mRNA Relative Expression of VFA Absorption-Related Genes in Rumen Dorsal Sac Tissue

Rumen dorsal sac tissue samples were ground to powder in liquid nitrogen using an 8 cm diameter ceramic mortar. Thirty-five to seventy milligrams of tissue powder were placed in 1.5 mL EP tubes containing 750 L Trizol and homogenized (Polytron, PT 1200E) to extract total RNA. Extracted RNA was reverse-transcribed to cDNA using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa), and cDNA concentration was measured. Target gene sequences for monocarboxylate transporter 1 (MCT1), monocarboxylate transporter 4 (MCT4), anion exchanger 2 (AE2), down-regulated in adenoma (DRA),

Na^+/H^+ exchanger 3 (NHE3), and the reference gene ribosomal protein L13 (RpL13) were obtained from NCBI. Primers were designed using Primer3 online software (Table 3) and synthesized by Beijing Liuhe Huada Gene Technology Co., Ltd. Quantitative analysis was performed by fluorescence quantitative PCR using the synthesized cDNA as template, and results were calculated using the 2^(-ΔΔCT) method.

1.5 Data Processing and Statistical Analysis

Data were initially processed using Excel 2010 and analyzed by one-way ANOVA using SPSS 22.0. Duncan' s multiple comparison test was used for post-hoc analysis. Results are expressed as means ± standard deviation. Differences were considered significant at $P < 0.05$.

2.1 Effects of Dietary Flaxseed Cake Replacement on Growth Performance

As shown in Table 4 , ADG and ADFI in groups I and II did not differ significantly from the CK group ($P > 0.05$), but ADFI in group III was significantly reduced ($P < 0.05$). The feed-to-gain ratio in group II was significantly lower than in the CK group ($P < 0.05$), while no significant differences were observed between groups I and III and the CK group ($P > 0.05$).

2.2 Effects of Dietary Flaxseed Cake Replacement on Rumen Digestive Enzyme Activities

As presented in Table 5 , dietary flaxseed cake replacement had no significant effect on α-amylase activity ($P > 0.05$). Carboxymethyl cellulase, β-glucosidase, and protease activities in groups II and III were significantly higher than in the CK group ($P < 0.05$). Xylanase activity in group III was significantly higher than in groups CK and I ($P < 0.05$). Pectinase activity in group III was significantly higher than in all other groups ($P < 0.05$), with no significant differences among the other groups ($P > 0.05$).

2.3 Effects of Dietary Flaxseed Cake Replacement on Rumen Fermentation

As shown in Table 6 , dietary flaxseed cake replacement had no significant effect on rumen pH ($P > 0.05$). Acetate, butyrate, and total VFA concentrations in group II were significantly higher than in groups CK, I, and III ($P < 0.05$), with no significant differences among these three groups ($P > 0.05$). Propionate concentration and acetate-to-propionate ratio were not significantly affected by flaxseed cake inclusion level ($P > 0.05$).

Compared with the CK group, NH_3-N concentration was significantly decreased ($P < 0.05$) and MCP concentration was significantly increased ($P < 0.05$) in all three flaxseed cake groups, with no significant differences among these groups

($P > 0.05$). Lactate concentration was not significantly affected by dietary treatment ($P > 0.05$).

2.4 Effects of Dietary Flaxseed Cake Replacement on Expression of VFA Absorption-Related Genes in Rumen Epithelial Tissue

As shown in Figure 1 [Figure 1: see original paper]-A and 1-B, mRNA relative expression of MCT1 and MCT4 in group II was significantly higher than in groups CK and III ($P < 0.05$) but did not differ significantly from group I ($P > 0.05$). Dietary treatment had no significant effect on AE2 mRNA expression ($P > 0.05$) (Figure 1-C). DRA mRNA expression did not differ significantly between groups CK and I ($P > 0.05$) but was significantly lower in groups II and III compared with CK ($P < 0.05$) (Figure 1-D). NHE3 mRNA expression in group III was significantly higher than in the CK group ($P < 0.05$), with no significant differences among groups CK, I, and II ($P > 0.05$) (Figure 1-E).

