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Research Article

Effects of Dietary Supplementation of Guanidinoacetic Acid in Soybean or Canola Meal Based Diets on Growth Performance and Development of Ascites in Broiler Chickens

Shahabodin Zafari Naeini*, Fariborz Khajali, Behnam Ahmadipour and Hosein Hassanpour

Department of Animal Science, Shahrekord University, Shahrekord, Iran

***Corresponding Author:** Shahabodin Zafari Naeini, Department of Animal Science, Shahrekord University, Shahrekord, Iran.

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Abstract

This study assessed the effects of dietary supplementation of Guanidinoacetic acid (GAA; commercial form CreAMINO®) in soybean (SBM) or canola meal (CM) based diets on performance, carcass characteristics, ascites mortality, blood and plasma parameters, internal organs, gut morphology and ascites indices in broiler chickens raised under the hypobaric and cold condition for 42 days. Also, tried to determine the ability of CM to serve as a dietary replacement for SBM for growing and finishing stages of broilers.

A total of 600 male broilers (Ross 308) were reared at high altitudes under cool conditions. A Maize-SBM based diet was formulated for the starting (1 to 10 days of age; without GAA supplement for all birds), SBM and CM diets for the growing (11 to 24 days of age; used GAA) and the finishing (25 to 42 days of age; used GAA) stages according to Nutrition Specifications for ROSS 308 (target live weight 2.50 - 3.00 kg) recommendations. This experiment was conducted as a completely randomized design that chicks were allocated to 8 treatments with 5 replicates of 15 birds. The 8 diets were (1) SBM, (2) SBM+ 0.6g/kg GAA, (3) SBM + 1.2g/kg GAA, (4) SBM + 2.4g/kg GAA and (5) CM, (6) CM + 0.6g/kg GAA, (7) CM + 1.2g/kg GAA, (8) CM + 2.4g/kg GAA.

Feed Conversion Ratio (FCR) in all individual stages and overall (42 d) was not affected by adding GAA to SBM diets, while FCR of birds fed on CM diet was worse than SBM and even was exacerbated with high levels (2.4g/kg) of GAA in the overall study period, significantly. The development of ascites in broiler chickens fed on CM diet containing 0.6 or 1.2g GAA/kg was lower than other CM based diets. Adding GAA had not significant impact on abdominal fat and thigh yield in neither SBM nor CM groups. The carcass yield was not affected by diet protein source (SBM or CM) or adding GAA (0.6 and 1.2g/kg diet; $P > 0.05$) but adding 2.4g/kg GAA to the CM diets decreased this parameter, dramatically. In contrast, breast yield increased in SBM group by adding GAA ($P < 0.05$ for 0.6 and 2.4g/kg diet). The weight of liver and heart were decreased with inclusion of GAA at level 1.2g/kg for CM diets. Enrichment of diets with GAA alleviated the adverse effects of Hypobaric-cold stress as reflected by reduction in blood hematocrit, Malondialdehyde (MDA), Heterophil/lymphocyte and right ventricle to total ventricle ratio (RV/TV) at 42 d of age. Addition of GAA (0.6g/kg for SBM and 1.2 or 2.4g/kg for CM groups) to the diet of birds reared under Hypobaric-cold stress resulted in a higher jejunal villus surface area compared to those fed on control diets.

The results of this research indicate that GAA supplementation in diets did not affect the performance of birds fed SBM based diet as well as CM diets, but adding the high level of GAA (2.4g/kg) in the CM based diet exacerbated the adverse effects of hypobaric and cold stress on performance, gut development and ascites syndrome, significantly. In addition, GAA supplementation at 1.2g/kg improved RV:TV, heart weight, hematocrit, MDA, NO in birds fed on CM based diet and raised under hypobaric and cold conditions, while higher levels of GAA had mortal effects on broilers in CM groups.

