Gastrointestinal tract development in fattening lambs fed diets with different amylose to amylopectin ratios

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Gastrointestinal tract development in fattening lambs fed diets with different amylose to amylopectin ratios

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Abbreviations: GIT, gastrointestinal tract; NDF, neutral detergent fiber; ADF, acid detergent fiber; VFA, volatile fatty acid; IGF-1, Insulin-like growth factors I; IGF-1R, Insulin-like growth factors receptors; TS, tapioca starch; MS, maize starch; WS, wheat starch; PS, pea starch; RT-PCR, real-time PCR; DM, dry matter; CP, crude protein.

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Thirty-six, 7-day-old male lambs of similar weights were used in the current study to
examine the effects of dietary amylose to amylopectin ratios (amylose/amylopectin) on
gastrointestinal development in fattening lambs fed concentrates with different sources of
starch (tapioca, wheat, maize and pea) and amylose/amylopectin (0.12, 0.23, 0.24 and 0.48,
respectively). The maize starch and wheat starch diets improved weight and volume of
gastrointestinal. The pea starch diet significantly increased papillar height ($P<0.001$), papillar
surface area ($P=0.019$), and density of papillae ($P=0.001$) in the rumen. Additionally, the pea
starch diet significantly enhanced villus height, crypt depth, and villus surface area and villus
to crypt ratio ($P<0.05$) in small intestine. Expressions of insulin-like growth factors I (*IGF-I*)
and insulin-like growth factors receptors (*IGF-IR*) significantly increased in the duodenum
mucosa ($P=0.021$, $P=0.006$, respectively), jejunum mucosa ($P=0.002$, $P=0.005$, respectively),
and ileum mucosa ($P=0.003$, $P<0.01$, respectively) in the pea starch diet group. The results of
the present study show that maize starch and wheat starch accelerated physical development
of rumen and intestine compartments, whereas pea starch increased development of their
some morphological parameters, possibly through enhanced expression of genes such as
IGF-I and *IGF-IR* in fattening lambs.

Keywords: growing lambs; digestive system; morphological development; starch

Postnatal development of the gastrointestinal tract (GIT) is vital for the health and productivity of ruminant animals. Intestinal development is particularly important during the process of transitioning from neonatal reliance on nutrients supplied from milk to nutrients supplied from solid feed (cereal grains and hay). In pre-weaning ruminants, solid feed intake, especially high carbohydrate diets, stimulates rumen microbial proliferation and volatile fatty acid (VFA) production, subsequently initiating rumen development (Harrison et al. 1960). The effects of different dietary treatments on gastrointestinal development and growth performance have been investigated in pre-weaning ruminants; for example, the effects of high carbohydrate diets (Suárez et al. 2006), dietary starch levels (Wang et al. 2009), grain processing (Lesmeister and Heinrichs 2004), and different starch sources (Khan et al. 2007).

Starch is composed of linear glucose polymers (amylose) and highly branched polymers (amylopectin). The amylose/amylopectin ratio varies in cereal grains and is correlated with starch digestibility; high levels of amylose are less digestible (Svihus et al. 2005). Slower rates of digestion increase the amount of starch passing through the rumen and influence the site of starch digestion along the GIT. These variations may affect the quantity and proportion of VFA in the GIT and influence its development. To develop starch sources with suitable amylose/amylopectin ratios for fattening lambs, a better understanding of GIT development is necessary. This would improve weaning strategies and foster better growth performance of post-weaning lambs. It is hypothesized that starch with different amylose/amylopectin ratios will influence gastrointestinal development of fattening lambs. Here, we fed fattening lambs diets containing different amylose/amylopectin ratios and investigated morphological development in the GIT and the expression of genes related to GIT development and growth.

MATERIALS AND METHODS

All experimental animals were cared for according to the Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Science.

