Original research article

Effects of dietary leucine supplementation on the gene expression of mammalian target of rapamycin signaling pathway and intestinal development of broilers

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ABSTRACT

This experiment was to investigate the effects of dietary leucine supplementation on the gene expression of mammalian target of rapamycin (mTOR) signaling pathway and intestinal development of broilers. A total of 384 one-day-old broilers were randomly assigned into 4 treatments with 6 replicates (16 broilers per replicate). Broilers in these treatment groups were offered the following diets with 1.37, 1.77, 2.17 and 2.57% of leucine. These diet treatments were named 1.37TM, 1.77TM, 2.17TM, and 2.57TM. The experiment lasted 21 days and all birds had free access to feed and water. Results indicated that there was no significant difference in body weight, average daily gain and average feed intake among all treatments (P > 0.05). The broiler duodenal villus height in 2.57TM was the lowest, but the highest occurred in 1.37TM on d 7 and 14 (P < 0.05). The villus height in the jejunum and ileum increased along with leucine level from 1.37 to 2.17%. The villus height of jejunum was significantly higher in 2.17TM than in 1.37TM on d 7 and 14, and the ratio of villus height to crypt depth (V:C) in the duodenum, jejunum and ileum increased significantly (P < 0.05) on d 21. The gene expression level of mTOR in the duodenum decreased with increasing leucine level and was higher in 1.37TM than in 2.57TM on d 7 and 14 (P < 0.05). On d 14 and 21 of the trial, the expression of S6K1 in the duodenum was higher in 2.57TM than in 1.37TM (P < 0.05), and the expression of mTOR, S6K1 in the jejunum and ileum increased with increasing leucine level form 1.37 to 2.17%, whereas a significant difference occurred between 1.37TM and 2.17TM (P < 0.05). In conclusion, the addition of leucine fails to enhance the growth performance of broilers. However, leucine can improve intestinal development by enhancing villus height and V:C ratio in the jejunum and ileum. Moreover, the expression of mTOR, S6K1 increased as the level of dietary leucine was elevated from 1.37 to 2.17%.

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1. Introduction

Leucine is one of branched-chain amino acids, which belong to essential amino acids for both animals and human (Li et al., 2011; Wu et al., 2014). As an essential amino acid, leucine has certain biological properties, such as providing energy, regulating protein, carbohydrate and lipid metabolism, adjusting immune function and mRNA translational origination, thereby leucine can affect nutrient metabolism and animal growth (Suryawan et al., 2012a,b). Presently, the regulatory functions of amino acids in nutrition or physiology as well as the mechanism at cellular or molecular levels have attracted increasing attention of scientists (Kim and Wu, 2009; Katane et al., 2008). It has been confirmed that leucine can enhance muscle protein synthesis via promoting protein synthesis and inhibiting its degradation (Dardevet et al., 2000), Li et al. (2009) and Palii et al. (2009) suggested that leucine supplementation in low-protein meal could regulate muscle protein synthesis and...
animal growth, stimulate growth hormone secretion, and adjust gene expression. Moreover, leucine plays a critical role in enhancing translational efficiency, which refers to inducing the activation of mammalian target of rapamycin (mTOR) and its downstream pathway leading to mRNA translation and relative protein synthesis (Wu et al., 2010; Kimball and Jefferson, 2006). Although growing evidence shows beneficial effects of leucine in the regulation of protein synthesis in skeletal muscle (Anthony et al., 2002; Escobar et al., 2007; Wilson et al., 2010; Boutry et al., 2013; Deng et al., 2014; Columbus et al., 2015), few studies have determined the effects of dietary supplementation of L-leucine on the development and performance of young broilers, thereby not so much information is available regarding the effects of leucine on the intestinal development and activation of translation initiation. In order to evaluate the potential benefits of leucine supplementation on neonatal growth, it is essential to assess the effects of leucine on protein synthesis not only in skeletal muscle, but also in other tissues of the neonate, considering the tissue-specific effect of leucine on stimulating protein synthesis. As the small intestine is the first tissue to encounter the nutritional intervention, determining the effects of a diet supplemented with leucine on translation and protein synthesis in this tissue is essential for future understanding of the role that leucine may play in neonatal growth. Hence, L-leucine may be a biological active nutrient that can regulate the development and maturation of the small intestine. Provision of L-leucine at an earlier age may improve the intestinal development and regulate expression of genes and signaling pathways to affect absorption and metabolism of dietary nutrients in animals (Yin et al., 2010; Zhang et al., 2013). Therefore, different levels of dietary leucine were prepared to explore the potential effects of leucine on the intestinal development and gene expression of mTORsignaling pathway of broiler.