Keywords: Performance; Guanidinoacetic Acid; Blood Indices; Gut Health; Canola; Pulmonary Hypertension

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Introduction

Feed constitutes the greatest proportion of costs associated with poultry industry, and most of that cost is related to the high price of protein-furnishing ingredients. So poultry producers increasingly look for alternative feed ingredients to use as substitutes. Canola is one of the most important oilseed crops in many countries like Canada, China and India (Top three Countries producing rapeseed; 10). Canola meal (CM) is a by-product of canola seed. In many studies, broiler chickens fed high CM diets had lower feed intake (FI) and body weight gain (BWG) compared to the birds fed a soybean meal (SBM) based diet [22,42]. Although CM is lower in protein content compared with SBM, with regard to amino acid (AA) content, CM contains more Methionine (Met) and Cysteine (Cys) but less Lysine (Lys) and Arginine (Arg), which the last could be of importance when introducing CM to broiler diets at high inclusion rates because the Arg content of CM is approximately two-thirds of that of SBM [21]. Arg is the fifth limiting essential AA in most poultry diets and is needed for mainly bodily functions concerning maintenance (i.e., creatine production) and growth (i.e., protein synthesis) [45]. Due to the increased growth rate of modern broilers [12], lack of Arg *de novo* synthesis, chickens are highly dependent on dietary sources for this AA, so supplementation of Arg to CM-based diets has been shown to partly restore the growth performance [22]. Also, Arg plays a critical role in cardiopulmonary hemodynamics and reduced mortality from ascites [40,48]. One way to relieve the formulation pressure of CM based diet may be providing Guanidinoacetic acid (GAA) that can spare the use of Arg for Cre synthesis. GAA is a compound formed from Arg and Gly, and is produced via chemical synthesis from glycine cyanamide commercially as CreAMINO®. Under Hypobaric Hypoxia GAA supplementation had the potential to enhancing growth and preventing right ventricular hypertrophy (RVH) in broiler chickens [1]. Either GAA supplemented as an Arg replacement [8], or as spare Arg in Arg-deficient diets, has been reported to both, but also to improve growth performance in Arg-adequate diets [28]. If the price of GAA is less expensive than, or equal to, commercially available Arg, then GAA would be more beneficial to supplement because of the improvement that observed in Arg-adequate diets. Thus, the poultry farmers may prefer GAA as an Arg replacement, relieving the necessity for Arg supplementation in today poultry diets.

Materials and Methods

Birds and Experimental Facility

This experiment was conducted in a poultry house (2,100 m above sea level) of Shahrekord University in Shahrekord, Iran, where the partial pressure of oxygen is estimated to be 145 mm Hg. The experimental chicks were kept, maintained and treated according to accepted standards for the human treatment of animals. There were 40 litter pens in the house, and 15 chicks (Ross 308) were allocated to each pen (in total 600 day-old male broilers) that all pens had equal initial body weights (600 ± 10g). Then, diets were randomised to pens (8 diets/treatment). Birds were reared in 1.8 m^2 floor pens on 6 cm of wood shavings with a bell drinker and a feed trough with free access. The temperature of the experimental house was maintained at about 32°C, 25°C and 20°C during the first, second and third weeks, respectively, and 15°C thereafter as described previously (24). Birds were subjected to 23h light and 1h dark throughout the trial.

Treatments

Chicks received a commercial Maize-SBM diet formulated for the starting (1 to 10 d of age) stage (Table 1), then Maize-SBM and a Maize-CM diet for growing (11 to 24 d of age) and finishing (25 to 42 d of age) stages according to Nutrition Specifications for ROSS 308 (target live weight 2.50 - 3.00 kg) recommendations (Table 2) in mash form with the following supplementations: (I) SBM, (II) SBM + 0.6g/kg GAA (CreAMINO®, Evonik Degussa Gmbh, Essen, Germany), (III) SBM + 1.2g/kg GAA, (IV) SBM + 2.4g/kg GAA and (V) CM, (VI) CM + 0.6g/kg GAA, (VII) CM + 1.2g/kg GAA, (VIII) CM + 2.4g/kg GAA. Solvent-extracted CM was obtained from a local manufacturer and had low glucosinolate level (5.43 µmol/g obtained by analysis according to the procedure described in a study [18]. All diets had similar metabolizable energy and protein. Replacing SBM with CM tends to reduce the dietary electrolyte balance cause of a lower potassium concentration in CM *vs.* SBM [15]. Therefore, potassium carbonate was added to the CM-based diets to equalize the dietary electrolyte balance of all dietary treatments. The samples of feed ingredients and mixed diets were analyzed for CP and AA content. For that, duplicate samples of each ingredient or diet were subjected to 6 N HCl and hydrolyzed for 24h at 110°C [3]. All samples were analyzed for AA content by using an ion-exchange chromatograph (LKB 4141 Amino Acid Analyzer, LKB Biochrom Ltd., Cambridge, UK) after acid hydrolysis. Performic acid oxidation was done to determine sulfur amino acids as well [29]. The purity of CreAMINO® and molecular weight of GAA were 96% and 117.11g/mol, which accounts for 4.91, 9.83 and 19.67 mol of GAA in every 0.6, 1.2 or 2.4g/kg CreAMINO®, respectively.

