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Effect of dietary energy and nutrient levels on performance and liver expression of respiratory chain genes in broiler chickens

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Abstract: The interaction between dietary energy and key nutrients, and its impact on broiler performance and mitochondrial function, remains poorly understood. This study aimed to evaluate the effects of energy and nutrient (lysine, calcium, and phosphorus) levels on animal performance and liver expression of genes related to lipid metabolism and electron transport in broilers from 22 to 42 days of age. A total of 432 male Cobb 500 broiler chickens were distributed in a completely randomized design with three treatments, eight replications, and 18 birds per experimental unit. The first treatment (control) consisted of a diet based on corn and soybean meal with a metabolizable energy (ME) content of 12.70 MJ/kg of dry matter. The second treatment was a high-energy (HE) diet (13.33 MJ/kg) formulated by increasing the ME value of the control diet by 0.63 MJ/kg while maintaining the same levels of digestible lysine, calcium (Ca), and available phosphorus (P). The third treatment was a high-energy and high-nutrient (HEHN) diet, providing 13.33 MJ/kg and a 5% increase in digestible lysine, Ca, and P levels compared to the control diet. Animal performance and carcass yield were analyzed at 42 days of age. Expression levels of apolipoprotein B (*APOB*), NADH dehydrogenase subunit I (*ND1*), and cytochrome c oxidase (*COX1*) genes were also determined. The treatments exerted a statistically significant effect on weight gain (P = 0.0377). The HE diets resulted in a statistically significant improvement in feed efficiency compared with the control diet (P = 0.0017), but no significant effect was observed on carcass yield. Furthermore, the HEHN diet enhanced weight gain compared with the control. *COX1* and *ND1* expression were influenced by dietary energy level. In conclusion, the results indicate that HEHN diets improve performance and alter the expression of genes related to lipid metabolism and electron transport in broiler chickens from 22 to 42 days of age but do not affect carcass yield.

Keywords: amino acids, minerals, mitochondria, gene expression.

1. Introduction

A balanced diet offers significant advantages to animal performance, providing economic and environmental gains. From this perspective, metabolizable energy (ME) is a strategic nutritional factor, as it favors feed intake, improves feed conversion, and reduces body fat in birds. However, when altering the energy level of bird diets, it is essential to proportionally adjust the levels of amino acids and minerals to ensure optimal production efficiency (Jlali et al., 2020).

The minerals calcium and phosphorus and the amino acid lysine are crucial nutrients that need to be obtained in adequate amounts from the diet to support bird growth (Mansilla et al., 2020; Alagawany et al., 2021; Tran et al., 2021). However, more knowledge is needed about the effects of energy and nutrient interactions on the molecular mechanisms of mitochondrial energy production. It is known that nutritional factors are directly associated with the functioning of mitochondria (Jehl et al., 2019; Saheb et al., 2019), suggesting a relationship between energy performance and mitochondrial gene expression in chickens (Li et al., 2021; Poompramun et al., 2021; Dunislawska et al., 2023).

Studies have shown that dietary energy availability affects the expression of mitochondrial genes and that lysine enhances the expression of electron transport chain genes (Désert et al., 2018). These events are only possible because consumed nutrients are exported from the liver to extrahepatic tissues. In birds, the liver is a crucial organ in energy metabolism, as it captures fatty compounds obtained from the diet or through de novo synthesis and transfers them to extrahepatic tissues via very low-density lipoproteins (VLDL) (Hermier, 1997).

The synthesis and release of VLDL depend on numerous factors. Apolipoprotein B (ApoB) plays a vital role in lipid transport and energy metabolism, participating in the regulation of body weight and abdominal fat deposition in animals (Na et al., 2018; Suzuki et al., 2019). APOB gene expression levels vary according to the age and species of the bird. This gene can be easily detected in liver tissues (Li et al., 2018).

Diets containing optimum levels of amino acids, minerals, and energy contribute to enhancing performance and carcass quality (Johnson et al., 2020). Thus, there is likely a correlation between nutritional status and the expression of genes involved in nutrient transport and energy production, as evidenced by ATP synthesis via the electron transport chain. Given the foregoing, this study aimed to assess the effects of dietary energy and nutrient (lysine, Ca, and P) levels on animal performance and expression of genes related to lipid metabolism and electron transport, namely APOB, cytochrome c oxidase (COX1), and NADH dehydrogenase subunit I (ND1), in broilers from 22 to 42 days of age.

