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Effect of enzyme activated guanidinoacetic acid supplementation with different levels of metabolizable energy containing diets on serum biochemical profile and immunity index in laying hens

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Abstract

An experiment was undertaken to evaluate the effect of enzyme activated Guanidinoacetic acid supplementation with different levels of metabolizable energy containing diets on hematological, blood biochemical and immunity status in laying hens. Total one hundred sixty 24 week old layer (BV300) birds were randomly divided into five treatment groups on the basis of their live body weight having four replicates of eight birds each for 56 days. The dietary treatment includes control diet (T₁), 5% and 10% reduced ME diet with Guanidinoacetic acid (GAA) supplementation at 0.6 g/kg (T₂ & T₃) feed and enzyme activated Guanidinoacetic acid (eGAA) supplementation at 0.6 g/kg (T₄ & T₅) feed. The results revealed that all hematological parameters of laying hens showed non-significant differences ($p > 0.05$) among treatment groups. Blood biochemical constituents in laying hens revealed that the albumin, uric acid and AST were significantly differed among the treatment while other blood biochemical were comparable. The values of TAC, SOD, GSH-Px and MDA levels in laying hens were found at par ($p > 0.05$) among the treatment groups. Hence, it might be concluded that supplementation of enzyme activated GAA might efficient to cover the gap of energy reduction in the feed at either 5 or 10% in laying hens without affecting the health status of laying hens.

Keywords: Enzyme activated Guanidinoacetic acid, metabolizable energy, laying hens, blood biochemical profile, immunity status

Introduction

India ranks second in the world for egg production, fourth in meat production and it leads the world in milk production. The nation's total egg production is projected to reach 149.11 billion eggs in 2024-2025, representing a 3.18% increase over the previous year compared to predictions of 142.77 billion eggs in 2013-2014 (DOAH, 2025) [11]. Hybrid layers of today lays on an average of 310-320 eggs per year compared to 240-250 eggs 30 years back. Similarly, present day broilers attain 1.8 to 2.0 kg mean body weight with FCR of 1.6 to 1.8 in only 38 days (Rao, 2020) [25]. The per capita availability of eggs was increased from 62 nos/annum in 2014-15 to 106 nos/annum in 2024-25. The expansion of the poultry sector is significantly dependent on the availability of high-quality with required quantity of feed for both producers and consumers and at economic rate (Adejinmi *et al.*, 2011) [1]. In developing countries, poultry production industries face the challenge of raising feed costs by 60-70 percent (Tesfaye *et al.*, 2013) [28]. For economic reasons, this supply of nutrients should be at least cost and so we must supply only enough for requirements without there being any major excesses. In formulating diets, the necessary nutrients are energy, protein, fat, vitamins, minerals and water. Among these in poultry diets energy and some of the essential amino acid such as Methionine and Lysine are considered as limiting nutrients (Alagawany *et al.*, 2021) [3].

Metabolizable Energy (ME) is the part of energy remaining after faecal and urinary energy loss and represents the energy available for growth or reproduction. It is considered as the standard measure used to describe energy requirements of the birds and dietary content of energy (NRC, 1994) [23].

The dietary ME level is one of the major factors, which play an important role in regulating feed intake and feed efficiency in chickens (Lopez and Leeson, 2008) [19]. Guanidinoacetic acid is a stable and highly available feed supplement that is mainly used to boost energy utilisation. It is a direct precursor of creatine which has recently attracted attention as a feed additive due to its high thermal stability (Vranes *et al.*, 2017) [29]. The European Food Safety Authority (2009) [12] concluded that GAA, if administered at a dosage not higher than 600-800 mg GAA per kg feed could be considered as an effective and safe feed additive for chickens for improving performance characteristics. Hence, GAA has been officially registered as an animal feed additive by American and European regulatory agencies (Khajali *et al.*, 2020) [18]. Now a days, researchers are gaining more attention to evaluate the potential of enzyme activated Guanidinoacetic acid (eGAA) instead ordinary exogenous GAA for their role in energy metabolism. According to metabolic pathways of GAA in animals, the activity of transporter enzyme and methyl group has been enhanced to make activated GAA which can be quickly and efficiently convert into creatine in young animals or when animal suffer in stress condition. Therefore, this study was conducted to determine effects of supplementing enzyme activated GAA with different levels of ME on blood biochemical profile and immunity index in laying hens.

