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Effects of isovalerate supplementation on morphology and functional gene expression of small intestine mucosa in pre- and post-weaned dairy calves

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Abstract

The present study evaluated the effects of isovalerate supplementation on the development of the small intestinal mucosa in dairy calves. Forty-eight Chinese Holstein bull calves at 15 days of age and 45.1 ± 0.36 kg of body weight were assigned randomly to four groups. The treatments were control, low-isovalerate, moderate-isovalerate and high-isovalerate with 0, 3, 6 and 9 g isovalerate per calf per day, respectively. The study comprised 75 days with a 15-day adaptation period followed by a 60-day sampling period. Calves were weaned at 60 days of age. Six calves were chosen from each treatment at random and slaughtered at 30 and 90 days of age. The small intestine morphology and activities of amylase and trypsin improved significantly with increasing age. No interaction between treatments and age was observed. The small intestine length, mucosa layer thickness, villus height and crypt depth increased linearly with increasing isovalerate supplementation. However, the ratio of villus height to crypt depth was not affected by treatment. Activities of amylase and trypsin increased linearly. The lactase activity increased linearly during the 75-day period and for pre-weaned calves but was unaltered for post-weaned calves. The relative mRNA expressions of growth hormone receptor, insulin-like growth factor-1 receptor and sodium-glucose co-transporter-1 in the small intestine mucosa increased linearly, and a similar pattern was observed for the expression of peptide transporter-1 in the duodenum and proximal jejunum. The results suggested that small intestine development was promoted by isovalerate in a dose-dependent manner.

Introduction

In dairy farming practices, development of the small intestine is crucial for nutrient digestion and absorption in calves and is related positively to nutrient intake (Kreikemeier *et al.* 1990; McLeod & Baldwin 2000; Zitnan *et al.* 2003). Increases in energy, protein and volatile fatty acids (VFA) reaching the small intestine could improve the digestive function of the small intestine (Katoh & Yajima 1989; Swanson *et al.* 2000; 2002; Richards *et al.* 2003). Moreover, VFA, growth hormone (GH) and insulin-like growth factor-1 (IGF-1) promoted the development of the small intestine by stimulating epithelial cell proliferation and differentiation (Bühler *et al.* 1998; Baldwin 1999; Georgiev *et al.* 2003; Wang 2007). The regulatory effects of GH and IGF-1 depended on their respective receptors in the small intestinal mucosa (Georgiev *et al.* 2003; Howarth 2003). In line with this, Smith *et al.* (2002) found an enhanced abundance of growth hormone receptor (GHR) and insulin-like growth factor 1 receptor (IGF1R) in mRNA with increasing nutrient intake in calves. Furthermore, intestinal absorption function can be reflected by mRNA expression of peptide transporter 1 (PEPT1) and sodium-glucose cotransporter 1 (SGLT1) in the small intestine (Shirazi-Beechey *et al.* 1991; Liu *et al.* 2009*a*). Therefore, supplements which promote nutrient intake, rumen fermentation and microbial protein synthesis could be used to improve the development of the small intestine in calves.

Branched-chain VFA, including isobutyrate, isovalerate and 2-methyl butyrate, have positive impacts on rumen fermentation, cellulolytic bacteria populations, rumen enzyme activity, nutrient digestion and productive performance in steers (Misra & Thakur 2001; Liu *et al.* 2009*b*) and calves (Liu *et al.* 2016). In previous studies, dry matter (DM) intake, ruminal total VFA and butyrate production, nutrient digestibility and microbial protein synthesis in pre- and post-weaned calves increased with isovalerate supplementation (Liu *et al.* 2016). Based on these studies, it was hypothesized that the supplementation of isovalerate could

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promote the development of the small intestine. Therefore, the current study aimed to evaluate the effects of isovalerate supplementation on morphology, digestive enzyme activity and functional gene expression of the small intestine in pre- and post-weaned dairy calves.

