Effects of different starch source of starter on small intestinal growth and endogenous GLP-2 secretion in preweaned lambs¹

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ABSTRACT: The objective of this study was to investigate the effects of different sources of starch in starter feed on small intestinal growth and endogenous glucagon-like peptide 2 (GLP-2) secretion in preweaned lambs. Twenty-four 10-d-old lambs were divided into three groups that were treated with different iso-starch diets containing purified cassava starch (CS, $n = 8$), maize starch (MS, $n = 8$), and pea starch (PS, $n = 8$). At 56 d old, there was no significant difference in final body weight (BW) of lambs among the three groups. However, different starch source in starter significantly affected the average daily feed intake (ADFI) and average daily gain (ADG) of lambs among three groups. Compared with the CS and MS diets, the PS diet significantly increased the GLP-2 concentration in blood plasma ($P < 0.001$), the crypt depth of the jejunum ($P = 0.006$), and the villus height of the ileum ($P = 0.039$). Meanwhile, PS

diet significantly increased the mRNA expression of proglucagon and the glucagon-like peptide 2 receptor (GLP-2R) in the jejunum and ileum ($P < 0.001$). Furthermore, the PS diet significantly upregulated the mRNA expression of cyclin D1 ($P < 0.001$), cyclin E ($P = 0.006$), and cyclin-dependent kinases 6 (CDK6) ($P = 0.048$) in the jejunum and cyclin A ($P < 0.001$), cyclin D1 ($P < 0.001$), and CDK6 ($P = 0.002$) in the ileum. Correlation analysis showed that endogenous GLP-2 secretion was positively related to the mRNA levels of cell cycle proteins in small intestinal mucosa. In summary, all results showed that PS in starter feed promoted small intestinal growth that may, in part, be related to cell cycle acceleration and endogenous GLP-2 secretion in preweaned lambs. These findings provide new insights into nutritional interventions that promote the development of small intestines in young ruminants.

Key words: cell proliferation, glucagon-like peptide-2, intestinal epithelia, lamb, starch

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INTRODUCTION

In young ruminants, due to the immature physiological function of the rumen, higher amounts of nutrients may enter the small intestine to be digested and absorbed, which suggested that the small

intestine may play a more critical role than it does in adult ruminant animals (Wood et al., 2000; Baldwin et al., 2004). Thus, nutritional intervention strategies that promote small intestinal growth in preweaned ruminants may have great significance for animal performance. Studies indicated that different starch source of starter had a various rate and extent of digestion in the rumen, which resulted in different amounts of starch entering the small intestine, and then impacted the development of intestinal epithelia (Goni et al., 1996; Moharrery et al., 2014; Ren et al., 2016). However, the molecular mechanism of different starch source of starter affecting the small intestinal growth is unknown.

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Many previous studies demonstrated that intestinal development was, in part, regulated by the secretion of glucagon-like peptide-2 (GLP-2), which can be stimulated by nutrients, especially carbohydrates and lipids (Martin et al., 2005; Taylor-Edwards et al., 2011; Connor et al., 2015). Recent studies in nonruminants have demonstrated the molecular mechanism of GLP-2 promotes small intestinal growth (Dube and Brubaker, 2007; Drucker and Yusta, 2014). However, the specific mechanism of GLP-2 that promotes the proliferation of intestinal epithelium in ruminants was unclear. Connor et al. (2010) found a significant positive correlation between the mRNA expression of GLP-2 receptor (GLP-2R) and cyclin D1, which suggested that GLP-2 may promote the proliferation of small intestinal epithelial cells associated with increments of cyclin D1 expression in dairy cows.

Thus, we hypothesized that different starch source of starter may affect small intestinal growth, cell cycle progress, and endogenous GLP-2 secretion in preweaned lambs.

MATERIALS AND METHODS

The experimental design and procedures for this study were approved by the Animal Care and Use Committee of Nanjing Agricultural University following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988).

Animal Experimental Design

A total of 24 newborn Hu sheep lambs were selected in this study. The beginning date of experiment was adjusted for each lamb to account for different birth dates. The 24 lambs were divided into three groups with different feeding treatments: cassava starch (CS, amylose/amylopectin = 0.11, $n = 8$), maize starch (MS, amylose/amylopectin = 0.27, $n = 8$), and pea starch (PS, amylose/amylopectin = 0.44, $n = 8$). At 10 d of age, the lambs began to be separated from their mothers from 0400 to 1900 every day and placed in individual pens. The lambs received starter feed with different amylose-to-amylopectin ratios (CS, MS, and PS) ad libitum, and were fed with breast milk for 1 h at fixed times (0630, 1030, and 1530) during this period. Starter was offered twice daily in two equal amounts at 0430 h and 1200 h daily. When the DMI (dry matter intake) of the lambs' starter reached 200 g/animal⁻¹d⁻¹, the amount of starter did not rise any further. The lambs in CS, MS, and PS group maintained a 200 g/animal⁻¹d⁻¹ starter intake for an

average of 8, 9, and 7 d before slaughter, respectively. All lambs received oat hay (10.05 % crude protein, 28.71 % crude fiber) and water ad libitum. None of the lambs had access to the ewes' concentrate feed. The starter intake of lambs was recorded every day from 15 d of age. The body weight (BW) of lambs was measured weekly before the morning feeding. And, the average daily feed intake (ADFI) and average daily gain (ADG) were also calculated. The experimental starter diets were designed according to the nutrient requirements of Hu sheep lambs (NY/Y816-2004; Ministry of Agriculture of China, 2004). The nutrient compositions of the three starter diets are presented in Table 1.

