

Effect of different levels of protein concentrates supplementation on the growth performance, plasma amino acids profile and *mTOR* cascade genes expression in early-weaned yak calves

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Objective: This study evaluated the effects of different levels of protein concentrate supplementation on the growth performance of yak calves, and correlated the growth rate to changes occurring in the plasma- amino acids, -insulin profile, and signaling activity of mammalian target of rapamycin (*mTOR*) cascade to characterize the mechanism through which the protein synthesis can be improved in early weaned yaks.

Methods: For this study, 48 early (3 months old) weaned yak calves were selected, and assigned into four dietary treatments according to randomized complete block design. The four blocks were balanced for body weight and sex. The yaks were either grazed on natural pasture (control diet) in a single herd or the grazing yaks was supplemented with one of the three protein rich supplements containing low (17%; LP), medium (19%; MP), or high (21%; HP) levels of crude proteins for a period of 30 days.

Results: Results showed that the average daily gain of calves increased (0.14 vs 0.23-0.26 kg; $p < 0.05$) with protein concentrates supplementation. The concentration of plasma methionine increased ($p < 0.05$; 8.6 vs 10.1-12.4 $\mu\text{mol/L}$), while those of serine and tyrosine did not change ($p > 0.05$) when the grazing calves were supplemented with protein concentrates. Compared to control diet, the insulin level of calves increased ($p < 0.05$; 1.86 vs 2.16-2.54 $\mu\text{IU/mL}$) with supplementation of protein concentrates. Addition of protein concentrates up-regulated ($p < 0.05$) expression of *mTOR-raptor*, mammalian vacuolar protein sorting 34 homolog, the translational regulators eukaryotic translation initiation factor 4E binding protein 1, and S6 kinase 1 genes in both *Longissimus dorsi* and semitendinosus. In contrast, the expression of sequestosome 1 was down-regulated in the concentrate supplemented calves.

Conclusion: Our results show that protein supplementation improves the growth performance of early weaned yak calves, and that plasma methionine and insulin concentrations were the key mediator for gene expression and protein deposition in the muscles.

Keywords: Early Weaning; Supplementary Feeding; Protein Synthesis; Yak; Yak Calves Growth

INTRODUCTION

More than 41 million Tibetan sheep and 14 million yaks are raised on one of the world largest (129.3×10^6 ha) alpine-meadow grassland in the Qinghai-Tibetan plateau [1]. The grassland in the plateau is characterized by high altitude (3,000 to 5,400 m), low average annual temperature (-1°C to -5°C) and a prolonged winter period. Extreme cold conditions during winter result in an extended pasture dormant period (October to May), and a shorten pasture growth period of approximately 100 to 150 days. Under these harsh environment and prolonged pasture scarcity periods, yak plays a major role in the livelihood and food supply of the local population [2] and because of it, yak is commonly called as "the treasure of the plateau". It has been reported that yaks provide more than 90% of the milk and about 50% of the meat consumed in the region [2]. Yaks

are mainly raised on the natural pasture, and as such they are fattened during the short autumn season, but losses weight (~20%) during the subsequent winter feed scarcity period [3]. A systematic study by Long et al [2] find out that strategic feed supplementation during the pasture scarcity period can enhance milk yield, fertility and reduce calving interval in mature (5 to 13 years) yaks. However, little is known about the effect of concentrate supplementation on the growth performance of young calves.

Traditionally, calves are nursed by their dams for a very long time, ranging from 6 months to more than a year, in order to increase their survival rate during the winter feed scarcity period. However, due to the prolonged nursing, the mother yaks usually return to oestrous very late, and as a result the calving interval is usually delayed for more than 1.5 years. On top of that, the quantity of milk decreases as lactation and winter progresses, and the milk alone could not meet the nutrient requirements of rapid growing young calves. As a result, the calf growth slows down as the lactation progresses, and ultimately leads to weight loss during the late lactation period. Moreover, early weaning has been shown to be an effective way to improve the reproductive efficiency of cows. The postpartum anoestrous period of early weaned cows is 24 days shorter than nursing cows [4]. The early weaning also improves the body condition and pregnancy rate, and subsequently decreases the calving interval [5]. On the other hand, the growth performance of early weaned calves can be enhanced with concentrate supplementation. To the author's knowledge no systematic research has been conducted on the effects of concentrate supplementation on the growth of performance of yak calves.

