

## Dietary supplementation with $\beta$ -hydroxy- $\beta$ -methylbutyrate calcium during the early postnatal period accelerates skeletal muscle fibre growth and maturity in intra-uterine growth-retarded and normal-birth-weight piglets

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### Abstract

Intra-uterine growth restriction (IUGR) impairs postnatal growth and skeletal muscle development in neonatal infants. This study evaluated whether dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate Ca (HMB-Ca) supplementation during the early postnatal period could improve muscle growth in IUGR neonates using piglets as a model. A total of twelve pairs of IUGR and normal-birth-weight (NBW) male piglets with average initial weights (1.85 (SEM 0.36) and 2.51 (SEM 0.39) kg, respectively) were randomly allotted to groups that received milk-based diets (CON) or milk-based diets supplemented with 800 mg/kg HMB-Ca (HMB) during days 7–28 after birth. Blood and *longissimus dorsi* (LD) samples were collected and analysed for plasma amino acid content, fibre morphology and the expression of genes related to muscle development. The results indicate that, regardless of diet, IUGR piglets had a significantly decreased average daily weight gain (ADG) compared with that of NBW piglets ( $P < 0.05$ ). However, IUGR piglets fed HMB-Ca had a net weight and ADG similar to that of NBW piglets fed the CON diet. Irrespective of body weight (BW), HMB-Ca supplementation markedly increased the type II fibre cross-sectional area and the mRNA expression of mammalian target of rapamycin (*mTOR*), insulin-like growth factor-1 and myosin heavy-chain isoform *Iib* in the LD of piglets ( $P < 0.05$ ). Moreover, there was a significant interaction between the effects of BW and HMB on *mTOR* expression in the LD ( $P < 0.05$ ). In conclusion, HMB-Ca supplementation during the early postnatal period could improve skeletal muscle growth and maturity by accelerating fast-twitch glycolytic fibre development in piglets.

**Key words:**  $\beta$ -Hydroxy- $\beta$ -methylbutyrate calcium: Growth and development: Piglets: Skeletal muscle fibre

It has been well demonstrated that approximately 10% of human neonates might suffer from intra-uterine growth restriction (IUGR) at birth and remain small throughout their lives, which results in serious effects on their health<sup>(1,2)</sup>. Furthermore, there is some evidence that IUGR severely impairs the growth and development of skeletal muscle in neonates<sup>(3,4)</sup> and reduces the number of secondary and total muscle fibre, potentially resulting in decreased muscle mass and delayed skeletal muscle maturity<sup>(5,6)</sup>. More importantly, neonates with IUGR have altered muscle energy metabolism and function, which have been associated with a decreased ability for glucose uptake by muscle tissue and the development of insulin resistance as adults<sup>(7,8)</sup>. Research has documented that the growth and protein turnover of infants are higher during the neonatal period than that at any other period of life<sup>(9)</sup>, and a defect in normal muscle development during the

early postnatal period can influence later growth and health, as well as muscle contractile properties and metabolic maturation<sup>(10)</sup>. Therefore, the improvement of skeletal muscle development in IUGR piglets is of interest.

In recent years, considerable effort has been focused on improving the postnatal growth of IUGR neonates via nutritional regulation. Growing evidence indicates that both leucine and its metabolite ( $\beta$ -hydroxy- $\beta$ -methylbutyrate, HMB) stimulate protein synthesis in skeletal muscle through the activation of the mammalian target of rapamycin (*mTOR*) signalling pathway in piglets and rats<sup>(11,12)</sup>. In addition, studies have indicated that neonatal pigs infused with HMB (free acid) have higher skeletal muscle protein synthesis<sup>(13)</sup>. In addition, Moore *et al.*<sup>(14)</sup> reported that early post-hatch poultry fed with HMB had better muscle development via an increase in the mitotic activity of myogenic

**Abbreviations:** BW, body weight; CK, creatine kinase; CON, control; CSA, cross-sectional area; HMB,  $\beta$ -hydroxy- $\beta$ -methylbutyrate; HMB-Ca,  $\beta$ -hydroxy- $\beta$ -methylbutyrate calcium; *IGF-1*, insulin-like growth factor-1; IUGR, intra-uterine growth restriction; LD, *longissimus dorsi*; LDH, lactate dehydrogenase; NBW, normal birth weight; *MRF-4*, muscle regulatory factor 4; *mTOR*, mammalian target of rapamycin; MyHC, myosin heavy-chain.

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satellite cells. Moreover, HMB supplementation stimulated growth hormone–insulin-like growth factor (IGF) axis activity and decreased the rate of protein breakdown in a rodent model<sup>(15,16)</sup>. However, there are only limited data on the effects of dietary exposure to HMB on skeletal muscle growth and maturity and amino acid metabolism in IUGR neonates during the early postnatal period. Sufficient skeletal muscle growth is essential for lasting metabolic health. Because of the structural and physiological similarities in skeletal muscle between pigs and humans, this study used piglets to assess the effects of dietary supplementation with  $\beta$ -hydroxy- $\beta$ -methylbutyrate Ca (HMB-Ca) on skeletal muscle growth, the plasma amino acid profile, and the expression of muscle growth and development-related genes of IUGR neonates during the early postnatal period.

