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Hydroxy methionine analog and DL-methionine: effects on intestinal and reproductive tract histomorphometry of light laying hens during the production phase

[Metionina hidroxi análoga e DL-metionina: efeitos na histomorfometria intestinal e do aparelho reprodutivo de poedeiras leves na fase de produção]

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Abstract: The bioefficacy of calcium salt of 2-hydroxy-4-(methylthio) butanoic acid (HMBA-Ca) as a methionine source was evaluated against DL-methionine (DLM) in light-laying hens by assessing histomorphometric and hepatic parameters. A total of 1,080 Hy-Line W80 laying hens were randomly assigned to a 2×4+1 (two methionine sources; four supplementation levels: 0.46 %, 0.54 %, 0.56 %, and 0.58 %; and one control group without supplementation). Birds fed DLM exhibited increased villus width, height, surface area, and villus-to-crypt ratio ($p < 0.05$). The calculated bioefficacies were 99 %, 78 %, 83 %, and 79 %, respectively. Methionine supplementation reduced the probability of hepatic steatosis ($p < 0.05$) and decreased the hepatic lipid profile ($p < 0.05$). DLM increased epithelial thickness in the magnum, and the supplementation levels influenced the height and width of the uterine folds ($p < 0.05$). The corresponding bioefficacy values were 70 %, 131 %, and 76 %, respectively. Regardless of the source, methionine supplementation benefited the development of the intestinal mucosa, uterus, and magnum and reduced the incidence of hepatic steatosis in this study. Based on the bioefficacy results, DLM demonstrated greater bioavailability than HMBA-Ca. A DLM level of 0.58 %, which yielded the best results, is recommended.

Keywords: intestinal morphometry; hepatic fat; uterine folds.

Resumo: Avaliou-se a bioeficácia relativa do ácido 2-hidroxi-4-(metiltio)butanóico sal de cálcio (HMBA-Ca) em comparação a DL-metionina (DLM) aos níveis de metionina em poedeiras leves nos parâmetros histomorfométricos e hepáticos. Um total de 1080 poedeiras Hy-line W80 foram distribuídas aleatoriamente em arranjo fatorial: 2x4+1 (2 fontes, 4 níveis de suplementação cada: 0.46, 0.54, 0.56 e 0.58 % e 1 grupo isento de suplementação). No intestino, aves alimentadas com DLM tiveram maior largura, altura e área de superfície de vilo e relação vilo cripta ($p<0.05$). A bioeficácia foi de 99, 78, 83 e 79 %, respectivamente. A suplementação de metionina reduziu a probabilidade de esteatose ($p<0.05$). A suplementação proporcionou redução do perfil lipídico hepático ($p<0.05$). DLM apresentou maior espessura de epitélio do magno e os níveis influenciaram altura e largura

de dobra uterínica ($p<0.05$). A bioeficácia foi de 70, 131 e 76 %. A suplementação de metionina independente da fonte utilizada foi benéfica ao desenvolvimento da mucosa intestinal, do útero, magno, e menor incidência de esteatose hepática. De acordo com a bioeficácia, aves alimentadas com DLM apresentam maior biodisponibilidade em relação a HMBA-Ca. Contudo, recomenda-se o nível de 0.58 % de metionina com a DLM por ter proporcionado os melhores resultados.

Palavras-chave: morfometria intestinal; gordura hepática; dobras uterinas.

1. Introduction

Methionine is the primary limiting essential amino acid in corn-soybean meal-based diets for laying hens, and it significantly affects their productivity and feed efficiency. In addition to serving as a methyl group donor and precursor of cysteine and glutathione, methionine is directly involved in lipid metabolism, antioxidant defense, and maintenance of cellular integrity [1, 2]. Recent studies have demonstrated that varying dietary methionine levels can modulate metabolic processes, digestive function, the uterine environment, and immune responses, thereby influencing poultry performance and health [3].

Methionine supplementation is associated with changes in intestinal histomorphometry, including increased villus height, reduced villus-to-crypt ratio, and enhanced absorptive surface area, suggesting stimulation of epithelial cell proliferation and intestinal development [4]. Furthermore, methionine reduces oxidative stress and lipid peroxidation and may influence lipid parameters in serum and tissues, such as cholesterol and triglyceride levels [1]. Conversely, methionine deficient diets have been associated with hepatic fat accumulation, alterations in methylation pathways, and disruptions in lipid metabolism, predisposing birds to hepatic steatosis and other liver lesions [5].

From an industrial perspective, DL-methionine (DLM) and calcium salt 2-hydroxy-4-(methylthio)butanoic acid (HMBA-Ca) are the primary methionine sources used in feed formulations. Although both sources deliver biologically active methionine, they vary in chemical structure, with DLM containing amino groups and HMBA-Ca containing hydroxyl groups. Furthermore, their absorption, metabolism, and dose response characteristics in poultry differ [6]. These differences have resulted in conflicting estimates of equivalence between these sources, even when methionine availability is considered comparable. Therefore, relative bioefficacy is commonly used to compare their performance under practical production conditions [7].