Materials and Methods

Experimental details

The experiment on layers was carried out at Biotech Poultry

Farm, At. & Post, Vadod, Dist. Anand, Gujarat for 56 days (24th week to 31st week). Geographically, this experimental station is located at 22.30° N latitude, 73.19° E longitude and 39 meters above sea level with subtropical climatic conditions. A total one hundred and sixty (n = 160) layers of 24 weeks old of BV300 strain reared under cage system were used as experimental birds. They were weighed individually and randomly assigned into five homogenous groups on the basis of their live body weight. Each group (32 birds) comprised of 4 replicates (08 birds/replica). The 24 weeks old layers were weighed individually with standard electronic weighing balance and it was considered as initial body weight of layers. Dietary treatments include basal diet (T₁), 5% and 10% reduced energy diet with GAA supplementation at 0.06% (T₂ & T₃) feed and eGAA supplementation at 0.06% (T₄ & T₅) feed.

Birds were adapted to respective treatment diet for a week before the commencement of the actual data collection. Standard vaccination schedule was strictly followed. The required quantity of feed used in present experiment was formulated by using available feed resources and prepared in a single batch by uniform mixing of ingredients. Feed and water were provided with feeder and drinker channel. The entire replica under each group were separated to each other by kept one blank cage for avoid mixing of feed. Light bulbs were placed for the lighting system to increase the lighting period to 16 h per day in order to increase feed intake and laying. They were fed standard diet formulated as per BIS (2007) [7] by using ingredients listed in Table 1.

Table 1: Ingredients and nutrient composition of treatment diets

Ingredient (%)	Layer Phase I				
	T ₁	T ₂	T ₃	T ₄	T ₅
Corn	60.00	56.00	45.00	56.00	45.00
Soybean Meal	19.80	18.60	17.00	18.60	17.00
Rice Bran	7.00	13.34	25.94	13.34	25.94
Vegetable Oil	1.20	0.00	0.00	0.00	0.00
GAA	0.00	0.06	0.06	0.00	0.00
Enzyme Activated GAA	0.00	0.00	0.00	0.06	0.06
Calcium Carbonate	9.00	9.00	9.00	9.00	9.00
DCP	2.00	2.00	2.00	2.00	2.00
Sodium chloride	0.27	0.27	0.27	0.27	0.27
L-Lysine	0.19	0.19	0.19	0.19	0.19
DL-Methionine	0.16	0.16	0.16	0.16	0.16
Choline chloride	0.15	0.15	0.15	0.15	0.15
Trace mineral mixture	0.09	0.09	0.09	0.09	0.09
Vitamin premix	0.05	0.05	0.05	0.05	0.05
Toxin binder	0.09	0.09	0.09	0.09	0.09
Total	100.00	100.00	100.00	100.00	100.00
Nutrients					
CP	18.01	18.00	18.04	18.00	18.04
ME(Kcal/kg)	2603.00	2469.48	2340.48	2469.48	2340.48
EE	3.41	3.05	2.92	3.05	2.92
CF	4.10	5.20	7.36	5.20	7.36
CA	4.02	4.02	4.03	4.02	4.03
*Mineral-vitamin premix: Vitamin A (retinyl acetate)-12, 500 IU; Vitamin D3 (cholecalciferol)-3000IU; Vitamin E (DL- α -tocopheryl acetate)-60 IU; Vitamin K (menadione sodium bisulfite)-1.02 mg; Riboflavin-2.0mg; Pantothenate-8.0 mg; Niacin-6mg; Folic acid-0.5 mg; Biotin-0.10 mg; Thiamine-1 mg; Vitamin B12-20 mg; Mn-120 mg; Zn-80 mg; Fe-52 mg; Cu-15 mg; I-1.5 mg; Se-0.4 m					