Chemical Analysis

Samples of each lamb starter were analyzed for dry matter (ISO 6496), crude protein (ISO 15670),

Table 1. Ingredient and chemical composition of the starter diets (dry matter basis)

Items	Diet		
	CS	MS	PS
Ingredient composition, % DM ¹			
CS ^a	51.60	-	-
MS ^a	-	51.60	-
PS ^a	-	-	51.60
Soybean meal	28.00	28.00	28.00
Corn gluten meal	15.00	15.00	15.00
Soybean oil	1.20	1.20	1.20
Limestone meal	0.80	0.80	0.80
CaHPO ₄	1.80	1.80	1.80
NaCl, Salt	0.60	0.60	0.60
Mineral and vitamin supplement ^b	1.00	1.00	1.00
Nutrient composition			
DM ¹ , %	88.37	88.78	88.45
Metabolic energy ^c , MJ/kg DM ¹	11.31	11.43	11.26
Crude protein ^d , % DM ¹	25.10	25.15	25.35
Crude fat ^d , % DM ¹	3.55	3.80	3.67
Crude fiber ^d , % DM ¹	6.24	6.34	6.89
Crude ash ^d , % DM ¹	6.37	6.33	6.18
Total starch ^d , % DM ¹	45.36	45.92	45.86
Amylose ^d , % DM ¹	4.43	9.68	14.12
Amylopectin ^d , % DM ¹	40.93	36.24	31.75
Amylose/amylopectin	0.11	0.27	0.44

^aThe purity of cassava, corn and pea starch are 92.3%, 92.7%, and 92.5%, respectively (actual values for measurement).

^bContained 16% calcium carbonate, 102 g/kg of Zn, 47 g/kg of Mn, 26 g/kg of Cu, 1,140 mg/kg of I, 500 mg/kg of Se, 340 mg/kg of Co, 5,665,235 µg/kg of vitamin A, 21,459 µg/kg of vitamin D, and 15,768 mg/kg of vitamin E.

^cvalues were analyzed based on database of the NRC (2007).

^dThe actual values for measurement.

¹DM = dry matter

crude fat (ISO 6492), crude fiber (ISO 6865), and crude ash (ISO 5894), according to the Association of Analytical Chemists (AOAC, 1995). Starch content and amylose content was analyzed using Total Starch Kit and Amylose/Amylopectin Kit (Megazyme, Bray, Ireland).

Sample Collection

At 56 d of age, lambs were slaughtered in a local slaughterhouse 2 h after their first starter feeding. In order to prevent cross-contamination, we disinfected all of the tools and house before collecting samples, and used a complete set of germfree sampling tools for every lambs. Jugular vein blood (at least 5 ml) was collected using blood collection tubes containing 40 KIU Na-heparin/ml blood. An aliquot of blood was transferred to a chilled tube containing aprotinin (Phoenix Pharmaceuticals, Phoenix Europe GmbH, Karlsruhe, Germany) (54 KIU/ml blood) to inhibit the activity of proteases (especially the Dipeptidyl Peptidase IV). Plasma was harvested by centrifuging the blood samples at 10,000 g at 4 °C for 10 min and stored at −80 °C until GLP-2 concentration analysis. Immediately after blood collection, lambs were stunned by captive bolt and then exsanguinated from the carotid arteries. Immediately after slaughter, a representative sample of rumen digesta (at least 200 ml) was collected, and pH values were determined using a portable pH meter (HI 9024C; HANNA Instruments, Woonsocket, RI). The rumen digesta was then strained through four layers of cheesecloth. A sample of the ruminal fluid (10 ml) was preserved in 25% (wt/vol) of metaphosphoric acid (5 ml ruminal fluid to 1 ml metaphosphoric acid) and stored at −20 °C until later analysis for VFA (volatile fatty acid) concentration using capillary column gas chromatography (GC-14B, Shimadzu, Japan; capillary column film thickness: 30 m × 0.32 mm × 0.25 µm; column temperature = 110 °C; injector temperature = 180 °C; detector temperature = 180 °C) (Qin, 1982). A sample of the ruminal fluid (10 ml) was stored at −20 °C until later analysis for amylase activity using an Amylase Activity Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China). The entire small intestine (duodenum, jejunum, and ileum) including contents was weighed. A representative sample of jejunum and ileum digesta (at least 10 g) was stored at −20 °C until later analysis for amylase activity using an Amylase Activity Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China). A representative sample of small intestine sections (20 cm) were collected

from the midpoint of the jejunum (midpoint of the small intestine), and ileum (30 cm from the ileal-cecal junction) (Schneider et al., 2008; Ren et al., 2016), and immediately washed three times in ice-cold phosphate-buffered saline. The samples were divided into three portions. The first portion of the tissue sample was cut into smaller pieces (0.5 cm × 0.5 cm) and immediately frozen in liquid nitrogen for RNA extraction, and then analyzed for mRNA expression of GLP-2R. The second portion of tissue sample scraped from the underlying tissue using a germ-free glass slide was immediately frozen in liquid nitrogen for RNA extraction, and then analyzed for mRNA expression of proglucagon (GCG, a precursor of GLP-2) and cell cycle proteins. The third portion was cut into smaller pieces (1.0 cm × 1.0 cm) and immediately fixed in 4 % paraformaldehyde (Sigma, St. Louis, MO) for histomorphometric microscopy analysis.