Animals, Management, Feeding, and Treatments

Thirty-six hybrid male lambs (Northeast fine wool sheep × Merino) born in February 2013 were separated from their mothers at 7 days of age, weighed and grouped by body weight to four dietary treatments with nine lambs per group. The trial was conducted in a

farm during the spring period from February to mid-April (the weather data: daily maximum and minimum temperature were 3°C to -20°C), thus all the lambs were housed in wooden pens (0.7 × 1.5 m, bedded with wood shavings) in a room with a temperature of 10°C to 15°C. Water was provided *ad libitum*. During the experimental period (7-56 days of age) the lambs received milk by normally breast-feed on a regular schedule (3 times a day: 11:30-13:30, 16:30-18:30, 21:30-8:30 of the next day), experimental concentrates with different amylose/amylopectin ratios, and *Leymus chinensis* hay. The pens were designed with openings in the front and concentrates and hay were supplied in separate buckets and were provided *ad libitum* (15% refusals). The offered concentrate/hay ratio was 85/15, however most of the hay was left as refusal, which means that the major nutrient requirements for lambs came from milk and experimental concentrates. The chemical composition of *Leymus chinensis* was 91% for dry matter, 7% for crude protein, 47% for acid detergent fiber (ADF), 67% for neutral detergent fiber (NDF). The chopped length of the grass hay was approximately 3 cm.

The four experimental concentrates were formulated to be iso-starch, iso-nitrogenous, and iso-caloric, but with different sources of starch: tapioca starch (TS, amylose/amylopectin: 4.60/39.50=0.12), wheat starch (WS, amylose/amylopectin: 8.20/35.63=0.23), maize starch (MS, amylose/amylopectin: 8.36/35.50=0.24), and pea starch (PS, amylose/amylopectin: 14.63/30.24=0.48). The diets were formulated to meet the nutrient requirements from concentrate diet set by the NRC (2007) for lambs with medium growth speed. All starch sources were purchased from Shanghai Dragon and Food Co., Ltd (Shanghai, China). There were no differences between the four diets for other nutrient components. The ingredients and chemical composition of the experimental concentrates are shown in Table 1.

Measurements and Sampling

On day 56, twelve fattening lambs (three per treatment group) were slaughtered by

captive bolt stunning. The abdominal cavity was opened and the visceral organs were removed. The three parts of the stomach (reticulorumen, omasum, and abomasum), and the remainder of the GIT were separated and weighed with digesta. After removing digesta, the stomach was rinsed with cold saline and weighed. The volume displacement of each stomach compartment was determined by a technique reported by Stobo et al. (1966). Rumen tissues were sampled from the caudal portion of the caudoventral blind sac, cranial and caudal portions of the dorsal sac, cranial portion of the ventral sac, and ventral portion of the caudoventral sac. The following were used for morphological measurements: papillar height, papillar width, papillar surface area, and rumen muscularis thickness (Lesmeister et al. 2004; Mirzaei et al. 2014). Three 1-cm² samples from each rumen tissue were used to determine the density of the papillae (i.e., 15 measurements per lamb). All the rumen tissues were fixed in 4% formalin solution prior to subsequent measurements.

Three regions of the small intestine (duodenum, jejunum, and ileum), and the large intestine (cecum, colon, and rectum) were separated from mesentery and weighed with digesta. After removing digesta, the intestines were rinsed with cold saline and weighed. Small intestine sections (15–20 cm) from the midpoint of the duodenum (1 m from the pyloric sphincter), jejunum (midpoint of the small intestine), and ileum (1 m from the ileal–cecal junction) were collected for morphological analysis. As for the ruminal tissues, the small intestine tissues were fixed in 4% formalin solution prior to measurement. The intestinal canal was opened along its longitudinal axis and the mucosa tissue was harvested by gently scraping with a microscope slide; the collected tissue was snap-frozen in liquid nitrogen and stored at -80°C until used to obtain mRNA.