2. Materials and methods

2.1. Animals and diets

A total of 384 newly hatched healthy Arbor Acres male broilers (BW 45.46 ± 0.32 g) from a commercial hatchery (Hua Du Broiler Company, Beijing, China) were randomly assigned into 4 dietary treatments supplemented with leucine with 6 replicates per treatment (16 broilers per replicate). Experimental diets were formulated referring to the nutrient requirements for broilers published by the Ministry of Agriculture of the People’s Republic of China (2004). The Leucine percentages in the nutrient composition of these 4 treatments were 1.37% (1.37TM); 1.77% (1.77TM); 2.17% (2.17TM) and 2.57% (2.57TM). Broilers were raised in cages, with 23 h of light per day and ad libitum access to diets and water. Besides, regular management and poultry vaccination were conducted according to the recommended procedures in the broiler breeding manual. The broilers mental states, appetite and feces were observed, and mortality was recorded. Ingredients and nutrient composition of experimental diets were shown in Table 1. Experimental procedures followed the pertinent laws of animal protection approved by the Animal Care Advisory Committee of Feed Research Institute, Chinese Academy of Agricultural Sciences.

2.2. Sampling

On days 7, 14 and 21 of the trial, one bird with average body weight was taken from each replicate, weighed and slaughtered by bleeding the left jugular vein. The intestinal tissue was separated for sampling. Tissue samples (about 5 cm) were taken at the top of the duodenum (from the gizzard to the point of entry of the pancreo-biliary ducts), jejunum (from the pancreo-biliary ducts to the yolk stalk) and ileum (from yolk stalk to the ileo-cecal junction). Each piece of tissue was quickly rinsed in ice-cold phosphate-buffered saline (PBS) to clear away luminal contents, scraped for the histological analysis. jejunum and ileum were fixed in 4% neutral buffered formalin for 24 h and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin for histological analysis.

2.3. Growth performance

Fasting of broilers began at 2200 on d 20, but they had ad libitum access to water. The feed intake, ADFI, ADG and G:F were determined at 0800 on d 21 for each replicate.

Table 1. Ingredients and nutrient composition of experimental diets (DM basis).