Health status

Blood was collected randomly from eight birds of each group (two birds/replica) at the end of experiment for

collection of serum and plasma separately. Biochemical parameters viz. glucose, total protein, albumin, globulin, total cholesterol, triglycerides, uric acid and creatinine were

estimated from serum samples by using commercially available analytical kits (Q-Line Biotech Pvt. Ltd.) using spectrophotometer (Model No. 4001/1 Gensys 20, Thermo Fisher Scientific, USA). Hematological parameters viz. hemoglobin (Hb), packed cell volume (PCV), total leucocyte count (TLC) and total erythrocyte count (TEC) were analysed using auto-haematology cell counter (Exigo EOS H400 hematology analyzer, Spanga, Sweden). Antioxidant indices including superoxide dismutase (SOD), reduced glutathione (GSH-Px), lipid peroxidation (MDA), total antioxidant capacity (TAC) and Malondialdehyde (MDA) was carried out with the help of chicken ELISA kit (G Biosciences, Geno Technology, Inc.) and reading was undertaken in ELISA reader (Cyber ELISA-R01 microplate reader, Cyberlab corp., USA).

Statistical analysis

Data collected from the above experiments were analysed using SPSS (20.0) version. One-way analysis of variance

and Tukey least significant difference test for post-hoc comparisons (Snedecor and Cochran, 1994)^[27].

Results and Discussion

Hematological Parameters

The effects of dietary supplementation of enzyme activated Guanidinoacetic acid (eGAA) with different levels of metabolizable energy (ME) on hematological parameters of laying hens are presented in Table 2. All hematological parameters of layers showed non-significant differences ($p > 0.05$) among treatment groups. Although numerical improvements were observed in T₄ and T₅, especially for Hb, RBC, PCV and related erythrocyte indices. The highest values were recorded in T₄ indicating a potential hematopoietic enhancement due to the supplementation of eGAA which enhanced ATP availability via creatine may aid in maintaining hematological homeostasis under energy deficient conditions.

Table 2: Effect of eGAA supplementation with different levels of ME containing diets on hematological parameters of laying hens

Parameters	Treatment					SEM	P-Value
	T ₁	T ₂	T ₃	T ₄	T ₅		
Hb (g/dl)	8.50 ± 0.16	8.56 ± 0.27	9.06 ± 0.27	9.21 ± 0.23	9.13 ± 0.31	0.118	0.246
RBC (*10 ⁶ /μl)	4.25 ± 0.08	4.28 ± 0.14	4.53 ± 0.13	4.60 ± 0.11	4.56 ± 0.16	0.059	0.562
PCV (%)	25.50 ± 0.49	25.69 ± 0.82	27.19 ± 0.82	27.63 ± 0.69	27.38 ± 0.94	0.355	0.731
MCV (fl)	102.00 ± 1.96	102.75 ± 3.29	108.75 ± 3.29	110.50 ± 2.74	109.50 ± 3.75	1.422	0.334
MCH (pg)	38.25 ± 0.74	38.53 ± 1.23	40.78 ± 1.23	41.44 ± 1.03	41.06 ± 1.41	0.533	0.406
MCHC (g/dl)	29.75 ± 0.57	29.97 ± 0.96	31.72 ± 0.62	32.23 ± 0.80	31.94 ± 1.09	0.414	0.800
TLC (*10 ³ /μl)	17.00 ± 0.08	17.13 ± 0.13	18.13 ± 0.13	18.42 ± 0.11	18.25 ± 0.16	0.059	0.765