GLP-2 Level Measurement

The GLP-2 concentration of blood plasma was determined using commercial enzyme immunoassay kits (catalogue number: FEK-028-14; Phoenix Pharmaceuticals, Phoenix Europe GmbH, Karlsruhe, Germany) based on the method described by Moran et al. (2014). Samples were assayed in duplicate, and calibrator samples were included in each assay. The intra-assay variation was ±6%. Standard curves were constructed using GraphPad Prism 5 software (GraphPad Software Inc.). The Dipeptidyl Peptidase IV (DPPIV) inhibitor was included in the incubation media to avoid degradation of GLP-2 by the DPPIV enzyme present in the plasma.

Microscopic Study

Segments of jejunum and ileum were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at a 6 µm thickness, stained with hematoxylin and eosin, and mounted for analysis of villus height and crypt depth. Well-oriented and intact villi and crypts were examined for each animal. Measurements of villus height and crypt depth were made using a 40× objective lens, and at least 15 villi and 15 crypts were measured per animal. A minimum of five images were captured per section, with a minimum of three sections prepared per lambs. The microscopist was blinded to treatment conditions during the histomorphometric analysis. Image Pro Plus software (Media Cybernetics, Bethesda, MD) was used to measure predefined criteria previously described by

Moran et al. (2014). In brief, the crypt depth and the villus height were measured as the average distance from crypt base to crypt-villus junction and villus base to villus tip, respectively.

RNA Isolation and cDNA Synthesis

Total RNA was extracted with acid using Trizol (Takara Bio, Otsu, Japan) as described by Chomczynski and Sacchi (1987). The RNA concentration was then quantified using a Nanodrop spectrophotometer ND-1000UV-Vis (Thermo Fisher Scientific, Madison, WI). The absorption ratio (260/280 nm) of all samples was between 1.80 and 2.10, indicating high RNA purity. Aliquots of RNA samples were subjected to electrophoresis using a 1.4% agarose-formaldehyde gel to verify integrity. The concentration of RNA was adjusted to 1 µg/µl based on optical density and stored at -80 °C. Total RNA (1 µg) was carefully reverse-transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara Bio) according to the manufacturer's instructions.

Primer Design and qRT-PCR

Primer sets were designed to recognize and amplify conserved nucleotide sequences. Sheep cell cycle proteins (cyclins), cyclin-dependent kinases (CDKs), GCG, GLP-2R, and 18S ribosomal RNA (18S rRNA) cDNA sequences, and/or homologue(s) were identified using the BLAST (Basic Local Alignment Search Tool) computer program

(National Center for Biotechnology Information, Bethesda, MD). Primers were designed using the Primer 5 computer program (Whitehead Institute, Cambridge, MA). All of the primers were synthesized by Invitrogen Life Technologies (Shanghai, China). Real-time PCR analysis of target genes and 18S rRNA analysis were performed using the QuantStudio 5 Real-Time PCR Instrument (Applied Biosystems, Foster, CA) with fluorescence detection of SYBR green dye. Amplification conditions were as follows: 30 s at 95 °C followed by 40 cycles composed of 5 s at 95 °C, 34 s at 60 °C, 15 s at 95 °C, 60 s at 60 °C, and 15 s at 95 °C. Each sample contained 1–10 ng of cDNA in 2 × SYBR Green PCR Master Mix (Takara Bio) and 200 nmol/l of each primer in a final volume of 20 µl. All measurements were performed in triplicate. A reverse-transcription-negative blank of each sample and a no-template blank served as negative controls. Melt curve analysis showed no primer dimer formation in the assays. The PCR amplification efficiencies for all of the primers ranged between 92.1 and 104.2%. Delta Ct values were obtained by normalizing the Ct values of the target genes with 18S rRNA. The relative amount of each studied mRNA sample was analyzed according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). The primers and amplicon sizes of all genes are presented in Table 2.

Statistical Analysis

Results are presented as means with SE. Turkey's multiple range test in one-way analysis

