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Effects of Amylose to Amylopectin Ratio in Concentrate Feed on Rumen Bacterial Community in Lambs [1] Postprint

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Abstract

This study aimed to investigate the effects of dietary amylose to amylopectin ratio in concentrate on rumen bacterial community structure in lambs at different ages. Forty-eight newborn male lambs with no significant difference in body weight (P>0.05) were randomly allocated into 4 groups (3 replicates per group, 4 lambs per replicate). The amylose to amylopectin ratios in the concentrate were 0.12 (cassava starch group), 0.23 (corn starch group), 0.24 (wheat starch group), and 0.48 (pea starch group). The total experimental duration was 77 days. At 21, 35, 56, and 77 days of age, one lamb was randomly selected from each replicate for slaughter, and rumen fluid samples were collected. The diversity of rumen bacterial flora was determined and analyzed using PCR-denaturing gradient gel electrophoresis (DGGE). The results indicated that at 21, 35, 56, and 77 days of age, there were no significant differences in rumen bacterial diversity index, evenness, and richness among the different dietary amylose to amylopectin ratio groups (P>0.05). The rumen bacterial diversity index of 21-day-old lambs was significantly higher than that of 35-day-old lambs (P<0.05). The rumen bacterial diversity index of 56- and 77-day-old lambs increased to some extent, but remained numerically lower than that of 21-day-old lambs. Most bands recovered by DGGE belonged to the phyla Firmicutes and Bacteroidetes. The content of Selenomonas ruminantium in the rumen of wheat starch group lambs was relatively high at 56 days of age. It can be concluded that different amylose to amylopectin ratios in concentrate feed did not affect the structure of dominant rumen bacteria in lambs, but feeding a wheat starch diet promoted the growth of Selenomonas ruminantium in the rumen of 56-day-old lambs.

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Full Text

Effects of Amylose to Amylopectin Ratio in Concentrate on Rumen Bacterial Community in Lambs

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Abstract

This study investigated the effects of dietary amylose to amylopectin ratio in concentrate on rumen bacterial community composition in lambs at different developmental stages. Forty-eight newborn male lambs with no significant differences in body weight (P>0.05) were randomly allocated to four dietary groups (n=12 per group, with three replicates of four lambs each). The experimental concentrates contained different starch sources yielding amylose to amylopectin ratios of 0.12 (tapioca starch group), 0.23 (corn starch group), 0.24 (wheat starch group), and 0.48 (pea starch group). The trial lasted 77 days. At 21, 35, 56, and 77 days of age, one lamb per replicate was randomly selected for slaughter, and rumen fluid samples were collected for analysis. Rumen bacterial diversity was assessed using PCR-denaturing gradient gel electrophoresis (DGGE). The results demonstrated that dietary amylose to amylopectin ratio had no significant effect on bacterial diversity index, evenness, or richness in lambs at any of the four sampling time points (P>0.05). However, the diversity index in 21-day-old lambs was significantly higher than that in 35-day-old lambs (P<0.05), and although values increased again at 56 and 77 days of age, they remained numerically lower than those observed at 21 days. Sequence analysis of excised DGGE bands revealed that most belonged to the phyla Firmicutes and Bacteroidetes. Notably, *Selenomonas bovis* was found at higher abundance in the rumen of 56-day-old lambs fed the wheat starch diet. These findings indicate that while different amylose to amylopectin ratios in concentrate did not alter the structure of dominant rumen bacteria, wheat starch supplementation specifically promoted the growth of *Selenomonas bovis* in 56-day-old lambs.

Keywords: amylose to amylopectin ratio; lamb; PCR-denaturing gradient gel electrophoresis; rumen bacterial community; diversity index

Introduction

In lamb diets, forage typically constitutes a relatively low proportion because of its low dry matter content and poor fermentation efficiency in the rumen. Consequently, grain feed serves as the primary dietary component, with starch being its main nutrient. Starch is a high-molecular-weight polysaccharide composed of multiple glucose units linked by glycosidic bonds, existing in two molecular structures: amylose and amylopectin. Most starches contain both forms simultaneously [1-2]. Previous research has demonstrated that different amylose to amylopectin ratios can significantly affect lamb physiology. For instance, Liu [3] reported that feeding a high amylose to amylopectin ratio diet containing pea starch increased blood immunoglobulin concentrations and improved growth performance and meat quality in lambs. Ren et al. [4] investigated the effects of different amylose to amylopectin ratios on gastrointestinal tract development in fattening lambs and found that these ratios significantly influenced intestinal development. The rumen is one of the most important digestive organs in lambs, where nutrient digestion primarily depends on rumen microorganisms. These microbes exhibit substrate selectivity and can indirectly alter the rumen microenvironment by fermenting ingested substrates [5]. Therefore, different amylose to amylopectin ratios in concentrate may influence rumen microbial populations.

