Effects of dietary leucine and phenylalanine on pancreas development, enzyme activity, and relative gene expression in milk-fed Holstein dairy calves

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ABSTRACT

This study aimed to investigate the effect of dietary supplementation with leucine and phenylalanine on pancreas development, enzyme activity, and related gene expression in male Holstein calves. Twenty male Holstein calves [1 d of age, 38 ± 3 kg of body weight (BW)] were randomly assigned to 1 of the following 4 treatment groups with 5 calves in each group: control, leucine supplementation (1.435 g/L of milk), phenylalanine supplementation (0.725 g/L of milk), and leucine and phenylalanine (1.435 + 0.725 g/L of milk). The diets were made isonitrogenous with the inclusion of alanine in each respective treatment. The feeding trial lasted for 8 wk, including 1 wk for adaption and 7 wk for the feeding experiment. Leucine tended to increase the concentration of total pancreatic protein (mg/kg of BW). Phenylalanine increased the concentrations of plasma insulin, cholecystokinin, and pancreatic DNA (mg/g) and the expression of trypsin gene but decreased the pancreatic protein:DNA ratio and tended to decrease the pancreas weight (g/kg of BW). No differences were observed in total pancreatic DNA (mg/pancreas and mg/kg of BW), pancreatic protein (mg/pancreas), or activities of α -amylase, trypsin, and lipase. The relative expression levels of the genes encoding α -amylase and lipase did not differ among the 4 groups. The supplementation of both leucine and phenylalanine showed an interaction on the pancreas weight (g and g/kg of BW) and a tendency of an interaction on the pancreatic protein concentration (mg/g of pancreas and mg/kg of BW) and the plasma glucose concentration. Leucine tended to increase the size of the pancreatic cells, whereas phenylalanine tended to increase the number of pancreatic cells. However, neither AA affected the activities of the pancreatic enzymes of the calves. These results indicate that leucine and phenylalanine supple-

mentation in milk-fed Holstein calves differentially affect pancreatic growth and development.

Key words: calf, leucine, pancreas, phenylalanine

INTRODUCTION

The composition and content of dietary carbohydrates are key factors affecting rumen fermentation and the health and productivity of ruminants. To achieve the full genetic potential of lactating cows, diets rich in starch could be fed to increase milk production (Herrera-Saldana et al., 1990). The starch is first degraded in the rumen by amylolytic microbes to produce VFA, predominantly acetate, propionate, and butyrate, which are absorbed into the rumen wall. In ruminants, starch that resists microbial enzymatic degradation in the upper gut sections (i.e., forestomachs) may be termed bypass starch, undegradable starch, or ruminally resistant starch. More precisely, ruminally resistant starch is expected to resist degradation mechanisms in the rumen and be digested in the small intestine (Deckardt et al., 2013). About 18 to 42% of the starch bypasses rumen degradation and enters the small intestine (Owens et al., 1986), which could supply about 30% of total glucose needs (Huntington, 1997). Pancreatic α -amylase mediates the hydrolysis of rumen bypass starch to oligosaccharides or membrane-bound intestinal α -glucosidases, which hydrolyze these oligosaccharides to glucose in the small intestines. The released glucose is absorbed by the intestinal epithelial cells, providing the most important source of exogenous glucose for ruminants. The energy efficiency of starch in the small intestine is 42% higher than that in the rumen (Huntington et al., 2006). However, the digestibility of the small intestine for starch in dairy cows is on average 35 to 60% (Harmon et al., 2004), and thus some of the rumen bypass starch may not be digested, resulting in energy used inefficiently and health risks (Gressley et al., 2011). Zinn et al. (2002) revealed that the increase in digestibility depends on disruption of the protective protein matrix surrounding the starch

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granules rather than simply increasing starch solubility. Several studies have shown that insufficient secretion of pancreatic α -amylase is the limiting factor in the utilization of rumen bypass starch (Swanson et al., 2002; Harmon et al., 2004). Therefore, increasing the secretion of pancreatic α -amylase in dairy cows could be an approach to improve the energy supply and production performance of high-yielding dairy cows.