**Methods**

*Animal care and experimental design*

All procedures were in accordance with the guidelines set by the Animal Care and Use Committee of the Animal Nutrition Institute, Sichuan Agricultural University. The Ca salt (monohydrate) of HMB (purity 93%) was purchased from Jiangyin Sanyi Chemical Co. Ltd. Neonatal piglets from parities 2 and 3 of thirty sows were selected for this study. At day 7 after birth, a total of twelve pairs of IUGR (body weight (BW) 1.85 (SEM 0.36) kg) and normal-birth-weight (NBW) (BW 2.51 (SEM 0.39) kg) male piglets were weaned and assigned, according to BW, to groups fed the basal diets supplemented with 0 or 0.08% HMB-Ca. There were four treatment groups (birth weight/diet): IUGR/control (CON), IUGR/HMB, NBW/CON and NBW/HMB (six per group). All piglets were fed liquid diets every 3 h by bottle feeding from 7 and 28 d of age. All piglets were housed individually in metabolism cages (0.8×0.7×0.4 m) at an ambient temperature of 30°C in an environmentally controlled room and had free access to water.

*Formula milk*

The experimental diets used for the present study were prepared by adding 800 mg/kg HMB-Ca to the basal diet (Table 1). The basic milk replacement powder was formulated according to previous studies<sup>(17)</sup>. The dosage of supplemented HMB-Ca (800 mg/kg) was chosen according to previous studies on 7-d-old piglets<sup>(13)</sup>. The diets were prepared by mixing 1 kg of milk replacement powder (DM, 87.5%) with 4000 ml of water and were given to piglets via bottle feeding seven times per 24 h. The formula milk intake of the piglets was recorded daily. The average daily DM intake (ADMI) was calculated by multiplying the average daily intake of formula milk by its corresponding DM content. Formula milk intake was calculated as the difference between the offered amounts and the amount refused.

*Blood and tissue samples collection*

At day 29 after birth, blood samples (5 ml) were collected from the jugular vein after an overnight fast and immediately centrifuged for 10 min at 3500 g. The supernatants were stored at –20°C until analysis. All piglets were euthanised with an overdose

**Table 1.** Composition and nutrient content of the basal diet (87.5% DM basis)\*

Ingredients	%
Whole-milk powder (24% CP)	58.00
Whey protein concentrate (34% CP)	25.00
Casein	2.00
Coconut oil	10.00
Glucose	2.15
Dicalcium phosphate (18% P)	0.18
Choline chloride (50%)	0.10
Vitamin mixture†	0.10
Mineral premix‡	0.50
L-Lys.HCl (78.5%)	0.60
DL-Met (98.5%)	0.15
L-Thr (98%)	0.17
L-Trp (98%)	0.10
L-Arg (98.5%)	0.15
L-Ala (99.0%)	0.80
Total	100.00
<b>Nutrient content</b>	
Digestible energy (MJ/kg)	17.74
CP (%)	25.03
Ca (%)	0.88
Total P (%)	0.62
Available P (%)	0.62
Digestible Lys (%)	2.07
Digestible Met (%)	0.88
Digestible Leu (%)	2.40
Digestible Arg (%)	0.86

CP, crude protein; HMB-Ca,  $\beta$ -hydroxy- $\beta$ -methylbutyrate Ca.  
 \* The  $\beta$ -hydroxy- $\beta$ -methylbutyrate diet was prepared by supplementing the basal diet with 800 mg/kg HMB-Ca and removing the same amount of dicalcium phosphate.  
 † Vitamin premix provided per kg of powder diet: vitamin A, 0.94 mg; vitamin D<sub>3</sub>, 0.01 mg; vitamin E, 20 mg; vitamin K<sub>3</sub>, 1 mg; vitamin B<sub>12</sub>, 0.04 mg; riboflavin, 5 mg; niacin, 20 mg; pantothenic acid, 15 mg; folic acid, 1.5 mg; thiamin, 1.5 mg; pyridoxine, 2 mg; biotin, 0.1 mg.  
 ‡ Mineral premix provided per kg of powder diet: Zn, 90 mg; Mn, 40 mg; Fe, 90 mg; Cu, 6.0 mg; I, 0.2 mg; Se, 0.3 mg.

of anaesthesia according to the method described by Han *et al.*<sup>(17)</sup>. After death, the abdomen was opened, and the entire intestine was rapidly removed. Then *longissimus dorsi* (LD) muscle samples from the left half of the carcass were collected at the level of the twelfth/thirteenth ribs and cooled using liquid N<sub>2</sub> and stored at –80°C until subsequent analyses. For body composition analysis of the piglets, all internal organs were weighted, and the right half of the carcass was dissected into primary cuts, such as the loin, neck and ham, which were further manually separated into muscle tissue, subcutaneous adipose tissue, skin and bones after cooling overnight at 4°C, as described previously by Rehfeldt *et al.*<sup>(18)</sup>. The muscle tissue of the piglets consisted of the total amount of skeletal muscle and intramuscular fat, and the whole superficial fat layer consisted of the subcutaneous fat. Muscle samples for histochemical analysis were collected from LD on the right side of the carcass and cut into 2-cm<sup>3</sup> pieces (1.0×1.0×2.0 cm, parallel to the muscle fibres).

*Biochemical analyses*

The DNA of muscle samples was extracted using a QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer's instructions, and DNA quantification was performed using a NanoVue Plus spectrophotometer (GE Life Sciences). The protein concentration and the creatine kinase (CK) and lactate

dehydrogenase (LDH) activities of muscle samples were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the instruction manuals. Briefly, frozen muscle samples (approximately 50 mg) were homogenised in 450  $\mu$ l of 0.9% saline and then centrifuged at 3500 *g* for 10 min at 4°C. The protein content in the muscle supernatant was determined with the Coomassie Brilliant Blue dyeing method using bovine serum albumin as the standard and expressed as milligram per gram. CK activity was measured in the supernatants of the muscle homogenates at 1:10 and 1:5 dilution. The optical density in microplate assays was measured at 595 and 660 nm for protein concentration and CK activity, respectively, using a biochemical analyser (Multiskan Spectrum; Thermo Scientific). The plasma insulin concentration was analysed using a porcine insulin RIA kit (Tianjin Jiuding Medical Biological Engineering Co. Ltd).