Morphological Analysis

The morphological analyses of the rumen and small intestine tissues were performed using the biopsy technique reported by Wang et al. (2009). After rinsing with water, the

samples were dehydrated through an ethanol series (85%, 85%, 95%, 95%, 100%, 100%) for 50 min at each step. They were cleared twice in turpentine, saturated with paraffin, and embedded. Seven μm thick sections (10 sections from each sample) were stained with hematoxylin/eosin and analyzed under a light microscope (Olympus, Tokyo, Japan) at 50 $\times$ magnification. Digital images were captured using a color video camera (Olympus, Tokyo, Japan) and used to measure the heights and widths of the papillae and villus, rumen muscularis thickness, and crypt depth; the image analysis software of the Mshot Digital Imaging System (Guangzhou, China) was used to make these measurements. The area of the papillae was calculated as height $\times$ width and the area of the villus as (width at top + width at bottom) $\times$ height/2. The villus/crypt ratio was determined by comparison of villus height and crypt depth.

Chemical Analysis

Samples of each lamb diet and their refusal were analyzed for dry matter (ISO 6496), crude protein (ISO 15670), according to the Association of Analytical Chemists (AOAC, 1995). ADF and NDF content was determined according to the procedure of Van Soest (1991). Starch content was determined according to the procedure of Hall (2001). The diets were analyzed to determine the contents of amylose and amylopectin using the procedure described by Englyst et al. (1992). Calcium and phosphorus were measured by inductively coupled plasma emission spectroscopy using an Atom Scan 25 Plasma Spectroscopy (Thermo Jarrell Ash Corp., Grand Junction, CO) after acid digestion.

Freeze-dried mucosa samples were homogenized in liquid nitrogen. Total RNA was extracted from the homogenate using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) according to the instructions of the manufacturer and resuspended in RNase-free water treated with diethyl pyrocarbonate (Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany). The amount of RNA was measured using a Nano-Drop ND-1000 spectrophotometer

(Nano-Drop Technologies, Wilmington, DE). The integrity and purity of the RNA was assessed through optical density (ratio at wavelengths of 260 and 280 nm greater than 1.9 and less than 2.0) and by electrophoresis using ethidium bromide staining. mRNA samples were stored at -80°C until analysis. To determine the level of mRNA expression of insulin-like growth factor I (*IGF-I*) and insulin-like growth factor I receptor (*IGF-IR*), first-strand cDNA was generated using 1-µg samples of mRNA and a PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China) according to the manufacturer's recommendations. Real-time PCR (RT-PCR) primers for *IGF-I* (M30653) and *IGF-IR* (EF669473) were designed according to the mRNA sequences using Primer Premier 5.00 (Premier Biosoft International, Palo Alto, CA, USA) and based on the available sheep sequences in GenBank: *IGF-I* (forward, 5'-TATTT CAACA AGCCC ACG-3' and reverse, 5'-ACATC TCCAG CCTCC TCA-3'; 109 bp) and *IGF-IR* (forward, 5'-TGCCC GCCCA ACACC TA-3' and reverse, 5'-CCCGT TGCGG ATGAA GC-3'; 156 bp). The reference gene was sheep β-2-microglobulin (*B2M*) (EF489535) (forward, 5'-CGCCA GAAGA TGGAA AGC-3' and reverse, 5'-TGAAC TCAGC GTGGG ACA-3'; 168 bp). All primers were synthesized commercially by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (Shanghai, China). RT-PCR was performed in a 25-µL reaction mixture using a Line-Gene K Real-Time PCR Detection System and software (Bioer Technology, Hangzhou, China); the reaction mixture contained 2 µL first-strand cDNA and SYBR Green I (SYBR Green PCR Master Mix, Roche) as a fluorescent dye. Thermal cycling was performed with an initial denaturation step of 10 min at 95°C, followed by 40 cycles of 15 s at 94°C, 58°C for 60s, and a final extension at 72°C for 30 s. Relative quantification of gene expression was performed using 3 replicates for each sample and normalized against *B2M*. The sequence of each product was determined by dideoxy-mediated chain termination sequencing at Sangon Biotechnology, Inc. (Shanghai, China). Relative quantification of target gene expression was

calculated from the calibration curve and normalized against the expression of *B2M*, using the equation, $R = 2^{(CT(B2M)-CT(test))}$ (Livak and Schmittgen 2001).