| Item               | Group          |
|--------------------|----------------|
|                   | 1.37TM         | 1.77TM         | 2.17TM         | 2.57TM         |
| Ingredients, %     |                |                |                |                |
| Wheat              | 10.00          | 10.00          | 10.00          | 10.00          |
| Corn starch        | 38.51          | 39.41          | 40.22          | 41.04          |
| Extruded soybean   | 25.09          | 23.18          | 21.44          | 19.70          |
| Cottonseed meal    | 5.00           | 5.00           | 5.00           | 5.00           |
| Canola meal        | 6.00           | 6.00           | 6.00           | 6.00           |
| Rapeseed meal      | 3.00           | 3.00           | 3.00           | 3.00           |
| Rice bran          | 4.00           | 4.00           | 4.00           | 4.00           |
| Fish meal          | 3.00           | 3.00           | 3.00           | 3.00           |
| Salt               | 0.30           | 0.30           | 0.30           | 0.30           |
| CaHPO4             | 1.51           | 1.55           | 1.58           | 1.61           |
| L-Lys              | 0.34           | 0.40           | 0.45           | 0.50           |
| DL-Met             | 0.25           | 0.26           | 0.27           | 0.28           |
| L-Thr              | 0.13           | 0.15           | 0.18           | 0.21           |
| Lys                | 0.10           | 0.11           | 0.12           | 0.13           |
| L-Try              | 0.01           | 0.02           | 0.03           | 0.04           |
| Leu                | 0              | 0.50           | 0.95           | 1.41           |
| Ile                | 0.26           | 0.29           | 0.32           | 0.35           |
| Val                | 0.32           | 0.35           | 0.38           | 0.41           |
| Gly                | 0.04           | 0.11           | 0.17           | 0.23           |
| Tyr                | 0.01           | 0.04           | 0.05           | 0.09           |
| His                | 0.01           | 0.03           | 0.04           | 0.07           |
| Phe                | 0.06           | 0.09           | 0.12           | 0.19           |
| Arg                | 0.03           | 0.08           | 0.12           | 0.17           |
| Choline chloride   | 0.20           | 0.20           | 0.20           | 0.20           |
| Multi-minerals     | 0.04           | 0.04           | 0.04           | 0.04           |
| Multi-vitamin1     | 0.10           | 0.10           | 0.10           | 0.10           |
| Zeolite powder     | 0.57           | 0.67           | 0.79           | 0.86           |
| Total              | 100.00         | 100.00         | 100.00         | 100.00         |
| Nutrient composition2, % |                |                |                |                |
| ME, MJ/kg          | 12.34          | 12.34          | 12.34          | 12.34          |
| CP                 | 20.20          | 20.68          | 20.29          | 20.02          |
| CF                 | 5.83           | 5.50           | 5.20           | 4.90           |
| Lys                | 1.30           | 1.54           | 1.36           | 1.38           |
| Met                | 0.50           | 0.50           | 0.51           | 0.73           |
| Thr                | 0.84           | 0.84           | 0.82           | 0.84           |
| Try                | 0.23           | 0.22           | 0.21           | 0.22           |
| Arg                | 1.19           | 1.21           | 1.20           | 1.21           |
| Leu                | 1.33           | 1.74           | 2.15           | 2.58           |
| Ile                | 0.94           | 0.93           | 0.94           | 0.99           |
| Val                | 1.14           | 1.10           | 1.14           | 1.18           |
| Ca                 | 1.00           | 1.00           | 1.00           | 1.00           |
| Available phosphorus | 0.50           | 0.50           | 0.50           | 0.50           |

1 1.37TM – 1.37% leucine in the nutrient composition, etc; CF – crude fat.
2 Multi-minerals and multi-vitamin provided per kilogram of basal diets: VA 2, 500 IU, VD3, 400 IU, VE 10 IU, VK, 0.5 mg, VB1, 1.8 mg, VB2, 4.0 mg, VB6, 3.0 mg, VB12, 710 μg, pantothenic acid 11 mg, niacin acid 55 mg, folic acid 0.5 mg, biotin 0.12 mg, Cu 8 mg, Fe 80 mg, Zn 40 mg, Mn 60 mg, Se 0.15 mg, I 0.35 mg.
3 Values of ME, CF, Ca and available phosphorus were calculated and others were measured.
2.4. Intestinal morphology examination

Morphological analysis was performed on formalin-fixed intestinal samples that were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The sections were visualized using a light microscope. Villus height and crypts depth were measured and analyzed using Image-ProPlus 7.0 software, and 5 well-oriented villi and crypts were selected for measuring villus height and crypt depth. The ratios of villus height to crypt depth (V:C) were also calculated.

2.5. RNA isolation and real-time quantitative PCR

RNA was isolated from homogenized intestinal tissue samples with RNAprep pure Tissue Kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s protocol. Total RNA concentration was assessed spectrophotometrically (at 260 nm). The samples of OD260/OD280 that ranged between 1.8 and 2.0 were considered to be usable. Total RNA concentration was diluted into 200 to 500 μg/μL for the following reverse transcription. Additionally, RNA integrity was confirmed electrophoretically on agarose gel, and visualization of intact 5S, 18S and 28S ribosomal RNA bands were shown under UV light (Fig. 1).

RNA (1 μg) was reverse transcribed into complementary DNA (cDNA) using FastQuant cDNA Reverse Transcription Kit (Tiangen Biotech, Beijing, China) according to the manufacturer’s instruction. Samples were incubated at 42°C for 15 min and at 95°C for 3 min. Polymerase chain reaction (PCR) amplification of cDNA of each sample was performed using SYBR Green (Tiangen Biotech, Beijing, China) along with specific primers (Table 2) designed for each enterocyte gene through the Primer Premier 5.0 and eukaryotic translation initiation factor 4E binding protein 1.