Performance and ascites mortality

The weight of pens were recorded at 10, 24 and 42 d of age. Feed

Item (% unless noted)	Starter
Ingredient	
Corn	47.85
Soybean meal (42% CP)	40.03
Soy oil	5.54
Fish meal (60% CP)	3.00
Dicalcium phosphate	1.20
Oyster shell	1.40
Salt	0.35
DL-Methionine	0.15
L-Lysine HCl	0.15
Mineral supplement[1]	0.25
Vitamin supplement[2]	0.25
Calculated composition[3]	
AME (kcal/kg)	3,000.00
CP	23.0
Met + Cys	1.08
Lys	1.44
Arg	1.45
Ca	0.96
Available P	0.48
Na	0.19
K	0.90
Cl	0.25
Na+K-Cl (mEq/kg)	235

Table 1: Composition of the starting diet (Soybean meal) fed from 0 - 10 d of age.

[1] Provided the following per kilogram of diet: vitamin A (trans retinyl acetate), 3,600 IU; vitamin D3 (cholecalciferol), 800 IU; vitamin E (dl-α-tocopheryl acetate), 7.2 mg; vitamin K3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamine, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.

[2] Provided the following per kilogram of diet: Mn (from $MnSO_4 \cdot H_2O$), 40 mg; Zn (from ZnO), 40 mg; Fe (from $FeSO_4 \cdot 7H_2O$), 20 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 4 mg; I [from Ca (IO_3) $2 \cdot H_2O$], 0.64 mg; Se (from sodium selenite), 0.08 mg.

[3] Values in parentheses are amino acid levels obtained by analysis.

Item (% unless noted)	SBM	SBM+ 0.6 g/kg GAA	SBM+ 1.2 g/kg GAA	SBM+ 2.4 g/kg GAA	CM	CM+ 0.6 g/kg GAA	CM+ 1.2 g/kg GAA	CM+ 2.4 g/kg GAA
Ingredient								
Corn	52.88	52.82	52.76	52.64	40.50	40.44	40.38	40.26
Soybean meal (42% CP)	35.07	35.07	35.07	35.07	--	--	--	--
Canola meal (33% CP)	--	--	--	--	44.00	44.00	44.00	44.00
Soy oil	5.00	5.00	5.00	5.00	7.20	7.20	7.20	7.20
Fish meal (60% CP)	3.40	3.40	3.40	3.40	5.35	5.35	5.35	5.35
Dicalcium phosphate	1.40	1.40	1.40	1.40	0.90	0.90	0.90	0.90
Oyster shell	1.30	1.30	1.30	1.30	0.90	0.90	0.90	0.90

Salt	0.35	0.35	0.35	0.35	0.32	0.32	0.32	0.32
DL-Methionine	0.12	0.12	0.12	0.12	--	--	--	--
L-Lysine HCl	0.15	0.15	0.15	0.15	0.20	0.20	0.20	0.20
Mineral supplement[1]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin supplement[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Potassium carbonate	--	--	--	--	0.38	0.38	0.38	0.38
GAA	--	0.06	0.12	0.24	--	0.06	0.12	0.24
Calculated composition[3]								
AME (kcal/kg)	3100	3100	3100	3100	3098	3098	3098	3097
CP	21.50	21.50	21.50	21.50	21.49	21.49	21.49	21.49
Met + Cys	0.98	0.98	0.98	0.98	0.99	0.99	0.99	0.99
Lys	1.41	1.41	1.41	1.41	1.41	1.41	1.41	1.41
Arg	1.42	1.42	1.42	1.42	1.42	1.42	1.42	1.42
Ca	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.95
Available P	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Na	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
K	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Cl	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Na+K-Cl (mEq/kg)	232	232	232	232	232	232	232	232

Table 2: Composition of the growing diets fed from 11 - 24 d of age.