The pancreas is an important gland in the animal digestive system. Internal and external pancreatic secretions are essential for nutrient digestion and the regulation of several physiological processes. The pancreatic juice secreted by the pancreas is rich in bicarbonate and contains several types of digestive enzymes, including trypsin, pancreatic lipase, and pancreatic amylase, all of which are important hydrolases and play important roles in digestion and absorption (Rinderknecht et al., 1983). The secretion of pancreatic juice is affected by many factors, such as the age of the animal (Jiang et al., 2013), insulin (Baile et al., 1969), cholecystokinin (Owyang, 1994), dietary protein (Hara et al., 1996), and energy levels (de Dios et al., 1988). The effects of dietary protein and AA on the synthesis and secretion of trypsin have attracted much research interests. Richards et al. (2002) showed that in growing steers (initial BW = 379 kg) infused with starch (1,042 g/d) into the small intestine, the concentration of pancreatic α -amylase linearly increased in a dose-responsive manner to the amounts of infused casein (0, 50, 100, 150, and 200 g/d). Greater quantities of starch disappeared with increased casein infusion, and the infusion of 200 g/d of casein increased small intestinal starch disappearance by 226 g/d over the control. Swanson et al. (2008) obtained similar results by increasing the rumen bypass of soybean meal in yearling beef steers (initial BW = 510 kg). The studies of Liao et al. (2009) in Angus steers (initial BW = 260 kg) and Swanson et al. (2004) in Angus steers (initial BW = 290 kg) also revealed a positive dynamic relationship between the supply of protein and the secretion of pancreatic amylase. These authors also observed that a change in the ratio of the EAA to starch in the small intestine could change pancreatic amylase secretion. By infusing casein, EAA, NEAA, and glutamate into the duodenum, Brake et al. (2014) found that casein and EAA increased the secretion of pancreatic amylase without affecting the starch digestibility in the small intestine, whereas NEAA and glutamate increased the starch digestibility (steer initial BW = 259 kg). Yu et al. (2014) showed that both protein and AA could influence the activities of pancreatic enzymes and the expression of their genes in yearling ewes (initial BW = 297 kg) by altering pancreas development.

Amino acids traditionally were classified as nutritionally essential or nonessential for animals and humans based on nitrogen balance and growth (Wu, 2010). A key element of this classification is that all NEAA are assumed to be synthesized adequately in the body to meet the needs for protein synthesis (Wu, 2013). Therefore, Wu (2010) proposed a concept of functional AA, which is defined as those AA that participate in and regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of the organisms. Leucine can regulate protein synthesis and catabolism in animals by activating the mammalian target of rapamycin pathway (Boulton et al., 2013; Kim et al., 2013). Phenylalanine is an aromatic AA that can promote the secretion of cholecystokinin (CCK) through the calcium-sensing receptors (Liou et al., 2011). Cholecystokinin can promote the synthesis of pancreatic amylase, trypsin, and trypsinogen, stimulate the release of these pancreatic enzymes, and enhance their activities. Duodenal infusions of phenylalanine (Yu et al., 2013) and leucine (Liu et al., 2015) play a role in regulating the secretion of pancreatic α -amylase.

The above studies showed that AA play a regulatory role in pancreas secretion and the gene expression of pancreatic enzymes at both transcriptional and translational levels. We thus hypothesized that supplementation of leucine or phenylalanine further strengthens their roles in the regulation of pancreatic metabolism, leading to an increment of exogenous enzyme production. We noted that the aforementioned studies used cannulated animals to infuse the substrates via the cannula to avoid ruminal degradation, and influences of the surgery preparation on the results remain undefined. Therefore, the present study investigated the effects of supplemental leucine and phenylalanine on pancreas development, enzymatic activity, and transcriptional regulation in milk-fed Holstein dairy calves without any surgical preparation.

MATERIALS AND METHODS

The use of the animals and experimental protocols was approved by the Animal Care and Use Committee of the College of Animal Science and Technology, the Northwest A&F University (Yangling, Shaanxi, China).

Animals, Experimental Design, and Diets

Twenty male Holstein calves (1 d of age, 38 ± 3 kg of BW) were used in this study. Within 1 h after birth, each calf was fed 4.0 L of colostrum with an IgG concentration >50 g/L. The calves were randomly assigned

to 4 groups (5 calves/group). The calves in the control group received pasteurized milk only, whereas the calves in the treatment groups were fed milk supplemented with leucine (1.435 g/L of milk), phenylalanine (0.725 g/L), or both AA mixed (1.435 g of leucine/L and 0.725 g of phenylalanine/L), which were dissolved in the milk. The milk fed to all the groups was made isonitrogenous by adding alanine (1.366, 0.391, 0.975, and 0 g/L of milk for control, leucine, phenylalanine, and mixed treatments, respectively). The doses of leucine and phenylalanine for the experimental groups were based on the results of Yu et al. (2013) and Liu et al. (2015). The milk was sampled weekly and stored at -20°C , and the samples were pooled. The AA composition of the milk sample was determined by Evonik Nutrition and Care GmbH (Beijing, China) using ion-exchange chromatography with postcolumn derivatization with ninhydrin. Milk proteins were hydrolyzed with 6 M HCl for 24 h at 110°C , and free AA were quantified by measuring the absorption of reaction products with ninhydrin at 570 nm against their standards (Sadri et al., 2017). The mean concentrations are shown in Table 1. The amount of the AA supplemented (including the amounts of alanine added) was about 42% of the total AA present in the milk. The calves were fed individually.