Table 2. Primers for quantitative real-time PCR¹

Gene name	Gene ID	Primer sequence (5'→3')	Amplicon size, bp
GCG	NW_011943216.1	For: CGGAAGAAGTCAACATCGT R: TAAAGTCTCGGGTAGCAAG	119
GLP-2R	XM_004013306	For: GGCCTACAGATACTGCCTGTCT R: GATGTAGTTACGTGTGCAGTGA	244
Cyclin A	NC_019478.2	For: CTCTCCTATCACCGCCTGAC R: CTTTGGGGTCCAAGTTCTGC	144
Cyclin D1	NC_019478.2	For: CCTGCCGTCCATGCGGAA R: GAACTTCACATCTGTGGCAC	403
Cyclin E	XM_015100542	For: TGGCACCGATGTCTCTGTTC R: CCACACTGGCTTCTCACAGT	114
CDK2	NM_001142509.1	For: CCTAGCTTTCTGCCACTCTCAT R: TCACCACCTCGTGGGTATAAGT	153
CDK4	NM_001127269.1	For: GACCAAGACCTCAGGACGTATC R: CACCACTTGTCACCAGAATGTT	250
CDK6	XM_012177413.2	For: GATGGCTCTTACCTCAGTGGTT R: GGGTAGGGCAACATCTCTAGG	228
18S rRNA	KR264960	For: GATGGTAGTCGCCGTGCCTA R: TGCTGCCTTCCTTGGATGTG	107

¹GCG = proglucagon; GLP-2R = glucagon-like peptide 2 receptor; CDK2 = cyclin-dependent kinases 2; CDK4 = cyclin-dependent kinases 4; CDK6 = cyclin-dependent kinases 6; 18S rRNA = 18S ribosomal RNA.

of variance (ANOVA) was performed to assess statistical significance using SPSS software packages (SPSS version 18.0.1 for Windows; SPSS Inc, Chicago, IL). Correlation analysis was done using GraphPad Prism software version 5.00 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). Power calculations calculated using G*Power 3.1.9.2 before the start of the study had identified a required sample size of eight lambs per treatment group in order to enable detection of an effect size of 0.70 SD for most of the cognitive test scores with 80% power and a type I error of 5%, based on *F*-test using one-way ANOVA. Results with $P < 0.05$ were considered significant to identify differences among groups.

RESULTS

Animal Performance

The ADFI, ADG, and BW at different ages of lambs are presented in Table 3. There was no significant difference ($P = 0.810$) in birth weight among lambs in the CS group (3.23 ± 0.24 kg), MS group (3.31 ± 0.07 kg), and PS group (3.38 ± 0.14 kg).

The starter intake of lambs was recorded every day. The BW of lambs was measured weekly. Results showed no significant difference in BW among the three groups at the age of 1 wk ($P = 0.620$), 2 wk ($P = 0.875$), 3 wk ($P = 0.699$), 4 wk ($P = 0.313$), 7 wk ($P = 0.117$), and 8 wk ($P = 0.268$). But, PS-fed lambs had lower BW at the age of 5 wk ($P = 0.012$) and 6 wk ($P = 0.047$). There was no significant difference of ADG among lambs in the three groups at the age of 1 wk ($P = 0.949$), 2 wk ($P = 0.710$), 3 wk ($P = 0.105$), 6 wk ($P = 0.701$), 7 wk ($P = 0.584$), and 8 wk ($P = 0.222$). But, PS-fed lambs had lower ADG at the age of 4 wk ($P = 0.009$) and 5 wk ($P = 0.013$). Results showed no significant difference of ADFI among lambs in the three groups at the age of 5 wk ($P = 0.465$), 6 wk ($P = 0.452$), 7 wk ($P = 0.717$), and 8 wk ($P = 0.953$). But, PS-fed lambs had lower ADFI at the age of 3 wk ($P = 0.031$) and 4 wk ($P = 0.049$).

Physiological Parameters of the Rumen and Small Intestine

As shown in Table 4, there was no significant difference in ruminal pH ($P = 0.379$) among the three groups. The concentrations of total VFA

Table 3. Effect of different sources of starch in starter on the ADFI, ADG, and BW at different ages of lambs among three groups^a

Item	Age, week	CS	MS	PS	SEM	<i>P</i> -value
ADFI, g/d	3	21.34 ^{b,c}	24.46 ^b	9.48 ^c	2.75	0.031
	4	50.16 ^{b,c}	68.60 ^b	30.22 ^c	6.96	0.049
	5	104.10	108.83	86.59	7.12	0.465
	6	168.73	150.61	142.61	8.01	0.452
	7	193.13	185.20	185.37	4.01	0.717
	8	195.40	195.13	194.00	1.70	0.953
ADG, g/d	1	170.54	166.07	160.71	11.92	0.949
	2	198.21	176.79	183.93	10.37	0.710
	3	145.77	128.57	112.50	6.44	0.105
	4	180.82 ^b	171.43 ^b	133.93 ^c	7.03	0.009
	5	203.57 ^{b,c}	217.86 ^b	146.43 ^c	11.09	0.013
	6	218.37	198.21	205.36	9.54	0.701
	7	229.29	216.18	233.37	6.84	0.584
	8	248.21	219.64	260.71	9.80	0.222
BW, kg	1	4.42	4.69	4.43	0.12	0.620
	2	5.81	5.93	5.71	0.16	0.875
	3	6.82	6.83	6.50	0.17	0.699
	4	8.09	8.03	7.44	0.19	0.313
	5	9.52 ^b	9.55 ^b	8.46 ^c	0.18	0.012
	6	11.05 ^b	10.94 ^b	9.90 ^c	0.21	0.047
	7	12.66	12.46	11.54	0.24	0.117
	8	14.39	13.99	13.36	0.26	0.268

^aMean values within a column with unlike superscript letters (b and c) were significantly different ($P < 0.05$). Three groups were treated with different iso-starch diets containing CS ($n = 8$), MS ($n = 8$), and PS ($n = 8$).