The PCR reactions were performed in a final volume of 20 μL and performed as triplicate measurements of each sample via real-time quantitative PCR (Bio-Rad CFX96 Real-Time PCR, USA) under the conditions of 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, with a final extension at 72°C for 10 s. A melting curve analysis was generated following each real-time quantitative PCR assay to verify the specificity and purity of all PCR products. Data obtained from the PCR reactions provide the threshold cycle (Ct) value for each gene. The Ct value was the cycle number at which the reporter dye emission intensities rise above background noise (Huang et al., 2007). Ct of each enterocyte gene was normalized against that of β-actin (housekeeping gene). Furthermore, relative quantification of the target gene transcript with the reference gene (β-actin) was calculated by the 2−△△Ct method (Kenneth and Thomas, 2001).

2.6. Statistical analysis

Performance data (ADG, ADFI and G:F), intestinal morphology and fold change values (obtained from gene expression data for the different intestinal segments) were analyzed with one-way ANOVA against that of 1.37TM or 1.77TM using SPSS 19.0. If the effects of dietary leucine were significant, the means among the treatments were further compared using the LSD test. Data were presented as means ± SEM, and P < 0.05 was considered as significant. Moreover, data about expression of mRNA were transformed by function y = log10 x in advance of ANONA analysis for overcoming heterogeneity of variance.

3. Results

3.1. Growth performance

Broiler growth performance is presented in Table 3. No differences in ADG, ADFI and G:F of broilers were observed among the four treatments (P > 0.05). However, ADG and G:F were better in broilers for 2.17TM and 2.57TM compared with 1.37TM or 1.77TM (P < 0.05).

3.2. Intestinal morphology

There were no significant differences in the villus height, crypt depth and V:C ratio in the duodenum on d 7 (P > 0.05, Table 4).

Table 2

| Gene          | ACCN   | Primer sequence (5’→3’)                  | Product size, bp |
|---------------|--------|------------------------------------------|-----------------|
| β-actin       | NM_205511 | F: ATCCGGCCATCCCTGTGTTC                 | 120             |
|               |        | R: AGCCATGGCAATCTCGTTTC                 |                 |
| mTOR          | XM_417614 | F: CGTGATCAGCTTGGGAACCACC             | 220             |
|               |        | R: CTCCTGTCATCGCAACCTCA                |                 |
| S6K1          | NM_001030721 | F: CAATTTGCCCTCCCTCACTCA             | 175             |
|               |        | R: AAGCAGTCTCCACCTTCTGT                |                 |
| 4E-BP1        | XM_424384 | F: GCCAATCTAGGGTAAGAAAGA               | 146             |
|               |        | R: AACAGGAGGCTCAACAGG                  |                 |

ACCN = accession number; mTOR = mammalian target of rapamycin; S6K1 = p70 ribosomal protein S6 kinase 1; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein 1.

Table 3

| Item       | Group    | 1.37TM       | 1.77TM       | 2.17TM       | 2.57TM       |
|------------|----------|--------------|--------------|--------------|--------------|
| BW, g      |          | 747.94 ± 8.85 | 766.31 ± 41.76 | 779.33 ± 58.70 | 769.79 ± 11.69 |
| ADFI, g    |          | 47.02 ± 2.13  | 46.23 ± 2.38  | 46.35 ± 2.69  | 45.76 ± 1.64  |
| ADG, g     |          | 33.45 ± 0.42  | 34.33 ± 1.99  | 34.95 ± 2.80  | 34.49 ± 0.56  |
| G:F        |          | 1.49 ± 0.02   | 1.50 ± 0.06   | 1.45 ± 0.04   | 1.46 ± 0.03   |

1.37TM = 1.37% leucine in the nutrient composition, etc.
Effects of dietary leucine levels on the villus height, crypt depth and V:C ratio of broilers' duodenum at different days.