Several studies have shown that intestinal microbiota structure varies with age in monogastric animals [6-7], raising the question of whether similar age-related differences exist in rumen bacterial community structure in lambs. Currently, reports on the relationship between starch composition and rumen bacterial communities in ruminants of different ages remain limited. This study employed PCR-denaturing gradient gel electrophoresis (DGGE) to investigate how different amylose to amylopectin ratios in concentrate affect rumen bacterial communities in lambs at various ages. These findings are crucial for understanding how different starch compositions influence ruminant digestive health and provide a scientific basis for optimizing starch as a nutritional source to promote rumen and overall animal health.

1.1.1 Experimental Animals and Design

This experiment utilized a single-factor randomized design. Forty-eight healthy newborn male lambs with no significant differences in birth weight ($P>0.05$) were randomly divided into four groups, with three replicates per group and four lambs per replicate. The entire experimental period lasted 77 days. At 21, 35, 56, and 77 days of age, one lamb per replicate was selected for slaughter and subsequent measurements.

1.1.2 Experimental Diets

The concentrate formulation followed the nutrient requirements for fattening lambs recommended by NRC (1985). Tapioca starch (TS), corn starch (CS),

wheat starch (WS), and pea starch (PS) were used as the sole starch sources for each group, respectively, to create four experimental concentrates with consistent starch and nitrogen content and energy levels. All starch sources were food-grade (Shanghai Longfeng Food Co., Ltd.). The amylose and amylopectin contents in the concentrates were analyzed according to the method described by Englyst et al. [8]. The amylose to amylopectin ratios in TS, CS, WS, and PS were 0.12, 0.23, 0.24, and 0.48, respectively. The composition and nutrient levels of the experimental concentrates are presented in Table 1 .

Table 1 Composition and nutrient levels of experimental concentrates (air-dry basis)

Item	TS Group	CS Group	WS Group	PS Group
Ingredients/%				
Tapioca starch	55.00	-	-	-
Corn starch	-	55.00	-	-
Wheat starch	-	-	55.00	-
Pea starch	-	-	-	55.00
Soybean meal	23.00	23.00	23.00	23.00
Corn protein meal	11.00	11.00	11.00	11.00
Soybean oil	3.00	3.00	3.00	3.00
Limestone	2.00	2.00	2.00	2.00
$CaHPO_4$	1.50	1.50	1.50	1.50
NaCl	1.00	1.00	1.00	1.00
Premix[1]	3.50	3.50	3.50	3.50
Total	100.00	100.00	100.00	100.00
Nutrient levels[2]				
Total starch/%	55.12	55.08	55.15	55.09
Amylose/total starch/%	6.61	12.67	13.23	26.44
Amylopectin/total starch/%	48.51	42.41	41.92	28.65
Amylose/amylopectin	0.12	0.23	0.24	0.48
DM	87.32	87.45	87.38	87.41
CP	18.21	18.25	18.19	18.23
Ca	0.89	0.89	0.89	0.89
DE/(MJ/kg)	14.23	14.23	14.23	14.23

[1] One kg of premix provided the following: Zn 5,200 mg, Cu 1,200 mg, Mn 4,000 mg, Fe 6,000 mg, I 40 mg, Co 35 mg, Se 20 mg, VA 940 IU, VE 20 IU.
[2] DE was a calculated value, while others were measured values.

1.1.3 Animal Management

One week before the experiment, the sheep house was thoroughly cleaned, washed, and disinfected. Lambs were housed indoors and fed artificially by

bottle until 56 days of age. From 7 days postpartum, lambs had ad libitum access to their respective experimental concentrates and high-quality alfalfa hay, along with clean drinking water. Feeding occurred four times daily at 04:30, 10:00, 15:30, and 21:00. Throughout the experimental period, the barn was disinfected regularly, and lamb health status was monitored closely.