In young calf, the rate and transfer efficiency of absorbed amino acids into calf body is very high, which in turn is largely due to high rate of protein synthesis after feeding, and this response is particularly profound in the skeletal muscles. The high rate of protein deposition in the young calves is independently mediated by the rise in plasma amino acids and insulin hormone after protein feeding [6]. Recently it has been reported that in young animals the intracellular signalling mechanism, the mammalian target of rapamycin (*mTOR*), is activated by the rise of plasma insulin and amino acids concentration, which in turn increases the rate of protein synthesis [7-9]. In contrast, Rius et al [10] did not observe a consistent relationship between intracellular signalling activity and accelerated growth in calves by changing the protein contents of milk replacer. The present study was therefore designed to examine the effects of different levels of protein concentrates supplementation on the growth performance of early weaned yaks, and to correlate the growth performance with changes in i) plasma amino acids and insulin profile, and ii) signalling activity of *mTOR* cascade, in an effort to characterize the pathway through which the concentrate supplementation promotes protein synthesis in yak calves.

MATERIALS AND METHODS

Experimental site

The experiment was conducted at Qinghai Provincial Datong Yak Breeding Farm, located in an alpine-meadow grassland of Datong County (37°11' to 37°32' N, and 100°52' to 101°54' E) at an altitude of 3,293 m. The study was carried out during September and October 2011. The annual rain falls the area during 2011 was recorded as 463 mm, whereas the average temperature was recorded as 2.4°C, ranging from -31°C to 24°C. The main vegetation in the grasslands was lyme grass (*Elymus geminate*; with mean crude protein [CP] content of 10%±1%), which grows during summer months (May to October).

Animals, experimental design and feeding

Forty-eight, 3 months old yak (*Bos grunniens*), calves were assigned to four dietary treatments according to randomized complete block design. The calves were distributed over 4 blocks to balance for body weight (BW) and sex. The calves were either fed on natural grassland in Datong Yak Breeding Farm as a sole diet (control) or the grazing was supplemented with low (17%; LP), medium (19%; MP) or high (21%; HP) level of CP containing concentrates. The ingredients and chemical composition of the three concentrates is shown in Table 1. Prior to beginning of trial, all experimental animals were grazed on the pasture along with their mother yaks. Subsequently, calves were weaned, weight, labeled, and assigned to the four dietary treatments. The calves were

Table 1. Ingredient and chemical composition of concentrates fed to early weaned yak calves

Items	Treatments ¹⁾			
	Control	LP	MP	HP
Ingredient composition (%)				
Corn	-	69.0	63.2	57.0
Protein concentrate ²⁾	-	30.0	35.8	42.0
Minerals and vitamins mixture ³⁾	-	1.0	1.0	1.0
Chemical composition (% of DM)				
DM (%)	90.2	88.4	87.1	86.3
CP (N × 6.25)	8.2	17.2	19.1	21.2
NDF	49.8	15.4	17.3	18.6
EE	2.3	5.2	5.4	5.5
Ash	1.8	2.3	2.4	2.6
NFC ⁴⁾	37.9	59.9	55.8	52.1

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; EE, ether extract.

¹⁾ Control, only grazed on natural pasture; LP, supplemented with low crude protein (17%) concentrates; MP, supplemented with medium crude protein (19%) concentrates; HP, supplemented with high crude protein (21%) containing concentrates.

²⁾ The protein concentrate contained 8.9% blood meal, 16.7% corn gluten meal, 7.6% whey power, 23.8% feather meal, 33.3% corn dried distillers grains with solubles, 5.8% calcium carbonate, 2.4% potassium chloride and 1.5% sodium chloride.

³⁾ The mixture contained 35.0% calcium carbonate, 15.0% potassium chloride, 4.0% sodium chloride, 1.0% mineral premix (15% Fe, 10% Mn, 2% Cu, 700 mg/kg of Co, 800 mg/kg of I, and 1,200 mg/kg of Se), 1% vitamin premix (containing 6,000,000 IU of vitamin A, 1,000,000 IU of vitamin D, and 1,500 IU of vitamin E).

⁴⁾ Non-fiber carbohydrates, calculated as: $NFC = 100 - (CP + EE + ash + NDF)$.

adapted to the concentrate diets for two weeks before the data collection period. The concentrates were fed once a day between 1600 and 1700 h in out-of-doors colony houses (2.0×1.5 m), with 24 h/d access to clean drinking water. The adaptation period was 7 days, and the data collection period was one month.