Studies on light-laying hens have demonstrated the impact of adding methionine and partially substituting DL-methionine (DLM) with the calcium salt 2-hydroxy-4-(methylthio) butanoic acid (HMBA-Ca) on productivity, egg quality, and economic metrics [8]. However, there is limited information regarding the effects of different sources and levels of digestible methionine plus cysteine (Met+Cys) on intestinal and reproductive histomorphometry, as well as on hepatic characteristics such as total lipids, cholesterol, triglycerides, and hepatic steatosis. Therefore, this study aimed to assess the relative bioefficacy of HMBA-Ca with DLM at different levels of digestible Met+Cys by comparing the histomorphometry of the digestive and reproductive tracts and hepatic characteristics in light-laying hens.

2. Material and methods

2.1 Location, birds, housing, and experimental diets

The experiment was conducted at the Federal University of Paraíba (UFPB), Campus II, located at 06°57′48″ S latitude and 35°41′30″ W longitude at an altitude of 618 m in the state of Paraíba, Brazil. The Animal Use Ethics Committee of the UFPB granted approval for the use of animals in this study on November 19, 2019, under approval protocol no. 4161290819.

The experiment involved 1,080 commercial Hy-Line W80 laying hens, 42 weeks of age, with an initial average body weight of 1.600 ± 0.04 kg. The birds were housed in a conventional poultry house in metal cages (100 cm × 45 cm × 45 cm) with five birds per cage. Environmental conditions were monitored daily, with observed average maximum and minimum temperatures of 27.96 °C and 19.9 °C, respectively, and a relative humidity of 73 %. Food and water were provided ad libitum, and a lighting schedule of 17 h of light per day was followed.

The study employed a completely randomized design structured in a 2 × 4 + 1 factorial arrangement, consisting of two methionine sources (HMBA-Ca and DLM), four levels of digestible methionine plus cystine (Met+Cys) supplementation (0.46, 0.50, 0.54, and 0.58%) for each source, and an additional treatment without methionine supplementation, resulting in a total of nine treatments. Each treatment had 12 replicates, with 10 birds per experimental unit. The experimental diets were based on corn and soybean meal and formulated according to the Hy-Line W80 Management Guide (2016) recommendations, except for sulfur amino acids, which were adjusted according to Fickler et al. [9].

To achieve the desired levels of digestible Met+Cys (0.46, 0.50, 0.54, and 0.58 %), supplementation was increased by adding HMBA-Ca at 0.05, 0.10, 0.15, and 0.20 % of the diet, respectively. DL-methionine was included at 0.03, 0.07, 0.10, and 0.13 % of the diet to achieve the same digestible Met+Cys levels. Supplementation with DLM was based on a relative equivalence of 65 % compared to HMBA-Ca, which was considered to have 100 % equivalence. The ingredient compositions and calculated nutritional contents of the experimental diets are listed in Table 1.

Table 1. Composition of the experimental diets and calculated nutritional content of the basal diets for 42 to 52 weeks and 53 to 62 weeks of age (g/kg), based on a feed intake of 108 and 109 g for each phase, respectively.

Ingredients	42-52 weeks	53-62 weeks
Corn, 7,88 %	668.90	675.67
Soybean meal, 45,22 %	198.57	188.74
Limestone, 37 %	9.53	9.49
Soybean oil	94.98	100.22
Dicalcium phosphate, 19 %	18.74	16.72
Common salt	3.85	3.85
L-Lysine (Biolys®)	0,159	0,159
L-Threonine	1.70	1.59
L-Tryptophan (TrypAmino®)	0.18	0.11
Choline chloride, 60 %	0.01	0.07
Vitamin premix [1]	0.75	0.75
Mineral premix[2]	0.70	0.70
Antioxidant[3]	0.10	0.10
Inert	2.00	2.00
Calculated nutrient composition		
Metabolizable energy (MJ/kg)	2800	2800
Crude protein (%)	14.30	13.40
Calcium (%)	4.08	4.07
Available phosphorus (%)	0.50	0.44
SID lysine (%)	0.80	0.73
SID methionine (%)	0.20	0.19
SID methionine + cysteine (%)	0.40	0.39
SID threonine (%)	0.56	0.51
SID tryptophan (%)	0.17	0.15
SID arginine (%)	0.83	0.57
SID isoleucine (%)	0.62	0.52
SID leucine (%)	1.21	1.15
SID valine (%)	0.70	0.64

SID = standardized ileal digestible [1] Provided per kilogram of diet: 15.000.000 IU vitamin A; 1.500.000 vitamin IU D_3; 15.000 UI vitamin E; 2 g thiamine; 4 g riboflavin; 3 g pyridoxine; 0.015 g vitamin B12; 10 g D-pantothenic acid; 3 g vitamin K_3; 1 g folic acid. [2] Provided per kilogram of diet: 60 g Mn; 60 g Fe; 50 g Zn/ 10 g Cu; 2 g Coand 250 mg se. [3]Quantum Blue 5000.

2.2 Data and sample collection

At the conclusion of the experiment, eight birds from each treatment group were euthanized for tissue sampling. Tissue samples (approximately 1 cm in length) were collected from the central part of the duodenum and jejunum (specifically, the duodenal loop and from the bile duct entrance to Meckel's diverticulum), the middle lateral section of the uterus and magnum, and the left lobe of the liver. Samples were fixed in 10 % buffered formalin and processed using standard histological procedures for paraffin embedding. Histological sections were cut to a thickness of 5 µm, stained with hematoxylin and periodic acid–Schiff (PAS), and examined under a light microscope (Olympus BX60, Tokyo, Japan). The images were digitized and analyzed using Olympus cellSens Dimension image analysis software (Olympus, USA) [(10)].