Calculation and Statistical Analyses

The weights of stomach compartments and intestine segments, capacities of stomach compartments, morphological indexes of the rumen and small intestine, and the relative expression ratio of the mRNA data were analyzed as a randomized complete block design using the General Linear Model (SAS Institute, Inc., Cary, NC, USA). Lambs were blocked by the different dietary amylose/amylopectin treatments (TS, WS, MS, and PS). The difference in treatment means were tested using Turkey's multiple range test. This model was used for the data analysis:

$$Y_i = \mu + T_i + e_i$$

where Y_i is the dependent variable, μ is the treatment means, T_i is the effect of different dietary amylose/amylopectin treatments, i is the different treatments (TS, WS, MS, and PS), and e_i is the random residual error. Results are reported as least square means and pooled SEM values; statistical significance was set as $P < 0.05$.

RESULTS

The anatomical measurements made in the fattening lambs are presented in Tables 2, 3, and 4. The live weights of the whole stomach ($P = 0.002$), forestomach ($P = 0.025$) and reticulorumen ($P = 0.017$) were significantly higher in lambs fed the MS diet than in those fed the WS diet. Lambs fed the MS diet also had higher weights of rumen than those fed the TS diet ($P = 0.024$); the weight of the rumen was lowest in lambs fed the TS diet (Table 3). The ratio of dietary amylose/amylopectin did not have a significant effect on the weight of intestine ($P > 0.05$) (Table 3). The weight of the jejunum was higher in lambs fed the PS diet and MS diet than in those fed TS diet and WS diet ($P = 0.036$). By contrast, the weight of the ileum was lower in lambs fed the PS diet and MS diet than in those fed the TS diet and WS

diet ($P = 0.041$). The weight of the rectum was highest in lambs fed the WS diet ($P = 0.040$), followed by those fed the MS diet, the PS diet, and the TS diet. The capacity of the omasum was larger in lambs fed the TS diet than in those fed the MS diet ($P = 0.034$) (Table 4).

The effects of the four diets on the morphology of the rumen epithelium and small intestinal mucosa of fattening lambs are summarized in Tables 5 and 6. The height ($P < 0.001$), surface area ($P = 0.019$), and density ($P = 0.001$) of rumen papillae in the PS group were greater than those in the TS, MS, and WS groups (Table 5). In the small intestine, villus height ($P = 0.008$), crypt depth ($P = 0.012$), and surface area ($P = 0.003$) of the duodenum mucosa; crypt depth ($P = 0.025$) and surface area ($P = 0.001$) of the jejunum mucosa; and crypt depth ($P < 0.001$), villus/crypt ratio ($P = 0.004$), and villus surface area ($P = 0.036$) of the ileum mucosa were greater in lambs fed the PS diet than in lambs of the TS, MS, and WS groups (Table 6). The villus/crypt ratio in the duodenum mucosa was greatest in lambs fed the WS diet, followed by the MS, PS, and TS diets.

We examined the effects of the different diets on the expression of two genes *IGF-1* and *IGF-1R*, associated with early development (Figs. 1, 2). In the small intestine, the highest level of *IGF-1* expression was found in the duodenum ($P = 0.038$) in the PS group compared to the WS, MS, and TS groups (Fig. 1). The PS diet significantly increased the mRNA levels of *IGF-1* in the jejunum ($P = 0.002$) and ileum ($P = 0.003$). The level of expression of *IGF-1R* in the duodenum ($P = 0.016$), jejunum ($P = 0.005$), and ileum ($P < 0.001$) was highest ($P < 0.05$) in the PS group, followed by those fed WS, MS, and TS diets (Fig. 2).