3.1 Effects of Dietary Flaxseed Cake Replacement on Rumen Digestive Enzyme Activities

Dietary digestion in the rumen requires coordinated action of multiple digestive enzymes. Activities of carboxymethyl cellulase, β-glucosidase, xylanase, and pectinase reflect the rumen' s capacity to degrade dietary fiber, while amylase and protease activities indicate the ability to degrade starch and protein. Most digestive enzymes are intracellular and extracellular enzymes secreted by rumen microorganisms; this study investigated extracellular enzyme activities. Phytic phosphorus content in flaxseed cake ranges from 0.02% to 0.24%, whereas soybean meal contains up to 0.43% phytic phosphorus, which can bind to amylase and pepsin and affect rumen digestive enzyme activity. This study showed that increasing flaxseed cake replacement levels significantly elevated activities of carboxymethyl cellulase, β-glucosidase, xylanase, pectinase, and protease, consistent with findings by Wang et al. This effect may be attributed to several mechanisms. First, flaxseed cake is rich in α-linolenic acid, which can serve as a precursor to influence rumen microbial properties, and contains abundant B vitamins, both of which promote rumen microbial growth and increase enzyme production. B vitamins also function as coenzymes or major components of coenzymes, providing catalytic effects. Second, flaxseed gum can enhance the ability of rumen microorganisms to adhere to feed. Additionally, flaxseed gum absorbs water and expands, facilitating microbial attachment to feed surfaces, promoting microbial growth, and increasing digestive enzyme production.

3.2 Effects of Dietary Flaxseed Cake Replacement on Rumen Fermentation

Rumen pH is a comprehensive indicator reflecting rumen fermentation level and internal environment status, influenced by fermentation products such as VFA and lactate. In this study, no significant differences in rumen pH were

observed among groups, and all values remained within the normal range (5.5-7.5), consistent with findings by Benchaar et al.

Dietary carbohydrates fermented by rumen microorganisms produce VFA, which represents not only the end product of rumen carbohydrate utilization but also the primary form of energy utilization in ruminants. VFA yield and composition directly indicate rumen fermentation patterns and energy conversion efficiency. In this study, 12% flaxseed cake supplementation significantly increased acetate, butyrate, and TVFA concentrations, primarily because appropriate flaxseed cake levels enhanced fiber digestive enzyme secretion and activity, promoting acetate and butyrate production. However, the 18% flaxseed cake group showed significantly lower acetate, butyrate, and TVFA concentrations than the 12% group, possibly because excessive PUFA accumulation exceeded the hydrogenation capacity of some rumen microorganisms, thereby affecting rumen fermentation. Flaxseed cake supplementation did not significantly affect the acetate-to-propionate ratio, indicating no change in fermentation type. This finding does not contradict Martin et al.' s research showing that flaxseed can alter rumen fermentation type. Several factors may explain this discrepancy. First, under our experimental conditions, although CP content gradually decreased with flaxseed cake addition, it provided adequate nitrogen sources for carbohydrate-fermenting microorganisms because flaxseed cake protein has better water-holding and emulsifying properties. Additionally, the CP provided by flaxseed cake contains virtually no unusable prolamins but is rich in albumin, glutelin, and globulin, which can be effectively digested and utilized by rumen microorganisms. Flaxseed cake supplementation also provided balanced rumen-degradable protein (RDP) and rumen-undegradable protein (RUP), whereas soybean meal contains anti-nutritional factors such as phytic phosphorus, trypsin inhibitors, and soy lectins that reduce protein utilization efficiency. Furthermore, compared with flaxseed, the fat content and composition in flaxseed cake have changed, and the effects of dietary vegetable oil on rumen VFA depend on both the amount and type of fat added.