2.2.1 Histomorphometry

The following parameters were measured in the small intestine: villus width (mean of measurements taken at the base, mid-region, and apex), villus height (measured from base to apex), crypt depth (associated with the corresponding villi), villus-to-crypt ratio, villus surface area, and goblet cell density (number of cells per 2,000 µm of linear epithelium).

For magnum and uterus morphometry, the fold height, fold width, fold perimeter, and epithelial thickness were evaluated. For each sample, 16 measurements per variable were obtained, and mean values were calculated for all variables analyzed. For the liver, five photomicrographs per bird were digitized to assess hepatic steatosis by examining the number and size of cytoplasmic lipid vacuoles in hepatocytes, according to the method of Ishak et al. [11].

2.2.2 Hepatic lipid extraction

Total hepatic lipids were extracted according to the method described by Folch et al. [12], with some modifications, using homogenized liver samples (1.0 g) in a chloroform:methanol mixture (2:1, v/v). After separation of the organic phase and solvent evaporation, the total lipid content was determined gravimetrically and expressed as grams of fat per 100 g of liver on a wet weight basis.

Hepatic cholesterol and triglyceride levels were determined as described by Gilglioni et al. [13]. The lipid fraction was re-dissolved in 200 µL of 2 % Triton X-100 solution, followed by vortex mixing and heating at 55 °C until fully dissolved. The total cholesterol and triglyceride concentrations in the suspension were quantified using an enzymatic colorimetric method with specific commercial kits (Labtest Diagnóstica S.A.) [14] according to the manufacturer's instructions. The results, initially obtained in mg/dL, were subsequently converted to mg of total cholesterol or triglycerides per 100 g of wet liver, considering the suspension volumes used in the reactions, total volume of Triton X-100 used for resuspension, and mass of tissue used in the homogenate [13,14].

2.3 Statistical analysis

The data were subjected to analysis of variance (ANOVA). When a significant effect of the methionine source was detected ($p \leq 0.05$), the means were compared using a t-test at the 5 % probability level. All analyses were performed using the SAS® University Edition software.

To evaluate the effects of digestible Met+Cys levels, regression models were applied using Met+Cys levels as the independent variable and intestinal morphometry and reproductive characteristics as performance variables and hepatic parameters as response variables. Both linear and exponential models were evaluated, and the selected model demonstrated significant coefficients ($p \leq 0.05$), the best adjusted coefficient of determination, and biological plausibility of the response.

For the hepatic steatosis score, multivariate ordinal logistic regression analysis was performed using the cumulative logit procedure to estimate probabilities using R statistical software version 3.5.1 [15]. The bioefficacy of HMBA-Ca relative to DL-methionine (DLM) was estimated using an exponential linear model as described by Littell et al. (16), considering the digestible Met+Cys levels of each source as independent variables and the performance and/or morphometric variables as responses. Using this model, the relative bioefficacy of HMBA-Ca in relation to DLM was calculated, and 95 % confidence intervals were obtained [16].

3. Results

3.1 Intestinal morphometry

As shown in Table 2, birds fed DL-methionine (DLM) supplements exhibited greater villus height (VH), villus surface area (VSA), and number of goblet cells (GC) than those fed HMBA-Ca ($p < 0.05$). Increasing digestible methionine plus cystine (Met+Cys) levels resulted in a positive linear

effect on villus width (VW), VH, VSA, and crypt depth (CD) ($p < 0.05$), indicating a progressive improvement in intestinal morphological structures. A quadratic effect was observed for VSA and GC ($p < 0.05$), suggesting that the maximum duodenal morphological development was 0.63 % for VSA and 0.56 % for GC. A significant interaction between methionine source and Met+Cys level was observed for VW, VSA, and villus-to-crypt ratio (V:C) ($p < 0.05$).

Table 2. Effects of methionine sources, different methionine levels, and the interaction between HMBA-Ca and DLM on villus width (VW), villus length (VL), villus surface area (VSA), crypt depth (CD), villus-to-crypt ratio (V:C), and goblet cells (GC) in the duodenum of light laying hens.

Sources	**VW** (µm)	**VL** (µm)	**VSA** (cm³)	**CD** (µm)	**V:C** (µm)	**GC**
HMBA-Ca	220.2	1378.7[b]	0.94[b]	165.7	8.40[b]	92.1[b]
DLM	214.9	1588.8[a]	1.07[a]	164.1	9.77[a]	98.9[a]
p-value	0.531	<0.001	0.026	0.749	<0.001	<0.001
Levels (%)						
0.46	190.3	1398.8	0.84	156.1	8.98	79.8
0.50	217.7	1461.8	0.99	162.6	9.07	94.9
0.54	227.9	1511.8	1.07	169.6	9.06	102.1
0.58	234.4	1562.5	1.15	165.3	9.24	102.1
Regression						
Linear	0.001	0.001	<0.001	0.027	0.562	0.001
Quadratic	0.220	0.857	0.041	0.645	0.891	0.041
Interaction						
Levels x Source	0.019	0.362	0.025	0.788	0.038	0.891
S.E.M	31.52	127.74	0.18	19.39	1.12	13.51