The feeding trial lasted for 8 wk, with the first week for the adaptation. During the first week, the calves were manually bottle fed 3 L of milk twice daily. During the adaption period, AA supplementation was increased 20% daily until it reached 100% of the target supplementation on the sixth day. From wk 2 to 3, the calves were manually bucket fed 3.5 L of milk twice daily, and from wk 4 to 8 they were fed 4 L of milk twice daily. The milk offered to the calves was fully consumed without any refusal.

From the beginning of the third week, a starter feed was provided twice daily. The amount of feed offered to each calf was 210 g/d in wk 3 and gradually increased to 1,800 g/d in wk 8 to ensure that all calves consumed the feed without any refusal. The calves had free access to fresh drinking water. The commercial starter was provided by Purina (Purina Mills Start and Grow Sunfresh; St. Louis, MO). The ingredients and nutrient composition of the starter are shown in Table 2.

Sample Collection and Analysis

On the last day of wk 8, 20 calves were slaughtered after 16 h of fasting. The animals were killed by bleeding the jugular vein by a registered veterinarian. The slaughtering procedure proceeded as following: 4 calves/d, 1 from each treatment, sequentially ceased feeding in 1-h intervals, and after 16 h of fasting they were slaughtered in the same order. This was to ensure

Table 1. Concentrations (g/L) of AA in milk fed to calves

AA	Concentration
Arginine	1.429
Histidine	0.920
Isoleucine	1.744
Leucine	3.432
Lysine	3.055
Methionine	0.950
Phenylalanine	1.731
Threonine	1.804
Valine	2.372
Alanine	1.356
Aspartate	2.869
Cysteine	0.358
Glutamate	7.605
Tyrosine	1.846
Glycine	0.838
Serine	2.260
Proline	6.458

that each animal was fasted for the same period of 16 h. All 20 calves were slaughtered over 5 d, with random orders of 4 treatments on each day. Before slaughter, 10 mL of blood was collected from the jugular vein into a heparinized syringe and immediately centrifuged at $2,500 \times g$ for 15 min at 4°C . Then, plasma was collected into a 2-mL centrifuge tube. Aprotinin was added at a final concentration of 10,000 IU/mL. The plasma samples were stored at -80°C until analysis.

Plasma glucose was determined on the day of sampling using the glucose oxidase-peroxidase assay kit (Jiancheng, Nanjing, China) and a Synergy HT Multifunctional Microplate Reader (Power Wave XS2, BioTek, Winooski, VT). Plasma urea nitrogen, total

Table 2. Nutrient levels of the starter¹ fed to calves (% DM)

Item	Content, %
DM	87.06
CP	20.01
Crude ash	15.47
Starch	38.79
NDF	12.20
ADF	6.20
Calcium	0.70
Total phosphorus	0.38

¹The starter was purchased from Purina Co. (St. Louis, MO). It contained corn, soybean meal, wheat bran, sugar cane molasses, calcium hydrophosphate, stone powder, salt, L-lysine, vitamin A, vitamin D₃, vitamin E, copper sulfate, and ferrous sulfate. The AOAC International (1999) methods were used to determine the DM (method 930.5), CP ($N \times 6.25$; method 984.13), ash (method 942.05), calcium (method 927.02), and phosphorus (method 965.17) contents in the starter. The contents of NDF and ADF were sequentially determined according to the methods described by Van Soest et al. (1991), with heat-stable α -amylase (A3306, Sigma-Aldrich, St. Louis, MO) and sodium sulfite being used in the NDF procedure (inclusive of residual ash). The starch content was determined using an enzymatic method (α -amylase and amyloglucosidase) implemented in a commercial starch analysis kit (Megazyme International Ireland Ltd., Bray, Ireland). All analyses were performed in our laboratory.