Table 4. Effect of different sources of starch in starter on ruminal and blood parameters in lambs^a

Item	CS	MS	PS	SEM	P-value
Ruminal pH	5.50	5.63	5.73	0.07	0.379
Total VFA, mM	131.11 ^b	119.29 ^c	107.65 ^d	2.38	<0.001
Acetate, mM	75.07 ^b	70.86 ^c	63.13 ^d	1.24	<0.001
Propionate, mM	31.78 ^b	27.65 ^c	20.97 ^d	1.07	<0.001
Butyrate, mM	20.88 ^b	17.96 ^c	19.79 ^b	0.42	0.010
Other VFA ¹ , mM	3.38 ^{b,c}	2.81 ^c	3.75 ^b	0.14	0.011
Acetate, mol/100 mol of VFA	57.29 ^c	59.41 ^b	58.66 ^b	0.26	0.001
Propionate, mol/100 mol of VFA	24.16 ^b	23.18 ^b	19.48 ^c	0.50	<0.001
Butyrate, mol/100 mol of VFA	15.96 ^c	15.05 ^c	18.39 ^b	0.38	<0.001
Other VFA ¹ , mol/100 mol of VFA	2.58 ^c	2.36 ^c	3.02 ^b	0.13	<0.001
Acetate: Propionate	2.38 ^d	2.57 ^c	3.02 ^b	0.07	<0.001
<i>Blood parameters</i>					
Plasma GLP-2 ² , pg/ml	46.80 ^c	45.43 ^c	59.19 ^b	1.79	<0.001

^aMean values within a column with unlike superscript letters (b and c) were significantly different ($P < 0.05$). Three groups were treated with different iso-starch diets containing CS ($n = 8$), MS ($n = 8$), and PS ($n = 8$).

¹Other VFA = valerate + isobutyrate + isovalerate.

²GLP-2 = glucagon-like peptide 2.

($P < 0.001$), acetate ($P < 0.001$), and propionate ($P < 0.001$) were the lowest in PS-fed lambs, followed by MS-fed lambs, and then CS-fed lambs. The butyrate concentration was lower in MS-fed lambs compared to CS-fed and PS-fed lambs ($P = 0.010$). The concentration of other VFA was greater ($P = 0.011$) in lambs fed the PS diet compared to those fed the MS diets, and was no difference with those fed the CS diets. The acetate proportion was lower ($P = 0.001$) in CS-fed lambs than in MS-fed and PS-fed lambs. The propionate proportion of PS-fed lambs was lower ($P < 0.001$) than those of CS-fed and MS-fed lambs. The butyrate proportion ($P < 0.001$) and other VFA proportions ($P < 0.001$) were greater in lambs fed the PS diet than in lambs fed the CS and MS diets. The acetate-to-propionate ratio was the greatest in lambs fed the PS diet ($P < 0.001$) and lowest in lambs fed the CS diet. Compared with the groups on CS and MS diets, the PS diet group had significantly greater GLP-2 concentration in plasma ($P < 0.001$).

As shown in Table 5, the amylase activity in ruminal contents was not significantly different among the three groups ($P = 0.098$). The amylase activity in jejunal contents was the greater ($P = 0.007$) in PS-fed lambs compared to those fed the CS, and was no difference with those fed the MS diets. However, the amylase activity in ileal contents had no significant difference ($P = 0.163$) among the three groups.

Small Intestinal Morphology

As shown in Table 6. The entire small intestine (duodenum, jejunum, and ileum) including

Table 5. Effect of different sources of starch in starter on ruminal and small intestinal amylase activity in lambs^a

Item	CS	MS	PS	SEM	P-value
Rumen, U/mgprot ¹	1.76	1.73	1.43	0.07	0.098
Jejunum, U/mgprot	19.44 ^c	24.31 ^{b,c}	28.67 ^b	1.28	0.007
Ileum, U/mgprot	18.92	22.57	24.99	1.22	0.163

^aMean values within a column with unlike superscript letters (b and c) were significantly different ($P < 0.05$). Three groups were treated with different iso-starch diets containing CS ($n = 8$), MS ($n = 8$), and PS ($n = 8$).

¹U = unit of activity.

contents to BW, expressed as a percentage of live BW, had a trend of increase ($P = 0.072$) in PS group, compared with that in CS group. In the jejunum, crypt depth ($P = 0.006$) in the PS diet group was greater than that in the CS and MS groups significantly, while there was no significant difference in villus height ($P = 0.215$) or in villus height/crypt depth ($P = 0.437$) among the three groups. In the ileum, villus height ($P = 0.039$) in the PS and MS diet groups was greater than that in the CS group significantly. There was no significant difference in crypt depth ($P = 0.833$) among the three groups. Compared with CS group, the villus height/crypt depth had a trend of increase ($P = 0.068$) in PS group.