[1] Provided the following per kilogram of diet: vitamin A (trans retinyl acetate), 3,600 IU; vitamin D3 (cholecalciferol), 800 IU; vitamin E (dl-α-tocopheryl acetate), 7.2 mg; vitamin K3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamine, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.

[2] Provided the following per kilogram of diet: Mn (from $MnSO_4 \cdot H_2O$), 40 mg; Zn (from ZnO), 40 mg; Fe (from $FeSO_4 \cdot 7H_2O$), 20 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 4 mg; I [from $Ca(IO_3)2 \cdot H_2O$], 0.64 mg; Se (from sodium selenite), 0.08 mg.

[3] Values in parentheses are amino acid levels obtained by analysis.

Item (% unless noted)	**SBM**	**SBM+ 0.6 g/kg GAA**	**SBM+ 1.2 g/kg GAA**	**SBM+ 2.4 g/kg GAA**	**CM**	**CM+ 0.6 g/kg GAA**	**CM+ 1.2 g/kg GAA**	**CM+ 2.4 g/kg GAA**
Ingredient								
Corn	56.60	56.54	56.48	56.36	47.00	46.94	46.88	46.76
Soybean meal (42% CP)	32.90	32.90	32.90	32.90	--	--	--	--
Canola meal (33% CP)	--	--	--	--	41.00	41.00	41.00	41.00
Soy oil	6.00	6.00	6.00	6.00	7.20	7.20	7.20	7.20
Fish meal (60% CP)	1.00	1.00	1.00	1.00	2.60	2.60	2.60	2.60
Dicalcium phosphate	1.30	1.30	1.30	1.30	1.1	1.1	1.1	1.1
Oyster shell	1.40	1.40	1.40	1.40	0.90	0.90	0.90	0.90
Salt	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
DL-Methionine	0.15	0.15	0.15	0.15	--	--	--	--
L-Lysine HCl	0.10	0.10	0.10	0.10	0.16	0.16	0.16	0.16

Mineral supplement[1]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Vitamin supplement[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Potassium carbonate	--	--	--	--	0.30	0.30	0.30	0.30
GAA	--	0.06	0.12	0.24	--	0.06	0.12	0.24
Calculated composition[3]								
AME (kcal/kg)	3200	3200	3200	3200	3200	3200	3200	3200
CP	19.50	19.50	19.50	19.50	19.50	19.50	19.50	19.50
Met + Cys	0.90	0.90	0.90	0.90	0.90	0.89	0.89	0.89
Lys	1.16	1.16	1.16	1.16	1.16	1.16	1.16	1.16
Arg	1.28	1.28	1.28	1.28	1.28	1.12	1.12	1.12
Ca	0.92	0.92	0.92	0.92	0.92	0.92	0.92	0.92
Available P	0.40	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Na	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
K	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85
Cl	0.22	0.22	0.22	0.22	0.22	0.22	0.22	0.22
Na+K-Cl (mEq/kg)	221	221	221	221	221	221	221	221

Table 3: Composition of the Finisher diets fed from 25 - 42 d of age.

[1] Provided the following per kilogram of diet: vitamin A (trans retinyl acetate), 3,600 IU; vitamin D3 (cholecalciferol), 800 IU; vitamin E (dl-α-tocopheryl acetate), 7.2 mg; vitamin K3, 1.6 mg; thiamine, 0.72 mg; riboflavin, 3.3 mg; niacin, 0.4 mg; pyridoxin, 1.2 mg; cobalamine, 0.6 mg; folic acid, 0.5 mg; choline chloride, 200 mg.

[2] Provided the following per kilogram of diet: Mn (from $MnSO_4 \cdot H_2O$), 40 mg; Zn (from ZnO), 40 mg; Fe (from $FeSO_4 \cdot 7H_2O$), 20 mg; Cu (from $CuSO4 \cdot 5H_2O$), 4 mg; I [from $Ca(IO_3)2 \cdot H_2O$], 0.64 mg; Se (from sodium selenite), 0.08 mg.