Table 3. The PCR primers used to amplify genes encoding α -amylase, trypsin, and lipase

Item	Forward primer	Reverse primer
α -Amylase	GAAATGGCCGTGTGACAGAATTTA	ACAAAGACAAGTGCCCTGTCAGAA
Trypsin	TGTCTGCGGCTCACTGCTAC	GCTGGGATGGACGATACTCTTG
Lipase	GTGGAAGCAAATGATGGACAAG	TGGGTTGAGGGTGAGCAGA
18S rRNA ¹	ACCCATTCGAACGTCTGCCCTATT	TCCTTGATGTGGTAGCCGTTTCT

¹Housekeeping gene.

protein, albumin, and globulin were determined using commercial kits (Jiancheng). Insulin and CCK were determined using an ELISA kit (Cloud-Clone Corp., Katy, TX). The concentration of AA in plasma was measured by Evonik Nutrition and Care GmbH using ion-exchange chromatography with postcolumn derivatization with ninhydrin. The EAA pool included leucine, phenylalanine, arginine, histidine, isoleucine, lysine, methionine, threonine, and valine; the NEAA pool included alanine, aspartate, cysteine, glutamate, glutamine, glycine, serine, proline, and taurine. Total AA was the sum of EAA and NEAA.

Immediately after slaughter, the pancreas was separated and weighed. Then, a sample (3–5 g) was taken, frozen in liquid nitrogen, and stored at -80°C for evaluation of the pancreas development later on using the method of Crozier et al. (2008). Another pancreatic tissue sample (~ 2 g) was taken and homogenized in a homogenizing solution (containing 5 mM MgCl_2 and 0.1% Triton X-100, vol/vol) and sonicated for 15 s. The homogenate was centrifuged at $1,000 \times g$ at 4°C for 10 min, and then the supernatant was used for assays of the total protein and enzyme activities. The total protein concentration in the homogenate was determined using a protein detection kit (Jiancheng). The activities of α -amylase, trypsin, and lipase in the homogenate were determined using respective assay kits per the manufacturer's instructions without any modification (Jiancheng). The DNA was extracted and then quantified using a DNA quantification kit (Sigma-Aldrich). The total protein content in the pancreatic tissue was calculated against the calibration curve using bovine albumin as the standard. Concentration and content of DNA were used as an index of pancreatic hyperplasia (change in cell number), and protein:DNA ratios were used as indices of pancreatic hypertrophy (change in cell size; Swanson et al., 2008).

The expression levels of pancreatic α -amylase, trypsin, and lipase mRNA were determined using real-time quantitative PCR using respective specific primers (Table 3). Between 50 and 100 mg of pancreatic tissue was ground in liquid nitrogen, and the total RNA was isolated using the Animal Tissue Total RNA extraction kit (Tiangen Biotech, Beijing, China). The total RNA concentrations were quantified by absorbance

at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and sample integrity was determined by the ratio of absorbance at 260 nm to 280 nm, with all samples having a ratio ≥ 1.9 .

Reverse transcription (**RT**) was performed using the PrimeScript RT reagent kit (TaKaRa Biotechnology Co. Ltd., Dalian, China). Each RT reaction (20 μL total volume) contained 4.0 μL of $5\times$ PrimeScript buffer, 1.0 μL of PrimeScript RT enzyme mix I, 1.0 μL of oligo dT primer (50 μM), 1.0 μL of random 6 mers (100 μM), 1.0 μL of total RNA, and 12.0 μL of RNase-free distilled H_2O . Real-time quantitative PCR was performed using the SYBR Premix Ex TaqTM II kit (TaKaRa Biotechnology Co. Ltd.) to quantify the transcripts of the genes coding for pancreatic α -amylase, trypsin, and lipase. The real-time PCR reaction (25 μL total volume) contained 12.5 μL of SYBR Premix Ex TaqTM II, 1.0 μL of each primer, 1.0 μL of cDNA template, and 9.5 μL of double-distilled H_2O . The thermal cycles were as follows: initial denaturation at 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The amplification efficiency of each gene was validated by constructing a standard curve through serial dilutions of cDNA. All samples were run in triplicate, and 18S rRNA (the housekeeping gene) was selected as an endogenous control to normalize the target gene expression (White et al., 2012). The average cycle threshold values were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS (version 9.1; SAS Institute Inc., Cary, NC) using the following model:

$$Y_{jk} = \mu + L_j + P_k + LP_{jk} + \varepsilon_{jk},$$

where Y_{jk} is the response variable, μ is the overall mean, L_j is the fixed effect of leucine (j = with or without), P_k is the fixed effect of phenylalanine (k = with or without), LP_{jk} is the interaction of leucine and phenylalanine, and ε_{jk} is the residual error. The results were reported as the least squares means $\pm$ standard error

Table 4. Amino acid concentrations in plasma of male Holstein calves fed milk and the starter supplemented with leucine and phenylalanine (n = 5)

