Effect of dietary lysine on performance and expression of electron transport chain genes in the *pectoralis major* muscle of broilers


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The aim of this study was to evaluate the effect of dietary lysine on performance, protein deposition and respiratory chain gene expression in male broilers. A total of 252 Cobb 500 broilers were distributed, in a completely randomized design, into four treatments with seven replicates of nine birds per experimental unit. Experimental treatments consisted of diets based on corn and soybean meal, with four levels of digestible lysine: 1.016%, 1.099%, 1.182% and 1.265%. The increase in the level of digestible lysine in the diet provided higher weight gains, feed efficiency and body protein deposition. Birds fed the lowest level of dietary lysine (1.016%) showed a lower expression of genes such as NADH dehydrogenase subunit I (ND1), cytochrome b (CYTB) and cytochrome c oxidase subunits I (COX I), II (COX II) and III (COX III), displaying the worst performance and body protein deposition. This demonstrates the relationship existing between the expression of the evaluated genes and the performance responses. In conclusion, results indicate that broilers fed diets with higher levels of digestible lysine have increased messenger RNA expression of some genes coded in the mitochondrial electron transport chain (ND1, CYTB, COX I, COX II and COX III). It may be stated that diets with proper levels of digestible lysine, within the ‘ideal protein’ concept, promote the expression of genes, which increases the mitochondrial energy, thereby fostering body protein deposition and the performance of broilers in the starter phase.

Keywords: amino acids, genes, protein deposition, respiratory chain

Implications

Broiler nutrition is a strategic area for successful poultry production that has defined the requirements of nutrients essential for performance maximization. However, little is known about the effect of specific nutrients like amino acids on the metabolic events. Knowing the action of dietary lysine on gene expression opens the possibility of manipulating more-economic diets adjusted for meat production.

Introduction

Lysine is one of the most widely studied amino acids in broiler nutrition, mainly because it is the second most limiting amino acid for growth in corn- and soybean meal-based diets, after the first limiting methionine and cysteine (Fakhraei et al., 2010; Dozier and Payne, 2012). Because of its broad commercial availability and easy laboratory analysis, lysine has been considered the reference standard for the adjustment of the ideal protein, which has allowed the targeting for body protein deposition and decreased diversion to other metabolic pathways (Garcia et al., 2006). Hocquette et al. (2007) stated that among the amino acids, lysine probably has the most specific effect on the carcass composition and muscle growth.

Determining the digestible lysine requirement for broilers has been the target of many studies (Dozier et al., 2010; Corzo et al., 2012) aimed at improvements in performance responses by birds (Kidd et al., 1998) and body protein deposition (Fatufe et al., 2004; Wijtten et al., 2004). However, few studies have addressed the molecular mechanisms affected by the dietary lysine.

The knowledge of the energy requirement for the protein synthesis has been established, and this may require changes in mitochondrial energy production (Buttery and Boorman, 1976). However, little is known about the effect of nutrients, or more specifically, the amino acid lysine on the use or production of energy components. The cell production of high-energy molecules like ATP is a feature of the mitochondrial oxidative phosphorylation (OXPHOS), which is
composed of two subsystems: the electron transport chain (ETC), which involves complexes I to IV, and ATP synthase, known as complex V (Wallace, 2007).

Given the importance of the mitochondrial DNA (mtDNA) for the encoding of polypeptides that translocate protons via the OXPHOS complex (I, II, III, IV and V), it is of paramount importance to know its regulations in different climatic and/or nutritional conditions, as alterations in these complexes may reduce ATP production, causing a predisposition to the appearance of degenerative and metabolic diseases in humans (Wallace, 2005 and 2007), metabolic syndromes in poultry (Guan et al., 2007) and reduction of meat quality in cattle (Mannen et al., 2003).

Although studies have demonstrated the relationship between animal performance and the mitochondrial function, not much is known yet about the effect of levels of nutrients on the expression of ETC- and OXPHOS-related genes (Ojano-Dirainet al., 2007; Bottje and Carstens, 2009; Wang et al., 2012). The increased feed efficiency and protein deposition stimulated by lysine may lead to changes in the gene expression of ETC and OXPHOS, as feed efficiency is related to the mitochondrial function (Bottje et al., 2002; Iqbal et al., 2004; Wang et al., 2012).

Therefore, this study aimed to evaluate the effects of dietary digestible lysine on the messenger RNA (mRNA) expression of OXPHOS-related genes (NADH dehydrogenase subunit I (ND1), cytochrome b (CYTB), cytochrome c oxidase subunits I (COX I), II (COX II), III (COX III) and ATP synthase F0 subunit 6 (ATP6)) by relating it to body protein deposition and performance of broilers in the starter phase (8 to 21 days).