Means with different letters in the same column differ significantly according to the test T ($p \leq 0.05$). Regression equations for the levels: (VW: $\hat{Y} = 356.25x + 32.325$. $R^2 = 0.89$); (VL: $\hat{Y} = 1352.8x + 780.29$. $R^2 = 0.99$); (VSA: $\hat{Y} = -10.938x2 + 13.9x - 3{,}2361$. $R^2 = 0.99$. Maximum point: 0,63 %); (CD: $\hat{Y} = 87x + 118.21$. $R^2 = 0.62$) e (GC: $\hat{Y} = -2359.4x2 + 2639x – 634.86$. $R^2 = 0.99$. Maximum point: 0.56%) Availability =; RV:C: y= 120.9+197.5 *(1-e-(0.67x1+ 1.32x2+1.33x3)) GC: y = 44.0 +84.6 *(1-e-(0.72x1+1.92 x2+1.59x3)).

Methionine sources and Met+Cys levels significantly influenced VW, VSA, and V:C ratios (Table 3) ($p < 0.05$). Birds supplemented with HMBA-Ca showed a progressive increase in VW and VSA as Met+Cys levels increased, whereas birds receiving DLM maintained more constant values across supplementation levels. DLM resulted in higher VW and VSA values at 0.46 % Met+Cys; however, HMBA-Ca exhibited higher values from 0.54 % Met+Cys onward ($p < 0.05$). DLM resulted in higher V:C ratios at 0.46, 0.50, and 0.58 % Met+Cys levels ($p < 0.05$). Regression analyses indicated a linear response for VW and VSA within the HMBA-Ca source, with coefficients of determination (R^2) of 0.89 and 0.95, respectively.

Table 4 presents the effects of methionine sources (HMBA-Ca and DLM) and different Met+Cys levels on the morphology of the jejunum and ileum of light laying hens, including structural measurements of the villi (VL, VW, and VSA), crypt depth (CD), villus-to-crypt ratio (V:C), and number of goblet cells (GC). In the jejunum, the source of methionine did not affect VW, CD, or GC ($p > 0.05$). However, birds fed DLM-supplemented feed exhibited higher VH, VSA, and V:C ratios than those fed HMBA-Ca supplemented feed ($p < 0.05$). Increasing Met+Cys levels positively and linearly affected VH, VSA, and CD ($p < 0.001$), whereas VW, GC, and V:C were not affected by dietary Met+Cys levels.

Table 3. Effects of treatments on the interaction of villus width (VW), villus surface area (VSA), and villus-to-crypt ratio (V:C) on the duodenal morphology of light laying hens.

Variables		Levels (%)				Regression	
		0.46	**0.50**	**0.54**	**0.58**	**Linear**	**Quadratic**
VW µm	HMBA-Ca	170.7[b]	220.5	238.8	251.0	<0.001	0.120
	DLM	210.0[a]	214.9	217.0	217.8	0.637	0.862
	p-value	0.023	0.743	0.201	0.053		
VSA cm³	HMBA-Ca	0.66[b]	0.92	1.08	1.17	<0.001	0.217
	DLM	1.01[a]	1.06	1.07	1.13	0.240	0.921
	p-value	0.001	0.173	0.964	0.700		
V:C µm	HMBA-CA	8.16[b]	8.34[b]	8.56	8.55[b]	0.462	0.822
	DLM	9.79[a]	9.81[a]	9.56	9.93[a]	0.934	0.676
	p-value	0.008	0.016	0.101	0.024		

Means within the same column with different letters differ significantly according to the t-test. ($p < 0.05$). HMBA-Ca:VW: $\hat{Y} = 648.38x - 116.88$. $R^2 = 0.89$; VSA:$y = 4.225x - 1.2395$. $R^2 = 0.95$). Availability: VW: $\hat{Y} = 915.8+991.6*(1-e^{-(0.620x_1 + 2.10x_2 +1.21x_3)})$; VSA: $\hat{Y} = 0.25+1.40*(1-e^{-(0.70x_1 + 1.72x_2 +1.35x_3)})$ e V:C: $\hat{Y} = 6.0+ 5.5*(1-e^{-(1.22x_1 +1.98x_2 +1.12x_3)})$.

Table 4. Effects of methionine sources, different methionine levels, and the interaction between HMBA-Ca and DLM on villus width (VW, µm), villus length (VL, µm), villus surface area (VSA, cm³), crypt depth (CD, µm), villus-to-crypt ratio (V:C), and goblet cells (GC) in the jejunum and ileum of light laying hens.