The mRNA Expression of GCG and GLP-2R in Small Intestinal Tissue

The effects of different sources of starch in starter feeds on the mRNA expression of GCG

Table 6. Effect of different sources of starch in starter on small intestinal relative weight and small intestinal morphological indexes in lambs^a

Item	CS	MS	PS	SEM	P-value
Small intestinal relative weight ^b , %	2.02	2.24	2.41	0.18	0.072
Jejunum					
Villus height, μm	424.47	447.37	485.27	9.73	0.215
Crypt depth, μm	368.40 ^d	362.80 ^d	448.38 ^c	11.19	0.006
Villus height/Crypt depth	1.22	1.36	1.16	0.03	0.437
Ileum					
Villus height, μm	373.30 ^d	422.81 ^c	425.14 ^c	9.87	0.039
Crypt depth, μm	320.84	324.82	312.03	7.85	0.833
Villus height/Crypt depth	1.18	1.32	1.50	0.05	0.068

^aMean values within a column with unlike superscript letters were significantly different ($P < 0.05$). Three groups were treated with different iso-starch diets containing CS ($n = 8$), MS ($n = 8$), and PS ($n = 8$).

^bSmall intestinal relative weight, entire small intestine (duodenum, jejunum, and ileum) including contents/body weight.

and GLP-2R in small intestinal tissue are presented in Fig. 1. In the jejunum, the mRNA expression of GCG was greatest ($P < 0.001$) in lambs fed the PS diet, followed by those fed the MS diet and then the CS diet. The mRNA expression of GLP-2R was greatest ($P < 0.001$) in lambs fed the PS diet compared to lambs in the MS and CS diet groups. In the ileum, the expression of GCG and GLP-2R was greater ($P < 0.001$) in lambs fed the PS diet than that in lambs fed the MS and CS diets.

The mRNA Expression of Cell Cycle Proteins in Small Intestinal Mucosa

The effects of different sources of starch in starter feeds on the mRNA expression of cell cycle proteins in small intestinal mucosa in lambs are presented in Fig. 2. In the jejunum, compared with the CS diet group, the PS diet group had significantly increased mRNA levels of cyclin D1 ($P < 0.001$), cyclin E ($P = 0.006$), CDK6 ($P = 0.048$), and a trend of increase mRNA levels of cyclin A ($P = 0.081$); however, the mRNA expression level of CDK2 ($P = 0.248$) and CDK4 ($P = 0.921$) did not differ among the three groups. In the ileum, the PS diet group had significantly increased mRNA levels of cyclin A ($P < 0.001$), cyclin D1 ($P < 0.001$), and CDK6 ($P = 0.002$) compared with the CS and MS diet groups; However, the mRNA expression level of cyclin E ($P = 0.409$), CDK2 ($P = 0.267$) and CDK4 ($P = 0.299$) did not differ among the three groups.

Correlation Between the Endogenous GLP-2 Secretion and the mRNA Levels of Cell Cycle Proteins in Small Intestinal Mucosa

The relationships between the endogenous GLP-2 secretion and the mRNA levels of cell cycle

Figure 1. Effect of different sources of starch in starter on GCG and GLP-2R mRNA expression in lambs. Three groups were treated with different iso-starch diets containing CS ($n = 8$), MS ($n = 8$), and PS ($n = 8$). Quantitative RT-PCR results are expressed as relative mRNA expression (fold of CS group, normalized to 18S rRNA) as means, with their standard errors. Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$). GCG = Proglucagon; GLP-2R = glucagon-like peptide 2 receptor; 18S rRNA = 18S ribosomal RNA.

proteins in small intestinal mucosa are shown in Fig. 3. Correlation analysis revealed that there were positive correlations between the GLP-2 concentration in plasma and cyclin E mRNA expression in jejunum ($P = 0.017$; $r = 0.48$), cyclin A ($P = 0.049$; $r = 0.41$), and cyclin D1 ($P = 0.004$; $r = 0.56$) mRNA

Figure 2. Effect of different sources of starch in starter on cell cycle protein mRNA expression in lambs. Three groups were treated with different iso-starch diets containing CS ($n = 8$), MS ($n = 8$), and PS ($n = 8$). Quantitative RT-PCR results are expressed as relative mRNA expression (fold of CS group, normalized to 18S rRNA) as means, with their standard errors. Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$). CDK2 = cyclin-dependent kinases 2; CDK4 = cyclin-dependent kinases 4; CDK6 = cyclin-dependent kinases 6; 18S rRNA = 18S ribosomal RNA.

Figure 3. Correlation between the endogenous GLP-2 secretion and the mRNA levels of cell cycle proteins in small intestinal mucosa. The red color represents a significant positive correlation ($P < 0.05$; $r > 0.40$), the green color represents not significant correlation ($P > 0.05$) and the gray color shows that not conducted analysis of correlation. GCG = Proglucagon; GLP-2R = glucagon-like peptide 2 receptor; CDK2 = cyclin-dependent kinases 2; CDK4 = cyclin-dependent kinases 4; CDK6 = cyclin-dependent kinases 6; 18S rRNA = 18S ribosomal RNA.

expression in ileum. In the jejunum, the mRNA expression of GCG was positively correlated with the mRNA expression of cyclin D1 ($P = 0.007$; $r = 0.53$) and cyclin E ($P = 0.046$; $r = 0.41$); the mRNA expression of GLP-2R was positively correlated with the mRNA expression of cyclin D1 ($P = 0.026$; $r = 0.45$). In the ileum, the mRNA expression of GCG was positively correlated with the mRNA expression of cyclin A ($P = 0.001$; $r = 0.62$), cyclin D1 ($P < 0.011$; $r = 0.73$), and CDK6 ($P = 0.015$; $r = 0.49$); the mRNA expression of GLP-2R was positively correlated with the mRNA expression of cyclin A ($P = 0.006$; $r = 0.55$), cyclin D1 ($P < 0.001$; $r = 0.79$), and CDK6 ($P < 0.001$; $r = 0.71$) in the ileum.