Item	Treatment ¹				SEM	P-value		
	C	P	L	M		P	L	P × L
EAA, µg/mL								
Leucine	18.89	18.99	43.21	40.17	0.739	0.373	<0.001	0.259
Phenylalanine	9.60	19.09	10.51	19.79	0.467	<0.001	0.442	0.858
Arginine	44.20	42.53	44.54	43.26	0.952	0.387	0.839	0.931
Histidine	10.94	10.41	12.55	11.33	0.290	0.139	0.045	0.474
Isoleucine	12.58	11.80	12.00	13.09	0.320	0.718	0.668	0.190
Lysine	19.64	18.80	20.89	20.22	0.528	0.564	0.253	0.895
Methionine	3.89	3.87	4.08	4.41	0.112	0.415	0.103	0.465
Threonine	11.63	11.47	13.00	13.07	0.358	0.979	0.035	0.819
Valine	28.77	27.24	28.39	30.90	0.634	0.573	0.244	0.157
Total EAA	160.15	164.20	189.17	196.24	3.415	0.376	<0.001	0.913
NEAA, µg/mL								
Alanine	28.47	27.06	21.52	21.86	0.756	0.707	<0.001	0.664
Aspartate	11.21	10.52	11.91	12.36	0.305	0.875	0.058	0.422
Cysteine	0.22	0.23	0.14	0.17	0.022	0.476	0.242	0.929
Glutamate	17.97	14.98	16.11	14.59	0.394	0.005	0.156	0.383
Glutamine	46.45	43.30	44.85	44.03	0.986	0.357	0.807	0.640
Glycine	30.62	33.00	27.33	30.03	0.581	0.047	0.008	0.971
Serine	13.76	13.12	12.95	15.00	0.349	0.338	0.546	0.062
Proline	16.49	16.61	17.16	16.99	0.475	0.949	0.737	0.704
Taurine	15.36	14.82	15.06	17.36	0.398	0.475	0.285	0.094
Total NEAA	180.54	173.65	167.02	172.41	3.353	0.868	0.195	0.431
Total AA	340.69	337.85	356.19	368.64	6.15	0.685	0.089	0.635

¹C = control (pasteurized milk only); P = milk supplemented with phenylalanine (0.725 g/L); L = milk supplemented with leucine (1.435 g/L); M = milk supplemented with both leucine (1.435 g/L) and phenylalanine (0.725 g/L).

of the mean, and a treatment difference was declared significant at $P \leq 0.05$ or a trend at $P < 0.10$.

RESULTS

Effects of Leucine and Phenylalanine Supplementation on Plasma AA and Metabolite Concentrations

Table 4 shows the AA concentrations in plasma upon supplementation of leucine and phenylalanine.

The supplementations with leucine and phenylalanine increased their concentrations in plasma ($P < 0.01$). However, the supplementations had no effects ($P > 0.05$) on total protein, albumin, globulin, glucose, urea nitrogen concentration, and albumin:globulin ratio in plasma (Table 5). Phenylalanine increased the plasma insulin and CCK concentrations ($P < 0.05$). Phenylalanine and leucine showed a tendency of an interaction on plasma glucose concentration ($P = 0.058$), and leucine infusion alone tended to reduce glucose concentration compared with the control.

Table 5. Metabolite concentrations in plasma of male Holstein calves fed milk and the starter supplemented with leucine and phenylalanine (n = 5)

Item	Treatment ¹				SEM	P-value		
	C	P	L	M		P	L	P × L
Total protein, g/L	35.03	27.88	29.65	35.86	2.624	0.929	0.808	0.222
Albumin, g/L	21.23	17.83	20.33	23.42	1.522	0.960	0.453	0.302
Globin, g/L	13.80	10.05	9.33	12.44	1.648	0.925	0.756	0.314
Albumin/globin	1.94	1.76	2.22	2.55	0.222	0.875	0.249	0.568
Glucose, mmol/L	2.85	2.57	2.41	2.64	0.064	0.874	0.176	0.058
Urea N, mmol/L	3.53	3.05	3.38	3.24	0.202	0.453	0.967	0.685
Insulin, pg/mL	278.74	367.89	213.35	337.04	22.374	0.039	0.308	0.708
Cholecystokinin, pg/mL	4.57	5.57	3.25	3.21	0.328	0.015	0.479	0.441

¹C = control (pasteurized milk only); P = milk supplemented with phenylalanine (0.725 g/L); L = milk supplemented with leucine (1.435 g/L); M = milk supplemented with both leucine (1.435 g/L) and phenylalanine (0.725 g/L).