	VW	**VL**	**VSA**	**CD**	**V:C**	**GC**
			Jejunal morphology			
HMBA-Ca	157.1	908.6[b]	0.44[b]	109.8	8.30[b]	173.9
DLM	154.7	982.6[a]	0.53[a]	108.3	9.08[a]	176.7
p-value	0.993	<0.001	0.001	<0.001	0.016	0.958
Levels (%)						
0.46	157.0	822.8	0.45	101.5	8.14	173.8
0.50	154.6	909.7	0.46	107.2	5.50	176.6
0.54	156.0	1015.7	0.49	108.5	9.34	175.9
0.58	155.9	1034.2	0.54	118.6	8.77	175.0
Linear	0.939	<0.001	<0.001	<0.001	0.016	0.958
Quadratic	0.839	0.160	0.477	0.588	0.060	0.867
Sources x Levels	0.998	0.088	0.510	0.588	0.334	0.703
s.e.m	21.19	89.80	0.08	8.62	0.95	41.69
			Ileal morphology			
HMBA-Ca	152.5	774.0[b]	0.37	87.6	8.86	182.7
DLM	144.4	819.6[a]	0.37	92.2	9.01	191.4
p-value	0.176	0.021	0.902	0.054	0.620	0.245
Levels (%)						
0.46	139.9	753.7	0.32	87.1	8.65	182.6
0.50	142.7	803.9	0.36	87.8	9.16	192.0
0.54	151.5	814.6	0.38	90.5	9.21	185.2
0.58	159.6	814.8	0.40	94.1	8.72	188.4
Linear	0.013	0.028	0.001	0.030	0.839	0.747
Quadratic	0.065	0.200	0.701	0.538	0.088	0.672
Source x Levels	0.793	0.951	0.741	0.162	0.206	0.731
s.e.m	22.29	72.14	0.06	19.79	1.08	27.50

Means with different letters in the same column differ significantly according to the test. T ($p < 0.05$). Regression equations for the levels: Jejunum = (CV: $y = 1850.4x - 16.55$. $R^2=0.94$); (VSA: $y = 0.875x + 0.0025$. $R^2= 0.94$); (CD: $y = 133.9x + 39.44$. $R^2=0.90$). Availability = VL: $y = 557.5+677.4 *(1-e^{-(0.82x_1 +1.85x_2 +1.39x_3)})$; VSA: $y= 0.20+0.50 *(1-e^{-(0.93x_1 + 1.61x_2 +1.35x_3)})$ e VC: $y= 5.35+6.02 *(1-e^{-(1.10x_1 +1.79 x_2 +1.42x_3)})$.Regression equations for the levels: Ileum = (VW: $\hat{Y} = 170{,}05x + 60{,}064$; $R^2 = 0{,}96$); (VL: $\hat{Y} = 484{,}65x + 544{,}8$; $R^2 = 0{,}73$); (VSA: $y = 59.15x + 59.182$. $R^2= 0.92$); (CD: $y = 59.15x + 59.182$. $R^2 = 0{,}92$).Availability: VW: $y= 506.3+487.9 *(1-e^{-(0.87x_1 + 1.97x_2 +1.47x_3)})$.

In the ileum, VH exhibited a pattern similar to that of the jejunum, with higher values observed in birds fed DLM ($p < 0.05$). The other variables (VW, VSA, CD, V:C, and GC) were not affected by the source of methionine ($p > 0.05$). Digestible Met+Cys levels promoted linear increases in VW, VH, VSA, and CD ($p < 0.03$) but did not influence V:C or GC ($p > 0.05$). No significant interactions between methionine source and Met+Cys levels were observed in either intestinal segment ($P > 0.05$). Regression equations confirmed consistent linear responses for VH, VSA, and CD in the jejunum ($R^2 \geq 0.90$) and for VW, VH, VSA, and CD in the ileum ($R^2 \geq 0.73$), indicating that increased methionine availability favors the morphological development of the intestinal epithelium throughout the gastrointestinal tract.

3.2 Determination of hepatic lipid content

Increased digestible Met+Cys levels reduced the probability of moderate-to-severe hepatic steatosis ($p < 0.05$). The absence of hepatic steatosis was noted with an increase in methionine supplementation from both sources (Figure 1).

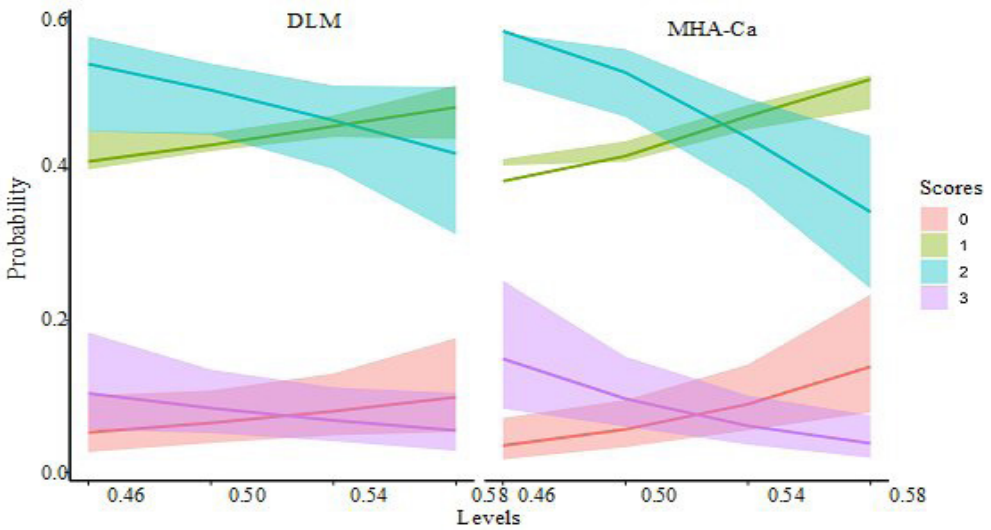

Figure 1. Effects of treatments on the evaluation of fatty liver. Score 0 (absent), 1 (mild), 2 (moderate), and 3 (advanced).