Rumen NH_3-N concentration is an important indicator of rumen nitrogen metabolism, primarily affected by rumen wall absorption, digesta passage rate, and MCP synthesis efficiency. Murphy et al. reported that the optimal NH_3-N concentration for rumen microbial growth is 6.3–27.5 mg/dL. This study showed that flaxseed cake replacement significantly reduced rumen NH_3-N concentration, consistent with findings by Jalc et al. This effect may be attributed to flaxseed gum promoting rumen wall absorption and digesta passage rate, and possibly enhancing NH_3-N incorporation into MCP. The theoretical basis for rumen energy-nitrogen synchronization is the selectivity, dependency, and timeliness of microorganisms for dietary nitrogen and energy, with MCP concentration being the most direct manifestation of energy-nitrogen synchronization. Synchronization indicates that the fermentation rate of fermentable organic matter (FOM) and the degradation rate of degradable nitrogen (RDN) are coordinated. This study demonstrated that flaxseed cake replacement significantly increased rumen MCP concentration, likely because

flaxseed cake provided a more suitable FOM/RDN ratio than soybean meal. This finding aligns with the observed promotion of NH_3-N conversion to MCP. Whether flaxseed cake replacement truly promotes NH_3-N conversion to MCP requires further investigation.

3.3 Effects of Dietary Flaxseed Cake Replacement on Expression of VFA Absorption-Related Genes in Rumen Epithelial Tissue

Continuous VFA production from rumen fermentation maintains a weakly acidic environment suitable for microbial growth. However, excessive VFA accumulation can decrease rumen pH and cause acidosis, reducing fiber degradation capacity and rumen epithelial absorption function. A small portion of VFA is neutralized by salivary bicarbonate, some enters the intestine with digesta, but approximately 85% is absorbed through the rumen wall into the blood. VFA is effectively absorbed by epithelial cells primarily as undissociated forms (HSCFA) via passive diffusion or as dissociated forms ($SCFA^-$) via exchange with HCO_3^- ions. During passive diffusion, lipophilic HSCFA molecules enter rumen epithelial cells and rapidly dissociate into H^+ and $SCFA^-$, increasing intracellular H^+ concentration and activating transporters such as monocarboxylate transporters (MCTs) or Na^+/H^+ exchangers (NHE) on the basolateral membrane to facilitate H^+, lactate, and ketone body efflux. During ion exchange, $SCFA^-$ transport depends on anion exchangers (AE) on the apical membrane or DRA on the basolateral membrane to mediate $SCFA^-/HCO_3^-$ exchange. The rate of VFA transport via ion exchange increases significantly with higher HSCFA concentration and lower pH in the rumen, but decreases with lower HCO_3^- concentration on the basolateral or apical membrane side. Research has shown that altering feeding levels in goats can change mRNA expression and activity of genes involved in VFA absorption in rumen epithelial cells.

This study showed that 12% flaxseed cake supplementation significantly increased MCT1 and MCT4 mRNA expression compared with the CK group, likely because the 12% level significantly increased butyrate concentration. Butyrate can stimulate rumen epithelial cell proliferation and, due to its high lipophilicity and predominantly undissociated state, increased HSCFA concentration promotes passive transport, thereby increasing MCT1 and MCT4 mRNA expression. This finding is consistent with results from Yan and Yang et al. However, compared with the 12% group, the 18% group showed significantly decreased MCT1 and MCT4 mRNA expression, possibly because anti-nutritional factor linamarin in flaxseed cake produces toxic hydrogen cyanide (HCN) in the rumen. Excessive CN^- inhibits intracellular respiratory enzyme activity, causing tissue hypoxia and affecting MCT1 and MCT4 mRNA expression. This study also found that both 12% and 18% flaxseed cake supplementation significantly decreased DRA mRNA expression, possibly because α-linolenic acid in flaxseed cake reduces CO_2 production in rumen fluid, thereby decreasing HCO_3^- concentration, weakening the ion exchange pathway, and reducing DRA mRNA expression. Whether α-linolenic acid and linamarin in flaxseed cake directly af-

fect VFA absorption gene expression requires further investigation.

4 Conclusion

Feeding diets containing 12% flaxseed cake reduced feed-to-gain ratio, increased rumen acetate, butyrate, and TVFA concentrations, decreased NH_3-N concentration, and increased MCP concentration in sheep. This supplementation level also enhanced activities of carboxymethyl cellulase, β-glucosidase, and protease, and increased mRNA expression of VFA absorption-related genes (MCT1, MCT4, and NHE3) while decreasing DRA mRNA expression. In conclusion, under the conditions of this study, replacing soybean meal with flaxseed cake at an appropriate proportion can improve rumen metabolism in sheep, with the optimal inclusion level being 12%.

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