During the experimental period, data was recorded daily on the amount of concentrate offered and refused by individual calf. The grazed grass was sampled weekly and concentrates after each batch for chemical analysis. At the end of growth trial, individual yak was weighed and average daily gain (ADG) was calculated.

Blood sampling, amino acids profile and insulin and insulin analysis

Before slaughtering of the experimental yaks, blood samples (20 mL per calf) were collected from 24 (6 from each dietary group) calves, via jugular vein puncture. The blood sample of each calf was immediately transferred to EDTA treated tubes, processed and stored at -20°C for analysis of insulin and amino acids profile.

For amino acid analysis, plasma samples were thawed for 2 h at 4°C. The thawed plasma samples were first deproteinized by the addition of an equal volume of sulphosalicylic acid (SSA, 20% wt/vol). The precipitated protein was separated by centrifugation at 10,000 g for 15 min at 4°C. The supernatant was removed, and re-centrifuged at 10,000 g for 15 min at 4 °C, to remove any remaining protein. The clear supernatant was collected, and divided into two equal portions, one part of which was hydrolyzed in 6 N HCl at 110°C for 24 h. Both sub-samples were then analyzed for amino acid profile using Hitachi 835-50 amino acid analyser (Hitachi, Tokyo, Japan). Standard amino acids (Shengshi Kangpu Institute of Chemical Technology, Beijing, China) were used for quantitative assessment of the recovery. The peptide-bound amino acids were calculated as the difference in amino acid contents before and after acid-hydrolysis. The porcine insulin immunoassay kit (Qcbio Science & Technologies Co., Ltd. Shanghai, China) was used to determine immune-reactive insulin concentration in the plasma.

Muscles samples collection

All animals were slaughtered at the end of experiment. Samples from the longissimus dorsi and semitendinosus muscles were collected within 30 minutes postmortem, and immediately transferred to liquid nitrogen for targeted genes expression examination.

Genes expression examination

RNA isolation and reverse transcription: Total RNA was isolated from longissimus dorsi and *semitendinosus* muscles samples of each yak, by sequential extraction with Trizol Reagent (Invitrogen, Carlsbad, CA, USA). RNase-free DNase treatment was used to preclude any contamination with trace genome DNA. Integrity of isolated RNA was confirmed by agarose gel electrophoresis. The sampled RNA concentration and purity was confirmed at approximately 260/280 nm using nucleic acid/protein analyser

(Beckman DU-800, Fullerton, CA, USA). Reverse transcription (RT) was carried out in a total volume of 10 µL, containing 2 µL 5×Prime script buffer, RT enzyme Mix, 0.5 µL oligo dT primer (50 µM), 0.5 µL Prime script, 4 µL total RNA, 0.5 µL random hexamer primers (100 µM) and 2.5 µL RNase-free ddH₂O. The RT reaction was performed at 37°C for 15 min, and 85°C for 5 s. A negative control was included during the RT reaction, in which reverse transcriptase was omitted.

Real-time polymerase chain reaction: Real-time polymerase chain reaction (PCR) was carried out to analyze mRNA expression in the longissimus dorsi and *semitendinosus* of yaks using SYBR Green PCR Mix (TaKaRa, Shiga, Japan). A total volume of 25 µL reaction system containing 12.5 µL SYBR Premix Ex Taq (2×), 2 µL cDNA and 9.5 µL ddH₂O and 0.5 µL each of forward and reverse primers (10 µM) were used for PCR reaction. The sequences for the forward and reverse primers are shown in Supplementary Table S1. The PCR conditions were as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 65°C for 30 s, and extension at 73°C for 30 s. Melting curve conditions were 95°C for 0 s, 50°C for 30 s and 95°C for 0 s (temperature change velocity 0.5°C/s). Amplification and melt curve analysis was carried out using iQ5 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA). Melting curve analysis was applied to confirm the specificity of each amplification product, and product size was quantified by electrophoresis on agarose gel. Negative controls were performed by substituting water for cDNA. The *β-actin* gene expression was used as a housekeeping gene to calculate the relative transcriptional levels of the target genes in each sample. The experiment was repeated three times.