Muscle tissue samples were measured for HMB content by a modification of the plasma and milk sample analysis method previously described by Deshpande *et al.*<sup>(19)</sup> and Ehling & Reddy<sup>(20)</sup>. Briefly, acidified muscle tissue homogenates were extracted with methyl-*t*-butyl ether for 2 h, and  $\alpha$ -hydroxy- $\alpha$ -methylbutyric acid (Sigma H40009; Sigma-Aldrich) was used as an internal standard. The extracting solution was centrifuged at 12000 *g* and 4°C for 5 min. Then, the supernatant was transferred to a clean test tube and evaporated to dryness by N flushing at 40°C. After drying, 4 ml of 0.10 M-HCl in 90:10 (v/v) water-acetonitrile was added to the same test tube. After a brief vortex mixing, the mixture was passed through an OASIS<sup>®</sup> MCX cartridge (60 mg) (Waters) and the eluate was collected. The HMB content was analysed via HPLC with MS (Agilent).

The plasma free amino acid content was determined according to the method described by Li *et al.*<sup>(21)</sup>. Briefly, 300  $\mu$ l of the plasma sample and 900  $\mu$ l of 10% sulphosalicylic acid were mixed and centrifuged at 12000 *g* and 4°C for 15 min. Then, the supernatant was filtered through a 0.22- $\mu$ m-pore-size PTFE syringe filter (Millipore) into a 2-ml auto-sampler vial and analysed for amino acid content by an automatic amino acid analyzer (Hitachi). Amino acid standard solutions type B and AN-II (Wako Pure Chemical Industries Ltd) were used for calibration.

### Histochemistry and microscopy

The LD samples were mounted on a cryostat chuck with a few drops of tissue-freezing medium, and then serial 10- $\mu$ m sections were cut at -20°C. Sections were stained with a typical ATPase stain following alkalisation (pH=9.40) and acidification (pH=4.20) to evaluate muscle morphology using a modification of the method of Guth & Samaha<sup>(22)</sup>. All sections were photographed using a digital microscope (Nikon), and the muscle fibres were counted in five randomly selected fields of known size (1.01 mm<sup>2</sup>, 200–300 fibres). The muscle fibre cross-sectional area (CSA) in the total area analysed was measured with Image-Pro Plus version 6.0 software (Media Cybernetics).

### Total RNA extraction and real-time RT-PCR

The genes detected in the muscle samples included myosin heavy-chain (MyHC) isoform (*I*, *Ia*, *Iix* and *Iib*), muscle

regulatory factor 4 (*MRF-4*), myogenin (*MyoG*), *IGF-1*, mTOR, myostatin (*MSTN*) and  $\beta$ -actin. The information about the primer pairs for the selected genes is summarised in Table 2. Total RNA was extracted from the frozen samples using TRIzol (Invitrogen) according to the manufacturer's protocol. Real-time quantitative PCR (qPCR) analysis was performed using the SYBR Green method, and the target genes were quantified using CFX manager version 1.1 software (Bio-Rad Laboratories). A commercial RT kit (TaKaRa) was used for the synthesis of complementary DNA (cDNA). The qRT-PCR reaction contained 5  $\mu$ l of SYBR Green supermix (TaKaRa), 1  $\mu$ l of cDNA, 0.5  $\mu$ l of each gene-specific primer and 3  $\mu$ l of double-distilled H<sub>2</sub>O. The following thermal cycling conditions were used: denaturation at 95°C for 15 s, followed by forty cycles of denaturation at 95°C for 5 s and annealing at 61.5°C for 30 s. To confirm the specificity of each product, a melting curve analysis (50–95°C with a heating rate of 0.1°C/s and continuous fluorescence measurement) was performed, and the size of the products was determined by agarose gel electrophoresis. The qPCR analysis of each sample was repeated three times. The relative mRNA abundances of the detected genes were calculated using the 2<sup>- $\Delta\Delta C_t$</sup>  method<sup>(21)</sup>.

### Tissue protein extraction and Western blot analysis

The frozen LD samples were lysed and the protein concentration was determined using a protein assay kit according to the manufacturer's instructions. Western blot analysis of fast-MyHC content using the anti-fast myosin skeletal heavy chain antibody (ab91506, diluted 1:5000; Abcam) was carried out as described previously<sup>(23)</sup>. The relative expression of the target protein was normalised to that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Bio-Rich 042, BMSX, diluted 1:20 000; Beijing BMSX Technology Co. Ltd), which was used as the internal standard. The band density of fast-MyHC was

**Table 2.** Primer sequences of the target genes

Target genes	Primer sequence (5'-3')	GenBank no.
<i>MyHC-I</i>	F: GTTTGCCAACTATGCTGGGG	NM_213855.1
	R: TGTGCAGAGCTGACACAGTC	
<i>MyHC-Ila</i>	F: CTCTGAGTTCAGCAGCCATGA	AB025260
	R: GATGTCTTGGCATCAAAGGGC	
<i>MyHC-Ilx</i>	F: TTGACTGGGCTGCCATCAAT	AB025262
	R: GCCTCAATGCGCTCCTTTTC	
<i>MyHC-Iib</i>	F: GAGGTACATCTAGTGCCCT	AB025261
	R: GCAGCCTCCCCAAAATAGC	
<i>MRF-4</i>	F: CCTTCGGTGCCCTTTCTCCAT	NM_001244672.1
	R: GAGTTATTTCTCCCCACTTCC	
<i>MyoG</i>	F: GACATCCCCCTACTTACCA	NM_001012406.1
	R: TCCCAGCCCCCTTATCTTC	
<i>mTOR</i>	F: CATTGGAGATGGTTTGGTGA	XM_003127584.3
	R: ATGGGATGTGGCTTGTGTA	
<i>IGF-1</i>	F: CACAGACGGGCATCGGGAT	FJ914498.1
	R: ACTTGGCAGGCTTGAGGGGT	
<i>MSTN</i>	F: TTTACCTGTTTATGCTGATTGTTG	AY448008.2
	R: TTTGCTAATGTTAGGAGCTGTTTC	
$\beta$ -Actin	F: CCAGCACGATGAAGATCAAGA	AY550069.1
	R: AATGCAACTAACAGTCCGCCTA	