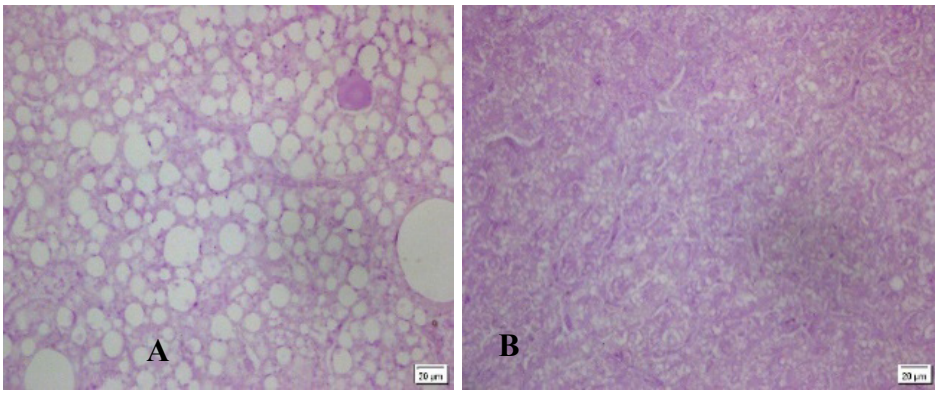

Figure 2. Hepatic histology of laying hens fed methionine. Photomicrographs show sections of hepatic tissue stained with hematoxylin and Schiff reagent from laying hens fed diets supplemented with 0.46 % digestible Met + Cys (A), showing marked steatosis with focal aggregates of inflammatory cells. The histology of livers supplemented with 0.58 % digestible Met + Cys shows a normal appearance (B).

The lipid profile results revealed that birds fed DLM had the lowest hepatic triglyceride concentrations ($p < 0.05$). In addition, increasing methionine supplementation reduced ($p < 0.05$) total hepatic lipid, triglyceride, and cholesterol levels (Table 5).

Table 5. Effects of treatments on the hepatic lipid profile (mg/dL).

	Total lipids	Triglycerides	Total cholesterol
HMBA-Ca	37.9	1.765[b]	199.3
DLM	39.2	1.562[a]	195.9
p-value	0.799	0.055	0.156
Levels (%)			
0.46	51.6	2.363	245.4
0.50	40.4	1.783	206.6
0.54	33.6	1.287	171.5
0.58	28.7	1.220	168.2
Linear regression	0.019	<0.001	0.009
Levels x Source	0.929	0.219	0.737
s.e.m	16.09	283.99	74.81

Means within the same column with different letters differ significantly according to the test. teste T. test ($p < 0.05$). Total lipids: $y = -188.75x + 136.73$. $R^2 = 0.96$; Triglycerides: $y = -9812.5x + 6765.8$. $R^2 = 0.91$; Cholesterol: $y = -666.75x + 544.64$. $R^2 = 0.90$.

3.3 Reproductive tract morphometry

The methionine source affected several variables of uterine and magnum morphology, as shown in Table 6. Birds supplemented with DLM exhibited greater uterine fold height (UFH) and width (UFW) compared to those receiving HMBA-Ca ($p < 0.05$). In contrast, HMBA-Ca supplementation resulted in a greater magnum fold height (MFH) ($p < 0.05$). Digestible Met+Cys levels had positive linear effects on UFH, UFW, MFH, magnum fold width (MFW), and magnum fold perimeter (MFP) ($p < 0.05$), indicating progressive increases in these variables with increasing dietary levels. Compared with DLM, the relative bioefficacy of HMBA-Ca was estimated to be 70, 72, 131, and 76 % for UFH, UFW, MFH, and magnum epithelial thickness (MET), respectively, based on the 95 % confidence intervals.

Table 6. Effects of methionine sources, different methionine levels, and the interaction between HMBA-Ca and DLM on uterine fold height (UFH, µm), uterine fold width (UFW, µm), uterine fold perimeter (UFP, cm³), magnum fold height (MFH, µm), magnum fold width (MFW, µm), magnum fold perimeter (MFP, cm³), and magnum epithelial thickness (MET, µm) in laying hens**.**

Sources	UFH (µm)	UFW (µm)	UFP (cm)	MFH (µm)	MFW (µm)	MFP (cm)	MET (µm)
HMBA-Ca	2.253[b]	493.2[b]	1.52	2.838[a]	1.082	2.64	28.0
DLM	2.538[a]	552.5[a]	1.59	2.685[b]	1.055	2.55	30.8
p-value	0.021	0.001	0.481	0.024	0.331	0.397	0.001
Levels (%)							
0.46	2.053	461.7	1.34	2.638	967.7	2.20	25.1
0.50	2.329	538.7	1.71	2.666	1.027	2.42	27.5
0.54	2.524	506.7	1.45	2.754	1.133	2.65	34.7
0.58	2.677	584.3	1.73	2.989	1.147	3.12	30.2
Linear	<0.001	0.001	0.053	0.001	<0.001	<0.001	<0.001
Quadratic	0.492	0.989	0.672	0.125	0.425	0.251	<0.001
Levels x Source	0.121	146.0	0.346	0.124	0.803	0.245	<0.001
s.e.m	329.48	85.01	0.39	247.93	105.78	0.39	2.85