Material and methods

All procedures applied in this experiment were approved by the Committee on Ethics and Animal Protection of Universidade Federal de Sergipe, Sergipe, Brazil.

Experimental design and animals

A total of 252 male broilers of the Cobb 500 commercial line, from 8 to 21 days of age, were used in this experiment. From 1 to 7 days of age, birds were reared in a masonry shed with concrete floor littered with wood shavings, where drinkers and feeders were available for free water and feed consumption. At 8 days of age, with an average initial BW of 177 ± 0.28 g, birds were housed in 0.3 m² metabolic cages, distributed, in a completely randomized design, into four treatments with seven replicates of nine birds per experimental unit. Treatments consisted of diets formulated following the nutritional recommendations suggested by Rostagno et al. (2011), with four levels of digestible lysine: 1.016%, 1.099%, 1.182% and 1.265%, for the period from 8 to 21 days of age (Table 1). Diets were prepared adopting the dilution technique, in which the experimental diets containing the highest and lowest levels of digestible lysine (1.265% and 1.016%, respectively) were combined with each other at the proportions of 66.67%/33.33% and 33.33%/66.67%, generating diets with intermediate levels (1.099% and 1.182%). Birds and the supplied feed were weighed weekly to determine feed intake, weight gain and feed efficiency. Mortality was checked daily to adjust the performance data. In case of mortality in any experimental unit, the diet reserved to that group would be weighed and considered as intake up to that moment.

Table 1 Ingredients and calculated nutritional composition of the initial (1 to 7 days) and experimental basal diets (8 to 21 days)

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>1 to 7 days</th>
<th>8 to 21 days</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Digestible lysine levels (%)</td>
<td></td>
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<td></td>
<td>1.016</td>
<td>1.265</td>
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</tbody>
</table>

Ingredients

- Corn (7.88%) 53.031 59.207 60.176
- Soybean meal (45%) 39.810 29.888 28.637
- Meat and bone meal (46%) – 4.924 4.961
- Corn gluten meal (60%) – 2.200 2.200
- Soybean oil 3.030 2.589 2.214
- Dicalcium phosphate 1.787 – –
- Limestone 1.217 0.384 0.379
- Sodium chloride 0.507 0.435 0.435
- L-Threonine (99%) 0.299 0.177 0.370
- L-Lysine HCl (78%) 0.126 0.046 0.325
- L-Threonine (98%) 0.043 – 0.154
- L-Methionine (99%) 0.162 0.100 0.100
- L-Lysine HCl (78%) 0.050 0.050 0.050
- Vitamin supplement1 0.100 0.100 0.100
- Mineral supplement2 0.050 0.050 0.050
- Chemical composition
  - CP (%) 22.500 20.800 20.800
  - Metabolizable energy (kcal/kg) 2980 3050 3050
  - Fat (%) 5.627 5.934 5.579
  - Crude fiber (%) 3.027 2.780 2.730
  - Ash (%) 2.994 2.620 2.560
  - Calcium (%) 1.000 0.880 0.880
  - Available phosphorus (%) 0.450 0.393 0.393
  - Digestible arginine (%) 1.442 1.297 1.262
  - Digestible lysine (%) 1.250 1.016 1.265
  - Digestible threonine (%) 0.890 0.642
  - Digestible methionine + cysteine (%) 0.900 0.731 0.911
  - Digestible methionine (%) 0.595 0.456
  - Digestible threonine (%) 0.810 0.680 0.822
  - Digestible tryptophan (%) 0.257 0.212 0.205
  - Digestible valine (%) 0.959 0.869 0.848
  - Potassium (%) 0.882 0.778 0.758
  - Sodium (%) 0.220 0.218 0.218

1The vitamin supplement contained per kg of diet: vitamin A = 7000 IU, vitamin D₃ = 2500 IU, vitamin E = 18 IU, vitamin K₃ = 1 mg, vitamin B₁ = 1.5 mg, vitamin B₃ = 5.5 mg, vitamin B₄ = 1.6 mg, vitamin B₁₂ = 12 μg, pantothenic acid = 10 mg, biotin = 0.05 mg, folic acid = 0.9 mg, nicotinic acid = 32.5 mg.
2The mineral supplement contained per kg of diet: iron = 30 mg, manganese = 60 mg, zinc = 50 mg, iodine = 2.5 mg, selenium = 0.25 mg.
Birds were maintained under a continuous light period (24 h/day), with feed and water available ad libitum. During the entire experimental period, the temperature and humidity of the broiler house were recorded for every 15 min using Thermo-Hygrometers (model ITR 157; Instrutherm Company, São Paulo, Brazil) placed at three different points in the shed (front, middle and back). The temperature ranged from 22.7°C in the morning to 28.5°C in the afternoon. Relative humidity in the rearing facility ranged from 81.0% in the morning to 68% in the afternoon.