Materials and methods

Animals and experimental design

The experimental protocol was approved by the Animal Care and Use Committee of Shanxi Agriculture University. Forty-eight Chinese Holstein bull calves at 15 days of age and 45.1 ± 0.36 kg body weight (BW) were assigned randomly to one of four groups, 12 calves per group. The treatments were: control (without isovalerate), low-isovalerate (LIV), moderate-isovalerate (MIV) and high-isovalerate (HIV) with 3, 6 and 9 g isovalerate per calf per day, respectively. The supplement of isovalerate (analytical grade, 98.5% of isovalerate) was purchased commercially (Shanghai Aladdin Biological Technology Co., LTD, Shanghai, China) and hand-mixed into milk for pre-weaned calves and into concentrate for post-weaned calves. The study lasted 75 days, including a 15-day adaptation period followed by a 60-day sampling period. Calves were weaned at 60 days of age. During the pre-weaning period, calves were offered whole milk (0.10 of BW) twice daily via nipple feeding at 08.00 and 15.00 h for 25 days. From days 26 to 30, the daily offer of milk was decreased by 50% and calves were fed a commercial concentrate *ad libitum*. The post-weaning diets consisted of 600 g/kg alfalfa hay and 400 g/kg commercial concentrate based on a DM basis and was offered *ad libitum*. The chemical composition of the feeds is shown in Table 1. Calves were housed in individual pens (2.5×3 m^2) and fresh water was available throughout the experimental period.

Data collection, sampling procedures and chemical analysis

Animals were weighed at 15, 30, 60 and 90 days of age. Milk, feed offered and refusals were recorded daily through the experimental period to calculate DM intake. Samples of milk, feed and refusals were collected once weekly for DM determination. Milk samples were preserved with 2-bromo-2-nitropropane-1, 3-diol and pooled for each group per period before chemical analysis. Feeds and refusals were dried in an oven at 55 °C for 48 h and ground to pass through a 1-mm screen with a mill (FZ102, Shanghai Hong Ji Instrument Co., Ltd., Shanghai, China) for chemical analysis. During the experimental period, six calves were chosen from each treatment at random, euthanized (lethal doses of pentobarbital sodium) and slaughtered at 30 and 90 days of age before the morning feeding. The entire digestive tract was removed and placed on ice. The small intestine was separated, laid out on a table and looped around pegs as described by Bauer *et al.* (2001). After the length of the small intestine was measured, small intestinal chyme and sections were collected from the duodenum (10 cm distal to the pyloric sphincter), proximal jejunum (10 cm distal to ligament of trites), distal jejunum (10 cm distal to ligament of trites) and ileum (10 cm proximal to the ileocecal junction). The chyme in the lumen of each site

Table 1. Ingredient and chemical composition of the diet (g/kg dry matter (DM))

Item	Milk	Alfalfa hay	Concentrate	Post-weaning diet
Ingredient composition				
Alfalfa hay				600.0
Maize grain, ground			500.0	200.0
Wheat bran			150.0	60.0
Soybean meal			205.0	82.0
Cottonseed cake			100.0	40.0
Calcium carbonate			12.5	5.0
Salt			10.0	4.0
Dicalcium phosphate			10.0	4.0
Mineral and vitamin mixture[a]			12.5	5.0
Chemical composition				
DM	128.6	912.8	943.2	924.5
ME (MJ/kg)[b]		9.2	13.9	11.2
Crude protein	244.9	177.3	194.1	182.8
Fat	330.1			
Lactose	355.0			
Neutral detergent fibre		526.8	250.1	417.1
Acid detergent fibre		395.4	134.9	293. 2
Calcium		12.9	7.1	10.5
Phosphorus		3.1	6.8	4.6

[a]Contained 8 mg Co, 700 mg Cu, 4000 mg Fe, 2400 mg Mn, 2400 mg Zn, 240 mg I, 120 mg Se, 600 000 IU vitamin A, 1 00 000 IU vitamin D and 3000 mg vitamin E per kg premix.
[b]Calculated from NRC (2001).

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was collected into 10 ml collection tubes and stored at −80 °C for enzyme activity analysis. Approximately 5-cm intestinal segments were removed, emptied and washed clean with normal saline. For morphometry, a 1×1 cm^2 area of the small intestine was retrieved. Three 1×1 cm^2 samples were collected and stored at −80 °C for RNA isolation and analysis. The entire process from animal stunning through to tissue collection generally took about 20 min. Milk samples were analysed for fat, true protein and lactose using infrared spectrophotometry (Foss 120 Milko-Scan, Foss Electric, Hillerød, Denmark) according to AOAC (1997, method 975.16) procedures. Analytical DM content of oven-dried samples was determined by drying at 135 °C for 3 h (AOAC 1997; method 930.15). Ash content was determined by combustion at 550 °C for 5 h. The content of neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the methods described by Van Soest *et al.* (1991) with heat stable alpha amylase and sodium sulphite used in the NDF procedure, and expressed inclusive of residual ash. Content of nitrogen (N) in the samples was determined by the Kjeldahl method (AOAC 1997; method 976.05) and multiplied by 6.25 to obtain protein content. Frozen chyme in the lumen was thawed and homogenized, and analysed using an amylase activity assay kit (C016, Nanjing Jiancheng Bioengineering Institute, Nanjing, China), a trypsin activity assay kit (A080-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and a lactase activity assay kit (A082-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) by a UV2100 spectrophotometer (Shanghai Wanning Precision Scientific Instruments co., LTD, Shanghai, China). One unit (U) of enzyme activity was defined as the amount of enzyme that hydrolysed 1 μM substrate/min at pH 7.20 and 37 °C for trypsin, amylase and lactase.