Means within the same column with different letters differ significantly according to the test. T. ($p< .05$).UFH: $y = 165.7x – 289.99$. $R^2 = 0.98$; UFW: $y = 839.1x + 86.56$. $R^2 = 0.70$; MFW: $y = 2852.9x + 1278.6$. $R^2 = 0.85$; MFH: $y = 1614.6x + 229.46$. $R^2 = 0.93$; MFP: $y = 7.475x – 1.2895$. $R^2 = 0.96$. HMBA-Ca: MET: $y = 2323.4x^2 + 2442.1x – 609.01$. $R^2 = 0.62$. Max = 0.53Availability: UFH: y= 1478+1865*(1-e-(0.94x1+ 1.60x2+1.12x3)); UFW: y= 263.3+486.6*(1-e-(0.93x1+ 1.66x2+1.21x3)); MFH: y= 2059+1215*(1-e-(1.37x1+1.35 x2+1.80x3)) MET: y= 15.92+22.66*(1-e-(0.87x1+ 1.92x2+1.46x3)).

A significant interaction between methionine sources and Met+Cys levels was observed for MET, with notable differences between HMBA-Ca and DLM, specifically at the 0.58 % level ($p < 0.001$) (Table 7). For the HMBA-Ca source, a quadratic response was observed ($p < 0.001$), where the epithelial thickness increased up to the 0.54 % Met+Cys level and then decreased to the highest level, with an estimated peak of 0.53 %. In contrast, birds supplemented with DLM exhibited a positive linear response to increasing Met+Cys levels ($p < 0.001$), resulting in progressively higher MET values, achieving the greatest thickness at 0.58 %.

Table 7. Effect of the interaction between HMBA-Ca relative to DLM and Met + Cys levels on the magnum epithelial thickness (MET) of light laying hens at 62 weeks of age.

		Methionine levels (%)				Regression	
		0.46	**0.50**	**0.54**	**0.58**	**Linear**	**Quadratic**
MET (µm)	HMBA-Ca	24.04	27.32	36.13	24.54[b]	0.037	<0.001
	DLM	26.29	27.84	33.32	35.99[a]	<0.001	0.607
	p-value	0.156	0.732	0.070	<0.001		

Lowercase letters differ among rows according to the t-test at the 0.5 % probability level. (P<0.05) HMBA-Ca: $\hat{Y} = 2323.4x^2 + 2442.1x - 609.01$; $R^2 = 0.62$. Máx = 0.53 %) DLM: $\hat{Y} = 86.45x - 14.094$; $R^2 = 0.95$).

4. Discussion

The gastrointestinal tract is one of the primary tissues involved in amino acid utilization, particularly in protein synthesis, cellular signaling, and antioxidant defense [17]. A substantial fraction of the ingested essential amino acids is consumed during first-pass intestinal metabolism, with methionine being one of the most extensively utilized amino acids by enterocytes [18]. This highlights the essential role of methionine in maintaining the integrity of the intestinal mucosa.

In the present study, digestible Met+Cys supplementation consistently improved intestinal morphometry, particularly in the duodenum and jejunum, as evidenced by increased VH, VW, and VSA, higher V:C ratios, and greater numbers of GC. The regression models predominantly showed positive linear or quadratic responses for these variables as Met+Cys levels increased, with the estimated maxima occurring near the highest supplementation levels tested. These findings suggest that methionine contributes to accelerated epithelial renewal, expansion of the absorptive surface, and strengthening of the mucosal barrier, which is consistent with previous findings on increased VH and absorptive area in quail and broiler chickens fed diets supplemented with increasing methionine levels [18].

The mechanisms underlying these morphological responses may be related to the antioxidant function of methionine and its role in regulating cell growth. The participation of methionine in glutathione synthesis aids in neutralizing reactive oxygen species and safeguarding mucosal cellular structures [18], whereas the formation of polyamines, such as spermidine and spermine, is directly associated with the proliferation of intestinal epithelial cells and increased villus renewal rates [19]. Additionally, enhanced transsulfuration activity, with the conversion of homocysteine into intermediates of the tricarboxylic acid cycle [20], may provide energy to proliferating cells, further supporting the development of the mucosa.

Conversely, diets containing lower methionine levels failed to sustain adequate intestinal mucosal growth, resulting in less developed villi and reduced villus-to-crypt ratios. This pattern is consistent with the hypothesis that methionine deficiency disrupts the amino acid balance in tissues, impairs mucosal redox status, reduces cellular proliferation, and increases susceptibility to apoptosis because of the limited availability of protective metabolites derived from its metabolism [21, 22].

Differences between methionine sources were more evident in the morphological parameters at lower supplementation levels. In general, DLM tended to promote greater VH and VSA in the duodenum and jejunum, as well as higher V:C ratios at specific levels, whereas HMBA-Ca produced equivalent or slightly lower responses under these conditions. These results suggest that under suboptimal Met+Cys levels, DLM exhibits greater relative bioefficacy in enhancing intestinal morphology. This difference is consistent with the chemical nature of the methionine sources and the intestinal transport systems involved: HMBA-Ca, as a monocarboxylate analog, is absorbed via H^+- or Na^+-dependent monocarboxylate transporters (MCT), whereas DLM is absorbed through specific sodium-dependent neutral amino acid transport systems [23]. At low supplementation levels, differences in transporter affinity and maximal transport rates may favor DLM [24], whereas HMBA-Ca may be partially diverted to microbial metabolism, reducing its availability to the enterocytes.