DISCUSSION

Animal Performance

In the current study, the BW at 8 wk of age in lambs was not affected by the different sources of starch in starter. These results are consistent with the studies discussed earlier (Ren et al., 2015). However, we found an interesting result that PS-fed lambs had a lower ADG than that in CS-fed and MS-fed lambs at the age of 4 wk and 5 wk. These results can probably be explained by the reason that the PS diet was difficult to be digested in the gastrointestinal tracts of newborn lambs, which may contribute to lower intake of starter feed, and then decreased the ADG of the lambs. The results indicated that different sources of starch affect the growth performance of lambs during preweaning, but the effects of different sources of starch on growth performance in the postweaning period need to be investigated further.

Ruminal and Small Intestinal Environment

Starch is an important energy source in animal feed (Orskov, 1986). The process of starch digestion in ruminants is different from nonruminants. In ruminants, starch is fermented into VFA in the rumen, which may provide energy for epithelia and the rest of the animal. In the present study, the concentrations of total VFA, acetate, and propionate were the lowest in PS-fed lambs, followed by MS-fed lambs, and then CS-fed lambs. It is suggested that different sources of starch in starter have a various rate and extent of digestion in the rumen, which could be attributed to the ratio of amylose to amylopectin, the size of starch granules and their surface areas, the degree of starch crystallinity (Oates,

1997). Goni et al. (1996) pointed that PS include amount of resistant starch and difficult digestion in gastrointestinal tract. Wang et al. (2016) found that higher content of resistant starch in diet have a lower concentration of total VFA, acetate and propionate in rumen of goats. Moreover, higher content of amylopectin in PS also maybe contribute to lower degradation rates in the rumen. Stevnebo et al. (2009) pointed out that the amylose proportion was negatively correlated with starch degradation in vitro. Ren et al. (2016) indicated that PS was difficult to degrade and affected the proportion and quantity of VFA in the rumen. It has been suggested that PS may had a lower digestion rate and that undegradable starch in the rumen will flow into the small intestine.

Previous study found that conventional yellow dent corn diet (higher ratio of amylose than waxy corn) had a lower starch disappearance in stomach and a higher amount of starch flowed into duodenum and ileum in sheep (Akay et al., 2002). In the current study, due to the insufficient small intestinal contents and large amount of water in contents, we are not able to measure the amount of starch in small intestinal contents. Therefore, we could not demonstrate directly that there was more rumen bypass and starch inflow into the small intestine in PS-fed lambs. However, we found that the PS diet significantly increased the activity of alpha amylase in the jejunum. In the small intestine, amylase breaks down starch into glucose, which is absorbed by the animal body (Noziere et al., 2014). Alpha amylase is synthesized and stored in zymogen granules until stimulation signals are received that trigger duodenum exocrine activities. Swanson et al. (2000) confirmed that high starch and high energy diets can increase the activity of alpha amylase and alpha amylase protein in lambs. Thus, we inferred that there maybe more rumen bypass and starch inflow into the small intestine in PS-fed lambs.

Morphology of Small Intestinal Tissue

The small intestine is the functional organ for the digestion and absorption of nutrients, and it is also an important immune organ in the mammals. The early life of lambs is an advantageous time to intervene the developmental profile of the small intestine by nutritional strategies (Baldwin et al., 2004). Khan et al. (2008) indicated that different starch source have different influences on the nutritional and physiological status of calves. Meanwhile, Ren et al. (2016) found that a PS diet significantly increased the villus height and crypt

depth in lambs. In the present study, we also found that PS diets increased the crypt depth of the jejunum and the villus height of the ileum in lambs. The changes of morphology may be beneficial to the absorption and utilization of nutrients.

