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Effects of Dietary Lysine Levels on the Plasma Concentrations of Growth-Related Hormones in Late-Stage Finishing Pigs

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Abstract

This study was undertaken to investigate the effects of dietary lysine on the plasma concentrations of three growth-related hormones in pigs. Nine late-stage finishing barrows were assigned to three dietary treatments according to a completely randomized experimental design (3 pigs/treatment). Three corn and soybean meal-based diets were formulated to contain three levels of lysine, which were 0.43, 0.71, and 0.98% for Diets 1 (lysine deficient), 2 (lysine adequate), and 3 (lysine excess), respectively. The feeding trial lasted 4 weeks, during which the pigs were allowed ad libitum access to the diets and water. After the 4 weeks, blood was collected and plasma samples were obtained. Then, the plasma concentrations of insulin, growth hormone (GH), and insulin-like growth factor 1 (IGF-1) were measured. No difference in the plasma concentration of insulin or GH among the three treatments was found ($P > 0.10$). However, the plasma IGF-1 concentration was lower ($P < 0.05$) in the pigs fed Diet 1 or 3 than fed Diet 2, suggesting that either dietary lysine deficiency or excess can lead to a lower concentration of plasma IGF-1. It was concluded that IGF-1, instead of insulin or GH, in the blood may be a key controlling growth factor in response to dietary lysine supply for regulating muscle growth in late-stage finishing pigs.

Keywords: lysine, hormone, blood plasma, finishing pig

1. Introduction

The biochemical process of protein turnover in the skeletal muscle of pigs is of great importance for the production of food protein for human consumption [1]. Some nutrients, such as glucose and fatty acids, play important roles not only as energetic substrates but also as cell

signaling molecules to regulate the protein turnover in animal body [2–4]. Similarly, besides their function as building blocks for body protein biosynthesis, some amino acids (AAs) can also function as cell signaling molecules regulating those metabolic pathways that are necessary for muscle protein accretion [5, 6]. The regulation of key signaling and metabolic pathways of muscle protein turnover by AAs (Wang et al., personal communication) is closely associated with the concomitant responses of some growth-related hormones [1, 7, 8].

The plasma concentrations of growth-related polypeptide hormones, such as insulin, growth hormone (GH; a.k.a. somatotropin), and insulin-like growth factor 1 (IGF-1), can be affected by animal nutritional status and, in turn, regulate cell and tissue growth and development in animal body [9–11]. Because these hormones are not fat-soluble, they cannot penetrate cell membranes into cytosol. Therefore, they exert cell signaling effects through binding to their corresponding receptors on the cell membranes, where they further activate cell signaling cascades to regulate gene expression and protein turnover [7, 12].

Lysine is the first limiting AA in typical grain-based swine diets [1, 13], and sufficient dietary lysine supply is critical for pig growth performance, especially the growth of skeletal muscle, the largest AA reservoir in the body [14–16]. According to some previous studies on growing pigs [14, 17], dietary lysine supplementation stimulated the insulin secretion, increased the plasma insulin concentration, but not the plasma concentrations of GH and IGF-1, in a dose-dependent manner. On nursery pigs, it was reported that the plasma IGF-1 concentration was reduced when animals were fed a diet lower in lysine level [18]. However, whether or not the effect of dietary lysine on the growth performance of finishing pigs is mediated via these growth-related hormones is still unknown. Therefore, the objective of this study was to investigate the effect of dietary lysine at different levels on the plasma concentrations of three key growth-related hormones, which were insulin, GH, and IGF-1, in late-stage finishing pigs.

2. Materials and methods

2.1. Animal trial and sample collection

All the experimental protocols involving caring, handling, and treatment of pigs were approved by Mississippi State University Institutional Animal Care and Use Committee. A total of nine crossbred (Large White × Landrace) barrows with an average initial body weight (BW) 94.4 ± 6.7 kg were housed in an environment-controlled swine barn at the Leveck Animal Research Center of Mississippi State University. The pigs were randomly assigned to nine individual feeding pens, and then were assigned to three dietary treatment groups according to a completely randomized experimental design. Each treatment consisted of 3 pen replicates ($n = 3$) with one pig per pen.