1.2 Sample Collection

At 21, 35, 56, and 77 days of age, one lamb per replicate was selected, exsanguinated via jugular venipuncture, and the abdominal cavity was opened to collect rumen contents. The contents were filtered through four layers of sterile gauze, aliquoted into sterile cryovials, immediately snap-frozen in liquid nitrogen, and stored at -80°C in the laboratory for subsequent microbial community analysis.

1.3 Rumen Fluid Genomic DNA Extraction and 16S rDNA V6-V8 Region Amplification

Genomic DNA was extracted from each sample using the cetyltrimethylammonium bromide (CTAB)/sodium dodecyl sulfate (SDS) method. Using the extracted genomic DNA as template, the universal primers U968-GC-F and L1401R were employed to amplify the 16S rDNA gene sequence. Primer information is presented in Table 2 .

Table 2 Information of primers

Primer	Sequence (5 -3)
U968F	AACGCGAAGAACCTTAC
L1401R	CGGTGTGTACAAGACCC
U968-GC-F	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAA

The GC hairpin structure is underlined.

The PCR amplification system (50 µL) contained: 10×PCR buffer 5 µL, dNTP (2.5 mmol/L) 3.2 µL, rTaq (5 U/µL) 0.4 µL, U968-GC-F (20 µmol/L) 1 µL, L1401R (20 µmol/L) 1 µL, template DNA 50 ng, and ddH_2O to 50 µL. The PCR program consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 20 s, and extension at 72°C for 40 s, with a final extension at 72°C for 7 min.

1.4 DGGE Analysis of PCR Products

Ten microliters of PCR product were analyzed by DGGE using 8% polyacrylamide gels with a denaturing gradient of 35%-62% in 1×TAE buffer. Electrophoresis was performed at 80 V and 60°C for 16 h, followed by silver staining.

1.5 Recovery and Sequencing of Dominant Bands from DGGE Profiles

Target bands were excised with a sterile scalpel, and DNA was recovered using the Omega gel extraction kit. Two microliters of the recovered product were used as template for re-amplification with primers U968F/L1401R (without GC clamp). The re-amplified DNA fragments were gel-purified, ligated into the pMD18-T vector, and transformed into DH5α competent cells. Positive clones were screened and sent to BGI for sequencing of the inserted bacterial 16S rDNA fragments.

1.6 Diversity Index, Similarity Coefficient, and Band Sequencing Analysis

Bacterial diversity index is a comprehensive indicator representing community species number, individual count, and evenness. Based on the number and intensity of bands in each DGGE lane, bacterial diversity index, evenness, and richness were calculated for each sample using Quantity One software for digital analysis. The formulas were as follows:

Evenness (E) = H/Hmax = H/ln(S)

Where: H is the diversity index; E is evenness; R is richness; pi is the proportion of intensity of a single band relative to the total intensity of all bands in that lane; N is the total intensity of all bands in a single lane; Ni is the intensity of the i-th band; and S is the total number of bands in a sample.

Similarity between samples was calculated using Dice coefficients:

Cs = 2j/(a+b)

Where: j is the number of common bands between samples A and B; a and b are the total number of bands in each sample, respectively.

Cloned band sequences were compared against the GenBank database for taxonomic identification and submitted to NCBI to obtain accession numbers.

1.7 Data Processing and Statistical Analysis

DGGE profile data were analyzed using SPSS 16.0 software for one-way ANOVA, followed by Duncan' s multiple comparison test. Results are expressed as mean ± standard deviation, with $P<0.05$ considered statistically significant.

2.1 DGGE Analysis of PCR Products

Total DNA was successfully extracted from rumen contents of lambs fed different amylose to amylopectin ratios at 21, 35, 56, and 77 days of age. Electrophoresis revealed high-molecular-weight genomic DNA with clear bands suitable for subsequent PCR amplification. Using universal primers targeting the V6-V8 region (U968-GC-F and L1401R), 16S rDNA fragments of approximately 480 bp were obtained for DGGE analysis.