The mRNA Expression of Cell Cycle Proteins in Small Intestinal Mucosa

From the presented data and the previous discussion, we concluded that PS in starter feed significantly promote the intestinal epithelia growth. In order to further confirm this result, we conducted qRT-PCR analysis to investigate the mRNA expression of cell cycle proteins in small intestinal mucosa. Cellular proliferation is controlled primarily by the regulation of the cell cycle, which consists of four distinct but sequential phases (the G1, S, G2, and M phase), and any change in the cell cycle will affect the rate of cell production (Shen et al., 2004). Moreover, the cell cycle is regulated by cyclins and CDKs (Lu et al., 2013). In the present study, the gene expression of cyclin D1, cyclin E, and CDK6 in the jejunum, and cyclin D1 and CDK6 in the ileum mucosa, increased with the PS diet. Previous study found that cyclin D1 combines with CDK4 or CDK6 to form complexes that are essential for the G1 phase (Mathew et al., 2010). The G1 phase is the key step in the eukaryotic cell division cycle. Shen et al. (2004) found that supplying a high metabolic energy diet can accelerate cell cycle progression of the rumen epithelial cells in goats due to upregulated cyclin D1. Filmus et al. (1994) also found that the elevated expression of cyclin D1 and/or complexes of cyclin D1-CDK4 led to a shortening of the G1-phase duration in rat intestine epithelial cells. Cyclin E, which associates with CDK2, is maximal in late G1, and the formation of active CDK2/cyclin E complex is essential for S phase entry (Okuda et al., 2000). Moreover, we found that the gene expression of cyclin A in ileum mucosa increased with the PS diet. Cyclin A plays an important role in the S phase and G2/M phase (Jeffrey et al., 1995). The cyclinA-CDK2 complexes, which assemble at the onset of the S phase, drive chromosome duplication through phosphorylation of key DNA replication factors (Kanakkanthara et al., 2016). Girard et al. (1991) also suggested that cyclin A plays a major role in the control of DNA replication in mammalian cells. However, the mRNA expression level of CDK2 and CDK4 did not differ among the three groups. Certainly, the changes in the cell cycle genes' mRNA expression cannot completely reflect the variations of cell cycle

progression in small intestinal epithelia. The effects of PS in starter feed on cell cycle protein expression need to be investigated further. Therefore, we suggest that cyclin A, cyclin D1, cyclin E, and CDK6 may important genes that facilitate small intestinal epithelial development by promoting cell cycle progression in the PS treatment.

The mRNA Expression of GCG and GLP-2R in Small Intestinal Mucosa

Glucagon-like peptide 2 (GLP-2) is a 33-amino acid peptide derived from the proteolytic cleavage of GCG by prohormone convertase 1/3 in enteroendocrine L cells (Burrin et al., 2001; Liu et al., 2008; Connor et al., 2015). The functions of GLP-2 include promoting intestinal development, enhancing intestinal barrier function, increasing blood flow, and acting as an anti-inflammatory agent (Dube and Brubaker, 2007; Taylor-Edwards et al., 2011). The most important function of GLP-2 is to promote the growth of intestinal mucosa and the repair of intestinal mucosa after injury. In the present study, we found that the mRNA expression of GCG and GLP-2R in small intestinal mucosa and the concentration of GLP-2 in blood were enhanced with PS diets. This result could be expected; it has been shown that PS diets may have more rumen bypass and starch inflow into the small intestine, which could stimulate the endogenous secretion of GLP-2. Many previous studies also showed that high energy nutrients can stimulate endogenous secretion of GLP-2 (Connor et al., 2015). For instance, carbohydrate feeding increases the concentration of GLP-2 in plasma of men (Xiao et al., 1999), and isolated perfused pig ileum stimulated with luminal glucose increased GLP-2 secretion (Orskov et al., 1986). When GLP-2 is combined with GLP-2R, it appears to promote the development of intestinal epithelia. Connor et al. (2010) detected the high expression of GCG and GLP-2R in the small intestine tissue of dairy cattle and confirmed that the GLP-2 signal system exists in the intestines of ruminants. We also found that the expression of GCG and GLP-2R in small intestinal epithelia was increased in PS diets. Taylor-Edwards et al. (2011) found that subcutaneous injection of GLP-2 can promote the growth of intestinal mucosa and blood circulation of calves. Connor et al. (2013) also confirmed that GLP-2 can be used as a drug by improving intestinal mucosal cell self-renewal to relieve diarrhea in calves. As discussed previously, we suggest that the amount of nutrition arriving at the small intestine in PS diet is

more than that in CS and MS diet; thus, the PS diet could stimulate the secretion of endogenous GLP-2. In addition, GLP-2 will promote the growth of small intestine epithelial cells by upregulating the mRNA expression of cell cycle proteins in small intestinal mucosa.

Furthermore, correlation analysis revealed a significant correlation between the endogenous GLP-2 secretion and the mRNA levels of cell cycle proteins. When GLP-2 combined with GLP-2R, it will promote the expression and synthesis of IGF-1 and accelerate cell cycles and promote the development of mucosa (Connor et al., 2015). Jasleen et al. (2002) reported that GLP-2 treatment of intestinal epithelial cell lines could promote the expression of cyclin A and cyclin D1 in vitro. Connor et al. (2010) also found that the expression of cyclin D1 and GLP-2R had a positive correlation in bovine small intestine. These results indicated that the cell cycle acceleration induced by PS diet in small intestinal epithelia may, in part, be related to changes in endogenous GLP-2 secretion and expression of its receptor in lambs. Nevertheless, the underlying mechanism of GLP-2 promotion of small intestinal epithelial proliferation still needs to be further evaluated.

CONCLUSIONS

In summary, all results showed that PS in starter feed was more beneficial to increase small intestinal (jejunum and ileum) villus height and crypt depth and also to promote cell cycle acceleration, which may, in part, be related to changes in endogenous GLP-2 secretion and expression of its receptor in preweaned lambs. These findings provide new insights into nutritional interventions to promote the development of the small intestine in young ruminants. Nevertheless, the effect of GLP-2 on the development of small intestine in lambs needs to be investigated in the future.

Conflict of interest statement. There are no conflicts of interest to declare.

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