Haematological parameters are indicators of the physiological status of livestock (Etim *et al.*, 2014)^[14]. In the support of present findings, chickens were fed low ME (2700 Kcal/kg) basal diets and the GAA at 0, 0.6, and 1.2 g/kg observed improved the health of broilers with no negative effects (Ayeni *et al.*, 2023)^[5] and hypothesized that GAA could functioned as an antioxidant in the blood to safeguard the structure of the erythrocyte membrane and increase the deformability and transportation capacity of erythrocytes. According to Oloruntola *et al.* (2019)^[24], the PCV value fall within the normal range for broiler chicken and suggested that GAA/eGAA supplementation with reduced dietary energy does not adversely affect erythropoiesis, oxygen-carrying capacity and immune cell profile. This indicates that GAA supplementation supports normal physiological and metabolic functions without

inducing hematological stress (Mousavi *et al.*, 2013)^[22]. In contrary, DeGroot *et al.* (2018)^[10] reported improved hematological profiles in broilers supplemented with GAA, attributing it to better metabolic efficiency and oxygen transport capacity.

Blood biochemical status

Blood biochemical status (Table 3) revealed that the albumin, uric acid and AST were significantly differed among the treatment in layer chicken while other blood biochemical were comparable. However, the overall biochemical profile remained within the normal physiological range (Kaneko *et al.*, 2008)^[17], indicating that supplementation of 0.06% enzyme-activated GAA even with reduced energy diets did not negatively affect liver and kidney function.

Table 3: Effect of eGAA supplementation with different levels of ME containing diets on blood biochemical parameters of laying hens

Parameters	Treatment					SEM	P-Value
	T ₁	T ₂	T ₃	T ₄	T ₅		
Glucose (mg/dl)	212.78 ± 9.40	222.53 ± 11.72	225.99 ± 11.44	226.18 ± 5.56	229.76 ± 31.94	7.171	0.962
Total Protein (g/dl)	6.26 ± 0.39	6.48 ± 0.43	6.43 ± 0.53	6.53 ± 0.58	6.39 ± 0.38	0.199	0.996
Albumin (g/dl)	3.58 ± 0.17 ^{ab}	3.56 ± 0.22 ^{ab}	3.15 ± 0.21 ^b	3.11 ± 0.23 ^b	3.88 ± 0.27 ^a	0.105	0.103
Globulin (g/dl)	2.68 ± 0.30	2.91 ± 0.32	2.83 ± 0.21	2.85 ± 0.31	2.50 ± 0.34	0.129	0.873
Albumin:Globulin	1.46 ± 0.17	1.32 ± 0.15	1.19 ± 0.16	1.21 ± 0.19	1.76 ± 0.26	0.087	0.236
Uric Acid (mg/dl)	4.21 ± 0.28 ^b	5.37 ± 0.42 ^b	5.58 ± 0.41 ^b	7.64 ± 0.46 ^a	7.81 ± 0.90 ^a	0.319	0.000
Creatinine (mg/dl)	0.43 ± 0.49	0.40 ± 0.53	0.41 ± 0.62	0.51 ± 0.25	0.55 ± 0.20	0.195	0.839
Total cholesterol (mg/dl)	77.89 ± 3.60	93.74 ± 6.87	83.75 ± 8.82	73.45 ± 5.47	79.00 ± 15.29	3.956	0.569
Triglyceride (mg/dl)	456.62 ± 71.50	432.16 ± 48.82	379.61 ± 35.42	307.21 ± 61.76	316.18 ± 42.86	24.680	0.207
ALT (U/L)	4.61 ± 0.14	4.56 ± 0.26	4.83 ± 0.37	4.90 ± 0.22	4.93 ± 0.32	0.320	0.821
AST (U/L)	188.24 ± 15.10 ^a	163.56 ± 51.02 ^b	182.90 ± 35.07 ^{ab}	190.43 ± 42.10 ^a	173.69 ± 25.04 ^{ab}	33.035	0.078

^{abc}Means with different superscript in a row differ significantly ($p < 0.05$)