A corn and soybean meal-based diet (a lysine-deficient diet; defined as Diet 1) was formulated to meet or exceed the NRC [13] recommended requirements of various nutrients, including crude protein (CP) and essential AAs, but not of lysine. Diet 2 (a lysine-adequate diet) and Diet 3 (a lysine-excess diet) were formulated by adding L-lysine monohydrochloride (Archer

Daniels Midland Co., Quincy, IL, USA) to Diet 1 at the expense of corn at the rates of 0.35% and 0.70%, respectively (**Table 1**). The total lysine contents (calculated, as-fed basis) in Diets 1, 2, and 3 were 0.43, 0.71, and 0.98%, respectively. To confirm the contents of major nutrients, samples of the three diets were submitted to the Essig Animal Nutrition Laboratory at Mississippi State University for proximate analysis, and the results are shown in **Table 2**. The AA contents of the three diets were analyzed by using the high-performance liquid chromatography methods [19] at Texas A&M University (College Station, TX, USA), and the results are also shown in **Table 2**.

	Diet 1	Diet 2	Diet 3
<i>Ingredients (%)</i>			
Corn	90.844	90.494	90.144
Soybean meal	6.400	6.400	6.400
Canola oil	0.800	0.800	0.800
L-Lysine-HCl (98.5%)	0.000	0.350	0.700
DL-Methionine (99%)	0.040	0.040	0.040
L-Threonine (98.5%)	0.090	0.090	0.090
L-Tryptophan (99%)	0.035	0.035	0.035
Limestone	0.650	0.650	0.650
Dicalcium phosphate	0.900	0.900	0.900
Salt	0.200	0.200	0.200
Mineral premix ²	0.033	0.033	0.033
Vitamin premix ³	0.008	0.008	0.008
Total	100.000	100.000	100.000
<i>Composition⁴</i>			
Metabolizable energy (kcal/kg)	3319	3323	3326
Crude Protein (%)	10.45	10.75	11.05
Total Lysine (%)	0.43	0.71	0.98
Total Methionine (%)	0.24	0.24	0.24
Total Threonine (%)	0.50	0.50	0.50
Total Tryptophan (%)	0.14	0.14	0.14
Total Ca (%)	0.46	0.46	0.46
Total P (%)	0.43	0.43	0.43

¹Diets 1, 2, and 3 were formulated to contain dietary lysine at 0.43%, 0.71%, and 0.98% (as-fed basis), respectively, of which Diets 2 and 3 were formulated by adding 0.35% and 0.70% L-lysine-HCl (Archer Daniels Midland Co., Quincy, IL) to Diet 1 at the expense of corn.

²The mineral premix contained 13.2% Ca, 1.0% Cu, 8.0% Fe, 5.0% Mn, 10.0% Zn, 500 ppm I, and 300 ppm Se.

³Each kilogram of vitamin premix contained the following: 22.05 million IU vitamin A, 3.31 million IU vitamin D3, 66,138 IU vitamin E, 88 mg vitamin B12, 220 mg biotin, 8,818 mg menadione, 15,432 mg riboflavin, 61,728 mg d-pantothenic acid, and 88,183 mg niacin.

⁴Calculated major nutrients.

Table 1. Ingredient and nutrient compositions (as-fed basis) of the three experimental diets fed to the late-stage finishing pigs¹.

Nutrient and Energy	Experimental Diet		
	Diet 1	Diet 2	Diet 3
Dry matter, %	87.10	87.10	87.10
Gross energy, kcal/kg	3,663	3,608	3,559
Crude protein, %	9.77	10.60	10.86
<i>Individual Amino Acids, %</i>			
Lysine	0.42	0.70	1.01
Aspartate	0.98	0.97	0.98
Asparagine	0.74	0.75	0.75
Glutamate	1.01	1.03	1.03
Glutamine	1.42	1.44	1.43
Serine	0.53	0.52	0.54
Histidine	0.34	0.33	0.34
Glycine	0.61	0.62	0.62
Threonine	0.50	0.51	0.50
Arginine	0.73	0.74	0.74
Alanine	0.81	0.83	0.82
Tyrosine	0.52	0.52	0.53
Tryptophan	0.14	0.13	0.14
Methionine	0.25	0.25	0.26
Valine	0.65	0.66	0.65
Phenylalanine	0.66	0.65	0.66
Isoleucine	0.52	0.52	0.53
Leucine	1.42	1.44	1.45
Cysteine	0.26	0.26	0.30
Proline	1.08	1.07	1.09

¹The energy, crude protein, and dry matter contents were analyzed at the Essig Animal Nutrition Laboratory, Mississippi State University (Starkville, MS, USA). The amino acid contents were analyzed at Texas A&M University (College Station, TX, USA).