Effects of Leucine and Phenylalanine Supplementation on Pancreas Development

No treatment difference ($P > 0.05$) was observed in the BW, pancreatic DNA content (mg/pancreas and mg/kg of BW), or pancreatic protein content (mg/pancreas; Table 6). Phenylalanine increased the pancreatic DNA content per gram of pancreas tissue ($P < 0.05$) and decreased the pancreatic protein:DNA ratio ($P < 0.05$), with a tendency to reduce the pancreatic weight per kilogram of BW ($P = 0.091$). Leucine tended to increase the total pancreatic protein content per kilogram of BW ($P = 0.068$). Leucine and phenylalanine showed interactions on the pancreatic weight (both total and per kilogram of BW; $P < 0.05$) and a tendency of an interaction on the pancreatic protein content (both per gram of pancreas tissue, $P = 0.063$, and per kilogram of BW, $P = 0.073$), and phenylalanine alone reduced the pancreatic weight and its protein content.

Effects of Leucine and Phenylalanine Supplementation on Pancreatic Enzyme Activity and mRNA Expression

Table 7 shows the activities of pancreatic α -amylase, trypsin, and lipase in calves upon supplementation of leucine and phenylalanine. The supplementation with either AA, individually or in combination, did not have effects on the activities of pancreatic α -amylase, trypsin, or lipase ($P > 0.05$). As shown in Table 7, leucine and phenylalanine had no effect on the relative expression levels of α -amylase and lipase genes ($P > 0.05$), but phenylalanine increased the relative expression of trypsin gene ($P < 0.05$). No interaction between leucine

and phenylalanine was observed on the activity or the expression levels of pancreatic α -amylase, trypsin, or lipase ($P > 0.05$).

DISCUSSION

The low starch digestibility in the small intestine in dairy cows is a factor that could restrict high production performance. Starch is a significant source of energy in high-concentrate diets for high-yielding dairy cows (NRC, 2001), but if dietary starch content is too high, its rapid degradation in the rumen may induce various metabolic diseases such as rumen acidosis. Many factors (e.g., starch form, quality, processing) affect starch digestibility in ruminant diets. For example, processing grains by application of various combinations of heat, moisture, time, and mechanical action could increase digestibility of starch by providing an opportunity for bacterial attachment to starch granules (Huntington, 1997). Owens et al. (1986) provide comprehensive summaries of the effects of grain source and processing methods on the site and extent of digestibility of starch. Energy efficiency of the starch utilization in the rumen of ruminants is lower than that in the small intestine by 42% (Yu et al., 2013). The starch that bypasses degradation in the rumen and enters the small intestine accounted for 35 to 50% of the total intake of starch, but the digestibility of the rumen bypass starch in the small intestine was only 35 to 60% (Harmon, 2009). Therefore, improvement in the digestibility of starch in the small intestine, thereby improving the production performance of ruminants, is important, especially for improving the feed conversion efficiency. The starch, protein, and fat entering the small intestine must first

Table 6. Pancreatic weight and contents of DNA and protein in male Holstein calves fed milk and the starter supplemented with leucine and phenylalanine (n = 5)

Item	Treatment ¹				SEM	P-value		
	C	P	L	M		P	L	P × L
BW, kg	79.2	79.1	78.5	75.5	0.78	0.625	0.395	0.175
Pancreatic weight								
g	56.67	40.00	51.67	58.00	2.198	0.255	0.156	0.017
g/kg of BW	0.72	0.51	0.66	0.77	0.028	0.091	0.374	0.009
Pancreatic DNA								
mg/g of pancreas	3.92	5.90	3.82	4.97	0.242	0.005	0.297	0.405
mg/pancreas	228.32	241.91	194.19	283.59	16.558	0.137	0.911	0.267
mg/kg of BW	2.90	3.06	2.48	3.75	0.211	0.106	0.747	0.201
Pancreatic protein								
mg/g of pancreas	75.54	84.66	81.35	75.94	1.831	0.619	0.696	0.063
mg/pancreas	4,219	3,341	4,212	4,370	163.0	0.284	0.134	0.129
mg/kg of BW	53.48	42.23	53.61	57.75	2.017	0.390	0.068	0.073
Protein:DNA	20.85	14.99	22.22	16.25	1.185	0.022	0.586	0.980

¹C = control (pasteurized milk only); P = milk supplemented with phenylalanine (0.725 g/L); L = milk supplemented with leucine (1.435 g/L); M = milk supplemented with both leucine (1.435 g/L) and phenylalanine (0.725 g/L).