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2. Materials and Methods

All experimental procedures were approved by the Animal Ethics Committee at the Federal University of Viçosa, Brazil (protocol No. 002/2015).

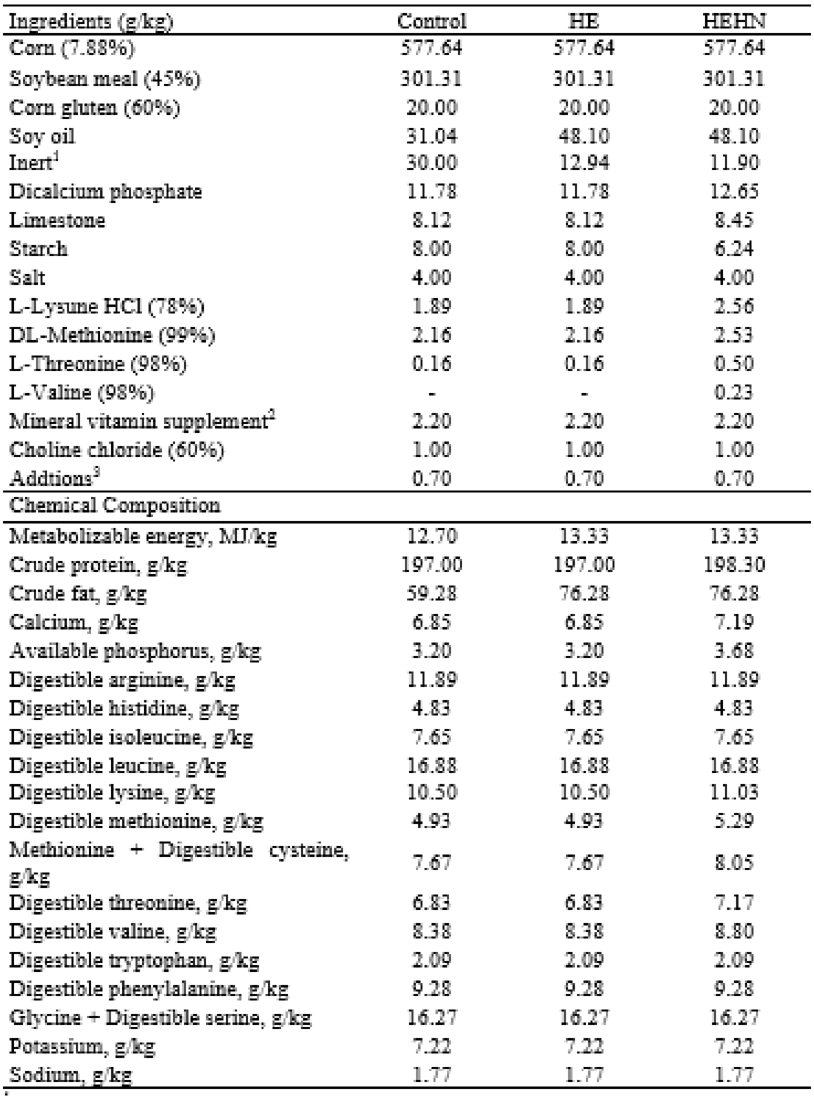
2.1. Experimental design and animals

A total of 432 male Cobb 500 broiler chickens aged 22 days were used in the experiment. Until 21 days of age, the birds were reared in a masonry shed with a concrete floor covered with wood shavings and equipped with drinkers and feeders. The birds received water and feed ad libitum. The feed used until 21 days of age was based on corn and soybean meal and contained 220 g/kg crude protein, 12.51 MJ/kg ME, 11.74 g/kg digestible lysine, 8.95 g/kg calcium (Ca), and 4.38 g/kg digestible phosphorus (P).

At 22 days of age, birds were distributed in a completely randomized design with three treatments, eight replications, and 18 birds per experimental unit. The birds had an average initial weight of 856 ± 6.0 g. Each experimental unit consisted of a 2 m2 cage equipped with drinkers and feeders.

Treatments were as follows: (i) a control diet containing 12.70 MJ/kg ME, 10.50 g/kg digestible lysine, 6.85 g/kg Ca, and 3.20 g/kg P (Table 1); (ii) a high-energy (HE) diet obtained by increasing the ME value of the control by 0.63 MJ/kg without altering the levels of digestible lysine, Ca, or P; and (iii) a high-energy, high-nutrient (HE-HN) diet obtained by increasing the ME value of the control by 0.63 MJ/kg and increasing the levels of digestible lysine, Ca, and P by 5%. The ratios of lysine to methionine + cysteine and of threonine to valine did not differ between treatments. The control diet was based on corn and soybean meal, following the recommendations of Rostagno et al. (2011) for 22- to 42-day-old broilers.