Chemical analysis

Prior to chemical analyses, the feed samples were ground through 1 mm screen. The ground samples were analyzed for the content of dry matter (method 934.01), CP (method 984.13, adopted for Kjeltac 2400 autoanalyzer; Foss Analytical A/S, Hillerød, Denmark), ether extract (method 954.02), neutral detergent fiber (method 2002.04), and ash (method 942.05) according to the standard methods of AOAC [11].

Statistical analysis

The data on growth performance, plasma amino acids and insulin profile and mTOR cascade gene expression profile were analyzed using PROC MIXED procedure of the SAS (Version 9.3, SAS Institute, Inc., Cary, NC, USA). The model used for the analysis was as follow,

$$Y = \mu + T_i + R_j + \varepsilon_{ij}$$

Where Y is the dependent variable, μ is the population mean for the response variable; T_i is the fixed effect of diet (diets = 4), R_j is the random effect of block, and ε_{ij} was the random error

associated with the observation ij.

When significant ($p < 0.05$) effects were observed, post-hoc analyses were conducted on the least-squares means to compute pair-wise differences among the means, using the Tukey-Kramer test adjusted for multiple comparisons. Means with different superscript letters were obtained with “pdmix 800 SAS macro” [12].

RESULTS AND DISCUSSION

Growth performance of yaks fed different levels of protein concentrates

Data on the growth performance of yak calves supplemented with different levels of protein concentrate is summarized in Table 2. Supplementation of protein concentrates increased the harvest BW (69.8 vs 74.3-76.2 kg; $p < 0.05$) and ADG (0.14 vs 0.23-0.26 kg; $p < 0.01$) of yaks. Among the supplemented groups, the harvest BW was not significantly different due to the level of CP in the concentrates. However, ADG differed due to the level of CP, and the highest ($p < 0.05$; 0.026) ADG was observed in calves supplemented with the MP concentrate. The ADG on MP concentrate was 11.5% higher than that of HP concentrate, and 12.0% higher than that of LP concentrate. These results show that the content of protein and protein to energy ratio of MP concentrate was more favourable for the growth of yak calves. No published data is available on the growth performance of yak calves; therefore, no direct comparisons could be made here. In general, the present results show that supplementation of concentrates to early weaned yak calves can enhance their growth performance, and at the same time it can minimize the energy deficiency in mother yaks during the winter feed scarcity periods. Moreover, it has been shown that early weaned beef calves are more resistant to stress later in the life [13], and had higher feed efficiency compared with normal weaned calves [5]. Overall, our results show that early weaning of yak calves is feasible and concentrate supplementation improves their growth performance.

Plasma amino acids profile of yaks fed different types of protein concentrates

The amino acids profile of early weaned grazing yak calves is presented in Table 3. In young calves, amino acids are not only regarded as important raw materials for body protein synthesis,

but plasma amino acids concentration is also widely accepted to be important regulators of feed intake and metabolic pathways [14]. Among the essential amino acids and nonessential amino acids, only methionine concentration was significantly higher ($p < 0.05$; 8.6 vs 10.1-12.4 $\mu\text{mol/L}$) in the plasma of calves supplemented with protein concentrates than the calves receiving no concentrate (control). Lysine and methionine are the first limiting amino acids in rapid growing young ruminants, as such the increase in the plasma methionine concentration can explain the higher ADG in calves supplemented with protein concentrates. Our results are consistent with the findings of Rius et al [10], who observed a higher concentration of methionine in plasma, due to a higher rate of protein deposition in the muscles of dairy calves fed with higher level of protein in milk replacers. Recently, methionine is reported to function as a regulator for initiation and elongation of mRNA during protein synthesis [15]. This means that the increase in plasma methionine concentration have stimulated protein synthesis in yak calves in this study. Surprisingly, the lysine concentration for grazing animal was not higher ($p > 0.05$) than the supplemented groups. This was in contrast to that observed by Xue et al [3] and Robinson et al [16], who reported an increase in plasma lysine concentration in calves supplemented with rumen protected lysine. Furthermore, no differences ($p > 0.05$) in threonine and isoleucine concentration were obtained for the grazing calves. Over the last decade, leucine and the other two-branch chained amino acids, isoleucine and valine, has received increasing attention due to its growth promoting activities in calves [17,18]. Despite significant difference in ADG among the four treatments in the present study, no marked differences were observed for the concentration of plasma branch chained amino acids. This discrepancy may be due to the intrinsic genetic variation or due to the type and amino acid composition of the yak ruminal microbial population. Further systematic research is required to explicitly understand this phenomenon.