| Item Group | 1.37TM | 1.77TM | 2.17TM | 2.57TM |
|------------|--------|--------|--------|--------|
| Villus height, µm | | | | |
| d 7 | 623.99 ± 23.68 | 619.57 ± 12.03 | 614.15 ± 24.43 | 611.69 ± 12.73 |
| d 14 | 892.32 ± 37.59b | 853.38 ± 54.93b | 868.09 ± 27.40b | 807.85 ± 50.52a |
| d 21 | 901.24 ± 70.54 | 884.90 ± 50.95 | 907.51 ± 24.77 | 874.19 ± 40.84 |
| Crypt depth, µm | | | | |
| d 7 | 100.59 ± 7.40 | 102.89 ± 9.28 | 99.76 ± 5.98 | 96.67 ± 8.90 |
| d 14 | 158.87 ± 8.68b | 127.36 ± 5.74b | 128.40 ± 7.23b | 137.63 ± 13.44a |
| d 21 | 164.13 ± 7.55bc | 151.12 ± 11.39b | 138.50 ± 11.57b | 176.42 ± 9.90c |
| V:C | | | | |
| d 7 | 6.22 ± 0.32 | 6.06 ± 0.47 | 6.17 ± 0.40 | 6.37 ± 0.50 |
| d 14 | 5.62 ± 0.23a | 6.70 ± 0.17b | 6.78 ± 0.31b | 5.90 ± 0.49a |
| d 21 | 5.49 ± 0.30ab | 5.87 ± 0.34b | 6.01 ± 0.75b | 4.96 ± 0.11a |

1.37% leucine in the nutrient composition, etc; V:C = the ratio of villus height to crypt depth.

*Within a row, means with different superscripts differ significantly (P < 0.05).

Effects of dietary leucine levels on the gene expression of the mammalian target of the rapamycin signaling pathways in the small intestine.

| Item | Group | 1.37TM | 1.77TM | 2.17TM | 2.57TM |
|------|-------|--------|--------|--------|--------|
| mTOR | | | | | |
| d 7 | 1.96 ± 0.04a | 1.72 ± 0.42ab | 1.58 ± 0.24ab | 1.46 ± 0.08a |
| d 14 | 2.66 ± 0.37b | 2.31 ± 0.15ab | 2.16 ± 0.21a | 1.93 ± 0.15a |
| d 21 | 2.42 ± 0.22 | 2.49 ± 0.31 | 2.51 ± 0.33 | 2.38 ± 0.18 |
| S6K1 | | | | | |
| d 7 | 2.43 ± 0.54 | 2.40 ± 1.28 | 3.07 ± 1.89 | 2.20 ± 0.88 |
| d 14 | 3.68 ± 0.28 | 3.48 ± 0.30a | 3.44 ± 0.12a | 3.13 ± 0.37a |
| d 21 | 4.06 ± 0.20a | 3.56 ± 0.27ab | 3.60 ± 0.45b | 3.48 ± 0.28a |
| 4E-BP1 | | | | | |
| d 7 | 2.54 ± 0.69 | 1.97 ± 0.89 | 2.72 ± 1.31 | 2.24 ± 0.83 |
| d 14 | 3.08 ± 0.58ab | 2.42 ± 0.36a | 2.49 ± 0.30ab | 2.55 ± 0.19ab |
| d 21 | 5.56 ± 0.93b | 4.68 ± 0.46b | 4.37 ± 0.25a | 4.22 ± 0.17a |

1.37% leucine in the nutrient composition, etc; mTOR = mammalian target of rapamycin; S6K1 = p70 ribosomal protein S6 kinase 1; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein 1.

*Within a row, means with different superscripts differ significantly (P < 0.05).

Effects of dietary leucine levels on the villus height, crypt depth and V:C ratio of broilers’ ileum at different days.

| Item Group | 1.37TM | 1.77TM | 2.17TM | 2.57TM |
|------------|--------|--------|--------|--------|
| Villus height, µm | | | | |
| d 7 | 220.55 ± 18.19 | 233.14 ± 19.12 | 239.39 ± 21.52 | 232.17 ± 16.76 |
| d 14 | 264.06 ± 22.01a | 302.63 ± 20.31b | 306.12 ± 9.42b | 295.11 ± 17.78b |
| d 21 | 362.62 ± 26.81ab | 372.00 ± 13.80ab | 395.85 ± 12.81b | 346.62 ± 31.16b |
| Crypt depth, µm | | | | |
| d 7 | 67.21 ± 9.77 | 64.52 ± 2.56 | 71.12 ± 10.64 | 70.19 ± 9.70 |
| d 14 | 86.95 ± 8.94b | 76.94 ± 9.00ab | 67.14 ± 3.79a | 74.50 ± 10.32a |
| d 21 | 136.19 ± 16.82b | 98.46 ± 6.84d | 93.04 ± 1.94a | 122.41 ± 20.25b |
| V:C | | | | |
| d 7 | 3.32 ± 0.30 | 3.61 ± 0.26 | 3.42 ± 0.52 | 3.34 ± 0.27 |
| d 14 | 3.05 ± 0.23a | 3.96 ± 0.28b | 4.57 ± 0.21c | 3.99 ± 0.32b |
| d 21 | 2.68 ± 0.28a | 3.80 ± 0.30b | 4.26 ± 0.12c | 2.88 ± 0.41a |