Protein deposition
To determine the body protein at time 0 (8 days), 10 chicks were selected and slaughtered by cervical dislocation. To determine the body protein deposition over the experimental period, seven birds from each treatment with a weight close to the average of the group (±10%) were selected at 21 days. Of the selected birds, three were fasted for ~8 h to empty the digestive tract, and slaughtered by cervical dislocation. The remaining four birds were slaughtered by the same method, without previous feed deprivation, and samples of the pectoralis major muscle were collected from these animals. These four birds had their intestinal tract washed with distilled water for removing the intestinal content and were regrouped into the respective treatment for analysis of protein deposition.

Selected birds were slaughtered by cervical dislocation and ground individually with feathers and entrails in a cutter machine for 10 min. After the material was homogenized, a 300-g sample was collected and stored in a freezer at −10°C until analyses. The Kjeldahl method (AOAC, 2000) was employed to determine the protein. Daily body protein deposition values were calculated, considering the difference in the amount of protein deposited in the 8- to 21-day period.

Gene expression
For the evaluation of gene expression, four birds were used per treatment, at 21 days of age. Immediately after the slaughter, a sample of the pectoralis major muscle was collected from the center of the left-lateral position and immersed in an RNAholder solution (BioAgency Biotecnologia, São Paulo, SP, Brazil) to stabilize and preserve the mRNA expression of the tissue at the time of collection, kept at 4°C for 24 h, and stored at −20°C until the RNA extraction.

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer recommendations. The RNA concentration was determined in a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was checked in 1% agarose gel stained with 1% agarose gel stained with 1% ethidium bromide and visualized under UV light. Total RNA was stored at −70°C until its use. All materials were treated by spraying with an RNaseExterminator decontaminant solution (BioAgency Biotecnologia). The complementary DNA was synthesized from the samples of the pectoralis major muscle using a SuperScript™ III First-Strand Synthesis SuperMix Kit (Invitrogen, Carlsbad, CA, USA), following the procedures recommended by the manufacturer.

Quantitative real-time (qRT) PCR reactions were performed by SYBR Green detection (SYBR Green PCR Master Mix; Applied Biosystems, Carlsbad, CA, USA). The primers utilized for the amplification reactions for the target genes (ND1, CYTB, COX I, COX II, COX III and ATP6) were obtained from the nucleotide sequences of mRNA of Gallus gallus from the Ensembl database (http://www.ensembl.org/Gallus_gallus). These sequences were used to design the primers using PrimerQuest, available at http://www.idtdna.com/Scitoools/Applications/primerquest, provided by Integrated DNA Technologies Inc. (Coralville, IA, USA). The mRNA levels of these target genes (Table 2) were normalized to the average hydroxymethyl-bilane synthase and hypoxanthine phosphoribosyltransferase 1 levels (ΔCt), in accordance with Nascimento et al. (2015).

Statistical analyses
Performance and protein deposition data were analyzed using the GLM procedure of SAS software (SAS Institute Inc., Cary, NC, USA). When significant effects were detected (P < 0.05), means were compared using the Student–Newman–Keuls test. Results are expressed as means and standard errors. Cq data were analyzed statistically using the %QPCR_MIXED SAS® macro statement (https://msu.edu/~steibelj/JP_files/QPCR.html) developed by commands on SAS PROC MIXED, which considers the independent random effects for target and reference genes in each biological replicate (Steibel et al., 2009).

Results
Performance and protein deposition
In the evaluation period from 8 to 21 days of age, increasing the level of inclusion of digestible lysine in the diets improved weight gain, feed efficiency and protein deposition (P < 0.05) (Table 3). The use of 1.016% digestible lysine provided lower weight gains and feed efficiency when compared with the increasing levels of lysine in the diets. Similar responses were found for feed efficiency and body protein deposition.

Gene expression
Results of relative expression were interpreted by contrasting with pairwise comparison between treatments. The results of the qRT-PCR analysis in the pectoralis major muscle of broilers at 21 days of age showed significant differences in gene expression between the dietary treatments. Birds fed diets formulated with 1.099% digestible lysine showed a 3.6 and 3.04 times higher expression of COX II (P < 0.05) and COX III (P < 0.10) genes, respectively, than those fed 1.016% digestible lysine (Figure 1). Similarly, birds receiving diets with 1.265% digestible lysine displayed a greater ND1 gene expression than birds fed 1.099% (P < 0.01) and 1.182% (P < 0.10) lysine. For the gene from OXPHOS complexes III (CYTB) and IV (COX I), a significant expression was observed when the diet containing 1.265% lysine was used compared with other dietary lysine levels (Figure 1).

