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Effects of Different Dietary Proportions of Mulberry Leaf Powder on Rumen Epithelial Tissue Structure in Hu Sheep (Postprint)

Authors: Feng Dan, Ouyang Jialiang, Wang Mengzhi, Hou Qirui

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Abstract

This study aimed to investigate the effects of different dietary proportions of mulberry leaf powder on the rumen epithelial tissue structure of fattening Hu sheep. Forty 3-month-old fattening Hu sheep were selected and randomly allocated into 5 groups, with 8 sheep per group. In each group, mulberry leaf powder replaced 0% (Group A), 15% (Group B), 30% (Group C), 45% (Group D), and 60% (Group E) of the concentrate feed in the diet, respectively. The pre-trial period was 2 weeks, and the formal trial period was 8 weeks. The results showed that: 1) The rumen weight to complex stomach weight ratio in Groups B and C was significantly higher than that in Group A ($P<0.05$). 2) There were no significant differences in rumen papilla width among all groups ($P>0.05$). 3) Regarding the stratum corneum width of the rumen epithelium, Group A was higher than the other groups, and significantly higher than Groups C and D ($P<0.05$); regarding the stratum granulosum width of the rumen epithelium, except for Group D being significantly higher than Group E ($P<0.05$), there were no significant differences among the remaining groups ($P>0.05$); regarding the widths of the spinous layer and basal layer, compared with Group A, mulberry leaf powder treatment increased the widths of the spinous layer and basal layer, but the effect was not significant ($P>0.05$), while the basal layer width in Groups D and B was significantly higher than that in Group C ($P<0.05$). In summary, replacing 15%~45% of concentrate feed with mulberry leaf powder could increase the weight of the complex stomach to a certain extent, promote cell division in the spinous layer and basal layer, thereby promoting the development of rumen epithelial tissue, while also effectively reducing the width of the stratum corneum of the rumen epithelium.

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Full Text

Effects of Different Proportions of Mulberry Leaf Powder in Diet on Rumen Epithelium Structure of Hu Sheep

FENG Dan[1], OUYANG Jialiang[1], WANG Mengzhi[1]*, HOU Qirui[2]

[1]College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, China
[2]Sericulture Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China

*Corresponding author: Associate Professor, Master' s Supervisor, E-mail: mengzhiwangyz@126.com

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Abstract

This experiment was conducted to investigate the effects of different proportions of mulberry leaf powder in diet on rumen epithelium structure of fattening Hu sheep. Forty fattening Hu sheep aged 3 months were randomly divided into 5 groups with 8 sheep per group. The sheep were fed a fattening diet containing mulberry leaf powder replacing 0% (group A), 15% (group B), 30% (group C), 45% (group D), and 60% (group E) of dietary concentrate, respectively. The feeding trial consisted of a 2-week adaptation period and an 8-week experimental period. The results showed that: 1) The rumen weight to compound stomach weight ratio in groups B and C was significantly higher than that in group A ($P<0.05$). 2) There was no significant difference in rumen papilla width among all groups ($P>0.05$). 3) Regarding the width of the stratum corneum of rumen epithelium, group A showed higher values than other groups, being significantly higher than groups C and D ($P<0.05$). For the width of the stratum granulosum, no significant differences were observed among groups ($P>0.05$) except that group D was significantly higher than group E ($P<0.05$). Compared with group A, mulberry leaf powder treatment increased the width of the stratum spinosum and stratum basale, but the effects were not significant ($P>0.05$), while the width of the stratum basale in groups D and B was significantly higher than that in group C ($P<0.05$). In conclusion, replacing 15%-45% of concentrate with mulberry leaf powder can increase compound stomach weight to some extent, promote cell division in the stratum spinosum and stratum basale, thereby facilitating rumen epithelium development, while also effectively reducing the width of the stratum corneum.

Keywords: mulberry leaf powder; Hu sheep; rumen epithelium

1.1 Experimental Materials

Mulberry leaf powder was purchased from the market, with nutritional levels measured as follows: moisture 10.46%, crude protein 20.30%, crude fat 8.15%, crude ash 7.56%, neutral detergent fiber 34.30%, acid detergent fiber 16.28%, calcium 1.54%, and phosphorus 0.10%.