However, as Met+Cys levels meet or exceed the needs of birds, the differences between the methionine sources become less pronounced, with both sources effectively supporting intestinal mucosal development and with comparable productive performance. In this context, a comprehensive analysis of the data revealed that DLM was more beneficial at lower supplementation levels, whereas at Met+Cys levels close to 0.58 %, both sources were functionally equivalent for most variables, with DLM showing only minor and isolated advantages for certain indicators.

The productive performance results closely reflected the observed morphological changes. Increasing Met+Cys levels positively and linearly affected egg production, egg weight, egg mass, eggs per hen, and feed conversion. Quadratic responses indicated optimal points near 0.59 % Met+Cys levels for egg mass and egg weight and 0.62 % for feed conversion per egg mass [8]. This pattern suggests that improved integrity and absorptive capacity of the gastrointestinal tract translate into enhanced nutrient utilization and, consequently, improved productivity. The absence of significant effects of methionine source on performance variables, despite differences in some morphological characteristics, supports the conclusion that at adequate supplementation levels, HMBA-Ca and DLM can be used interchangeably to support performance, provided that their relative bioefficacy is appropriately considered in diet formulation.

In addition to its effects on the gastrointestinal tract, Met+Cys supplementation significantly influenced hepatic lipid metabolism and development of steatosis. An inverse relationship has been observed between dietary methionine levels and hepatic fat accumulation, with methionine-deficient diets associated with higher lipid content and a greater incidence of moderate-to-advanced steatosis. In contrast, increasing Met+Cys supplementation reduced hepatic triglyceride and cholesterol levels. These findings are consistent with the established role of methionine as a precursor of phosphatidylcholine, which is a key structural component of very-low-density lipoproteins (VLDL). Insufficient phosphatidylcholine synthesis impairs hepatic triglyceride export, resulting in lipid accumulation in hepatocytes [25, 26]. Improvements in hepatic lipid profiles may also be associated with increased lipoprotein lipase activity and the contribution of cysteine-derived taurine, a downstream product of methionine metabolism, to bile acid conjugation and micelle formation, ultimately enhancing lipid digestion and absorption [27, 28].

In the reproductive tract, increasing Met+Cys levels facilitated enhanced development of uterine and magnum morphometry, as evidenced by increased fold height, width, and perimeter, as well as a thicker epithelium in the magnum. Given that amino acids play a crucial role in processes related to productive and reproductive performance [29] and that the uterus exhibits

luminal concentrations of methionine that exceed plasma levels during specific physiological phases [30], it is plausible that increased dietary methionine enhances the supply of this amino acid and its metabolites to the uterine lumen and magnum. This, in turn, may support the secretory activity and deposition of eggshell and albumin.

Magnum epithelial thickness exhibited a significant interaction between methionine source and supplementation level, with a quadratic response for HMBA-Ca and linear response for DLM. For HMBA-Ca, the epithelial thickness increased up to intermediate Met+Cys levels and declined at the highest level, suggesting an optimal response at approximately 0.53 %. In contrast, DLM elicited a consistent linear increase across the evaluated range, with the greatest epithelial thickness observed at 0.58 % Met+Cys. These results indicate a more stable and progressive effect of DLM on magnum epithelial development, potentially reflecting its enhanced capacity for mucus and albumin secretion. This interpretation is supported by previous reports describing increased albumin deposition and reduced oviposition intervals in birds exposed to elevated methionine levels [31].

Overall, the present findings demonstrate that methionine supplementation, irrespective of the source, is beneficial for intestinal mucosal development, uterine and magnum morphometry, and modulation of hepatic lipid metabolism, resulting in positive effects on the productive performance of light-laying hens. Within the evaluated range, higher Met+Cys levels generally elicited the most pronounced responses across all variables. In terms of relative bioefficacy, DLM was more effective at lower supplementation levels, whereas at levels approaching the upper requirement range, HMBA-Ca and DLM exhibited comparable effects on most of the parameters. This suggests a degree of flexibility in source selection, provided that formulation adjustments adequately account for the differences in availability and responses across supplementation levels.

5. Conclusion

In conclusion, Met+Cys supplementation improved the productive performance and intestinal morphometry of laying hens, with 0.58 % being the most efficient dietary level. DLM showed superior responses at lower supplementation levels, whereas both methionine sources were effective at adequate dietary concentrations.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Data availability statement

The data will be made available upon request from the corresponding author.

Author contributions

Conceptualization: T.S. Ferreira. Data curation: T.S. Ferreira. Formal analysis: T.S. Ferreira and I.N. Kaneko. Investigation: M.N. Soares. Methodology: T.S. Ferreira and J.V.C. Silva. Project administration: M.N. Soares. Supervision: F.G.P. Costa. Writing – original draft: T.S. Ferreira. Visualization: S.G. Pinheiro and R.F.B. Júnior. Writing – review and editing: M.N. Soares, S.G. Pinheiro, and R.F.B. Júnior.

Generative AI use statement

The authors did not use generative Artificial Intelligence tools or technologies in the creation or editing of any part of this manuscript.

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