*MyHC*, myosin heavy chain; F, forward; R, reverse; *MRF-4*, muscle regulatory factor 4; *MyoG*, myogenin; *mTOR*, mammalian target of rapamycin; *IGF-1*, insulin-like growth factor-1; *MSTN*, myostatin.

normalised to that of GAPDH, and fast-MyHC protein content was presented as the fold change relative to that of the NBW piglets in the control group.

**Statistical analysis**

All data were checked for a normal distribution and homogeneous variance. In addition, all data were analysed using the Proc MIXED procedure (SAS Institute Inc.) according to the following statistical model  $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \epsilon_{ijk}$  ( $i=1,2; j=1,2; k=1,2, \dots, n_{ij}$ ), where  $Y_{ijk}$  is the response variable,  $\mu$  the overall mean,  $\alpha_i$  the effect of BW ( $i=IUGR$  or NBW),  $\beta_j$  the effect of HMB ( $j=CON$  or HMB),  $(\alpha\beta)_{ij}$  the interaction between BW and HMB and  $\epsilon_{ijk}$  represents the random error, which was assumed to be  $N(0, \sigma^2)$ . Piglets were considered as the experimental unit. Differences between groups were analysed using the general linear model procedure

followed by Duncan's test. The results for muscle fibre CSA and fast-MyHC protein expression are presented as the mean values with their standard errors, and other data in tables are presented as the mean and pooled standard errors. Probability values  $<0.05$  were considered statistically significant, and values between 0.05 and 0.1 were considered to indicate trends.

**Results**

**Performance and body composition of piglets**

The results of piglet performance and body composition are presented in Tables 3 and 4. At the onset of the experiment (day 7 after birth), no differences were observed in the BW of IUGR and NBW piglets between the two dietary treatments, and the final BW and BW gain of the IUGR piglets were both lower than those of their NBW counterparts ( $-36\%$ ,  $P < 0.01$ ).

**Table 3.** Effect of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on the growth performance of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets (Mean values with their standard errors)

Parameters	CON		HMB		SEM	P		
	IUGR	NBW	IUGR	NBW		BW	HMB	BW x HMB
Initial weight (kg)	1.84 <sup>c</sup>	2.79 <sup>a</sup>	1.99 <sup>b,c</sup>	2.44 <sup>a,b</sup>	0.19	0.002	0.599	0.196
Final weight (kg)	5.80 <sup>b</sup>	8.09 <sup>a</sup>	6.40 <sup>b</sup>	8.52 <sup>a</sup>	0.46	<0.001	0.279	0.864
Net weight (kg)	3.96 <sup>c</sup>	5.29 <sup>a,b</sup>	4.41 <sup>b,c</sup>	6.08 <sup>a</sup>	0.32	<0.001	0.078	0.610
ADG (g/d)								
Days 7–14	95 <sup>b</sup>	132 <sup>a,b</sup>	138 <sup>a,b</sup>	175 <sup>a</sup>	17	0.021	0.043	0.981
Days 14–21	114 <sup>c</sup>	180 <sup>a,b</sup>	96 <sup>b,c</sup>	228 <sup>a</sup>	25	0.001	0.542	0.204
Days 21–28	358 <sup>b</sup>	438 <sup>a,b</sup>	402 <sup>a,b</sup>	465 <sup>a</sup>	29	0.029	0.247	0.768
Days 7–28	189 <sup>c</sup>	252 <sup>a,b</sup>	210 <sup>b,c</sup>	290 <sup>a</sup>	15	<0.001	0.077	0.605
ADMI (g/d)								
Days 7–14	79 <sup>b</sup>	115 <sup>a</sup>	82 <sup>b</sup>	116 <sup>a</sup>	8	0.001	0.784	0.879
Days 14–21	118 <sup>b</sup>	176 <sup>a</sup>	102 <sup>b</sup>	196 <sup>a</sup>	14	<0.001	0.913	0.235
Days 21–28	209 <sup>c</sup>	264 <sup>a,b</sup>	236 <sup>b,c</sup>	292 <sup>a</sup>	16	0.004	0.105	0.988
Days 7–28	135 <sup>b</sup>	185 <sup>a</sup>	140 <sup>b</sup>	201 <sup>a</sup>	10	<0.001	0.318	0.598
FCR*								
Days 7–14	0.86	0.89	0.66	0.67	0.08	0.754	0.022	0.932
Days 14–21	1.20	1.05	1.30	0.87	0.21	0.184	0.852	0.518
Days 21–28	0.59	0.60	0.59	0.63	0.01	0.094	0.305	0.532
Days 7–28	0.72 <sup>a</sup>	0.74 <sup>a</sup>	0.67 <sup>b</sup>	0.69 <sup>a,b</sup>	0.01	0.188	0.006	0.664

CON, control; BW, body weight; ADG, average daily weight gain; ADMI, average daily DM intake; FCR, feed conversion ratio.

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* FCR was calculated by dividing the ADMI by its corresponding ADG.