1.37% leucine in the nutrient composition, etc; V:C = the ratio of villus height to crypt depth.

*Within a row, means with different superscripts differ significantly (P < 0.05).
There were no significant differences in mRNA expressions of mTOR, S6K1 and 4E-BP1 in the jejunum among all treatments on d 7 ($P > 0.05$, Table 8). On d 14, mTOR, S6K1 and 4E-BP1 expressions were higher in 2.17TM than in the other treatments ($P < 0.05$), and mTOR expression level was 40 and 17% higher in 1.77TM and 2.57TM than in 1.37TM ($P < 0.05$); S6K1 expression was higher in 2.17TM than in the other treatments ($P < 0.05$), and there were no obvious difference among the other three treatments ($P > 0.05$); 4E-BP1 expressed much highly in 217TM compared with 1.37TM ($P < 0.05$). As a whole, mTOR, S6K1 and 4E-BP1 mRNA expressions increased in 2.17TM compared with 1.37TM ($P < 0.05$).

### Table 8
Effects of dietary leucine levels on the gene expression of mTOR, S6K1 and 4E-BP1 of broilers' jejunum at different days.

| Item   | Day | Group     | mRNA expression |
|--------|-----|-----------|-----------------|
| mTOR   | 7   | 1.37TM    | 1.63 ± 0.15     |
|        | 14  | 1.37TM    | 1.79 ± 0.07     |
|        | 21  | 1.37TM    | 2.16 ± 0.10     |
|        | 7   | 1.77TM    | 2.17 ± 0.34     |
|        | 14  | 1.77TM    | 1.89 ± 0.20     |
|        | 21  | 1.77TM    | 1.93 ± 0.16     |
| S6K1   | 7   | 2.17TM    | 2.12 ± 0.25     |
|        | 14  | 2.17TM    | 2.02 ± 0.12     |
|        | 21  | 2.17TM    | 2.04 ± 0.12     |
| 4E-BP1 | 7   | 2.17TM    | 2.12 ± 0.25     |
|        | 14  | 2.17TM    | 2.02 ± 0.12     |
|        | 21  | 2.17TM    | 2.04 ± 0.12     |

1.37TM – 1.37% leucine in the nutrient composition, etc; mTOR – mammalian target of rapamycin; S6K1 – p70 ribosomal protein S6 kinase 1; 4E-BP1 – eukaryotic translation initiation factor 4E binding protein. a,b,c Within a row, means with different superscripts differ significantly ($P < 0.05$).

As shown in Table 9, on d 7, mTOR expression in the ileum was 22 and 20% higher in 2.17TM than in 1.37TM and 1.77TM ($P < 0.05$), respectively; but no significant difference appeared among all treatments with regard to the gene expressions of S6K1 and 4E-BP1 ($P > 0.05$). On d 14, mTOR and S6K1 mRNA expressions were lower in 1.37TM than in the other treatments ($P < 0.05$). On d 21, expression of mTOR was higher in 2.17TM than in 1.37TM and 2.57TM ($P < 0.05$); S6K1 and 4E-BP1 mRNA expressions were higher in 2.17TM and 2.57TM than in another two treatments ($P < 0.05$). Therefore, compared with 1.37TM, mRNA expressions of mTOR, S6K1 and 4E-BP1 increased in 2.17TM ($P < 0.05$), which was similar to the result observed in the jejunum.