DISCUSSION

The results of the present study show that MS and WS accelerated physical development of rumen and intestine compartments, whereas PS increased development of their some morphological parameters such as papillae height, villus height, crypt depth and villus surface area, possibly through enhanced expression of genes such as *IGF-1* and *IGF-1R* in fattening

lamb. In the study reported previously (Ren et al., 2015), the PS diet tended to increase average daily gain (136, 144, 126 and 165g/lamb for TS, MS, WS and PS, respectively) and concentrate intake (185, 204, 191 and 212 g/d for TS, MS, WS and PS, respectively) compared to the other three starch sources in the fattening lambs. Therefore, these results indicate that development of morphological parameters of GIT and its segments for fattening lambs in the postnatal period is more critical. Recent studies identified specific interactions between nutrients and gene expression in the digestive tract that serve to increase visceral organ mass and directly change nutrient metabolism (Baldwin et al. 2004). In general, the digestibility of starch is determined by two major high molecular weight carbohydrate components, namely, amylose and amylopectin, and the nutritional value of starch depends to a considerable extent on the ratio of amylose to amylopectin (Camp et al. 2003; Doti et al. 2014). In ruminants, the early consumption post-natally of a feed starter promotes rumen development by stimulating the fermentation process (Naeem et al. 2012). Development of a viable digestive function within the gastrointestinal tract is required to initiate the maturation of the rumen epithelia and small intestine. This maturation process involves the differential expression of numerous genes regulating both physical and metabolic characteristics in the various tissues. Development of the GIT is therefore very sensitive to dietary nutrition during the early growth stage (Sun et al. 2014), suggesting that the amylose/amylopectin ratio of different starch sources might influence development of the GIT.

After beginning to take solid feed and the subsequent establishment of ruminal fermentation, the rumen of the young animal undergoes both physical and metabolic development (Baldwin et al. 2004). During the process of transitioning lambs from their neonatal reliance on nutrients supplied from milk to nutrients supplied from solid feed (cereal grains and hay), the mass and volume of the gastrointestinal tract grow rapidly and its function improves rapidly to mature into a fully functioning digestive system. Thus, the

greater weight of the stomach of the lambs on the PS diet compared to the TS diet and the greater weight of the stomach in lambs on the MS diet compared to the WS diet may be ascribed to greater physical stimuli resulting from increased consumption of solid feed and especially daily starch intake. Indeed as reported previously (Ren et al., 2015), average daily starch intakes of TS, MS, WS and PS lambs were 81.4, 90.0, 84.4 and 93.4 g, respectively. The physical structure of the feed is also likely to have a large influence on the development of the rumen muscularization and volume (Norouzzian and Valizadeh 2014).

Among the most dramatic physiological challenges to young ruminants is the development of rumen morphology, which is critical for nutrient utilization (Baldwin et al. 2004; Odongo et al. 2006). Amylopectin-rich starches are easily digested compared to those high in amylose. Diets with different amylose/amylopectin ratios influence the starch fermentation pattern and, thus, the relative amounts of short-chain VFA in the rumen are altered and ruminal development may be affected (Baldwin et al. 2004; Khan et al. 2008; Chen et al. 2013). Results from this study showed that the height, surface area, and density of papillae in the rumen were highest in lambs on the PS diet. We suggest that these changes are attributable to the increased level of chemical stimuli. The GIT epithelium is responsible for many physiological functions, including absorption, transportation, and metabolism of nutrients (Baldwin 1998). Increases in the height of the papillae enhance the absorptive capacity of the rumen. Additionally, the occurrence of the larger papillar surface areas and higher papillar densities in the PS diet (high amylose/amylopectin ratio) are indicative of a larger ruminal absorptive area. These changes contribute to increases in the supply of VFA to the rumen epithelium, and thus stimulate the further development of the rumen. The growth of papillae is an important aspect of the physical development of the rumen (Khan et al. 2008). The establishment of rumen fermentation promotes the development of the ruminal epithelium, and ultimately enhances the presence of VFA in the ruminal lumen to promote

normal papillary development. Previous studies have reported that the stimulatory effects of different VFA vary, with butyrate being the most stimulatory followed by propionate (Tamate et al. 1962). The amylose/amylopectin ratio influences the formation of resistant starch and is negatively correlated with starch digestion (Sajilata et al. 2006; Svihus et al. 2005). In the present study, the highest amylose/amylopectin ratio was present in the PS diet (0.48) and this might have caused the lowest rate of starch digestion and affected the quantity and proportion of VFA in the rumen, leading to greater stimulation of rumen development. Higher dietary amylose/amylopectin ratios might have provided higher levels of chemical stimuli and, thereby, resulted in greater height, surface area, and density of papillae in the rumens of lambs on the PS diet, followed by those fed MS, WS, and TS diets.