**Table 4.** Effects of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on body composition of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets (Mean values with their standard errors)

Parameters	CON		HMB		SEM	P		
	IUGR	NBW	IUGR	NBW		BW	HMB	BW x HMB
Hot carcass weight (kg)	3.88 <sup>b</sup>	5.66 <sup>a</sup>	4.18 <sup>b</sup>	5.71 <sup>a</sup>	0.33	<0.001	0.605	0.724
Internal organ (%)	5.52	5.61	5.33	5.46	0.12	0.374	0.186	0.857
Muscle tissue (%)	60.58 <sup>a,b</sup>	58.84 <sup>b</sup>	62.43 <sup>a</sup>	61.44 <sup>a,b</sup>	1.00	0.198	0.044	0.719
Subcutaneous								
Fat (%)	13.32	14.72	12.63	13.32	0.77	0.197	0.199	0.651
Bone (%)	16.03	15.66	15.60	14.86	0.87	0.538	0.492	0.838
Skin (%)	10.07	10.77	9.35	10.38	0.67	0.223	0.429	0.815

CON, control; BW, body weight.

<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).



However, the IUGR piglets fed HMB-Ca milk exhibited a net weight and average daily weight gain (ADG) similar to that of the CON-fed NBW piglets. Regardless of BW, piglets in the HMB group also showed an increased ADG (+14%,  $P < 0.10$ ) and a decreased feed conversion rate (-7%,  $P < 0.01$ ) from day 7 to 28 in comparison with that of the control pigs. In addition, HMB-Ca treatment increased ( $P < 0.05$ ) the relative ratio of skeletal muscle by 3.1 and 4.4% for the IUGR and NBW piglets, respectively, compared with that of the piglets fed the control diet.

**Plasma amino acid and insulin concentrations, and muscle  $\beta$ -hydroxy- $\beta$ -methylbutyrate levels of piglets**

As shown in Table 5, irrespective of BW, piglets supplemented with HMB-Ca milk had an increased HMB concentration in their LD ( $P < 0.01$ ) relative to that of piglets fed CON milk. Piglets fed the HMB-Ca supplemented milk also had an elevated plasma insulin concentration ( $P < 0.01$ ) relative to that of their CON counterparts. In addition, in comparison with the content in the CON piglets, dietary HMB-Ca treatment increased plasma leucine and branched-chain amino acid (BCAA) content ( $P < 0.05$ ). Similarly, plasma essential amino acid (EAA) and non-essential amino acid (NEAA) content showed increased levels ( $P < 0.05$ ) in piglets from the HMB group relative to the levels in the CON groups, but no interaction ( $P > 0.05$ ) between BW and HMB treatment was observed. Furthermore, the plasma urea content

of the IUGR piglets was lower than that of the NBW piglets ( $P < 0.01$ ).

**Biochemical and histological properties of the longissimus dorsi of piglets**

Data for CK and LDH activity, DNA and protein content, and the muscle fibre CSA of the skeletal muscle from the piglets are shown in Table 6 and Fig. 1. Regardless of BW, both the CK ( $P < 0.05$ ) and LDH activity ( $P < 0.05$ ) of the LD were greatly increased in piglets fed HMB-Ca milk compared with that in piglets fed CON milk. Furthermore, the mean type II fibre CSA of the LD of piglets fed HMB-Ca milk was significantly increased ( $P < 0.01$ ) relative to that of pigs supplemented with the CON milk, and the type I fibre CSA of the LD of IUGR piglets was lower than that of NBW piglets ( $P < 0.01$ ).

**Gene expression in the longissimus dorsi of piglets**

As shown in Table 7, regardless of BW, piglets fed HMB milk had significantly higher *MyHC-IIb* mRNA levels in the LD ( $P < 0.05$ ) than did pigs fed CON milk. Furthermore, compared with the levels of the CON group, the mRNA levels of *IGF-1*, *mTOR* and *MRF-4* in the LD were up-regulated in piglets fed HMB-Ca milk ( $P < 0.05$ ). However, there was no significant interaction between the effects of BW and HMB treatment on the mRNA abundance of these genes in the LD, except on *mTOR* ( $P < 0.05$ ).

**Table 5.** Effects of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on the plasma amino acid profile and muscle HMB level of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets (Mean values with their standard errors)

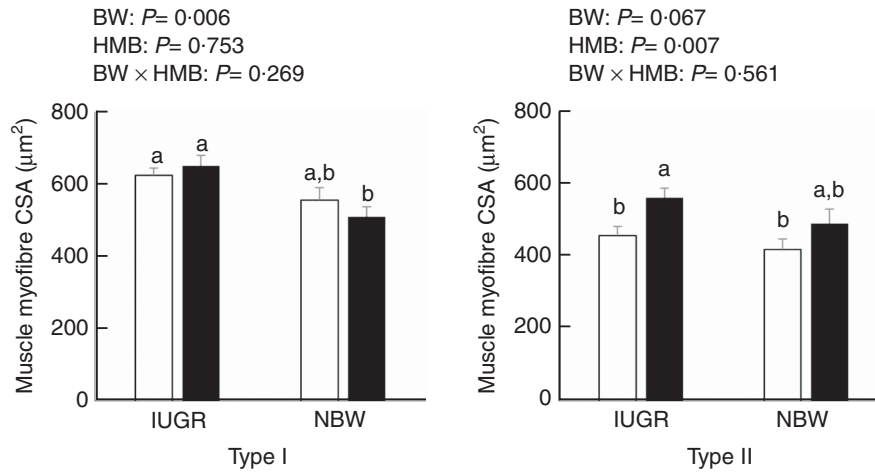
	CON		HMB		SEM	P		
	IUGR	NBW	IUGR	NBW		BW	HMB	BW x HMB
Muscle HMB (nmol/g tissue)	6.04 <sup>b</sup>	5.28 <sup>b</sup>	8.77 <sup>a</sup>	8.83 <sup>a</sup>	0.44	0.434	<0.001	0.366
Plasma insulin ( $\mu$ U/ml)	7.89 <sup>b,c</sup>	6.53 <sup>c</sup>	10.56 <sup>a,b</sup>	12.97 <sup>a</sup>	1.00	0.607	<0.001	0.077
Plasma urea (mmol/l)	4.97 <sup>b</sup>	7.52 <sup>a</sup>	5.21 <sup>b</sup>	7.27 <sup>a</sup>	0.39	<0.001	0.990	0.540
Plasma amino acid (nmol/ml)								
Leu	209 <sup>a</sup>	156 <sup>b</sup>	224 <sup>a</sup>	223 <sup>a</sup>	17	0.126	0.026	0.141
BCAA	675 <sup>a,b</sup>	577 <sup>b</sup>	750 <sup>a</sup>	713 <sup>a</sup>	87	0.094	0.013	0.436
EAA	1670 <sup>a,b</sup>	1560 <sup>b</sup>	1819 <sup>a,b</sup>	1909 <sup>a</sup>	202	0.917	0.019	0.306
NEAA	2440 <sup>b</sup>	2570 <sup>b</sup>	3512 <sup>a</sup>	3208 <sup>a</sup>	281	0.515	<0.001	0.118