The plasma insulin concentration of yaks fed with different types of protein concentrates

Plasma insulin concentration of grazing yak calves increased ($p < 0.05$; 1.86 vs 2.16-2.54 $\mu\text{L/mL}$) with supplementation of protein concentrates (Table 3). In agreement with our findings Bartlett et al [19] observed a linear increase in plasma insulin concen-

Table 2. The growth performance of yak calves fed different levels of protein concentrates

Item	Concentrate supplements ¹⁾				SEM	p-value Control vs Treatments
	Control	LP	MP	HP		
Initial BW (kg)	65.6	67.4	67.2	68.7	1.82	0.932
Harvest BW (kg)	69.8 ^b	74.3 ^a	74.9 ^a	76.2 ^a	6.98	0.035
ADG (kg)	0.14 ^c	0.23 ^b	0.26 ^a	0.25 ^{ab}	0.018	0.003

SEM, standard error of the mean; BW, body weight; ADG, average daily gain.

¹⁾ Control, only grazed on natural pasture; LP, supplemented with low crude protein (17%) concentrates; MP, supplemented with medium crude protein (19%) concentrates; HP, supplemented with high crude protein (21%) containing concentrates

^{a-c} Values within a row with different superscripts differ significantly at $p < 0.05$.

Table 3. Plasma amino acids and insulin profiles of yak calves fed different levels of protein concentrates

Item	Concentrate supplements ¹⁾				SEM	p-value Control vs Supplements
	Control	LP	MP	HP		
Essential amino acids (μmol/L)						
Lysine	95.4	89.3	87.2	90.1	9.81	0.063
Methionine	8.6 ^b	11.3 ^a	12.4 ^a	10.1 ^a	1.23	0.024
Threonine	77.2	81.8	80.2	80.9	4.39	0.241
Leucine	98.5	98.2	101.6	96.3	12.47	0.577
Isoleucine	55.7	61.9	59.2	57.3	7.54	0.711
Phenylalanine	27.8	28.2	30.6	28.1	4.12	0.615
Valine	144.2	147.6	142.1	143.2	8.67	0.719
None essential amino acids (μmol/L)						
Serine	86.3	102.3	97.1	101.2	12.23	0.165
Glutamate	68.3	67.5	67.1	72.7	8.28	0.429
Alanine	180.1	188.1	176.4	182.1	16.47	0.217
Tyrosine	28.2	33.2	35.2	32.4	4.99	0.417
Proline	70.5	67.6	68.2	72.3	6.76	0.331
Hormone concentration (μIU/mL)						
Insulin	1.86 ^c	2.16 ^b	2.52 ^a	2.24 ^b	0.019	0.016

SEM, standard error of the mean.

¹⁾ Control, only grazed on natural pasture; LP, supplemented with low crude protein (17%) concentrates; MP, supplemented with medium crude protein (19%) concentrates; HP, supplemented with high crude protein (21%) containing concentrates.

^{a-c} Values within a row with different superscripts differ significantly at $p < 0.05$.

tration when calves were fed with increasing contents of CP in milk replacer. The increase in plasma insulin concentration with supplementations of the protein concentrates is in line with the increase in ADG. The rise in plasma insulin concentration has been reported to promote protein synthesis through activation of mTOR pathway. Dennis et al [20] observed a synergistic effect of rise in plasma amino acids and insulin concentrations on the rate of protein deposition in growing animals. In the present study, it could not be unequivocally established that the growth performance of yak calves was influenced by insulin alone or in combination with amino acids. Additional, *in vitro* experiments need to be conducted to confirm whether methionine promote protein synthesis alone or in combination with insulin.

Gene expression profile of yaks fed different types of protein concentrates

The protein synthesis related genes expression profile in longissimus dorsi and *semitendinosus* muscles of grazing yak calves, as affected by feeding of different types of protein concentrates, is presented in Table 4, 5. Recent research shows that the mTOR complex 1 (*mTORC1*) plays a central role in the regulation of protein synthesis [21]. The *mTORC1* cascade is in turn upstream-regulated by chemotrophic and physiological inputs such as amino acids and insulin hormone. In order to characterize through which pathway, the amino acids initiate mTOR cascade reactions, three known upstream amino acids initiating signal factors were studied (Table 4, 5). However, no differences ($p > 0.05$) were observed among the dietary treatments for two of the upstream factors,