Although the most dramatic physical changes during development are associated with the rumen epithelium, changes in intestinal development also occur in response to nutritional changes in the diet (Baldwin et al. 2004; Wang et al. 2009). The initiation of solid feed is the main reason for the physical changes in the intestine. The results of intestinal weight among different group indicate that there may be other factors that affect the weight of the intestine. It was previously reported that the physical form of starch, its relationship to proteins, and cellular integrity of starch-containing units also affect development of the GIT (Khan et al. 2008).

The results here indicated that the PS diet increased the morphological development of the small intestine mucosa. Changes in the development of enterocytes and in the structure of villi determine the digestive and absorptive capacity of the small intestine (Wang et al. 2009). In the pre-weaning period, intestinal cells in fattening lambs undergo rapid proliferation to enable digestion and absorption of nutrients from the maternal milk and feeds. Villus height and crypt depth are direct indicators of the intestinal environment and may be used to estimate intestinal health. Changes to the morphology of the small intestine, such as increased

villus height and width, and crypt depth improve its absorptive function (Baldwin et al. 2004; Wang et al. 2009).

The maturation of the GIT is the result of the differential expression of numerous genes regulating both physical and metabolic characteristics of the tissue (Baldwin et al. 2004). In this study, the mRNA levels of *IGF-1* and *IGF-1R* in lambs fed different diets were examined in the mucosa of the small intestine. *IGF-1* is an important regulator of the activity of the small intestine that influences the proliferation of many cell types (including intestinal epithelial cells) and plays a fundamental role in growth, development, and metabolism (Lowe 1991; MacDonald 1999). The somatotrophic axis involving *IGF-1* and insulin affects gastrointestinal tract development and especially the proliferation and maturation of enterocytes (Ménard et al. 1999). These effects are consistent with those of the PS diet on small intestine development. *IGF-1*-mediated improvements in villus morphology may result in better intestinal health and function. IGF-1 affects gastrointestinal tract morphology and function only after binding to its mucosal receptor, IGF-1R (Lund 1994). Thus, the higher level of *IGF-1R* in lambs fed the PS diet suggests the possibility of increased IGF-1 function in the morphological development of the small intestine. The results reported here indicated that the diet with the highest amylose/amylopectin ratio (PS diet) promoted the development of the small intestine by enhancing the expression of *IGF-1* and *IGF-1R*.

CONCLUSIONS

MS and WS accelerated physical development of rumen and intestine compartments, whereas PS increased development of their some morphological parameters such as papillae height, villus height, crypt depth and villus surface area, possibly through enhanced expression of genes such as IGF-1 and IGF-1R in fattening lambs. Therefore, a diet with a high amylose/amylopectin ratio will increase the GIT development of fattening lambs and may contribute to better growth performance in the future.

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404 **Figure captions**

405 **Fig. 1.** Insulin-like growth factors I (*IGF-I*) mRNA expression in the small intestinal mucosa of fattening
406 lambs fed diets containing different amylose/amylopectin. The expression of *IGF-I* mRNA relative to
407 β -2-microglobulin (*B2M*) mRNA is expressed as the mean $\pm$ standard error of each region of the small
408 intestinal. Values without similar letters are significantly different ($P < 0.05$).

409

410 **Fig. 2.** Insulin-like growth factors receptors (*IGF-IR*) mRNA expression in the small intestinal mucosa of
411 fattening lambs fed diets containing different amylose/amylopectin. The expression of *IGF-IR* mRNA
412 relative to β -2-microglobulin (*B2M*) mRNA is expressed as the mean $\pm$ standard error of each region of
413 the small intestinal. Values without similar letters are significantly different ($P < 0.05$).

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