CON, control; BW, body weight; BCAA, branched-chain amino acid; EAA, essential amino acid; NEAA, non-essential amino acid.  
<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

**Table 6.** Effects of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on biochemical properties of the *longissimus dorsi* of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets (Mean values with their standard errors)

Parameters	CON		HMB		SEM	P		
	IUGR	NBW	IUGR	NBW		BW	HMB	BW x HMB
DNA ( $\mu$ g/g)	746	882	859	827	51	0.314	0.575	0.116
Protein (mg/g)	153	138	138	130	6	0.125	0.149	0.651
DNA/protein ( $\mu$ g/mg)	4.90	6.40	6.37	6.55	0.52	0.125	0.136	0.223
CK (IU/mg protein)	4.88	4.90	5.19	5.26	0.15	0.741	0.038	0.863
LDH (IU/mg protein)	1.64 <sup>a,b</sup>	1.51 <sup>b</sup>	1.78 <sup>a,b</sup>	2.15 <sup>a</sup>	0.17	0.488	0.039	0.166

CON, control; BW, body weight; CK, creatine kinase; LDH, lactate dehydrogenase.  
<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).



**Fig. 1.** Effects of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on the muscle fibre cross-sectional area (CSA) of the *longissimus dorsi* of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets. Values are means, with their standard errors represented by vertical bars. <sup>a,b</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ). □, Control; ■, HMB; BW, body weight.

**Table 7.** Effects of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on the mRNA level of the myosin heavy-chain (*MyHC*) isoform and myogenic genes in the *longissimus dorsi* of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets (Mean values with their standard errors)

Genes	CON		HMB		SEM	P		
	IUGR	NBW	IUGR	NBW		BW	HMB	BW x HMB
<b>MyHC isoform genes</b>								
<i>MyHC-I</i>	1.20	1.00	1.25	1.23	0.12	0.382	0.260	0.451
<i>MyHC-IIa</i>	0.96	1.00	0.92	1.02	0.12	0.532	0.929	0.771
<i>MyHC-IIx</i>	0.78	1.00	0.74	0.86	0.07	0.026	0.196	0.507
<i>MyHC-IIb</i>	1.26 <sup>a,b</sup>	1.00 <sup>b</sup>	1.30 <sup>a,b</sup>	1.56 <sup>a</sup>	0.13	0.975	0.040	0.067
<b>Myogenic genes</b>								
<i>MRF-4</i>	0.86 <sup>b</sup>	1.00 <sup>a,b</sup>	1.14 <sup>a,b</sup>	1.26 <sup>a</sup>	0.11	0.248	0.023	0.902
<i>MyoG</i>	0.95	1.00	0.91	1.11	0.10	0.222	0.743	0.464
<i>mTOR</i>	1.12 <sup>b</sup>	1.00 <sup>b</sup>	1.77 <sup>a</sup>	0.96 <sup>b</sup>	0.13	0.003	0.032	0.018
<i>IGF-1</i>	0.97 <sup>b</sup>	1.00 <sup>b</sup>	1.33 <sup>a</sup>	1.17 <sup>a,b</sup>	0.11	0.559	0.021	0.375
<i>MSTN</i>	0.81	1.00	0.93	0.99	0.12	0.332	0.663	0.597

CON, control; BW, body weight; *MRF-4*, muscle regulatory factor 4; *MyoG*, myogenin; *mTOR*, mammalian target of rapamycin; *IGF-1*, insulin-like growth factor-1; *MSTN*, myostatin. <sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

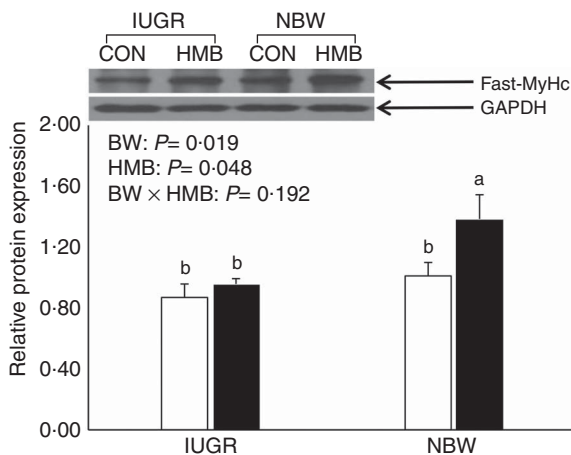
### Protein expression of fast-myosin heavy chain in the *longissimus dorsi* of piglets

The data for fast-MyHC protein content in the LD of piglets are presented in Fig. 2. Regardless of BW, the fast-MyHC protein content was significantly higher in the LD of piglets fed HMB-Ca milk than in the LD of piglets fed CON milk ( $P < 0.05$ ). However, dietary HMB-Ca supplementation did not increase fast-MyHC protein content in the LD of IUGR piglets.