[3] Values in parentheses are amino acid levels obtained by analysis.

intake (FI), BW gain (BWG), and feed: gain (FCR) were calculated for the 1 to 10 d, 11 to 24 d, 25 to 42 and overall study period (1-42 d) and mortality adjusted FCR were calculated based on former research [7]. Ascites mortalities were noted as distinguished by the aggregation of fluid in the abdominal cavity and pericardium [44] and the right ventricular weight: total ventricular weight ratio (RV:TV), that values greater than 0.25 are considered as pulmonary [38].

Carcass and internal organ parameters

Data obtained at carcass processing included live BW, hot carcass weight, abdominal fat, breast and thigh weights (% of BW), as well as internal organs weight (% of BW), inclusive liver, spleen, Bursa and hearts then RV/TV ratio calculated.

Blood and plasma parameters

At 42 d of age, 2 birds/pen (10 birds/treatment) that had BW within approximately 5% of the average pen BW were selected. Then blood samples (3 mL) were collected from the brachial vein in heparinized syringes and after centrifuging at 2,500 × g for 10 min at 25°C plasma was obtained. It was used for the measurement of plasma NO (nitrate +nitrite) according to described [14] by adding 250 μL of plasma to 1 mL of Griess reagent. The Griess reagent was a mixture (1:1) of 1% sulfanilamide in 5% phosphoric acid and 0.1% 1-naphthylethylenediamine, giving a red-violet color in presence of nitrite, the stable form of NO. The absorbance was measured at 540 nm by means of a Corning 480 spectrophotometer (Corning Inc., New York, NY). An aliquot of blood from each bird was immediately prepared for blood smears: spread on microscope slides, dried and fixed with methanol. Smears were stained with May- Grunwald and Giemsa stains. The slides were observed under light microscope and 100 white blood cells were numerated in each sample, for the differentiation of white blood cells count. Then Heterophil/lymphocyte (H/L) ratio were calculated. Samples of blood were collected in micro hematocrit tubes for measuring hematocrit. The uric acid (UA) concentration was analyzed according to previous studies [11], and malondialdehyde (MDA) concentration, as biomarker of oxidative stress, was assayed by the method of Nair and Turner [33]. The analyses of urea and creatine plasma were performed by spectrophotometric methods (Alcyon 300, USA) using commercially available kits (Pars Azmun, Tehran, Iran).

Assessment of intestinal morphology

To assess the intestinal morphometry, 2.0-cm segments from the midpoint of the duodenum and jejunum and the distal end of the lower ileum were dissected, flushed with PBS (pH 7), fixed in Clark fixative for 45 min, and stored in ethyl alcohol (50%). Each segment was placed in periodic acid-Schiff for staining. Muscle layers were separated from mucosa; rows of villi were cut in sagittal sections and transferred to glass slides and covered with a coverslip. These samples were examined with a microscope using an optical lens (1,000×). Villus height was measured from the top of the villus to the top of the lamina propria. Surface area was calculated using the formula $2\pi \times (VW/2) \times VL$, where VW is villus width and VL is villus length [52].

Statistical analysis

The Data of the experiment were analyzed in a completely randomized design by GLM procedure of SAS (2002) software and Duncan's multiple range test was used to compare and separate treatment means. The statistical model used for performance data was $Yij = \mu + Ti + eij$. For blood and carcass data, the model was $Yijk = \mu + Ti + eij + \varepsilon ijk$. In these models, Yij and Yijk are observations; μ is the general location parameter (i.e., the mean); Ti is the effect for being in treatment i; eij is random error; and εijk is subsampling error. The mortality data underwent several types of transformations including arcsin and square root before analysis. The differences between the means were significant at $P \leq 0.05$.

Results

Growth Performance and ascites mortality

The results of this study indicated that Feed conversion Ratio (FCR) and feed intake (FI) for SBM diets group in all starting, growing and finishing stages, as well as overall (1-42 d) were not affected by GAA supplementation (Table 4). In contrast, the FI of birds fed on CM diet increased in high levels (2.4g/kg) of GAA, while the weight gain (WG) did not grow as well, so the worst FCR in finishing stage (25-42 days) and overall rearing period (42 days) were observed in CM+2.4g/kg GAA. The best FCR (1-42 days) for both groups (SBM and CM) was in birds fed on 0.6g/kg GAA as supplement while they were not significant ($P > 0.05$). Adding high levels (1.2 and 2.4g/kg) of GAA to the CM treatments exacerbated the FCR (2.097and 2.229, respectively) significantly. The average ascites mortality in broiler chickens fed on CM diet containing 2.4g GAA/kg was higher than other CM or SBM based diets.