The DGGE profiles of rumen bacterial 16S rDNA PCR products are shown in Figure 1 [Figure 1: see original paper]. In PCR-DGGE analysis, intense bands represent dominant microbial populations, the number of bands indicates bacterial richness, and band positions reflect bacterial species. The results revealed abundant bands in lambs fed different amylose to amylopectin ratios across all ages. Common dominant bands were observed across groups at 21, 35, 56, and 77 days of age (e.g., bands 1, 4, 5, 6, 8, 9, and 10), while characteristic bands appeared at specific ages (e.g., bands 2, 3, and 7 at 21 and 56 days). Variations in band intensity, number, and position among different lanes indicated differences in rumen bacterial community structure both between dietary treatments at the same age and between ages within the same dietary treatment.

2.2 Diversity Analysis of Bacterial 16S rDNA V6-V8 Region PCR-DGGE Profiles

Diversity analysis of the PCR-DGGE profiles is presented in Figure 2 [Figure 2: see original paper]. No significant differences in rumen microbial diversity index were observed among dietary groups at 21, 35, 56, or 77 days of age ($P>0.05$). Regarding the effect of age, both diversity index and richness decreased with increasing age, with 21-day-old lambs showing significantly higher values than 35-day-old lambs ($P<0.05$). Although values increased slightly at 56 and 77 days, they remained numerically lower than those at 21 days. No significant differences in bacterial evenness were detected among ages ($P>0.05$).

2.3 Similarity Analysis of Bacterial 16S rDNA V6-V8 Region PCR-DGGE Profiles

The effects of dietary amylose to amylopectin ratio on rumen bacterial similarity in lambs at 21, 35, 56, and 77 days of age are presented in Tables 3-6 , respectively. The influence of age on bacterial similarity is shown in Table 7 . The results revealed no clear within-group similarity among lambs fed different amylose to amylopectin ratios at any age; however, within-group similarity coefficients tended to increase with age. In contrast, parallel samples from the same age group exhibited relatively high similarity.

2.4 Sequence Analysis of Common and Characteristic Bands from DGGE Profiles

Ten bands were excised from the DGGE gel (Figure 1) and sequenced, with results summarized in Table 8 . Band 1 was common to all dietary groups at 21 days of age and showed highest similarity to *Butyricimonas virosa*. Band 4 was common at 35 days and was most similar to *Prevotella ruminicola*. Bands 5 and 6 were common at 56 days, showing closest matches to *Propionispira arcuata* and *Selenomonas sputigena*, respectively. Bands 8, 9, and 10 were common at 77 days, with closest matches to *Ruminococcus bromii*, *Prevotella multisaccharivorax*, and *Prevotella dentalis*, respectively.

Characteristic bands included band 2, which appeared in two replicates of the PS group at 21 days but was absent in other groups, showing highest similarity to *Butyricimonas paravirosa*. Band 3 was relatively abundant in the TS group at 21 days and was most similar to *Prevotella marshii*. Band 7 was most abundant in the WS group at 56 days and was identified as *Selenomonas bovis*. Most sequenced bands belonged to the phyla Firmicutes and Bacteroidetes. Notably, band 2 showed only 88% similarity to the closest cultured species (*Butyricimonas paravirosa*) but 96% similarity to uncultured bacteria, suggesting it may represent a novel uncultured bacterial species. Similarly, band 3 exhibited only 90% similarity to *Prevotella marshii* but 93% similarity to uncultured rumen bacteria, indicating it may represent a novel uncultured rumen bacterial species.

Table 8 Comparison of genomic sequences in bands of DGGE by sequencing and BLAST analysis

Band No.	Closest strain	GenBank accession No.	Homology/%	Closest group
1	*Butyricimonas virosa*	NR_{041691}	96	Bacteroidetes
2	*Butyricimonas paravirosa*	NR_{126195}	88	Bacteroidetes
2	Uncultured bacterial	EF445218	96	Bacteroidetes
3	*Prevotella marshii*	NR_{113111}	90	Bacteroidetes
3	Uncultured rumen bacteria	HQ399714	93	Bacteroidetes
4	*Prevotella ruminicola*	NR_{102887}	99	Bacteroidetes
5	*Propionispira arcuata*	NR_{134138}	98	Firmicutes
6	*Selenomonas sputigena*	NR_{025115}	97	Firmicutes
7	*Selenomonas bovis*	NR_{044111}	98	Firmicutes
8	*Ruminococcus bromii*	NR_{025930}	99	Firmicutes
9	*Prevotella multisaccharivorax*	NR_{041285}	98	Bacteroidetes
10	*Prevotella dentalis*	NR_{102481}	97	Bacteroidetes