### Discussion

The primary aim of the present study was to investigate the influence of the dietary supplementation of HMB-Ca to neonates during the early postnatal period on skeletal muscle growth and development. Piglet performance, to a great extent, is influenced by milk intake, which correlates with maternal milk yield and birth weight<sup>(24,25)</sup>. Therefore, male piglets with similar low and normal BW were used for IUGR and NBW groups, respectively, in this

experiment. We found no significant difference in ADMI between dietary treatments during the entire experimental period, and IUGR piglets showed lower ADMI and ADG compared with those of NBW piglets. It has been shown in previous studies that the poor growth of IUGR piglets during the neonatal period is because of a number of factors, including reduced nutrient intake<sup>(26)</sup>. Interestingly, IUGR piglets fed HMB-Ca milk had a net weight and ADG similar to that of CON-fed NBW piglets in the present study. HMB is a metabolite of leucine, which is used to promote the growth of lean body mass following exercise, and, in particular, it is involved in disease-related muscle wasting<sup>(27,28)</sup>. HMB has been shown to increase muscle protein synthesis through the mTOR signalling pathways while reducing proteolytic processes, which is similar to the response of skeletal muscle to leucine<sup>(29)</sup>. In the current study, HMB-Ca treatment improved the performance of both IUGR and NBW piglets, which is in agreement with the findings of Nissen *et al.*<sup>(30)</sup>, who reported that, when fed to sows at 2 g/d from day 108 of gestation to day 21 of lactation, HMB-Ca resulted in an increase in the weaning BW of piglets. In addition, in this



**Fig. 2.** Effects of dietary  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) calcium supplementation on the fast-myosin heavy-chain (MyHC) protein levels in the *longissimus dorsi* of intra-uterine growth restriction (IUGR) and normal-birth-weight (NBW) piglets. Values are means, with their standard errors represented by vertical bars. <sup>a,b</sup> Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ). □, Control; ■, HMB; BW, body weight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

experiment, piglets fed HMB-Ca milk had an increased percentage of skeletal muscle relative to that of CON piglets, which is consistent with the findings of Qiao *et al.*<sup>(31)</sup>, who found that, when fed to broiler chicks at a dosage of 0.1%, HMB-Ca significantly improved breast muscle yield and resulted in less abdominal fat. Similarly, Pimentel *et al.*<sup>(32)</sup> reported that feeding of HMB at a dosage of 320 mg/kg BW to rats induced a significant increase in the weight of the extensor *digitorum longus* and *soleus*. Studies have also suggested that a piglet's growth and protein turnover are higher during the neonatal period<sup>(15)</sup>, and the majority of this growth comprises skeletal muscle growth, which represents 30% of the total body mass<sup>(33)</sup>. These observations indicate that increased performance might be obtained by improving skeletal muscle growth for piglets during the early postnatal period.

The secondary aim of the present study was to explore the mechanisms underlying the direct response of skeletal muscle growth in IUGR and NBW piglets to the dietary HMB-Ca supplementation, which is critical for the development of strategies to improve the growth and health of low-birth-weight neonates. It is well known that insulin and glucose, as well as amino acids, especially leucine, are the main anabolic promoters resulting in protein synthesis in the skeletal muscle of piglets<sup>(34)</sup>, and higher plasma insulin, glucose and amino acid levels can induce greater rates of muscle protein synthesis<sup>(35)</sup>. In the present study, HMB-Ca supplementation significantly increased insulin levels in IUGR and NBW piglets. However, the insulin levels were not significantly different between CON and HMB-fed IUGR piglet groups. A possible reason for this is that IUGR piglets have been shown to exhibit a small degree of insulin resistance during days 2 and 28 postnatal<sup>(36,37)</sup>, leading to higher fasting plasma insulin levels in IUGR piglets. Moreover, dietary HMB supplementation significantly affected the plasma amino acid metabolism of piglets, including increased plasma leucine, BCAA, EAA and NEAA content. It appears that the stimulation of protein synthesis is dependent on signals related to the concentration of extracellular, rather than

intramuscular, EAA<sup>(38)</sup>. Interestingly, we observed that piglets fed HMB-Ca milk had increased levels of HMB in their LD in this study. This effect was also demonstrated in the study by Wheatley *et al.*<sup>(13)</sup>, who found that, when piglets were infused with HMB at 100 or 400  $\mu$ mol/kg BW per h for 1 h, an increased HMB level was observed in the LD. This may provide the important evidence to explain why HMB enters the skeletal muscle tissue and promotes protein synthesis. In addition, the plasma urea content was not affected by dietary HMB-Ca treatment in the piglets in this study, providing further evidence that HMB might not promote protein breakdown.

In addition, we found that the LD of piglets supplemented with HMB-Ca milk showed an increase of LDH and CK activities, and had higher type II muscle fibre CSA. Lefaucheur *et al.*<sup>(10)</sup> reported that early postnatal malnutrition reduced the glycolytic capacity and CSA of fast-twitch fibres, leading to a delay in muscle maturation. Previous studies have also indicated that a higher LDH activity, which is a marker for the anaerobic glycolytic fibre, is found in fast glycolytic fibres than in slow oxidative fibres<sup>(39)</sup>, and that LDH activity is related to fibre CSA<sup>(40)</sup>. Furthermore, fast glycolytic fibres exhibit both a higher specific CK activity (a marker of differentiation in skeletal muscle development) and a greater phosphocreatine content than slow-twitch fibres<sup>(41)</sup>. Therefore, it is possible that the switch to a more anaerobic metabolism in the LD of pigs directly contributes to muscle fibre growth and maturity in response to dietary supplementation with HMB-Ca.