Table 7. Activity of α -amylase, trypsin, and lipase and the expressions of their genes in the pancreas of male Holstein calves fed milk supplemented with leucine and phenylalanine (n = 5)

Item	Treatment ¹				SEM	P-value		
	C	P	L	M		P	L	P × L
Enzyme activity, U/mg of protein								
α-Amylase	297.24	387.35	248.66	257.80	25.45	0.098	0.343	0.437
Trypsin	243.99	208.65	251.39	290.18	11.93	0.081	0.943	0.140
Lipase	2,327	2,613	2,253	3,215	187.04	0.490	0.115	0.380
Gene expression ²								
α-Amylase	1.38	1.36	0.67	1.07	0.152	0.533	0.122	0.503
Trypsin	0.69	2.37	0.82	1.18	0.189	0.016	0.181	0.104
Lipase	1.41	0.76	1.08	1.08	0.237	0.501	0.983	0.498

¹C = control (pasteurized milk only); P = milk supplemented with phenylalanine (0.725 g/L); L = milk supplemented with leucine (1.435 g/L); M = milk supplemented with both leucine (1.435 g/L) and phenylalanine (0.725 g/L).

²Fold change relative to 18S rRNA.

be digested (or hydrolyzed) into their monomers (i.e., glucose, AA, and fatty acids, respectively) before they can be absorbed. During the digestion process a variety of enzymes are secreted by the pancreas, including pancreatic α -amylase, trypsin, and lipase. Studies have shown that the insufficient secretion of pancreatic α -amylase is considered a factor limiting the starch digestion in the small intestine of ruminants (Owens et al., 1986; Harmon, 2009; Brake et al., 2014; Liu et al., 2015). Therefore, research on the pancreas has been focused on enhancing the synthesis and secretion of its digestive enzymes.

The protein synthesis of each pancreatic enzyme (e.g., α -amylase, trypsin, and lipase) in preruminant calves is specifically regulated, and the changes of the levels of mRNA specific for these enzymes appear unspecific with the age of animals (Zabielski et al., 1999). To understand possible regulatory mechanisms of individual functional AA rather than changes of overall dietary protein levels, we used the esophageal groove reflex, which exists in suckling calves, to investigate the effects of leucine and phenylalanine on the development of the pancreas in dairy calves, and both AA were dissolved in the milk to feed to the calves. In milk-fed calves (preruminant), liquid feeds (whole milk or milk substitute diet) usually directly enter the abomasum. Hence, leucine and phenylalanine added in the milk can be directly absorbed in the intestine of calves. This was supported by the increments of leucine and phenylalanine concentrations in plasma of the calves, and we anticipated that the increased supplies of leucine and phenylalanine would have an influence on pancreas development, enzyme activity, and relative gene expression in milk-fed calves in this study.

Cholecystokinin is a class of peptides secreted by intestinal mucosal I cells, and it is formed from pro-cholecystokinin (Nilaweera et al., 2010). Cholecystoki-

nin is a potent stimulant of the synthesis and release of pancreatic enzymes, and it regulates the pancreatic enzyme secretion by directly acting on the animal acinar cells or by stimulating the vagal nerve (Murphy et al., 2008). Phenylalanine can effectively stimulate the release of CCK through calcium-sensitive receptors (Ballinger and Clark, 1994). The study of Yu et al. (2013) on dairy goats found that a duodenal infusion of phenylalanine could promote the secretion of pancreatic enzymes by increasing the CCK concentration in the blood. It was noted in this study that plasma CCK was increased by phenylalanine but decreased by leucine, supplemented either alone or with phenylalanine. The promoting effect of phenylalanine on CCK is consistent with the findings of the previous study (Yu et al., 2013), supporting the stimulatory effect of phenylalanine on CCK.

Insulin is a protein hormone that is secreted by the pancreatic islet β -cells, and its secretion is stimulated by endogenous or exogenous substances such as glucose, lactose, ribose, arginine, and glucagon (Ensafi et al., 2015). This hormone can reduce the blood sugar in animals. This study shows that leucine had no significant effect on plasma insulin level. This is consistent with the findings of Sans et al. (2006), who showed that leucine had no significant effect on plasma insulin. Hashimoto and Hara (2004) also indicated that after receiving a leucine-rich diet for 7 d, mice decreased the secretion of pancreatic α -amylase while maintaining the insulin content in the blood. These results differ from reports by Xu et al. (2001) and Yang et al. (2015), who found stimulatory effects of leucine on insulin, possibly because the sustained high concentration of leucine in the blood increased the level of hypoxia-inducing factor 1- α in pancreatic cells by activating the mammalian target of rapamycin C1 pathway, thereby inhibiting the differentiation of pancreatic endocrine precursor cells

into islet β -cells (Rachdi et al., 2012). The results of this study also showed that phenylalanine alone could significantly increase the concentration of plasma insulin, mainly because CCK can enhance the insulin secretion. This result is in accordance with Rasmussen et al. (1990), who revealed that CCK can act synergistically through 2 functional classes of neurohumoral agonists (gastric inhibitory polypeptide and glucagonlike peptide 1 activate adenylate cyclase) to enhance insulin secretion.