Light microscopy and morphometry

Small intestinal tissue (1×1 cm^2 surface) was fixed in 4% formalin solution. After rinsing with water, the small intestinal tissue was dehydrated in a graded series of ethanol (70, 80, 85, 90, 95% and absolute ethanol), cleared twice with xylene, saturated and embedded in paraffin. Serial sections were then cut at 4 μm thicknesses, ten slices of each sample, deparaffinized in xylene, dehydrated, stained with haematoxylin and eosin, and observed under a light microscope. Muscular layer thickness, mucosa layer thickness, villus height and crypt depth of small intestine were determined by the computer operated *Image C* picture analysis system (Intronic GmbH, Berlin, Germany) and the IMES analysis program (Image-Pro Plus, version 6.0, Media Cybernetics Inc., Bethesda, MD, USA), using a colour video camera (Sony 3 CCD, CCD, Sony Electronics Inc., Tokyo, Japan) and a light microscope (Axiolab, Carl Zeiss Jena, Germany). The mucosa is the innermost layer of intestinal wall and is made up of epithelium, lamina propria and muscularis mucosae. The muscular layer is the third layer of the intestinal wall from the lumen outwards and comprises both longitudinal and circular smooth muscle that also helps with continued peristalsis and the movement of digested material along and out of the gut. In between the two layers of muscle lies the myenteric plexus.

Extraction of RNA and quantitative real-time polymerase chain reaction

Total RNA was extracted from the small intestine by using a Total RNA isolation kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quality and concentration of the isolated RNA were determined by using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The ratios of absorbance at 260 and 280 nm of all preparations were ~2.0. The integrity of RNA was checked by denaturing agarose gel electrophoresis and ethidium bromide staining. The iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) was used to synthesize cDNA from 500 ng total RNA of each sample per 10-μl sample reaction according to the manufacturer's instructions. Reaction conditions were 15 min at 37 °C and 5 min at 85 °C. Negative control reactions without reverse transcriptase were performed on each sample to detect possible contamination of genomic DNA or environmental DNA. The abundance of mRNA for GHR, IGF-1R, PEPT1 and SGLT1 was quantified by quantitative real-time polymerase chain reaction (qRT–PCR) using the iCycler and the iQ-SYBR green detection (Bio-Rad Laboratories, Hercules, CA, USA). The primer sets used for real-time PCR were forward primer (5′–3′) CAG ACA AAT CAC TCC ACC AA, reverse primer (5′–3′) GAA GGG CAC CAC CAG GAG T for 18s rRNA as reference gene, forward primer (5′–3′) AAA TTC ACC AAG TGC CGT TC, reverse primer (5′–3′) GGG GCA TTC TTT CCA TTC TT for GHR (GenBank No. AF044258, 141 bp), forward primer (5′–3′) CCT CAT TAT TCC TGC TAA CCA A, reverse primer (5′–3′) AGA TAG AAG AGA TGC GAG GAG GAT for IGF-1R (GenBank No. JN204287, 159 bp), forward primer (5′–3′) TGG CTG GGG AAG TTC AAG AC, reverse primer (5′–3′) TCC TGG CCC TCT TCA AA for PEPT1 (GenBank No. NM001099378, 239 bp), forward primer (5′–3′) TTT CTG GGG CCA TAT TCA TC, reverse primer (5′–3′) CGT CTG CAA GGT GTC TGT GT for SGLT1 (GenBank No. AF508807, 137 bp). Subsequent qPCR was performed on a MxPro-Mx3000P multiplex quantitative PCR systems (Stratagene, La Jolla, CA, USA) at a minimum in triplicate. A reaction mixture (20 μl) consisted of 2 μl cDNA, 10 μl SYBR Premix Taq™ II (TaKaRa Biotechnology Co., Ltd., Dalian, China), 0.8 μl PCR Forward Primer, 0.8 μl PCR Reverse Primer, 0.4 μl ROX Reference Dye II and 6.0 μl dH_2O. The PCR was performed under the following cycle conditions: 1 cycle of 95 °C for 20 s, 45 cycles of 95 °C for 20 s, and annealing temperature for 30 s and 62 °C for 20 s, followed by a melting curve analysis (Denman & McSweeney 2006). Fluorescence detection was performed at the end of each denaturation and extension step. The relative quantity of mRNA for GHR, IGF-1R, PEPT1 and SGLT1 were done as a proportion of 18S rRNA according to the equation:

$$\text{Relative quantification} = 2^{-(\text{Ct target genes} - \text{Ct total reference genes})}$$

where C_t represents threshold cycle.