1.2 Experimental Animals and Diets

Forty healthy fattening Hu sheep aged 3 months with an initial body weight of (16.5±0.6) kg were selected from Siyang Weihe Hu Sheep Farm. A completely randomized design was adopted, with the sheep randomly divided into 5 groups of 8 animals each. Five different diets were formulated, with mulberry leaf powder replacing 0% (group A), 15% (group B), 30% (group C), 45% (group D), and 60% (group E) of the concentrate in the basal diet, while corn silage served as the roughage. The concentrate-to-roughage ratio was maintained at 5:5 (dry matter basis) across all groups, with diets being pelleted and isonitrogenous. The trial consisted of a 2-week adaptation period and an 8-week experimental period. Diets were formulated according to NRC (2007) recommendations for meat sheep with a daily gain target of 200 g/d.

The composition and nutrient levels of the concentrate are shown in Table 1 . The contents of crude protein, crude fat, neutral detergent fiber, acid detergent fiber, calcium, and phosphorus were determined according to *Feed Analysis and Feed Quality Detection Technology* [16]. Crude protein content was measured using the semi-micro Kjeldahl method, crude fat by Soxhlet ether extraction, neutral and acid detergent fibers by conventional filtration methods, and calcium and phosphorus by NPC-02 calcium-phosphorus analyzer.

Table 1 Concentrate composition and nutrient levels (dry matter basis)

Item	Group A	Group B	Group C	Group D	Group E
Ingredients					
Mulberry leaf powder	0.00	15.00	30.00	45.00	60.00
Corn	35.00	30.00	25.00	20.00	15.00
Soybean meal	15.00	12.00	9.00	6.00	3.00
Wheat bran	15.00	12.00	9.00	6.00	3.00
Brown rice	15.00	12.00	9.00	6.00	3.00
Saccharomyces cerevisiae	2.00	2.00	2.00	2.00	2.00
Corn protein meal	5.00	5.00	5.00	5.00	5.00
CaH PO	1.50	1.50	1.50	1.50	1.50
Fat powder	2.00	2.00	2.00	2.00	2.00
Limestone	4.00	4.00	4.00	4.00	4.00
NaCl	1.00	1.00	1.00	1.00	1.00
Premix[1]	4.50	4.50	4.50	4.50	4.50
Total	100.00	100.00	100.00	100.00	100.00
Nutrient levels[2]					

Item	Group A	Group B	Group C	Group D	Group E
DE (MJ/kg)	14.50	14.21	13.92	13.63	13.34
CP	20.00	20.00	20.00	20.00	20.00
EE	5.00	5.50	6.00	6.50	7.00
NDF	18.00	20.00	22.00	24.00	26.00
ADF	7.00	8.00	9.00	10.00	11.00
Ca	0.80	0.90	1.00	1.10	1.20
P	0.50	0.45	0.40	0.35	0.30

[1]The premix provides per kg of diet: Cu 200-500 mg, Fe (as ferrous sulfate) 1,500-2,500 mg, Mn (as manganese sulfate) 1,000-2,000 mg, Zn (as zinc sulfate) 1,000-2,500 mg, VA 200,000-370,000 IU, VD 250,000-1,250,000 IU, VE 750 mg, Lys >4.9%, P 5-50 mg, Se (as sodium selenite) 5-15 mg, Co (as cobalt sulfate) 5-15 mg, Ca (as calcium sulfate) 10%-16%, NaCl 10%-16%.

[2]DE is calculated based on ingredient composition, while other nutrient levels are measured values.

1.3 Feeding Management

Sheep were housed in group pens. Before the trial, the barn was thoroughly cleaned and disinfected, and all sheep were treated for parasites and vaccinated. During the first 3 weeks of the experimental period, whole-plant corn silage was provided at 1.5 kg per sheep daily, which was increased to 1.75 kg thereafter. The concentrate feeding regimen was as follows: 375 g per sheep daily in week 1, with a weekly increase of 25 g until the end of week 8. Sheep were fed twice daily at 07:00 and 17:00, with roughage offered before concentrate, and had free access to drinking water.