Table 2. The analyzed nutrient compositions (as-fed basis) of three experimental diets fed to the late-stage finishing pigs¹.

The feeding trial lasted a total of 4 weeks, during which the pigs were allowed ad libitum access to the experimental diets and fresh water. All the pigs, feeders, waterers, and room temperature were checked two to three times on a daily basis (6:00 am to 7:00 pm). After the 4-week trial period, blood samples were collected by venipuncture of the jugular veins of individual pigs (10 mL/pig) in the early morning (6:00–8:00 am). Immediately after collection, the blood

samples were kept onto ice until the plasma was separated by centrifugation (at $800 \times g$) of the vacutainer tubes at 4°C for 16 min. The plasma samples were then stored in 200 μL aliquots at -80°C until the laboratory analysis of hormones was conducted.

2.2. Laboratory analyses of the growth-related hormones

2.2.1. Analysis of plasma insulin

The plasma concentration of insulin was measured in duplicate using a porcine insulin ELISA kit (ENZO Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions. Briefly, the plasma samples were first diluted (1:3) to remove matrix interference. The diluted samples were then added to a pre-coated insulin ELISA plate (100 μL /well/sample) and incubated on an orbital shaker (Forma 420; Thermo Fisher Scientific Inc., USA) at room temperature for 1 h, allowing the liquid to be thoroughly mixed. The plate was then rinsed four times with wash buffer (200 μL /well) and blotted on lint free paper towels after each rinse. After the final blot, the primary insulin antibody solution was added to the plate (100 μL /well) and incubated on the shaker at room temperature for 1 h. Residual primary antibody on the plate was then rinsed and blotted as described above. One hundred microliter of blue solution of horseradish peroxidase (HRP) conjugate was added to each well, and the plate was incubated on the shaker at room temperature for 30 min. After incubation, the plate was rinsed and blotted again to remove the residual blue conjugate if any. Then the HRP substrate solution was added to the plate (100 μL /well) and the plate was incubated on the shaker at room temperature for 30 min again. Finally, the reaction stop solution was added to the plate (100 μL /well), and the optical density (OD) value of the solution was measured at 450 nm using a microplate reader (SpectraMax Plus 384; Molecular Devices, San Francisco, CA, USA). The insulin concentration of each sample was calculated based on the OD values of the standard curve of known concentrations using Curve Expert 1.4 computer program (<http://www.curveexpert.net>).

2.2.2. Analysis of plasma growth hormone

The plasma concentration of GH was measured in duplicate using a porcine GH ELISA kit (Cloud-Clone Corp., Wuhan, China) according to the manufacturer's instructions. Briefly, the plasma samples were added to a pre-coated GH ELISA plate (100 μL /well/sample) and incubated at 37°C for 2 h. Then, the liquid was removed from each well, the "Detection Reagent A" working solution was added to each well (100 μL /well), and the plate was incubated at 37°C for 1 h. The plate was then rinsed three times with wash buffer (350 μL /well) and blotted on lint free paper towels after each rinse. Following the final blot, the "Detection Reagent B" working solution was added to the plate (100 μL /well), which was incubated at 37°C for 30 min. The plate was rinsed and blotted again for five times as described above. The substrate solution was then added to the plate (90 μL /well), and the plate was incubated at 37°C for 15 min with protection from light. Finally, the reaction stop solution was added to the plate (50 μL /well), and the OD value of the solution in each well was measured at 450 nm using a microplate reader (SpectraMax Plus 384). The GH concentration of each sample was calculated based on its OD values against the standard curve of known concentrations using Curve Expert 1.4 computer program (<http://www.curveexpert.net>).

2.2.3. Analysis of plasma insulin-like growth factor 1

The plasma concentration of IGF-1 was measured in duplicate using a human IGF-1 ELISA kit (ENZO Life Sciences) according to the manufacturer's instructions. Briefly, each plasma sample was mixed in a solution of 100% ethanol (1:5) and 2 N hydrochloric acid (7:1) and incubated at room temperature for 30 min to dissociate IGF-1 from IGF binding proteins. The binding proteins were then pelleted by centrifugation at $9900 \times g$ for 5 min at room temperature. The supernatant was removed and neutralized with an equal volume of the neutralizing reagent. The supernatant sample was then diluted to a final concentration of 1:35 with the assay buffer.