Statistical analysis

Data were analysed using the mixed model procedure of SAS (Proc Mixed; SAS 2002) with a 2 (age) × 4 (isovalerate supplementation) factorial arrangement of treatments. The treatment with different levels of isovalerate supplementation and age was considered as a fixed effect, while animals within treatment were considered as random effects. In addition, linear and quadratic orthogonal contrasts were tested using the CONTRAST statement of SAS with coefficients estimated based on the

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Table 2. Effects of isovalerate supplementation on small intestine length, muscular layer thickness and mucosa layer thickness in pre- and post-weaned dairy calves

	Treatments[a]					Contrast[b], *P*		
Item	Control	LIV	MIV	HIV	SE	Treatment	Linear	Quadratic
Intestine length								
Duodenum (cm)								
30 days of age	59.7	60.0	61.7	60.7	0.38	0.253	0.155	0.399
90 days of age	82.7	86.9	88.5	87.5	0.71	0.042	0.031	0.121
Jejunum (m)								
30 days of age	1.71	1.73	1.85	1.79	0.021	0.118	0.101	0.107
90 days of age	2.39	2.75	2.78	2.75	0.052	0.025	0.011	0.116
Ileum (cm)								
30 days of age	20.5	23.5	24.0	21.1	0.85	0.589	0.772	0.120
90 days of age	32.5	41.2	44.5	45.0	0.95	0.027	0.023	0.691
Total length (m)								
30 days of age	1.79	1.82	1.94	1.83	0.022	0.132	0.103	0.102
90 days of age	2.51	2.87	2.91	2.88	0.054	0.041	0.034	0.205
Mucosa layer thickness (μm)[c]								
Duodenum								
30 days of age	219	229	232	238	2.6	0.015	0.009	0.611
90 days of age	299	320	323	346	5.4	0.007	0.001	0.821
Proximal jejunum								
30 days of age	167	179	188	197	4.2	0.011	0.005	0.796
90 days of age	224	240	246	254	3.3	0.008	0.001	0.101
Distal jejunum								
30 days of age	368	384	417	434	10.4	0.018	0.014	0.980
90 days of age	551	598	624	648	12.5	0.007	0.001	0.635
Ileum								
30 days of age	346	358	376	387	5.3	0.006	0.001	0.614
90 days of age	474	503	529	534	9.2	0.014	0.010	0.397
Muscular layer thickness (μm)[c]								
Duodenum								
30 days of age	101	104	108	118	2.0	0.315	0.267	0.969
90 days of age	187	193	209	223	16.3	0.298	0.231	0.638
Proximal jejunum								
30 days of age	74	76	78	85	1.9	0.275	0.354	0.126
90 days of age	146	148	153	156	3.8	0.513	0.401	0.942
Distal jejunum								
30 days of age	361	379	388	391	7.4	0.374	0.303	0.464
90 days of age	239	243	248	254	2.6	0.471	0.411	0.886
Ileum								
30 days of age	181	192	215	216	5.8	0.403	0.312	0.576
90 days of age	309	318	324	326	2.8	0.352	0.228	0.522

[a]Control, low-isovalerate (LIV), moderate-isovalerate (MIV) and high-isovalerate (HIV) with 0, 3, 6 and 9 g isovalerate per calf per day, respectively.
[b]The main effect of age (30 *v.* 90 days) was significant ($P < 0.05$), and interactions between treatment and age was not significant.
[c]The mucosa is the innermost layer of intestinal wall and is made up of epithelium, lamina propria and muscularis mucosae. The muscular layer is the third layer of the intestinal wall from the lumen outwards and comprises both longitudinal and circular smooth muscle that also helps with continued peristalsis and the movement of digested material out of and along the gut. In between the two layers of muscle lies the myenteric plexus.

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isovalerate application rates. Effects of the factors were declared significant at $P < 0.05$.