Table 4. mTOR cascade genes expression profile profile in yak calves longissimus dorsi fed with different levels of protein concentrates

Items	Concentrate supplements ¹⁾				SEM	p-value Control vs Supplements
	Control	LP	MP	HP		
<i>mTOR-raptor</i>	1.03 ^b	2.63 ^a	2.78 ^a	3.02 ^a	0.342	0.012
<i>MAP4K3</i>	1.96	2.42	1.76	1.88	0.474	0.365
<i>IPMK</i>	1.24	1.64	1.42	1.77	0.313	0.414
<i>hVps34</i>	0.82 ^b	2.25 ^a	2.62 ^a	2.75 ^a	0.316	0.035
<i>p62</i>	3.67 ^a	0.95 ^b	0.89 ^b	0.78 ^b	0.455	0.016
<i>4EBP1</i>	1.12 ^b	2.07 ^{ab}	2.85 ^a	3.03 ^a	0.332	0.032
<i>S6K1</i>	0.98 ^b	1.65 ^a	1.78 ^a	2.07 ^a	0.274	0.447

SEM, standard error of the mean; *mTOR1-raptor*, regulatory associated protein of mTOR, complex 1; *MAP4K3*, mitogen-activated protein kinase kinase kinase kinase; *IPMK*, inositol polyphosphate monokinase; *hVps34*, mammalian vacuolar protein sorting 34 homolog; *p62*, sequestosome 1; *4EBP1*, eukaryotic translation initiation factor 4E binding protein 1; *S6K1*, ribosomal protein S6 kinase polypeptide 1.

¹⁾ Control, only grazed on natural pasture; LP, supplemented with low crude protein (17%) concentrates; MP, supplemented with medium crude protein (19%) concentrates; HP, supplemented with high crude protein (21%) containing concentrates.

^{ab} Values within a row with different superscripts differ significantly at $p < 0.05$.

Table 5. mTOR cascade genes expression profile in yaks semitendinosus fed different levels of protein concentrates

Items	Concentrate supplements ¹⁾				SEM	p-value Control vs treatments
	Control	LP	MP	HP		
<i>mTOR-raptor</i>	0.86 ^b	2.25 ^a	2.29 ^a	2.36 ^a	0.184	0.031
<i>MAP4K3</i>	1.33	1.59	1.65	1.94	0.123	0.487
<i>IPMK</i>	1.64	1.57	1.46	1.84	0.058	0.784
<i>hVps34</i>	0.65 ^b	2.17 ^a	2.47 ^a	2.58 ^a	0.224	0.016
<i>p62</i>	3.22 ^a	0.84 ^b	1.21 ^b	1.46 ^b	0.417	0.024
<i>4EBP1</i>	1.17 ^b	2.58 ^a	3.02 ^a	3.57 ^a	0.076	0.012
<i>S6K1</i>	0.87 ^b	2.24 ^a	2.63 ^a	2.88 ^a	0.212	0.017

SEM, standard error of the mean; *mTOR1-raptor*, regulatory associated protein of mTOR, complex 1; *MAP4K3*, mitogen-activated protein kinase kinase kinase kinase; *IPMK*, inositol polyphosphate monokinase; *hVps34*, mammalian vacuolar protein sorting 34 homolog; *p62*, sequestosome 1; *4EBP1*, eukaryotic translation initiation factor 4E binding protein 1; *S6K1*, ribosomal protein S6 kinase polypeptide 1.

¹⁾ Control, only grazed on natural pasture; LP, supplemented with low crude protein (17%) concentrates; MP, supplemented with medium crude protein (19%) concentrates; HP, supplemented with high crude protein (21%) containing concentrates.

^{ab} Values within a row with different superscripts differ significantly at $p < 0.05$.