The diluted samples in the assay buffer were added to a pre-coated IGF-1 ELISA plate (100 μ L/well/sample) and incubated on the orbital shaker (Forma 420, Thermo Fisher Scientific Inc.) at room temperature for 1 h, allowing the liquid to be thoroughly mixed. The ELISA plate was rinsed five times with the wash buffer (200 μ L/well) and blotted on lint free paper towels after each rinse. After the final blot, the primary IGF-1 antibody solution was added to the plate (100 μ L/well) and the plate was incubated on the shaker at room temperature for 2 h. Residual primary antibody on the plate was then rinsed and blotted again as described earlier. Then, the blue solution of HRP conjugate was added to the plate (100 μ L/well) and the plate was incubated on the shaker again at room temperature for 30 min. After incubation, the plate was rinsed and blotted to remove any residual blue conjugate. The HRP substrate solution was then added to the plate (100 μ L/well), which was incubated on the shaker at room temperature for 30 min. Finally, the reaction stop solution was added to the plate (100 μ L/well), and the OD value of the solution in each well was measured at 450 nm using a microplate reader (SpectraMax Plus 384). The plasma IGF-1 concentration of each sample was calculated based on OD values against the standard curve of known concentrations using Curve Expert 1.4 computer program (<http://www.curveexpert.net>).

2.3. Statistical analysis

The plasma concentration of each hormone of each sample was averaged from 2 values of the duplicate measurements and then subjected to analysis of variance (ANOVA) for a completely randomized experimental design using the general linear model (GLM) procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with dietary lysine level being the main effect and individual pigs being the experiment units. Three means of the treatments were separated by the protected *t*-test using the LSMEANS/PDIFF option in the GLM procedure. Probability values (*P*) less than 0.05 were considered as significant differences and the *P* values between 0.05 and 0.10 were considered as tendencies to be different.

3. Results and discussions

3.1. Lysine effect on the plasma insulin concentration

As shown in **Figure 1A**, there are no differences in the plasma insulin concentrations (*P* = 0.25) among the three dietary treatments, which suggests that the plasma insulin