Results

Small intestine morphology

No interaction between treatment and age was observed for small intestine morphology (Tables 2 and 3). The small intestine length, mucosa layer thickness, villus height and crypt depth were greater ($P < 0.05$) in post-weaned calves compared with pre-weaned calves. The muscular layer thickness was also greater ($P < 0.05$) in the duodenum, proximal jejunum and ileum, but was less ($P < 0.05$) in the distal jejunum of post-weaned calves compared with pre-weaned calves. The small intestine length was unaltered for pre-weaned calves but increased linearly ($P < 0.05$) for post-weaned calves with increasing isovalerate supplementation. Hence, overall small intestine length also increased linearly ($P < 0.05$). The muscular layer thickness of the small intestine was unaltered, but the mucosa layer thickness increased linearly ($P < 0.05$) with increasing isovalerate supplementation in calves. Similarly, the villus height and crypt depth of the small intestine increased linearly ($P < 0.05$), but the ratio of villus height to crypt depth was not affected by isovalerate supplementation in calves.

Activities of lactase, amylase and trypsin in the small intestine chyme

No interaction between treatments and age was observed for the activities of lactase, amylase and trypsin in small intestine chyme (Table 4). Lactase activity was lower ($P < 0.05$), but amylase activity was higher ($P < 0.05$) in post-weaned calves compared with pre-weaned calves. However, the activity of trypsin was lower ($P < 0.05$) in the duodenum and higher ($P < 0.05$) in proximal

Table 3. Effects of isovalerate supplementation on villus height, crypt depth and the ratio of villus height to crypt depth of small intestine in pre- and post-weaned dairy calves

	Treatments[a]					Contrast[b], *P*		
Item	Control	LIV	MIV	HIV	SE	Treatment	Linear	Quadratic
Villus height (μm)								
Duodenum								
30 days of age	73	74	77	82	1.2	0.009	0.001	0.145
90 days of age	109	116	124	131	2.9	0.008	0.001	0.946
Proximal jejunum								
30 days of age	66	70	75	79	2.0	0.015	0.012	0.954
90 days of age	111	120	126	137	3.1	0.007	0.001	0.875
Distal jejunum								
30 days of age	86	96	103	106	2.5	0.008	0.001	0.112
90 days of age	124	129	134	139	2.4	0.024	0.020	0.981
Ileum								
30 days of age	73	82	90	97	2.7	0.005	0.001	0.869
90 days of age	112	122	130	134	2.8	0.009	0.001	0.162
Crypt depth (μm)								
Duodenum								
30 days of age	53	55	58	59	1.3	0.047	0.043	0.785
90 days of age	76	88	90	97	2.6	0.008	0.002	0.459
Proximal jejunum								
30 days of age	43	45	48	56	1.8	0.009	0.001	0.321
90 days of age	75	77	83	88	2.2	0.010	0.001	0.504
Distal jejunum								
30 days of age	64	67	70	72	2.4	0.006	0.001	0.415
90 days of age	91	91	94	96	1.8	0.031	0.028	0.636
Ileum								
30 days of age	54	61	67	69	2.0	0.007	0.001	0.427
90 days of age	85	91	96	98	1.5	0.009	0.001	0.115

[a]Control, low-isovalerate (LIV), moderate-isovalerate (MIV) and high-isovalerate (HIV) with 0, 3, 6 and 9 g isovalerate per calf per day, respectively.
[b]The main effect of age (30 *v.* 90 days) was significant ($P < 0.05$) for villus height and crypt depth, and interaction between treatments and age was not significant ($P > 0.05$).

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Table 4. Effects of isovalerate supplementation on activities of lactase, amylase and trypsin in the small intestine chyme of pre- and post-weaned dairy calves

	Treatments[a]					Contrast[b], *P*		
Item	Control	LIV	MIV	HIV	SE	Treatment	Linear	Quadratic
Lactase (U/g)								
Duodenum								
30 days of age	1315	1355	1408	1418	19.4	0.043	0.048	0.669
90 days of age	290	297	308	311	2.9	0.213	0.101	0.444
Proximal jejunum								
30 days of age	1388	1403	1560	1531	16.1	0.025	0.012	0.483
90 days of age	838	856	865	877	9.1	0.201	0.182	0.866
Distal jejunum								
30 days of age	329	488	549	645	34.8	0.018	0.009	0.486
90 days of age	37.4	38.0	39.2	39.4	2.45	0.242	0.173	0.806
Ileum								
30 days of age	298	313	342	359	4.1	0.032	0.017	0.368
90 days of age	34.6	35.7	37.3	38.1	3.38	0.267	0.136	0.582
Amylase (U/g)								
Duodenum								
30 days of age	0.29	0.30	0.31	0.32	0.006	0.024	0.015	0.253
90 days of age	0.31	0.34	0.37	0.38	0.011	0.031	0.017	0.705
Proximal jejunum								
30 days of age	0.26	0.28	0.29	0.30	0.005	0.010	0.002	0.392
90 days of age	0.29	0.31	0.35	0.38	0.010	0.048	0.042	0.608
Distal jejunum								
30 days of age	0.32	0.34	0.38	0.39	0.010	0.021	0.014	0.247
90 days of age	0.37	0.39	0.41	0.42	0.004	0.047	0.034	0.868
Ileum								
30 days of age	0.35	0.36	0.37	0.37	0.016	0.029	0.011	0.425
90 days of age	0.43	0.44	0.45	0.45	0.014	0.041	0.020	0.613
Trypsin (kU/g)								
Duodenum								
30 days of age	2.20	2.22	2.71	3.56	0.170	0.015	0.008	0.584
90 days of age	1.41	1.44	2.04	2.29	0.116	0.013	0.005	0.481
Proximal jejunum								
30 days of age	3.75	4.46	5.46	5.83	0.244	0.042	0.021	0.122
90 days of age	5.93	6.34	6.47	6.75	0.195	0.021	0.009	0.466
Distal jejunum								
30 days of age	3.39	3.54	3.70	3.72	0.051	0.016	0.008	0.400
90 days of age	5.40	5.50	5.66	5.76	0.046	0.043	0.031	0.384
Ileum								
30 days of age	2.12	2.54	2.97	3.15	0.036	0.029	0.012	0.573
90 days of age	4.13	4.43	5.10	5.73	0.063	0.045	0.034	0.824