### Table 9
Gene expression in the ileum of broilers for different dietary leucine levels.

| Item   | Day | Group     | mRNA expression |
|--------|-----|-----------|-----------------|
| mTOR   | 7   | 1.37TM    | 1.13 ± 0.18     |
|        | 14  | 1.37TM    | 1.18 ± 0.15     |
|        | 21  | 1.37TM    | 0.83 ± 0.09     |
| S6K1   | 7   | 1.37TM    | 1.34 ± 0.11     |
|        | 14  | 1.37TM    | 1.79 ± 0.11     |
|        | 21  | 1.37TM    | 0.99 ± 0.22     |
| 4E-BP1 | 7   | 1.37TM    | 1.67 ± 0.27     |
|        | 14  | 1.37TM    | 2.05 ± 0.11     |
|        | 21  | 1.37TM    | 1.36 ± 0.18     |

1.37TM – 1.37% leucine in the nutrient composition, etc; mTOR – mammalian target of rapamycin; S6K1 – p70 ribosomal protein S6 kinase 1; 4E-BP1 – eukaryotic translation initiation factor 4E binding protein. a,b,c Within a row, means with different superscripts differ significantly ($P < 0.05$).

### 4. Discussion

#### 4.1. Growth performance

Erwan et al. (2009) found that the addition of 0.5 and 0.67% L-leucine to the diets resulted in 2.18 and 2.34% of the respective diet (CP 20%), but did not show a significant effect on feed intake, weight gain and feed conversion ratio over the d 21 to 42 feeding period. Similarly, the addition up to 3.23 or 4% of L-leucine did not cause a significant decrease in growth performance of broilers or induce toxicity in start broilers (Penz et al., 1984; Farran and Thomas, 1990; Edmonds and Baker, 1987). In our study, when leucine was added up to 2.57%, it did not show significant effects on the growth performance, which was in agreement with the literature.

#### 4.2. Intestinal morphology

Villus height and crypt depth are critical factors affecting animal’s ability to absorb nutrients in the intestine. The V:C ratio can reflect the intestinal function synthetically. Besides, an increase in the V:C ratio means improved development of the intestine, whereas a decrease implies the opposite. Zhang et al. (2013) suggested that the lower protein would lead to significantly decreased villus height in the duodenum, jejunum and ileum as well as the villus height in the duodenum. In our study, we avoided the influence from protein level on intestinal morphology by providing the same protein level diets. About one third of amino acids are catabolized by the small-intestinal epithelial cells, while the remaining amino acids entered the cycle system, and used by extra-intestine (Chen et al., 2009). There were unambiguous effects of leucine on the small intestinal morphology, mainly on the villous epithelium (Sheibak et al., 2007, Sun et al. (2015) reported that the supplemental L-leucine amounted up to 200% of L-leucine intake from sow’s milk by 7-day-old pigs for 2 weeks. They further demonstrated that L-leucine supplementation significantly increased villus height in the duodenum, and elevated the V:C ratio in the duodenum and ileum. Moreover, mRNA levels for L-leucine transporters (SLC6A14, SLC6A19 and SLC7A9) and the abundance of the system B0− neutral AA transporter protein increased in the jejunum with leucine-supplemented piglets. Collectively, these results indicate that L-leucine supplement improved intestinal development and whole-body growth in sucking piglets. Our data showed that V:C ratio in the duodenum and jejunum increased with the increasing dietary leucine level from 1.37 to 2.17%, which agreed with other studies. In addition, the crypt depth of the ileum in 2.17TM was lower than that in the 1.37TM, which may result from enhanced migration of epithelial cells in the crypt via activating the mTOR signaling by leucine (Rhodes et al., 2008). Besides, the villus height in jejunum and ileum increased, whereas decreased in the duodenum. The differences may be caused by the difference in experimental protocols, such as species, additive dosage or the used method of leucine (Alpers, 1972) and the structure of the small intestine. Furthermore, the villus height was concerned with the account of cells. Here, we observed that the villus height in the ileum and ileum increased. This indicated that the intestinal epithelial cell proliferation accelerated, possibly leading to rapid protein synthesis. Therefore, it was possible that the increase of villus height may be the representation of rapid protein synthesis. This proposition was consistent with the result of Yin et al. (2010) who suggested that intestinal protein synthesis as well as the weight of intestinal mucosa decreased when pigs consumed diets lacked branched-chain amino acids. On the contrary, 0.27% L-leucine supplementation (1.61% dietary leucine) enhanced protein synthesis in the proximal small intestine, and...
improved intestinal growth. Additional study suggested that 4% dietary leucine administration facilitated protein synthesis in the jejunum of piglets, and promoted the intestinal growth. To a certain extent, the above studies could support our speculation about the nature of increased villus height. In present study, we observed a trend of decrease in the duodenal villus height, which was in agreement with the results of Coeffier et al. (2011), who reported that supplementation of maltodextrins with leucine was able to decrease intestinal protein synthesis and total proteasome activity in the duodenum of human. Overall, the villus height and crypt depth were better in 2.17TM than in the other treatments, whereas the V:C ratio significantly declined as the level of leucine increased to 2.57%, which suggested that 2.57% of leucine supplementation had a negative effect on the small intestinal growth.