Effect of lysine on broiler gene expression
In this study, the diets with increasing levels of digestible lysine provided significant increases in weight gain, feed efficiency and body protein deposition, corroborating other studies (Leclercq, 1998; Fatuf et al., 2004; Wijtten et al., 2004). The observed weight gain has the direct participation of muscle growth, which is influenced by the balance of dietary amino acids, more specifically lysine. Another important aspect that increases weight gain in birds is the tissue hydration, as 3 g of water is retained per gram of protein deposited (Leenstra, 1986). In this study, there was an average increase of 19% body water gain in birds fed diets with higher lysine levels (data not shown).

In the experimental period, the body protein deposition of 14.7 g/bird per day observed with the digestible lysine level of 1.265% was 14.3% higher than the lowest level utilized (1.016%). Rostagno et al. (2011) recommended 1.174% digestible lysine in the diet for broilers in the period from 8 to 21 days of age, which can explain why lysine deficiency compromises protein deposition, as was observed by other authors (Fatuf et al., 2004; Wijtten et al., 2004). It should be noted that protein deposition depends on the dynamics of the synthesis and degradation processes. In this context, studies suggest that the increased protein deposition with increasing lysine levels in the diet may be a result of the increased synthesis, reduced degradation or changes in both components of the protein turnover (Buttery and Lindsay, 1980; Urdaneta-Rincon and Leeson, 2004). Within the ‘ideal protein’ concept, lysine undoubtedly promotes muscle growth; however, other amino acids such as leucine, glycine and arginine are essentially important for the protein synthesis, especially given their influence on the expression of mechanistic target of rapamycin, an important signaling pathway that regulates the protein synthesis (Wu et al., 2014).

Broilers, more efficient in feed conversion and weight gain, may show changes in the expression of ETC genes that cause alterations in the use of nutrients (Iqbal et al., 2004 and 2005; Ojano-Dirain et al., 2007). These studies demonstrate that problems in ETC with inefficiency in mitochondrial ATP production are associated with birds with low efficiency in converting nutrients into body components, resulting in low performance. The weight gain and protein deposition observed in this study were related to the alteration in the expression of ETC genes in the pectoralis major muscle of broilers. This observation is supported by the lower expression of ETC genes on the reduced level of digestible lysine in the diet, which probably reduced the availability of ATP in the cells.

The pectoralis major muscle is made up of glycolytic fibers and has few mitochondria compared with muscles with oxidative fibers; however, it has an ~60% higher ATP content (Pearson and Young, 1989). This ATP is important for both rapid contraction metabolism of the glycolytic fibers and because of the need for protein deposition. Protein deposition is a process that demands a large amount of energy.
Table 3  Effects of the digestible lysine levels on performance and body protein deposition of male broilers

<table>
<thead>
<tr>
<th>Digestible lysine (%)</th>
<th>1.016</th>
<th>1.099</th>
<th>1.182</th>
<th>1.265</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>BWG (g)</td>
<td>863(^{ab})</td>
<td>11.561</td>
<td>873(^{ab})</td>
<td>14.910</td>
</tr>
<tr>
<td>FE (g : g)</td>
<td>0.724(^{b})</td>
<td>0.007</td>
<td>0.746(^{ab})</td>
<td>0.008</td>
</tr>
<tr>
<td>PD (g/bird per day)</td>
<td>12.86(^{b})</td>
<td>0.179</td>
<td>13.49(^{ab})</td>
<td>0.264</td>
</tr>
</tbody>
</table>

**BWG =** BW gain; **FE =** feed efficiency; **PD =** protein deposition.
\(^{ab}\)Values within a row with different superscript letters differ significantly at \( P < 0.05 \).

In conclusion, our findings indicate that broilers fed diets with higher levels of digestible lysine increased the mRNA expression of some genes coded in the mitochondrial ETC (\( ND1, CYTB, COX I, COX II \) and \( COX III \)). It may be stated that diets with proper levels of digestible lysine, within the ‘ideal protein’ concept, promote the expression of genes, which increases the mitochondrial energy, thereby fostering body protein deposition and the performance of broilers in the starter phase.
Acknowledgments

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References


Buttery PJ and Lindsay DB 1980. Protein deposition in animals. Butterworth Publishers, Woburn, MA, USA.