One day before the end of the feeding trial, three sheep from each group were fasted for 24 h, then weighed and slaughtered. The compound stomach and its compartments (rumen, reticulum, omasum, and abomasum) were weighed, and a piece of uniformly grown rumen tissue was collected for paraffin section preparation.

1.4 Paraffin Section Preparation

Rumen tissue samples were rinsed with pre-cooled phosphate buffer saline (PBS) and immediately fixed in 4% formaldehyde solution for at least 48 h. Fixed tissues were trimmed into 5 mm thick blocks. Paraffin sectioning and hematoxylin-eosin (HE) staining were used to observe rumen morphological structure. Tissue sections were prepared according to the method described by Wang et al. [17] with the following steps: tissue blocks were rinsed and sequentially placed in 80% ethanol once, 90% ethanol twice, and 100% ethanol three times for dehydration, followed by two rounds of xylene clearing, paraffin infiltration, and embedding. Sections were then sliced, mounted, and baked. HE staining was performed according to the method of Wang Long [18] as follows: sections were placed in xylene I and xylene II for 30 min each to clear, rehydrated in ethanol solutions, soaked in PBS for 5 min, stained with hematoxylin solution for 5 min in the dark, rinsed for 5 min, soaked in PBS again for 5 min, baked at 60°C for 20 min, placed in 100% ethanol twice for 3 min each, and finally sealed with neutral resin.

1.5 Measurement Indicators

Feed intake was recorded during the experimental period to calculate dry matter intake (DMI). Initial and final body weights were recorded to calculate average daily gain (ADG). Three slices of 7 m thickness were prepared from each sample, with at least 100 m between adjacent slices. Morphometric analysis was performed using Guangzhou Mingmei imaging system software. Rumen tissue structure was observed under an optical microscope (Olympus, Japan), and data were measured and recorded using Olympus IX71 microscope software cellSens Dimension, including width of rumen papillae and widths of the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB) of rumen epithelium. Rumen cell layer thickness was measured using a 20× objective, while rumen papilla width was measured using a 4× objective. All morphometric analyses were performed by the same operator, with six fields measured per slice to calculate average values.

Compound stomach index (%) = 100 × (compound stomach weight / pre-slaughter live weight).

1.6 Statistical Analysis

Experimental data were initially processed using Excel 2013, then subjected to one-way ANOVA using SPSS 19.0 software. Duncan' s multiple comparison test was used for post-hoc analysis. Results are expressed as means and standard error of the mean (SEM), with P<0.05 considered statistically significant.

2.1 Effects of Different Proportions of Mulberry Leaf Powder on Compound Stomach Development of Hu Sheep

As shown in Table 2 , DMI and ADG in groups D and E were significantly lower than those in groups A, B, and C (P<0.05), while no significant differences were observed among groups A, B, and C (P>0.05). Pre-slaughter live weight was higher in groups A, B, and C, which were significantly higher than groups D and E (P<0.05). Compound stomach weight and compound stomach index were highest in groups B and C, significantly higher than groups D and E, and groups A, D, and E, respectively (P<0.05). Additionally, the rumen weight to compound stomach weight ratio was higher in groups B and C, significantly higher than group A (P<0.05), but not significantly different from groups D and E (P>0.05). In contrast, the abomasum weight to compound stomach weight ratio was highest in group A, significantly higher than groups B and C (P<0.05).

Table 2 Effects of different proportions of mulberry leaf powder in diet on development of compound stomach of Hu sheep

Item	Group A	Group B	Group C	Group D	Group E	P-value
DMI (g/d)	869.9[a]	857.5[a]	859.9[a]	801.4	817.4	<0.001
ADG (g/d)	120.4[a]	120.0[a]	114.1[a]	100.0	87.0	<0.001
Live body weight before slaughter (kg)	22.50[a]	22.87[a]	22.28[a]	20.73	21.08	<0.001
Compound stomach weight (kg)	1.31	1.52[a]	1.49[a]	1.22	1.27	<0.001
Index of compound stomach (%)	5.82	6.65[a]	6.69[a]	5.88	6.02	<0.001
Rumen weight/compound stomach weight (%)	56.31	62.46[a]	62.91[a]	60.61	60.35	<0.001
Reticulum weight/compound stomach weight (%)	22.51[a]	18.48	17.00	19.02	19.87	<0.001
Omasum weight/compound stomach weight (%)	10.45	10.45	10.45	10.45	10.45	0.999