[a]Control, low-isovalerate (LIV), moderate-isovalerate (MIV) and high-isovalerate (HIV) with 0, 3, 6 and 9 g isovalerate per calf per day, respectively.
[b]The main effect of age (30 *v.* 90 days) was significant ($P < 0.05$), and interaction between treatments and age was not significant ($P > 0.05$).

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jejunum, distal jejunum and ileum in post-weaned calves compared with pre-weaned calves. Lactase activity was unaltered for post-weaned calves but increased linearly ($P < 0.05$) for pre-weaned calves with increasing isovalerate supplementation. Hence, overall lactase activity also increased linearly ($P < 0.05$) in calves. Similarly, the activities of amylase and trypsin increased linearly ($P < 0.05$) with increasing isovalerate supplementation in calves.

Functional gene expression

No interaction between treatments and age was observed for mRNA expressions of GHR, IGF1R, PEPT1 or SGLT1 in the small intestine mucosa (Tables 5 and 6). Expression of GHR, IGF1R, PEPT1 and SGLT1 was lower ($P < 0.05$) in post-weaned calves compared with pre-weaned calves and relative expression of GHR, IGF1R and SGLT1 increased linearly ($P < 0.05$) with increasing isovalerate supplementation in calves. However, the relative expression of PFPT1 only increased linearly ($P < 0.05$) in the duodenum and proximal jejunum, and tended to increase ($P < 0.10$) in the distal jejunum and ileum in calves.

Discussion

Small intestine morphology

The increase in intestine length, mucosa layer thickness, villus height and crypt depth in calves were due to the increased expressions of GHR, IGF-1R, PEPT1 and SGLT1 in the small intestine mucosa. The results indicated that small intestine development was enhanced by isovalerate supplementation. Previous studies found that DM intake, ruminal VFA concentration, butyrate production and nutrient digestion increased with isovalerate supplementation in steers and calves (Liu *et al.* 2009*b*; 2016), which could explain the improved small intestine morphology in the

Table 5. Effects of isovalerate supplementation on relative mRNA expression of growth hormone receptor (GHR) and insulin-like growth factor 1 receptor (IGF1R) in the small intestine mucosa of pre- and post-weaned dairy calves (percentage of 18s rRNA)

	Treatments[a]					Contrast[b], *P*		
Item	Control	LIV	MIV	HIV	SE	Treatment	Linear	Quadratic
GHR mRNA								
Duodenum								
30 days of age	0.32	0.34	0.39	0.44	0.014	0.034	0.013	0.357
90 days of age	0.16	0.20	0.27	0.30	0.017	0.025	0.003	0.578
Proximal jejunum								
30 days of age	0.25	0.31	0.36	0.40	0.016	0.019	0.007	0.433
90 days of age	0.15	0.19	0.23	0.24	0.011	0.024	0.008	0.389
Distal jejunum								
30 days of age	0.14	0.15	0.18	0.18	0.005	0.046	0.035	0.349
90 days of age	0.06	0.07	0.09	0.10	0.006	0.038	0.029	0.233
Ileum								
30 days of age	0.06	0.07	0.09	0.09	0.003	0.041	0.026	0.211
90 days of age	0.04	0.06	0.06	0.07	0.002	0.027	0.007	0.347
IGF1R mRNA								
Duodenum								
30 days of age	0.06	0.07	0.08	0.08	0.003	0.022	0.009	0.119
90 days of age	0.04	0.04	0.04	0.05	0.001	0.035	0.024	0.488
Proximal jejunum								
30 days of age	0.10	0.11	0.12	0.13	0.005	0.033	0.015	0.985
90 days of age	0.06	0.07	0.08	0.10	0.004	0.029	0.011	0.458
Distal jejunum								
30 days of age	0.04	0.08	0.09	0.10	0.007	0.017	0.007	0.514
90 days of age	0.02	0.03	0.04	0.05	0.004	0.013	0.004	0.683
Ileum								
30 days of age	0.04	0.04	0.04	0.05	0.003	0.037	0.027	0.923
90 days of age	0.02	0.02	0.03	0.03	0.002	0.024	0.009	0.197