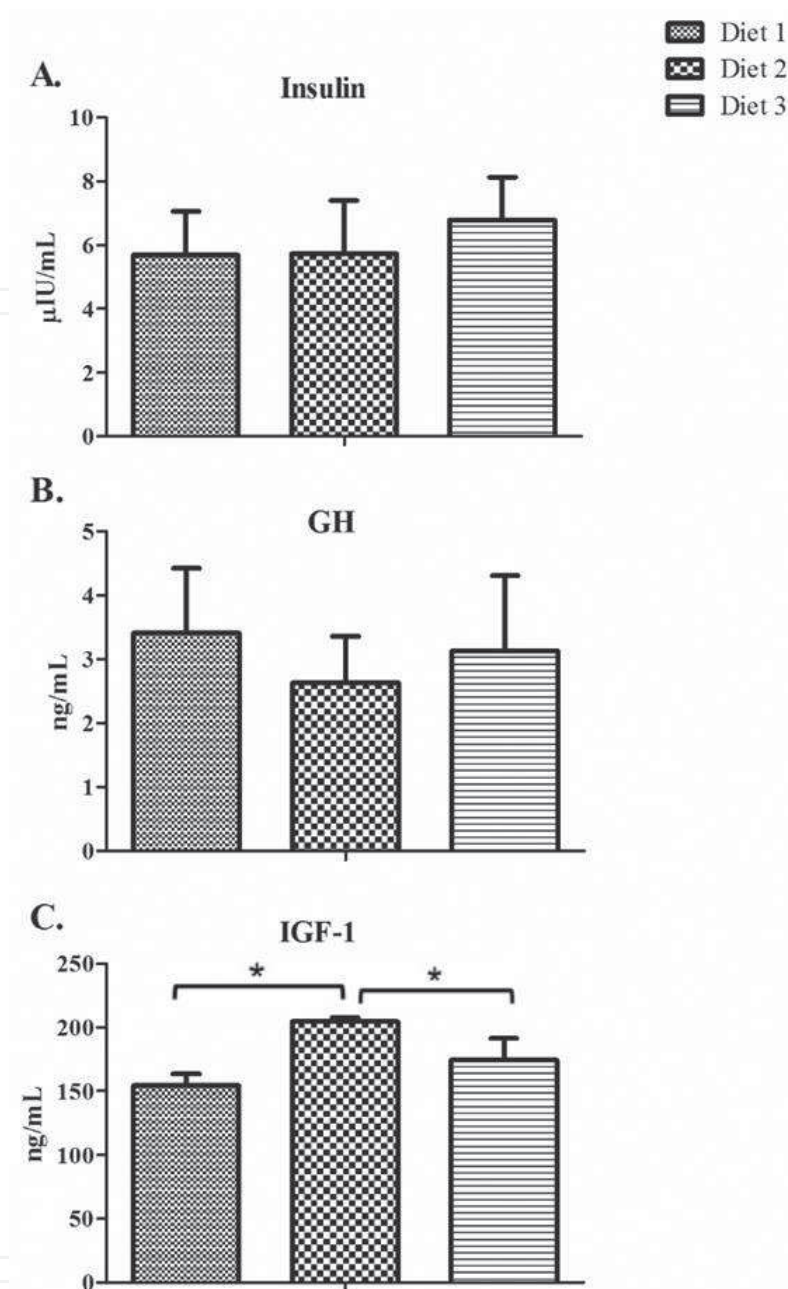


Figure 1. ELISA analyses of the plasma concentrations of (A) insulin, (B) growth hormone (GH), and (C) insulin-like growth factor 1 (IGF-1) in the late-stage finishing pigs fed a lysine-deficient diet (Diet 1), a lysine-adequate diet (Diet 2), or a lysine-excess diet (Diet 3). Shown on the y-axis are the hormone concentrations (μIU/mL or ng/mL). Bars denote means ±SD. All measurements were carried out in duplicate. The *signs denote differential concentrations between two treatment groups ($P < 0.05$).

level of the late-stage finishing pigs was not affected by the dietary lysine concentration, at least at the range from 0.43 to 0.98% (Table 1). As it is known, insulin plays a critical role in the metabolism of nutrients such as carbohydrates, lipids and proteins, and is a primary acute anabolic coordinator of nutrient partitioning [20]. As a signaling molecule, insulin can activate the insulin signaling transduction pathway, leading to an increase in phosphatidylinositol 3-kinase (PI3K) activity followed by an increase in protein kinase B

(PKB/Akt) activity. The PKB/Akt activity is associated with the phosphorylation and inhibition of the tuberous sclerosis complex (TSC2) and further the mTOR (mechanistic target of rapamycin) kinase activity to regulate protein turnover in skeletal muscle and other tissues [21, 22].

Amino acids and insulin can independently stimulate protein synthesis in skeletal muscle [23, 24]. However, dietary AAs or protein can also affect insulin secretion, although insulin is secreted primarily in response to the elevated blood glucose concentration [20, 25]. The reduction in dietary CP concentration has been shown to decrease plasma insulin concentration in growing pigs [10, 26]. In addition, it was observed that a leucine-induced stimulation of protein synthesis in the skeletal muscle of rats was facilitated by a transient increase in the blood concentration of insulin [27, 28].

Different AAs may have different capacities in stimulating insulin secretion. An intravenous administration of 30 g of AA mixtures or of certain individual AAs to healthy human subjects (19–27 years old) induced prompt and large increases in the level of plasma insulin [25]. While a mixture of 10 essential AAs, or the cationic lysine or arginine alone, appeared to be the most potent, cationic histidine was the least potent, and no obvious common physicochemical property or configuration could characterize the more potent or less potent AAs [25]. In pigs, it was reported that dietary administration of lysine also had a stimulating effect on insulin secretion, and this stimulation is in a dose-dependent manner. For example, the growing barrows fed diets containing 0.45 and 0.75% total lysine showed no difference in plasma insulin concentration, but the plasma insulin concentration was increased by 39% when dietary lysine concentration was raised to 0.98% [14]. Similarly, while the dietary concentration of total lysine at 0.71, 0.95, or 1.20% did not show any influence on plasma insulin concentration in growing pigs, 1.45% lysine in the diet significantly increased the plasma concentration of insulin [17]. In this present study on the late-stage finishing pigs, dietary lysine did not show any effect on the plasma insulin level, and there might be a few reasons responsible for the discrepancy between our results and those reported in the literature: (1) the late-stage finishing pigs may not be as sensitive as young humans or young growing pigs in response to AA stimulation, (2) our dietary lysine concentrations (**Table 1**) might not be high enough to stimulate the release of insulin, and (3) the different blood sample collection time relative to the time of AA administration or feed intake might cause the differences.