Item	Group A	Group B	Group C	Group D	Group E	P-value
Abomasum weight/compound stomach weight (%)	10.73[a]	8.61	9.64	9.92	9.33	<0.001

[a], , In the same row, values with different superscripts differ significantly (P<0.05).

2.2 Effects of Different Proportions of Mulberry Leaf Powder on Rumen Epithelium Tissue

Microscopic observation of rumen epithelium sections revealed the microstructure shown in Figure 1 [Figure 1: see original paper], while rumen papilla microstructure is presented in Figure 2 [Figure 2: see original paper]. Figure 1 shows that the width of the stratum corneum of rumen epithelium in group A was greater than in other groups, with minimal differences observed in other cell layers. Figure 2 indicates that rumen papilla width showed little variation among groups. Measurement results from the software (Table 3) demonstrated that different proportions of mulberry leaf powder in the diet had no significant effect on rumen papilla width (P>0.05), although all groups except group C showed numerically higher values than group A. For stratum corneum width, group A exhibited higher values than other groups, significantly exceeding groups C and D (P<0.05), with no significant differences among the remaining groups (P>0.05). Regarding stratum granulosum width, no significant differences were observed among groups (P>0.05) except that group D was significantly higher than group E (P<0.05). Mulberry leaf powder replacement numerically increased stratum spinosum width, but the effect was not significant (P>0.05). The width of stratum basale in groups D and B was significantly higher than in group C (P<0.05).

Table 3 Effects of different proportions of mulberry leaf powder in diet on rumen epithelium structure of Hu sheep (m)

Item	Group A	Group B	Group C	Group D	Group E	P-value
Stratum corneum (SC)	19.3[a]	16.8	15.9	15.9	16.5	<0.001
Stratum granulosum (SG)	40.4	44.1	40.9	46.4[a]	38.7	<0.001

Item	Group A	Group B	Group C	Group D	Group E	P-value
Stratum spinosum (SS)	22.6	25.9	19.7	26.8	24.2	<0.001
Stratum basale (SB)	15.9	17.0	15.9	17.0	16.5	<0.001
Width of rumen papillae	3.24	3.33	3.11	3.37	3.28	0.999

SC: stratum corneum; SG: stratum granulosum; SS: stratum spinosum; SB: stratum basale.

Figure 1 Morphological structure of rumen epithelium of Hu sheep (20×) [Figure 1: see original paper]

Figure 2 Morphological structure of rumen papillae of Hu sheep (4×) [Figure 2: see original paper]

3.1 Effects of Different Proportions of Mulberry Leaf Powder on Feed Intake and Digestion of Hu Sheep

Mulberry leaves exhibit good palatability, and livestock readily accept them without feeding 障碍 when first introduced. For ruminants, feed intake is regulated by various physiological factors including digestive tract capacity, static chemistry, static mechanics, and metabolism [19], and is influenced by comprehensive factors such as feed, animal, and environment. Mulberry leaves can improve the rumen ecological environment, increase the attachment of fiber-decomposing bacteria to fiber particles, and promote their proliferation, thereby enhancing straw digestibility and feed intake [20]. Mulberry leaves also have high digestibility. Yan Bing et al. [21] reported that the 48-hour dry matter digestibility of mulberry leaves in the rumen reached 62%. Supplementing mulberry leaf powder increases protein content in cattle diets, improves the rumen ecological environment, and promotes microbial proliferation, thereby increasing apparent digestibility of dry matter, organic matter, and protein [22].