It is also known that muscle development in postnatal piglets is regulated by transcription and growth factors, such as the myogenic differentiation factor (*MyoD*) family, which comprises four transcription factors (*MyoD*, myogenic factor 5 (*Myf-5*), *MyoG* and *MRF-4*), and *IGF-1* and 2 (*IGF-2*), which might both stimulate the proliferation of myoblasts<sup>(42,43)</sup>. *MyoD* and *Myf-5* are expressed constantly up to the 3rd week after birth, and their expression is followed by a decline in 50-d-old pigs. However, the level of *MRF-4* level does not show statistically significant differences between different stages of development<sup>(44,45)</sup>. Therefore, we used *MRF-4*, *MyoG*, *IGF-1* and *MSTN* as the main target genes to evaluate the response of skeletal muscle fibre development to dietary HMB-Ca supplementation. It appears that the transcription factor *MRF-4* regulates the final differentiation of myotubes, and this gene has a 10-fold higher postnatal expression than the other genes of the *MRF* family<sup>(46)</sup>. In this study, a higher level of *MRF-4* mRNA expression was observed in the LD of piglets fed HMB-Ca milk than in the LD of the CON-fed piglets. Furthermore, the feeding of milk supplemented with HMB-Ca significantly increased the *IGF-1* and *mTOR* mRNA levels in the LD of piglets, which agrees with the results of Kim *et al.*<sup>(47)</sup>, who reported a significant *IGF-1* mRNA increase in the *soleus* (+33%) when old, female Sprague-Dawley rats were administered HMB orally at a dosage of 0.48 g/kg per d. Moreover, we found that there was a significant interaction between the effects of HMB and BW on *mTOR* mRNA expression in the LD in this study. It has been suggested that the activation of the mTOR signalling pathway in skeletal muscle is under the control of the arginine family of amino acids and leucine<sup>(48)</sup>. In the current study, an increased leucine content was observed in IUGR piglets fed the HMB-Ca diet than

in CON-fed NBW piglets, which might also explain the increased expression of *mTOR* in IUGR pigs fed the HMB-Ca diet. However, at present, it is unclear why dietary HMB-Ca supplementation significantly increased *mTOR* expression in IUGR piglets but not in NBW piglets. Xu *et al.*<sup>(49)</sup> also reported a higher *mTOR* protein expression in IUGR piglets than in NBW piglets in response to dietary leucine supplementation. Further studies should be conducted in relation to the specific mechanisms of protein synthesis that are mediated by HMB in IUGR piglets.

It appears that marked changes occur in the skeletal muscle growth of piglets during the neonatal period, including rapid myofibril protein accretion<sup>(50)</sup>, changes in MyHC polymorphism, increases in aerobic and glycolytic metabolism and hypertrophy of muscle fibres<sup>(51)</sup>. However, limited studies exist that evaluate the direct impact of dietary HMB-Ca supplementation to piglets on the MyHC isoform mRNA levels of skeletal muscle. In this experiment, HMB-Ca treatment significantly increased *MyHC-IIb* mRNA and fast-MyHC protein levels in the LD of piglets. Research has suggested that high muscularity is positively related to a high abundance of the *MyHC-IIb* transcript<sup>(52)</sup>, and that from days 7 to 180 after birth the *MyHC-IIb* mRNA levels show a steadily increasing trend. Moreover, on day 30, *MyHC-I* and *MyHC-IIb* mRNA abundances are at their lowest<sup>(53)</sup>. On the basis of previous reports and the data from this experiment on the CK and LDH activities, *MyHC-IIb* and *IGF-1* mRNA levels, fast-MyHC protein content, the increased muscle growth and maturity in pigs fed HMB-Ca milk could be because of the increased levels of *MyHC-IIb* mRNA in fast glycolytic fibres, which enhances skeletal muscle fibre differentiation of piglets. However, dietary HMB-Ca supplementation did not increase *MyHC-IIb* mRNA or fast-MyHC protein expression in IUGR piglets. It has been suggested that newborn piglets exposed to IUGR have accelerated skeletal muscle development with a precocious type II to type I conversion and, as a consequence, an increase in the proportion and maturation of type I fibres<sup>(54,55)</sup>. Similarly, Fahey *et al.*<sup>(56)</sup> reported that fibre type shifts in the early neonatal period after maternal dietary restriction in sheep exhibited either a relative increase in type I oxidative fibres or a relative decrease in glycolytic type II fibres. The increased MyHC transition towards type I fibres during the neonatal period for IUGR piglets might result in decreased *MyHC-IIb* mRNA and fast-MyHC protein levels, and lower LDH activity in the LD in response to dietary HMB-Ca supplementation. The potential molecular mechanisms underlying the different effect on the growth of skeletal muscle in IUGR and NBW piglets fed an HMB diet should also be investigated further.

In conclusion, the findings of the present study suggest that dietary supplementation of HMB-Ca contributes to muscle fibre growth and maturity by accelerating fast-twitch glycolytic fibre development in IUGR and NBW pigs during the early postnatal period. The ability of HMB to stimulate gains in lean mass in young or neonatal animal has been largely ignored, and this study may have important implications for the postnatal skeletal muscle growth of IUGR and NBW piglets during the neonatal period. Future studies are needed to investigate the effects of different levels of HMB-Ca supplementation on lean mass gain in early postnatal piglets.

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The authors declare that there are no conflicts of interest.

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