4.3. Gene expression related to the rapamycin signaling pathways in the small intestine

It has been confirmed that leucine could promote protein synthesis in muscles by activating the rapamycin signaling pathways. In addition, according to Suryawane et al. (2012), neither leucine nor rapamycin had any effect on the activation of the upstream mTOR regulators, AMP-activated protein kinase and protein kinase B in skeletal muscle. The activation of eukaryotic initiation factor 2 (eIF2α) and elongation factor 2 was not affected by leucine or rapamycin, indicating that these two pathways were not limiting steps of leucine-induced protein synthesis. These results suggested that leucine stimulated muscle protein synthesis in neonatal animals by inducing the activation of mTOR and its downstream pathway leading to mRNA translation. In the small intestine, data from Wilson et al. (2011) indicated that long-term leucine infusion did not have an effect on either S6K1 and 4E-BP1 expressions or the protein synthesis in the jejunum of neonatal pigs. However, additional trials in vitro revealed that leucine could up-regulate S6K1 and 4E-BP1 activation in intestinal epithelial cells (Ban et al., 2004; Rhodes et al., 2008). Similarly, our data showed that mTOR and S6K1 mRNA expressions in the jejunum and ileum increased with the increasing level of dietary leucine from 1.37 to 2.17%, which were consistent with those reports for broilers (Rhoads and Wu, 2009), neonatal pigs (Murgas Torrazza et al., 2010) and rats (Naomoto et al., 2005). Meanwhile, the villus height in the jejunum and ileum were increased, which refer to the amount of cells. Thus, we speculated that leucine administration may promote intestinal protein synthesis by inducing the activation of mTOR and its downstream pathway, which facilitated the intestinal epithelial cell proliferation, leading to increased villus height, improving the growth of the small intestine. Nevertheless, mRNA expressions of mTOR, S6K1 and 4E-BP1 did not increase, in fact, tended to decrease, when the dietary leucine was added up to 2.57%. It was likely that the mechanism of this amino acid signal acceptance might be satiable, considering the manner of leucine-induced activation of S6K1 depended on the stimulation time and the concentration of amino acid (Ban et al., 2004). Therefore, adding leucine to diets beyond a certain range will not increase mRNA expressions of mTOR, S6K1 and 4E-BP1 in a linear fashion. Besides, our data showed that mRNA expression of mTOR in the duodenal mucosa tended to decline with the increasing dietary leucine level. Furthermore, mTOR mRNA expression was significantly higher in 2.57TM than in 1.37TM, which agreed with the leucine effects on the villus height in the duodenum. The reasons for the different effects of leucine on the different intestinal segments are still unclear. Perhaps, different segments of the gut might have different requirements for specific nutrients.

5. Conclusions

Leucine supplementation has no effects on growth performance in broilers. Moreover, leucine supplementation in neonatal broilers improves intestinal development as indicated by increased villus height as well as mTOR, S6K1 mRNA expressions in the jejunum and ileum. The V:C ratio in the duodenum, jejunum and ileum was elevated with the increased dietary leucine level from 1.37 to 2.17%. It is possible that leucine administration may promote intestinal protein synthesis by inducing the activation of mTOR and its downstream pathway, which facilitates the intestinal epithelial cell proliferation, leading to the increased villus height and improved the growth of the small intestine. However, supplementation of leucine to diet with 2.57% will inhibit intestinal growth.

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