Item	Diet								
	SBM	SBM+ 0.6 g/kg GAA	SBM+ 1.2 g/kg GAA	SBM+ 2.4 g/kg GAA	CM	CM+ 0.6 g/kg GAA	CM+ 1.2 g/kg GAA	CM+ 2.4 g/kg GAA	SEM
0-10 d of age									
Feed intake (g/bird)	182.2	180.0	182.2	180.8	182.2	181.2	182.0	179.8	1.46
Weight gain (g/bird)	134.5	134.9	135.1	136.1	134.8	134.3	134.8	134.8	0.81
Feed:gain (g:g)	1.345	1.341	1.340	1.330	1.345	1.347	1.343	1.342	0.0080
11-24 d of age									
Feed intake (g/bird)	988.4	972.9	1002.7	972.4	991.5	1005.2	1006.0	974.9	16.78
Weight gain (g/bird)	604.6^{ab}	580.7^{b}	630.7^{a}	580.2^{b}	521.9^{c}	539.5^{c}	521.2^{c}	527.8^{c}	13.55
Feed:gain (g:g)	1.641^{b}	1.675^{b}	1.590^{b}	1.676^{b}	1.907^{a}	1.867^{a}	1.931^{a}	1.848^{a}	0.0435
25-42 d of age									
Feed intake (g/bird)	2844.1^{b}	2699.3^{b}	2925.1^{b}	2871.5^{b}	2792.8^{b}	2764.2^{b}	2818.7^{b}	3214.5^{a}	98.65
Weight gain (g/bird)	1566.9^{a}	1565.6^{a}	1660.8^{a}	1606.2^{a}	1286.4^{b}	1325.0^{b}	1214.8^{b}	1260.6^{b}	60.32
Feed:gain (g:g)	1.822^{d}	1.727^{d}	1.767^{d}	1.789^{d}	2.182^{bc}	2.084^{c}	2.320^{b}	2.554^{a}	0.0489
1-42 d of age									

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Feed intake (g/bird)	4013.5^{b}	3853.3^{b}	4108.8^{ab}	4024.9^{b}	3965.3^{b}	3950.4^{b}	4005.8^{b}	4370.5^{a}	103.32
Weight gain (g/bird)	2344.6^{a}	2319.8^{a}	2465.2^{a}	2361.0^{a}	1981.4^{b}	2037.4^{b}	1909.3^{b}	1961.8^{b}	63.42
Feed:gain (g:g)	1.716^{d}	1.662^{d}	1.669^{d}	1.705^{d}	2.005^{c}	1.937^{c}	2.097^{b}	2.229^{a}	0.0276
Total mortality (%)	22^{ab}	19^{ab}	11^{b}	13^{b}	23^{ab}	21^{ab}	19^{ab}	32^{a}	5.68
Ascites mortality (%)	20^{ab}	18^{ab}	10^{b}	10^{b}	22^{ab}	20^{ab}	16^{ab}	28^{a}	5.14

Table 4: Effects of GAA supplementation to Soybean Meal or Canola meal-based diets on broiler growth performance[1].

[a-c] Means in the same row with different letters are significantly different ($P < 0.05$).

[1] Each mean represents values from 5 replicates.

Item	Diet								
	SBM	**SBM+ 0.6 g/kg GAA**	**SBM+ 1.2 g/kg GAA**	**SBM+ 2.4 g/kg GAA**	**CM**	**CM+ 0.6 g/kg GAA**	**CM+ 1.2 g/kg GAA**	**CM+ 2.4 g/kg GAA**	**SEM**
Carcass yield (% of BW)	70.5^{a}	71.6^{a}	70.8^{a}	71.0^{a}	70.9^{a}	71.4^{a}	70.7^{a}	68.6^{b}	0.55
Breast yield (% of BW)	26.42^{bc}	28.75^{a}	27.14^{ab}	28.57^{a}	24.17^{d}	23.95^{d}	24.60^{cd}	23.29^{d}	0.703
Thigh yield (% of BW)	27.09	26.89	26.92	28.08	28.43	26.92	27.85	27.77	0.616
Abdominal Fat (% of BW)	1.65^{abc}	1.57^{bc}	1.43^{bc}	1.36^{c}	1.99^{a}	1.78^{ab}	1.65^{abc}	1.72^{abc}	0.125

Table 5: Effect of GAA supplementation to Soybean meal or Canola meal-based diets on carcass characteristics of broilers at 42 d of age[1].