The absence of significant variation ($p > 0.05$) in biochemical indicator suggests that protein metabolism was not markedly influenced by dietary supplementation of GAA (Michiels *et al.*, 2012) [21]. These findings agree with Fosoul *et al.* (2018) [16] and Majdeddin *et al.* (2020) [20], who observed improved protein metabolism and serum protein profiles in broilers receiving GAA under energy-deficient conditions. In contrast, Emami *et al.* (2017) [13], Cordova

Noboa *et al.* (2018b) [08] and Al-Abdullatif *et al.* (2024) [02] did found significant difference ($p > 0.05$) in albumin concentration between treatment groups when GAA was supplemented at 0.6 g/kg to chicken which indicates the bioavailability of dietary protein and albumin were similar during the whole experimental period.

Immunity Index

Table 4: Effect of eGAA supplementation with different levels of ME containing diets on serum antioxidant status of laying hens

Parameters	Treatment					SEM	P-Value
	T ₁	T ₂	T ₃	T ₄	T ₅		
TAC (umol/L)	2.79 ± 0.38	2.38 ± 0.43	2.28 ± 0.29	2.47 ± 0.34	2.99 ± 0.42	0.164	0.641
SOD (ng/ml)	0.64 ± 0.35	0.63 ± 0.11	0.80 ± 0.18	0.81 ± 0.13	0.79 ± 0.25	0.109	0.955
GSH-Px (umol/L)	3.62 ± 0.74	4.02 ± 0.71	3.11 ± 0.51	5.00 ± 1.57	4.82 ± 0.80	0.414	0.586
MDA (umol/L)	559.92 ± 115.55	465.83 ± 83.94	243.92 ± 30.81	612.36 ± 81.31	613.52 ± 98.33	66.677	0.378

The values of all serum antioxidant markers (Table 4) in laying hens were found at par ($p > 0.05$) among the treatment groups which indicates that dietary eGAA did not notably alter the oxidative balance in chickens. Similar comparable trends were observed by Salah *et al.* (2020) [26] where non-significant linear increase in the liver GSH level with the increase in the dietary supplements of the GAA. However, the GAA supplements resulted in a significant reduction in the liver MDA levels. The positive effects of GAA on the liver antioxidant activity may be attributed to the expected higher creatine level. Similarly, the study of Azizollahi *et al.* (2024) [6] provides evidence that the administration of GAA results in a decrease in MDA levels and a numerical increase in TAC value. Previous studies have also highlighted the potential antioxidant effects of supplemental GAA in low to moderate quantities (Amiri *et al.*, 2019; Cui *et al.*, 2022) [4, 9].

In contrast to present findings, Ezzat *et al.* (2018) [15] observed that as increased the level of GAA there were parallel increased the superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity on betaine (1600 mg/kg) and GAA (600, 800 and 1000 mg/kg) supplementation with basal diet in laying hens. Likewise, Wang *et al.* (2012) [30] demonstrated that dietary GAA improved antioxidant status by elevating total antioxidant capacity and the activities of several antioxidant enzymes. This could be due to GAA-related metabolites (creatine and arginine) might be able to quench free radicals after GAA ingestion, suggesting indirect antioxidant effect of GAA utilization.

Conclusion

It was concluded that supplementation of GAA and eGAA with reduction level at 5 and 10% in ME level did not affect the hematological indicators of laying hens. Moreover, serum glucose, total protein, globulin, albumin: globulin, creatinine, total cholesterol and triglycerides remained statistically similar between treatment groups. While, albumin and uric acid were statistically higher in T₅ group containing eGAA with 10% reduced ME diets. Further, liver enzymes, ALT remained unchanged but AST became significantly differed ($p < 0.05$) in treatment group. In addition, the serum antioxidant including total antioxidant capacity (umol/L), Superoxide Dismutase (ng/ml), Glutathione Peroxidase (umol/L) and Malondialdehyde (umol/L) in laying hens were revealed comparable. Thus, eGAA supplementation might have potential to cover the

gap of energy reduction in the feed at 5 and 10% in layer chicken without affecting the performance of laying hens.

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