The birds were maintained under continuous light (24 h photoperiod) and had ad libitum access to food and water during the experimental period. The average temperature during the experiment was 24.7 °C.



Ingredients (g/kg)	Control	HE	HEHN
Corn (7.88%)	577.64	577.64	577.64
Soybean meal (45%)	301.31	301.31	301.31
Corn gluten (60%)	20.00	20.00	20.00
Soy oil	31.04	48.10	48.10
Inert[1]	30.00	12.94	11.90
Dicalcium phosphate	11.78	11.78	12.65
Limestone	8.12	8.12	8.45
Starch	8.00	8.00	6.24
Salt	4.00	4.00	4.00
L-Lysune HCl (78%)	1.89	1.89	2.56
DL-Methionine (99%)	2.16	2.16	2.53
L-Threonine (98%)	0.16	0.16	0.50
L-Valine (98%)	-	-	0.23
Mineral vitamin supplement[2]	2.20	2.20	2.20
Choline chloride (60%)	1.00	1.00	1.00
Addtions[3]	0.70	0.70	0.70
Chemical Composition			
Metabolizable energy, MJ/kg	12.70	13.33	13.33
Crude protein, g/kg	197.00	197.00	198.30
Crude fat, g/kg	59.28	76.28	76.28
Calcium, g/kg	6.85	6.85	7.19
Available phosphorus, g/kg	3.20	3.20	3.68
Digestible arginine, g/kg	11.89	11.89	11.89
Digestible histidine, g/kg	4.83	4.83	4.83
Digestible isoleucine, g/kg	7.65	7.65	7.65
Digestible leucine, g/kg	16.88	16.88	16.88
Digestible lysine, g/kg	10.50	10.50	11.03
Digestible methionine, g/kg	4.93	4.93	5.29
Methionine + Digestible cysteine, g/kg	7.67	7.67	8.05
Digestible threonine, g/kg	6.83	6.83	7.17
Digestible valine, g/kg	8.38	8.38	8.80
Digestible tryptophan, g/kg	2.09	2.09	2.09
Digestible phenylalanine, g/kg	9.28	9.28	9.28
Glycine + Digestible serine, g/kg	16.27	16.27	16.27
Potassium, g/kg	7.22	7.22	7.22
Sodium, g/kg	1.77	1.77	1.77

Table 1 – I ngredients and nutritional composition of experimental diets.

[1] Washed sand. [2] Guarantee levels per kilogram of product: vitamin A - 10,000,000 IU; vitamin D3 - 2,000,000 IU; Vitamin E - 30,000 IU; Vitamin B1 - 2.0g; vitamin B6 - 4.0 g; Pantothenic Acid - 12.0 g; Biotin - 0.10 g; Vitamin K3 - 3.0 g; folic acid - 1.0 g; Nicotinic Acid- 50.0 g;

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Vitamin B12 - 15,000 mcg; Selenium - 0.25 g; and Manganese 16.0 g; Iron - 100.0 g; Zinc - 100.0 g; Copper - 20.0 g; Cobalt - 2.0 g; Iodine - 2.0 g; and Vehicle q.s.p. - 1.000g. [3]Additives: Salinomycin 12% = 0.55 g/kg; Avilamycin = 0.05 g/kg; Antioxidant = 0.10 g/kg.

2.2. Animal performance and carcass yield

Birds and feed were weighed at the beginning and end of the experiment to determine feed intake, weight gain, and feed efficiency. Mortality was recorded daily to adjust performance data. At 42 days of age, eight birds per treatment were selected according to average weight, fasted for 8 h, stunned by electronarcosis, and slaughtered to determine carcass, breast, abdominal fat, and liver yields in relation to fasted weight.

2.3. RNA extraction and complementary DNA (cDNA) synthesis

For gene expression analysis, four birds per treatment were selected according to average weight and slaughtered by cervical dislocation at 42 days of age. Immediately after slaughter, a 5 g sample of the right lobe of the liver was collected and stored in liquid nitrogen at −70 °C until RNA extraction.

Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany), following the manufacturer's recommendations. RNA concentration was determined on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed by electrophoresis on a 1% agarose gel, followed by staining with 10% ethidium bromide and visualization under ultraviolet light. Total RNA was stored at −70 °C until use. cDNA synthesis from liver samples was performed using the SuperScript™ III First-Strand Synthesis Super Mix kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. cDNA concentration was determined on a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative real-time PCR reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Primers for apolipoprotein B (*APOB*), NADH dehydrogenase subunit I (*ND1*), cytochrome c oxidase (*COXI*), beta-actin (*ACTB*), and eukaryotic translation elongation factor 1 alpha 2 (*EFF1*) genes were designed using PrimerQuest (http://www.idtdna.com/SciTools/Applications/primerQuest) from *Gallus gallus* mRNA nucleotide sequences stored in Ensembl (http://www.ensembl.org/Gallus_gallus) and NCBI (http://www.ncbi.nlm.nih.gov/gene) databases. *ACTB* and *EFF1* were used as reference genes. All primers used in this study are described in Table 2. Amplification efficiencies (90% to 110%) were similar between target genes. Analysis of dissociation curves did not reveal nonspecific PCR products, such as primer dimers, indicating the reliability of the data in estimating mRNA expression. All analyses were performed in duplicate, and results are expressed in arbitrary units (AU).

Gene	Accession Number	Primer Sequence	Amp. (bp)[1]
ApoB[2]	ENSGALG00000016491	*F* – GCCGTTTGACTGGGAGTACA *R* – TCTTCCCATTTCCTGGTGCC	126
ND1	ENSGALG00000042750	*F* – AGAAGGAGAGTCAGAGCTAGTC *R* – CTTGGGTTCAGGAATAGGACG	135
COX 1	ENSGALG00010000017	*F* – TCCTCATTACTGCCATCCTAC *R* – GGTGTTGGTATAGGATTGGGTC	139
ACTB	ENSGALG00000009621	*F* – ACCCCAAAGCCAACAGA *R* – CCAGAGTCCATCACAATACC	136
EFF-1	NM_204157.2	*F* – GCCCGAAGTTCCTGAAATCT *R* – AACGACCCAGAGGAGGATAA	102

[1] Amplicon (base pairs).

[2] *ApoB* Apolipoprotein type B, *ND1* NADH dehydrogenase subunit I, *COX1* Cytochrome c oxidase, *ACTB* Beta-actin and *EFF-1* Eukaryotic Translation Elongation Factor 1 alpha 2. *F* Forward, *R* Reverse.

Table 2 – Primer pairs used to analyze gene expression.

2.4. Statistical Analysis

Performance and carcass composition data were analyzed using the GLM procedure of SAS software (SAS Institute Inc., Cary, NC, USA). Means were compared by Tukey's test at the 5% significance level. Gene expression data are expressed in AU, estimated by the $2^{-\Delta Ct}$ method (Livak, 2001). Duncan's test was used for multiple comparisons between the relative expression of target genes.

3. Results

A 0.63 MJ/kg increase in ME, with or without nutrient supplementation, promoted a significant improvement in feed efficiency ($P < 0.05$) compared with the control treatment (Table 3). Although there was no difference in feed intake, birds fed the HEHN diet had greater weight gain (2.033 kg) than birds fed the control diet (1.927 kg), but did not differ from birds fed the HE diet (1.967 kg). Carcass, breast, abdominal fat, and liver yields were not significantly influenced ($P > 0.05$) by treatment.

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	Treatments[1]			MSE[2]	P value
	Control	HE	HEHN		
Weight gain, kg	1.927^{b}	1.967^{ab}	2.033^{a}	0.0767	0.0377
Feed intake, kg	3.328	3.313	3.344	0.0921	0.8066
Feed conversion, kg/kg	0.579^{b}	0.594^{a}	0.608^{a}	0.0142	0.0017
Carcass yield, %	85.89	86.23	84.81	1.488	0.1607
Breast, %	30.95	29.73	30.22	1.871	0.4417
Abdominal fat, %	1.10	1.35	1.34	0.523	0.5736
Liver, %	1.58	1.76	1.63	0.190	0.1928

[1] Control group (Control) = 12.70 MJ/kg; high-energy (HE) = 13.33 MJ/kg no nutrient correction; high-energy and high-nutrient (HEHN) = 13.33 MJ/kg with nutrient correction (Lys, Ca.Pd).

[2] MSE – Mean standard error;

[a-b] Means within the same line followed by different superscript letters differ significantly at $P<0.05$ by the Tukey test.

Table 3 – Effects of energy levels and dietary nutrients on performance and carcass yields of male broilers (22-42 d).