[a]Control, low-isovalerate (LIV), moderate-isovalerate (MIV) and high-isovalerate (HIV) with 0, 3, 6 and 9 g isovalerate per calf per day, respectively.
[b]The main effect of age (30 *v.* 90 days) was significant ($P < 0.05$), and interaction between treatments and age was not significant.

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Table 6. Effects of isovalerate supplementation on relative mRNA expression of peptide transporter (PEPT1) and sodium-glucose cotransporter 1 (SGLT1) in the small intestine mucosa of pre- and post-weaned dairy calves (percentage of 18s rRNA)

	Treatments[a]					Contrast[b], *P*		
Item	Control	LIV	MIV	HIV	SE	Treatment	Linear	Quadratic
PEPT1 mRNA								
Duodenum								
30 days of age	0.07	0.08	0.08	0.09	0.004	0.012	0.003	0.500
90 days of age	0.06	0.06	0.07	0.07	0.002	0.023	0.011	0.360
Proximal jejunum								
30 days of age	0.12	0.12	0.14	0.15	0.005	0.043	0.048	0.892
90 days of age	0.08	0.09	0.10	0.12	0.006	0.011	0.003	0.787
Distal jejunum								
30 days of age	0.07	0.08	0.09	0.09	0.004	0.076	0.082	0.458
90 days of age	0.05	0.06	0.07	0.08	0.004	0.069	0.073	0.497
Ileum								
30 days of age	0.05	0.06	0.07	0.08	0.008	0.089	0.071	0.646
90 days of age	0.04	0.04	0.05	0.05	0.002	0.078	0.064	0.752
SGLT1 mRNA								
Duodenum								
30 days of age	0.06	0.07	0.08	0.09	0.005	0.011	0.005	0.966
90 days of age	0.05	0.05	0.06	0.06	0.003	0.033	0.029	0.619
Proximal jejunum								
30 days of age	0.06	0.07	0.08	0.09	0.004	0.022	0.017	0.394
90 days of age	0.03	0.06	0.06	0.08	0.005	0.018	0.015	0.379
Distal jejunum								
30 days of age	0.04	0.05	0.05	0.05	0.003	0.019	0.012	0.699
90 days of age	0.01	0.02	0.02	0.02	0.004	0.031	0.017	0.184
Ileum								
30 days of age	0.03	0.03	0.04	0.04	0.004	0.028	0.015	0.506
90 days of age	0.01	0.02	0.02	0.03	0.005	0.032	0.021	0.457

[a]Control, low-isovalerate (LIV), moderate-isovalerate (MIV) and high-isovalerate (HIV) with 0, 3, 6 and 9 g isovalerate per calf per day, respectively.
[b]The main effect of age (30 *v.* 90 days) was significant ($P < 0.05$), and interaction between treatments and age was not significant.

present study. Similarly, other studies reported that small intestine development was promoted by an increased DM intake and nutrient digestion in calves (Kreikemeier *et al.* 1990; Zitnan *et al.* 2003). Additionally, the literature has demonstrated that ruminal VFA could provide fuel for the small intestine, stimulate intestine mucosal cell mitosis and accelerate blood flow in the small intestine (Baldwin 1999; Topping & Clifton 2001). Moreover, some studies reported that the length and mass of the small intestine increased with oral administration of VFA in sheep (Baldwin 1999), villus height of jejunum was enhanced by increased rumen VFA production in goats (Wang *et al.* 2009), and mitotic index of the upper jejunum increased with sodium butyrate supplementation in calves (Guilloteau *et al.* 2009). Furthermore, the increased villus height and crypt depth suggested that the digestion and absorption capacity of the small intestine was enhanced with isovalerate supplementation (Kreikemeier *et al.* 1990; Wang *et al.* 2009). Because of the similar magnitude of increase in villus height and crypt depth, the ratio of villus height to crypt depth was unaltered with isovalerate supplementation. According to McLeod & Baldwin (2000), increasing energy intake and dietary forage content resulted in increased intestinal growth by cellular hyperplasia. The improved small intestine development in post-weaned calves compared with pre-weaned calves was due to a higher solid feed intake.