namely, mitogen activated protein kinase kinase kinase (*MAP4K3*) and inositol polyphosphate monokinase (*IPMK*). The *MAP4K3* is regulated by amino acids, but not by the hormone insulin [22], and act as upstream stimulating factor to further start Rag GTPases, and subsequently up regulate the *mTOR* [23]. The *IPMK* is a physiological cofactor of *mTOR*, which determines *mTORC1* stability and amino acids induced *mTOR* signalling [24]. Notably, significant differences were obtained in mammalian vacuolar protein sorting 34 homolog (*hVps34*) in both longissimus dorsi ($p < 0.05$; 0.82 vs 2.25-2.75) and *semitendinosus* ($p < 0.05$; 0.65 vs 2.17-2.58) muscles. The *hVps34* has been recognized as upstream regulator of *mTOR*, and this pathway is distinctly regulated by amino acids [25]. In the present study the *hVps34* expression was increased by 206% in longissimus dorsi and 270% in *semitendinosus* in the grazing yak calves supplemented with protein concentrate. Our results are supported by the findings of Tesseraud et al [26], who reported that the protein synthesis in avian QM7 myoblasts was up-regulated by *hVps34*. Byfield et al [27] and Gulati et al [28] also reported that *hVps34* up-regulate protein synthesis. These findings show that *hVps34* is the most sensitive signalling factor that is up-regulated by nutrient amino acids (methionine in this study), and the *hVps34* subsequently initiate *mTORC1* cascade reaction to promote protein synthesis and animal growth performance.

Shvets et al [29] reported that the deficiency of amino acids supply to the cells upregulate *sequestosome 1* (*p62*) that initiate cell mobilization (protein degradation) through ubiquitin-proteasome system pathway. The abundance of *p62* decreased in longissimus dorsi ($p < 0.05$; 3.67 vs 0.78-0.95) and *semitendinosus* ($p < 0.05$; 3.22 vs 0.84-1.46) of the grazing yak calves with the supplementation of protein concentrates. This means that *p62* is down regulated by the increase in plasma amino acids (methionine) concentration and by the process of protein accretion in the muscles. Our results are consistent with the findings of Geetha et al [30], who argued that *p62* was a transporter, responsible for shuttling substrates to proteasome.

The expression of *mTOR-raptor* ($p < 0.05$) increased with the supplementation of protein concentrate to the control diet (grazing). As a result, the expression profiles of two outputs of *mTOR* pathway, the translational regulators, namely, eukaryotic translation initiation factor 4E binding protein 1 (*4EBP1*), and S6 kinase 1 (*S6K1*) were also up-regulated. The *4EBP1* and *S6K1* followed the pattern of changes in plasma methionine and insulin. However, it could not be unequivocally established that whether the methionine act alone or together with insulin to stimulate upstream inputs, the *mTOR*, and ultimately the outputs of cascade reactions in yak-calves to improve protein synthesis and growth performance, and deserve further investigations.

In summary, our results showed that supplementation of CP rich (17% to 19%) concentrates to early weaned grazing yak calves increased their harvest BW (69.8 vs 74.3-76.2 kg; $p < 0.05$) and ADG (0.14 vs 0.23-0.26 kg; $p < 0.05$). The concentrate supplementation increased ($p < 0.05$) the concentration of plasma methionine (8.6 vs 10.1-12.4 $\mu\text{mol/L}$) and lysine. Further investigations revealed that the addition of protein concentrates up-regulated ($p < 0.05$) the expression of *mTOR-raptor*, *hVps34*, *4EBP1*, and *S6K1* genes in both longissimus dorsi and *semitendinosus* muscles. In contrast, the expression of *p62* was down-regulated in the concentrate supplemented calves. Our results show that plasma methionine and insulin concentrations were the key mediator for gene expression and protein deposition in the muscles, and more attention should be give to methionine content of yak calves feed.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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REFERENCES