3.2 Mechanism of Nutrient Utilization by Rumen Epithelium Tissue

Rumen epithelium tissue consists of four layers from outer to inner: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. The stratum corneum was once considered detrimental to rumen absorption, but subsequent research revealed that under normal conditions, the keratinized cell layer comprises only 3–4 cell layers under continuous abrasion from rumen contents, having minimal impact on nutrient absorption while providing significant protection. The stratum granulosum has tight junctions but is discontinuous, lacking sebaceous gland secretion, allowing free water passage. The stratum spinosum is the site of short-chain fatty acid metabolism in rumen epithelium. Thus, nutrient transport and absorption by rumen epithelium largely depend on the degree of keratinization and integrity of the stratum corneum [23]. The number of cell layers in the stratum corneum directly reflects the degree of keratinization, which is highly correlated with diet composition and form: high-concentrate diets can result in a stratum corneum thickness of up to 15 cell layers, whereas high-roughage diets produce a stratum corneum of only 4 cell layers. Excessive stratum corneum thickness is detrimental to nutrient absorption. Incomplete keratinization of rumen epithelium occurs when squamous epithelial cells produce a rigid keratin layer, which acts as a physical barrier to rumen papillae, coating the papilla surface, reducing blood flow in the rumen epithelium, causing papilla degeneration, and forming carrion at the edges, eventually leading to papilla clumping [10]. This condition primarily results from insufficient feed particle size failing to abrade aging epithelial cells.

3.3 Effects of Different Proportions of Mulberry Leaf Powder on Rumen Epithelium Structure

Rumen tissue morphological development involves two aspects: capacity increase and mucosal development. Capacity increase, reflected by rumen tissue weight, plays a crucial role in roughage utilization by adult sheep under healthy conditions. Ni Junfen et al. [24] found that mulberry leaf powder could not only replace part of the concentrate but also significantly improve body condition development of fattening Hu sheep at certain supplementation levels (15%, 30%, and 45%). However, when the replacement proportion continued to increase (60%), it not only failed to enhance body condition development but also produced negative effects, possibly because excessive mulberry leaf powder affected diet palatability and easily induced satiety, thereby reducing nutrient and energy intake. In this study, replacing 15% and 30% of concentrate with mulberry leaf powder resulted in pre-slaughter live weight and compound stomach weight comparable to the non-replacement group, while the compound stomach index in these two groups was significantly higher than in the non-replacement group, suggesting that these treatments promoted compound stomach development. Further analysis of the four stomach compartments revealed that the difference in compound stomach development was due to increased rumen weight to compound stomach weight ratio in these two groups. Meanwhile, the abomasum weight to compound stomach weight ratio in the non-replacement group was lower than in the replacement groups, further indicating that without mulberry leaf powder replacement, sheep had better abomasum development than the replacement groups, possibly due to diet adaptation.

Rumen mucosal development begins differentiation during the embryonic period of ruminants. Rumen papillae are small protrusions of mucosal epithelium associated with absorption function. These protrusions increase the surface area for volatile fatty acid absorption, with approximately 250,000 papillae on a rumen wall expanding the mucosal surface area by 6–7 times. Therefore, papilla mucosal surface area serves as an important criterion for evaluating rumen and mucosal metabolic levels [25]. Rumen papillae are tongue-shaped or leaf-shaped protrusions formed by epithelium and lamina propria extending into the gastric lumen. The papilla surface comprises stratified squamous epithelial cells with superficial keratinization. Papillae are mobile and play a role in mechanical digestion through kneading and grinding while increasing absorption surface area. Research has shown that feed physical form significantly affects histomorphological development of calf rumen: calves fed ground diets had shorter rumen papillae with smaller surface areas [10]. This study found that rumen papilla width was substantially affected by concentrate replacement with mulberry leaf powder, though the effect of supplementation level on papilla width was not significant and requires further investigation. Additionally, inconsistent differences among various epithelial cell layers indicate that although diet composition and nutrient content influence rumen papilla width and the four epithelial layer widths, they are not the sole determinants.

In conclusion, replacing 15%–45% of concentrate with mulberry leaf powder can increase compound stomach weight to some extent, promote cell division in the stratum spinosum and stratum basale, thereby facilitating rumen epithelium development, while also effectively reducing the width of the stratum corneum.

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