Activities of lactase, amylase and trypsin in the small intestine chyme

The increased activities of lactase, amylase and trypsin in the small intestine chyme indicated that the digestive function of the small intestine was improved with isovalerate supplementation.

An increased α-amylase was observed by increasing feed intake in calves (Kreikemeier *et al.* 1990), small intestine protein flow in steers (Richards *et al.* 2003) and dietary starch content in lambs (Swanson *et al.* 2000). Moreover, Swanson *et al.* (2002) found

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that increased small intestine protein flows enhanced the activities of α-amylase and trypsin in calves. Additionally, it has been demonstrated that short-chain fatty acids, especially butyrate can stimulate pancreatic amylase secretion (Katoh & Tsuda 1984; Katoh & Yajima 1989) via direct action on pancreatic acinar cells (Harada & Kato 1983). Therefore, the increased activities of lactase, amylase and trypsin in calves was also due to the increased DM intake, microbial protein synthesis and ruminal fermentation with isovalerate supplementation (Liu *et al.* 2009*b*; 2016), which provided sufficient starch, protein and VFA to the small intestine and promoted enzyme secretion. The lower lactase activity for post-weaned calves compared with pre-weaned calves further confirmed the findings of Siddons (1968), who reported that lactase activity decreased with increasing age in cows. Additionally, the unchanged lactase activity for post-weaned calves was attributed to the absence of lactose in post-weaned calves' diets compared with that of pre-weaned calves, and there was no milk for post-weaned calves. The increased secretion of amylase at 90 days of age could be expected due to the starch content of the post-weaning diet compared with 30 days of age.

Functional gene expression

The somatotrophin axis hormones, especially GH and IGF-1, are involved in the proliferation and maturation of enterocytes and play a key role in small intestine development (Bühler *et al.* 1998; Jehle *et al.* 1999; Georgiev *et al.* 2003). The regulatory effects of GH and IGF-1 depend on their respective receptor numbers and affinity in the small intestine mucosa (Georgiev *et al.* 2003; Howarth 2003). The increased relative mRNA expressions of GHR and IGF-1R in the small intestine mucosa was consistent with the increased blood concentrations of GH and IGF-1 with isovalerate supplementation in steers or calves (Liu *et al.* 2009*b*; 2016), and further indicated that small intestine development was promoted by isovalerate supplementation. According to Smith *et al.* (2002), hepatic mRNA expressions of GHR and IGF1 increased with increasing nutrient intake in calves. The increased GHR and IGF-1R expression should be attributed to the increased DM intake and ruminal fermentation seen with isovalerate supplementation (Bühler *et al.* 1998; Liu *et al.* 2009*b*; 2016). Nevertheless, Hammon & Blum (2002) found a greater IGF1R expression in the small intestine of calves fed milk compared with calves fed milk replacer. Flaga *et al.* (2012) observed that IGF1 mRNA levels in the duodenum and jejunum decreased after calves were 5 days old. Therefore, increased age and solid feed were the main reasons for the lower relative expressions of GHR and IGF1R in post-weaned calves compared with pre-weaned calves. The increased relative mRNA expressions of PEPT1 and SGLT1 in the small intestine mucosa indicated that the absorption function for small peptides and glucose was enhanced with isovalerate supplementation. The higher PEPT1 level in the proximal jejunum mucosa suggested that the proximal jejunum was the main site of small peptide absorption in the small intestine. Decreased SGLT1 expression with increasing age was consistent with the findings of Dyer *et al.* (1997) in lambs. Moreover, Shirazi-Beechey *et al.* (1991) attributed age-related declines in SGLT1 to decreased D-glucose content in the small intestine after rumen development. According to Liu *et al.* (2009*a*), expression of PEPT1 in the small intestine was enhanced with increasing concentrations of small peptides in the circulation. The decreased PEPT1 expression with increased age was also associated with rumen development, which increased feed protein degradation and decreased small peptides reaching the small intestine.

Conclusion

The supplementation of isovalerate increased the small intestine length, mucosa layer thickness, villus height, crypt depth, activities of lactase, amylase and trypsin and relative expressions of GHR, IGF1R, PEPT1 and SGLT1 in calves. The results suggested that morphology and function of digestion and absorption in the small intestine were promoted by isovalerate supplementation in a dose-dependent manner under the current experimental conditions.

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