The weight of the pancreas and the pancreas index can reflect the function of the body to a certain extent (Swanson et al., 2002, 2008; Yu et al., 2013). Swanson et al. (2002) found that postrumen infusion of casein enhanced the pancreatic α -amylase and trypsin activities in weaning calves as a result of increased pancreatic size. However, Yu et al. (2013) found that duodenal infusion of phenylalanine to adult goats did not alter the size of the pancreas in goats but rather increased the total α -amylase activity by increasing the α -amylase protein concentration. In the present study, the supplementation of phenylalanine alone in milk tended to decrease the weight of the pancreas but did not affect the protein concentration; in addition, the activity of α -amylase tended to be higher and trypsin activity tended to be lower, and the relative expression of trypsin gene was significantly higher. This result is not consistent with the findings of the previous studies. In Swanson et al. (2002), the infused casein in calves increased the small intestinal protein flow that increased pancreatic weight and thus total pancreatic α -amylase and trypsin activity, the effect of which might differ from that by infusion of a single AA. This premise is consistent with the notion that different AA may have different abilities to regulate the pancreas (Hashimoto and Hara, 2004; Brake et al., 2014). In the studies of Yu et al. (2013) and Swanson et al. (2008), older animals were used (adult goats and 1-yr-old castrated cattle, respectively). Older animals may respond to the same AA differently than younger animals with respect to pancreatic enzyme secretion. Swanson et al. (2008) found that when 1-yr-old castrated cattle were fed diets that differed in CP level, the diet with a higher protein level increased the concentration of pancreatic enzymes, whereas the size of the pancreas was not affected.

Although the size of the pancreas can be used to evaluate pancreas development, it does not necessarily reflect the level of function of the pancreas and does not explain the reasons for the differences in function. The number and size of the pancreatic cells can be used to evaluate the effect of AA on pancreas development (Swanson et al., 2008). In this study, supplementation with phenylalanine alone increased the concentration

of DNA in the pancreas and significantly decreased the protein:DNA ratio, suggesting that the number of pancreatic cells per unit of pancreatic weight was increased, but the cell size was decreased by phenylalanine supplementation. Intriguingly, no significant increase in the pancreatic enzyme activities accompanied the phenylalanine effect. This contradicts the results of Swanson et al. (2008) in yearling beef cattle and Yu et al. (2013) in adult goats, where they showed that the size, rather than the number, of the pancreatic cells affected the production ability of pancreatic digestive enzymes. This discrepancy may be attributed to the difference in the age of the animals used—that is, yearling cattle in Swanson et al. (2008), adult goats in Yu et al. (2013), and calves in this research. Conceivably, in growing ruminants that are still developing, phenylalanine may promote proliferation of pancreatic cells, whereas in adult ruminants phenylalanine can only increase the size of pancreatic cells. Leucine, alone and in combination with phenylalanine, tended to promote pancreatic protein per unit of BW, indicating that leucine may increase the size of pancreatic cells in calves.

The regulation of pancreatic enzyme expression is very complex. The possible mechanisms may include changes in tissue weight, mRNA levels of digestive enzymes, concentrations of digestive enzymes, and digestive enzyme posttranslational modifications (Swanson et al., 2002). In our study, phenylalanine supplementation significantly increased the relative expression of the trypsin gene of the pancreas. Such upregulation of the trypsin gene might be due to the increased CCK level observed in the blood. This has been proposed previously (Yu et al., 2013), and CCK can indeed significantly stimulate the pancreas enzyme activities, and gene expression of trypsin is relatively sensitive to changes in the diet. Leucine has been shown to regulate the secretion of pancreatic insulin (Yang et al., 2015). In this study, both leucine and phenylalanine showed no significant effect on the activity and gene expression of pancreatic α -amylase in calves, which is not consistent with the research results of Liu et al. (2015) in dairy cows and Yu et al. (2013, 2014) in adult dairy goats. Such discrepancy may be due to the differences in age of the animals and starch levels in the diets.

CONCLUSIONS

The results of this study show that supplementation with leucine or phenylalanine did not stimulate pancreatic α -amylase, trypsin, or lipase activity in milk-fed calves, but both AA changed the growth of pancreatic cells. Leucine increased the size of individual pancreatic cells, and phenylalanine increased the total number of

pancreatic cells. Phenylalanine increased circulating insulin and CCK, which may be an indication of pancreatic cell differentiation and function.

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