3.2. Lysine effects on the plasma concentrations of growth hormone and insulin-like growth factor 1

As shown in **Figure 1B**, there are no differences in the plasma GH concentrations ($P = 0.18$) among the three dietary treatments, which suggests that the plasma GH level of the late-stage finishing pigs was not affected by the dietary lysine concentration, at least at the range from 0.43 to 0.98% (**Table 1**). As shown in **Figure 1C**, however, the plasma IGF-1 concentration of the pigs fed either Diet 1 or Diet 3 was lower ($P < 0.05$) than that of the pigs fed Diet 2, which suggests that either dietary lysine deficiency or dietary lysine excess can lead to a lower level of plasma IGF-1 concentration in the late-stage finishing pigs.

In humans, it has been shown that the plasma level of GH can change in response to a number of physiologic stimuli, including plasma AAs [29]. Different single AAs intravenously administered into blood vary in their capacities to evoke a release of GH into the blood circulation [29]. Oral administration of lysine (1200 mg) plus arginine (1200 mg) to some healthy volunteers (male, aged 15–20 years) also provoked a release of GH and insulin into the blood, and this effect appeared to be specific to the combination of these two AAs because neither one demonstrated appreciable stimulating activity when administered alone [30].

Primarily consisting of GH, IGF-1, and their associated carrier proteins and receptors, somatotropin axis (a.k.a., GH-IGF-1 axis) is a very critical regulatory pathway for mammalian muscle growth and development, as well as the protein and lipid metabolism in various tissues [31–33]. The stimulating effect of GH on the growth and development of pig muscle has been reported by many previous *in vivo* studies with daily injection (i.m.) of exogenous porcine GH [34, 35], and it was hypothesized that the actions of GH on muscle growth are mediated by the insulin-like growth factors (IGFs). A great deal of evidence supports the view that the IGFs (especially, IGF-1) are important myogenic agents that could mediate the actions of GH, but this did not demonstrate that GH has no direct effect on the skeletal muscle to stimulate its growth [32].

Essential AAs, especially lysine, can promote swine muscle growth when sufficiently supplied, but it is unknown how or if its promotion effect on swine muscle growth is mediated via the GH-IGF-1 axis. This present study showed that the plasma concentration of IGF-1 in the late-stage finishing pigs fed either a lysine-deficient or lysine-excess diet was reduced ($P < 0.05$), although the plasma concentration of GH was not affected. These results support some previous studies conducted by Roy et al. [14] and Ren et al. [17], who reported that the dietary lysine level had no effect on the plasma GH concentration in growing pigs.

That the dietary lysine deficiency decreased the plasma IGF-1 concentration in the finishing pigs of this study supports the research conducted by Takenaka et al. [36] and Katsumata et al. [18], who showed that the plasma IGF-1 concentration was reduced when young rats (6 weeks of age) and nursery pigs were fed the diets lower in lysine. The previous studies on growing pigs, however, did not show that dietary lysine levels influence the plasma concentration of IGF-1 [14, 17].

The conflicting results regarding the effects of dietary lysine levels on the plasma IGF-1 concentration from different studies may be due to various factors, such as animal species used, growing stages of the animals, and the amounts of lysine provided in the diets. It has been known that a principal hormonal stimulus for IGF-1 production is GH [37]. However, it is interesting to find that the plasma IGF-1 concentration of the late-stage finishing pigs fed either a lysine-deficient or a lysine-excess diet was reduced when compared to the pigs fed a lysine-adequate diet, whereas the dietary lysine level had no effect on the plasma GH concentration. While the animal nutritional status is a key factor in regulating the activity and function of GH-IGF-1 axis [38], the regulation of IGF-1 release may be a key control point for animal muscle growth and nitrogen retention [9, 17]. In terms of animal growth performance, we have reported that there was no further improvement in the average daily gain when excess dietary lysine was provided to the late-stage finishing pigs [16]. Therefore, the lysine promotion effect on pig average daily gain might not be associated with the plasma GH, and

the plasma IGF-1 may be one of the limiting growth factors that restricts the pigs from further increasing in average daily gain or further increasing in muscle protein accretion in response to the dietary lysine provision above the NRC [13] recommended requirement.

4. Conclusions

The results generated from this study in late-stage finishing pigs suggest that dietary deficiency or excess of lysine did not affect the plasma concentrations of insulin and GH. However, the plasma concentration of IGF-1 was affected by the dietary lysine levels. In particular, either dietary lysine deficiency or excess led to a reduction in the plasma concentration of IGF-1 in the pigs relative to the pigs fed a lysine-adequate diet. Thus, it can be concluded that IGF-1, instead of insulin or GH, in the blood circulation may be a key controlling growth factor in response to dietary provision of lysine for regulating muscle growth in late-stage finishing pigs.

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