Gene expression analysis (Figure 1) showed that *APOB* expression tended to increase ($P = 0.674$) in animals fed HE (0.609 AU) and HEHN (0.665 AU) diets (13.33 MJ/kg ME) compared with birds fed the control diet (0.546 AU). Similarly, *ND1* expression tended to increase ($P = 0.052$) in birds fed the HE diet (3531.6 AU) compared with those fed HEHN (2501.5 AU) and the control diet (2038.1 AU). *COX1* expression in the liver was significantly higher ($P = 0.023$) in birds fed the HE diet (474.9 AU) than in birds fed HEHN and control diets, which did not differ from each other.

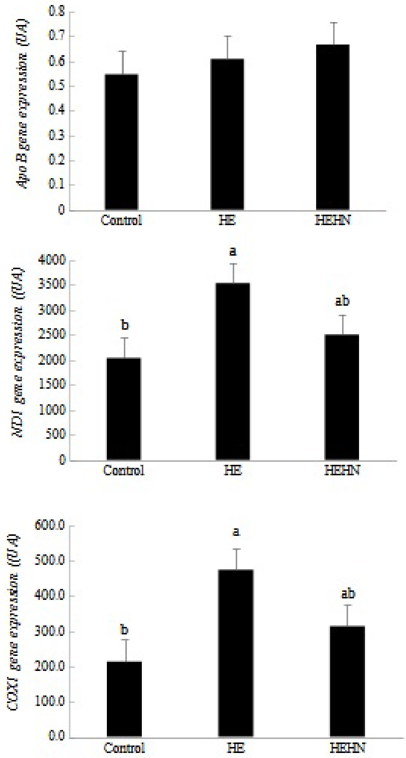

Figure 1 – R elative expression of genes related to the electron transport chain, Apolipoprotein type B (*ApoB*), NADH dehydrogenase subunit I (*ND1*), Cytochrome c oxidase subunit I (*COX I*) and metabolism and energy transport in the liver of broiler chickens at 42 days of age. Means with different letters a, b differ significantly ($P<0.05$). Control group (Control) = 12.70 MJ/kg; high-energy (HE) = 13.33 MJ/kg no nutrient correction; high-energy and high-nutrient (HEHN) = 13.33 MJ/kg with nutrient correction (Lys, Ca.Pd). Values are means with standard error.

4. Discussion

In this study, broiler diets with increased energy levels and nutrient supplementation promoted body growth and improved feed digestibility, in agreement with previous studies (Maharjan et al., 2020; Massuquetto et al., 2020; Barekatain et al., 2021). Although we did not observe variations in feed intake between diets, broilers fed the HEHN diet showed an increase in weight gain. This finding may be correlated with an increase in protein deposition. Protein deposition requires energy, consuming approximately 0.003

MJ per gram of deposited protein. Furthermore, protein deposition is directly influenced by the balance of dietary amino acids, with lysine being the major amino acid (Watanabe et al., 2020; He et al., 2021).

Nutrients such as Ca and P are essential for metabolism and the maintenance of bone structure in birds (Li et al., 2020), which supports weight gain. According to Lisnahan and Nubatonis (2021), diets containing Ca and P at a ratio of 4:1 and an energy level of 12.53 MJ/kg improve performance indicators and bone quality in broilers.

According to Massuquetto et al. (2020), increasing dietary energy levels (13.73 MJ/kg) through the addition of soybean oil, as performed in this study, improved weight gain and feed conversion in broilers. The importance of using soybean oil in poultry feed is underscored. It increases energy intake, thereby promoting an increase in net energy for maintenance and growth (Viñado et al., 2019). These effects might have been responsible for the improvement in feed efficiency in broilers fed the HE diet (13.33 MJ/kg).

Energy increment and nutrient supplementation were not sufficient to promote differences in carcass parameters. These findings are in agreement with those of Massuquetto et al. (2020), who did not observe an effect of dietary energy level on carcass yield in broilers. In the current study, we observed a trend toward increased weight gain and improved feed efficiency associated with increased liver *APOB* expression. ApoB, which is strongly expressed in the liver (Li et al., 2018), plays an essential role in the assembly and secretion of triglyceride-rich lipoproteins, such as VLDL and low-density lipoproteins, which transport lipids to extrahepatic tissues. Lipids available for mitochondrial beta-oxidation generate ATP (energy) for tissue growth, indicating a potential relationship between ApoB and broiler growth.