- Long R, Apori S, Castro F, Ørskov E. Feed value of native forages of the Tibetan Plateau of China. *Anim Feed Sci Technol* 1999;80:101-13.
- Long R, Zhang D, Wang X, Hu Z, Dong S. Effect of strategic feed supplementation on productive and reproductive performance in yak cows. *Prev Vet Med* 1999;38:195-206.
- Xue F, Zhou ZM, Ren LP, Meng QX. Influence of rumen-protected lysine supplementation on growth performance and plasma amino acid concentrations in growing cattle offered the maize stalk silage/maize grain-based diet. *Anim Feed Sci Technol* 2011;169:61-7.
- Houghton P, Lemenager R, Horstman L, Hendrix K, Moss G. Effects of body composition, pre- and postpartum energy level and early weaning on reproductive performance of beef cows and pre-weaning calf gain. *J Anim Sci* 1990;68:438-46.
- Arthington J, Kalmbacher R. Effect of early weaning on the performance of three-year-old, first-calf beef heifers and calves reared in the subtropics. *J Anim Sci* 2003;81:1136-41.
- Davis T, Suryawan A, Orellana R, Fiorotto M, Burrin D. Amino acids and insulin are regulators of muscle protein synthesis in neonatal pigs. *Animal* 2010;4:1790-6.
- O'Connor PMJ, Kimball SR, Suryawan A et al. Regulation of translation initiation by insulin and amino acids in skeletal muscle of neonatal pigs. *Am J Physiol-Endocrinol Metab* 2003;285:E40-53.
- Escobar J, Frank JW, Suryawan A, et al. Physiological rise in plasma leucine stimulates muscle protein synthesis in neonatal pigs by enhancing translation initiation factor activation. *Am J Physiol Endocrinol Metab* 2005;288:E914-21.
- Lansard M, Panserat S, Plagnes-Juan E, Seiliez I, Skiba-Cassy S. Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* 2010;39:801-10.
- Rius A, Weeks H, Cyriac J, et al. Protein and energy intakes affected amino acid concentrations in plasma, muscle, and liver, and cell signaling in the liver of growing dairy calves. *J Dairy Sci* 2012;95:1983-91.
- AOAC, Association of Official Analytical Chemists. *Official Methods of Analysis*. Association of Official Analytical Chemists. Washington, DC, USA: AOAC International; 1990.
- Saxton AM. A macro for converting mean separation output to letter groupings in PROC MIXED. Pages 1243-1246 in 23rd SAS User Group Intl. Cary, NC: SAS Institute; 1998.
- Arthington J, Spears J, Miller D. The effect of early weaning on feedlot performance and measures of stress in beef calves. *J Anim Sci* 2005;83:933-9.
- Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. *J Nutr Biochem* 2006;17:571-88.
- Appuhamy JA, Bell AL, Nayananjali WAD, Escobar J, Hanigan MD. Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. *J Nutr* 2011;141:1209-15.
- Robinson PH, Swanepoel N, Shinzato I, Juchem SO. Productive responses of lactating dairy cattle to supplementing high levels of ruminally protected lysine using a rumen protection technology. *Anim Feed Sci Technol* 2011;168:30-41.
- Yoshizawa F, Sekizawa H, Hirayama S, et al. Time course of leucine-induced 4E-BP1 and S6K1 phosphorylation in the liver and skeletal muscle of rats. *J Nutr Sci Vitaminol* 2001;47:311-5.
- Escobar J, Frank JW, Suryawan A, et al. Regulation of cardiac and skeletal muscle protein synthesis by individual branched-chain amino acids in neonatal pigs. *Am J Physiol-Endocrin Metab* 2006;290:E612-21.
- Bartlett K, McKeith F, VandeHaar M, Dahl G, Drackley J. Growth and body composition of dairy calves fed milk replacers containing different amounts of protein at two feeding rates. *J Anim Sci* 2006;84:1454-67.
- Dennis MD, Baum JI, Kimball SR, Jefferson LS. Mechanisms involved in the coordinate regulation of mTORC1 by insulin and amino acids. *J Biol Chem* 2011;286:8287-96.
- Laplanche M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* 2012;149:274-93.
- Findlay GM, Yan L, Procter J, Mieulet V, Lamb RF. A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. *Biochem J* 2007;403:13-20.
- Yan L, Mieulet V, Burgess D, et al. PP2A_{T61c} is an inhibitor of MAP4K3 in nutrient signaling to mTOR. *Mol Cell* 2010;37:633-42.
- Kim S, Kim SF, Maag D, et al. Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. *Cell Metab* 2011;13:215-21.
- Nobukuni T, Joaquin M, Roccio M, et al. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc Natl Acad Sci USA* 2005;102:14238-43.
- Tesseraud S, Bigot K, Taouis M. Amino acid availability regulates S6K1 and protein synthesis in avian insulin-insensitive QM7 myoblasts. *FEBS Lett* 2003;540:176-80.
- Byfield MP, Murray JT, Backer JM. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J Biol Chem* 2005;280:33076-82.
- Gulati P, Gaspers LD, Dann SG, et al. Amino acids activate mTOR complex 1 via Ca²⁺/CaM signaling to hVps34. *Cell Metabol* 2008;7:456-65.
- Shvets E, Fass E, Scherz-Shouval R, Elazar Z. The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes. *J Cell Sci* 2008;121:2685-95.
- Geetha T, Seibenhener ML, Chen L, Madura K, Wooten MW. p62 serves as a shuttling factor for TrkA interaction with the proteasome. *Biochem Biophys Res Commun* 2008;374:33-7.