The gastrointestinal microbiota of ruminants is predominantly composed of Fir-

micutes and Bacteroidetes [10-11]. Previous studies have shown that *Prevotella*, *Ruminococcus*, and Bacteroidetes are present in the rumen of 20-day-old lambs [12], and by 21 days of age, the rumen microbiota can already digest some feeds typically utilized by adult sheep [13]. As age and feed intake increase, facultative anaerobes are gradually replaced by obligate anaerobes [14], and although the dominant bacteria remain unstable during this period, Firmicutes and Bacteroidetes predominate [12]. Our sequencing results are consistent with these findings, as both common and characteristic bands belonged primarily to Firmicutes and Bacteroidetes.

Understanding rumen microbial diversity and population density is fundamental to improving rumen fermentation function. Rumen microbial community structure is influenced by host genetics, health status, and diet, and varies with geography, season, and feeding regimen [15]. In DGGE analysis, higher diversity indices indicate greater species diversity; evenness values approaching 1 suggest high uniformity and less dominance by specific species; and richness reflects the total number of species. Generally, higher microbial diversity correlates with greater ecosystem stability. Our study found no significant differences in rumen bacterial diversity index, evenness, or richness among lambs fed different amylose to amylopectin ratios at any age. These results differ from Zhang et al. [16], who reported that pig ileal digesta microbial diversity was significantly higher in soybean hull-fed pigs compared to wheat bran-fed pigs. However, our findings are consistent with Tian et al. [17], who found that diets with corn, wheat, or rice as the sole starch source did not affect intestinal microbial diversity or evenness in broilers. Given these inconsistent results regarding dietary modulation of gastrointestinal microbiota, our study suggests that different amylose to amylopectin ratios in concentrate do not significantly affect dominant rumen bacteria in lambs, though subtle compositional changes cannot be ruled out and warrant further investigation.

Mammals and poultry are born with relatively few gastrointestinal microbes, but microbial populations increase progressively with feed intake and environmental exposure. Our results regarding age effects showed that rumen bacterial diversity index was significantly higher at 21 days than at 35 days, then increased again at 56 and 77 days, though remaining numerically lower than at 21 days. This pattern of initial high diversity followed by a decrease and subsequent increase may reflect changes in diet and environment as lambs age, with increasing intake of concentrate and forage causing substantial shifts in rumen bacterial community structure, followed by gradual stabilization as the microbiota adapts.

Our combined DGGE and sequencing results revealed that Firmicutes and Bacteroidetes dominated the rumen of all four groups at each age, including species involved in nutrient metabolism. At 21 days, *Butyricimonas virosa* was detected in all groups. This species, isolated from rat feces, can utilize glucose and produces butyrate and isobutyrate as fermentation end-products [18]. We hypothesize that early supplementation with different starch sources may promote

B. virosa proliferation, producing short-chain fatty acids (particularly butyrate) that stimulate rumen development in young animals. At 77 days, *Ruminococcus bromii* was present in all groups. This species can degrade large carbohydrate molecules into monosaccharides or oligosaccharides that support the growth of other bacteria such as *Eubacterium*, *Bifidobacterium*, and *Anaerostipes* [19], suggesting that different starch sources at this age may play important roles in maintaining rumen health.

Additionally, we identified three characteristic bands: band 2 (representing an uncultured bacterium), band 3 (representing an uncultured rumen bacterium), and band 7 (representing *Selenomonas bovis*). *S. bovis*, isolated from yak rumen contents, can ferment arabinose, glucose, and mannose, producing acetate and propionate from glucose fermentation [20]. This species was detected in all three replicates of the WS group, indicating that wheat starch diet specifically promotes *S. bovis* growth in 56-day-old lambs.

Conclusions

Based on PCR-DGGE analysis, this study demonstrated that:

1. Different amylose to amylopectin ratios in concentrate had no significant effect on the diversity of dominant rumen bacteria in lambs; however, rumen bacterial diversity initially decreased and then increased with advancing age.
2. Feeding a wheat starch diet promoted the growth of *Selenomonas bovis* in the rumen of 56-day-old lambs.

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