Beta-oxidation of fatty acids in mitochondria depends on the action of carnitine palmitoyltransferase 1 (CPT1), which is mainly composed of lysine. Therefore, the presence of amino acids such as lysine may accelerate fatty acid oxidation. The 5% increase in lysine, Ca, and P in the HEHN diet was performed respecting the ideal amino acid ratio of methionine + cystine and threonine, which positively regulate body fat deposition in birds (Fouad and El-Senousey, 2014). Therefore, optimal nutrient adjustment of poultry diets can reduce problems related to fat deposition and improve carcass quality.

The increase in dietary Ca with the increase in supplementary fat confirms that adequate levels of Ca^{2+} are essential for *APOB* expression and energy metabolism. Since the 1980s, studies in rat hepatocytes have confirmed that Ca channel blockers reduce the synthesis and secretion of ApoB (Kwong et al., 1989). Recently, Zhang et al. (2021), when evaluating two levels of Ca and fat intake in the diet of male rats, confirmed that Ca supplementation reduces hepatic fat accumulation and increases mitochondrial Ca levels.

Although no differences in carcass, breast, or abdominal fat yields were observed, this does not mean that diets exerted no adverse effects. In modern broiler production systems, meat tenderness, low fat content, production viability, and animal welfare are all critical considerations. These factors are related to molecular transport, production, and use of mitochondrial energy for animal growth (Nematbakhsh et al., 2021; Wu et al., 2021). ND1 and COX1 are part of the mitochondrial respiratory chain. NADH dehydrogenases constitute respiratory complex I, being responsible for transferring electrons and pumping protons across the inner mitochondrial membrane from substrates bound to NADH. COX is part of complex IV of the electron transport chain, responsible for pumping protons and transferring electrons to oxygen, which binds to protons, forming water. Thus, alterations in molecules of the respiratory complex can interfere with the functioning of the respiratory chain and, consequently, with the energy efficiency of mitochondria.

Higher COX1 and ND1 expressions were observed in broilers fed the HE diet (13.33 MJ/kg). The reduced hepatic gene expression in birds fed the HEHN diet suggests that the additional nutrients supplied were exported from the liver to extrahepatic tissues, consistent with the role of the liver in coordinating lipid metabolism and lipoprotein transport in poultry (Suzuki et al., 2019; Ghafouri et al., 2021). Studies have shown that mitochondrial content and bioenergetic capacity vary between different types of muscle fibers in broilers, reflecting differences in oxidative potential and the utilization of energy substrates (Hakamata et al., 2018). Maharjan et al. (2021) confirmed that broiler chickens affected with breast myopathy, which requires tissue repair due to the intense rate of protein synthesis and degradation, use fatty acids transported from the liver as an energy source. Reverter et al. (2017) helped to explain the lack of differences in carcass and meat yields observed here. The authors observed the following ascending order of tissues in terms of mitochondrial content in male Cobb broilers: white adipose tissue (1.8%), thigh (3.1%), breast (4.0%), drumstick (13.9%), and heart (24.6%). Nevertheless, a highly positive and significant correlation was found between thigh and breast mitochondrial content, and a negative and significant correlation was observed between breast and abdominal fat mitochondrial content.

The results for weight gain and feed efficiency are related to the optimal ambience, health, and nutrition conditions of broilers. Ca supplementation was essential for mitochondrial energy production. Studies conducted in rats have confirmed that factors that alter the mitochondrial Ca^{2+} uniporter complex affect the flow of Ca^{2+} and, consequently, energy production (Kwong et al., 2018). This finding may have a strong correlation with the results observed in studies that reported that broilers affected by ascites showed mitochondrial Ca accumulation and reduced oxygen consumption (Miao et al., 2022; Li et al., 2022).

It is not possible to understand the dynamics of Ca without considering P, given that it is the second most required nutrient in birds. P in its inorganic form is kept within a narrow concentration range, as marked fluctuations in concentration may negatively affect various biochemical processes (Li et al., 2020). Increasing digestible lysine levels can influence the expression of genes involved in oxidative phosphorylation in broiler breast muscle. Cellular energy production results from a complex network of processes, in which multiple steps may modulate mitochondrial function. It is plausible that dietary treatments affected additional physiological mechanisms that contribute to energy efficiency, including the activity of ATP synthase in complex V and the role of translocases in the inner mitochondrial membrane, which mediate the transport of molecules such as ATP and ADP.

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5. Conclusion

The results indicate that HE diets (13.33 MJ/kg) increased the expression of *COX1* and *ND1*. Such an energy increase, combined with proportional supplementation of digestible lysine, Ca, and available P, increased *APOB* expression in the liver, in addition to improving the performance of broiler chickens from 22 to 42 days of age.

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