[a,b] Means in the same row with different letters are significantly different ($P < 0.05$).

[1] Except for ascites mortality, each mean represents values from 10 replicates.

[2] Right ventricle weight:total ventricle weight.

Item	Diet								
	SBM	**SBM+ 0.6 g/kg GAA**	**SBM+ 1.2 g/kg GAA**	**SBM+ 2.4 g/kg GAA**	**CM**	**CM+ 0.6 g/kg GAA**	**CM+ 1.2 g/kg GAA**	**CM+ 2.4 g/kg GAA**	**SEM**
Liver (% of BW)	2.59^{ab}	2.29^{b}	2.26^{b}	2.30^{b}	2.78^{a}	2.66^{ab}	2.30^{b}	2.62^{ab}	0.140
Spleen (% of BW)	0.11	0.11	0.18	0.12	0.11	0.12	0.13	0.10	0.023
Bursa (% of BW)	0.060^{ab}	0.045^{b}	0.069^{ab}	0.081^{a}	0.067^{ab}	0.055^{b}	0.060^{ab}	0.058^{ab}	0.0075
Heart (% of BW)	0.65^{c}	0.60^{c}	0.61^{c}	0.62^{c}	0.79^{a}	0.78^{ab}	0.67^{bc}	0.83^{a}	0.038
RV:TV[2]	0.280^{ab}	0.255^{b}	0.233^{b}	0.231^{b}	0.343^{a}	0.303^{ab}	0.251^{b}	0.262^{b}	0.0262

Table 6: Effect of GAA supplementation to Soybean meal or Canola meal-based diets on internal organ parameters of broilers at 42 d of age[1].

[a,b] Means in the same row with different letters are significantly different ($P < 0.05$).

[1] Except for ascites mortality, each mean represents values from 10 replicates.

[2] Right ventricle weight:total ventricle weight.

Item	Diet								SEM
	SBM	SBM+ 0.6 g/kg GAA	SBM+ 1.2 g/kg GAA	SBM+ 2.4 g/kg GAA	CM	CM+ 0.6 g/kg GAA	CM+ 1.2 g/kg GAA	CM+ 2.4 g/kg GAA	
Plasma NO (μmol/L)	11.79^{d}	13.84^{bcd}	14.77^{abc}	17.28^{a}	8.63^{e}	12.17^{cd}	13.45^{cd}	16.42^{ab}	0.939
Malondialdehyde (μmol/L)	4.61^{a}	3.61^{b}	3.02^{c}	3.73^{b}	4.76^{a}	3.91^{b}	3.58^{b}	3.82^{b}	0.107
Urea (μmol/L)	271.8	275.8	277.9	284.0	280.8	287.0	280.3	285.9	7.74
Uric acid (μmol/L)	330.8^{a}	287.7^{b}	234.2^{d}	281.3^{bc}	340.0^{a}	290.7^{b}	240.6^{d}	269.0^{c}	4.73
Creatine (μmol/L)	90.2^{b}	91.9^{b}	93.2^{b}	101.7^{a}	81.9^{c}	83.7^{c}	85.8^{c}	92.8^{b}	1.49
Hematocrit (%)	41.70^{ab}	40.40^{ab}	38.90^{b}	40.30^{ab}	42.10^{a}	39.30^{ab}	39.00^{b}	39.20^{ab}	0.953
Heterophil/lymphocyte	0.721^{a}	0.619^{abc}	0.460^{bc}	0.433^{c}	0.728^{a}	0.641^{ab}	0.586^{abc}	0.602^{abc}	0.0612

Table 7: Effect of GAA supplementation to Soybean meal or Canola meal-based diets on plasma parameters of broilers at 42 d of age[1].

^{a,b} Means in the same row with different letters are significantly different ($P < 0.05$).

[1] Except for ascites mortality, each mean represents values from 10 replicates.

[2] Right ventricle weight:total ventricle weight.

Item (mm unless noted)	Diet								SEM
	SBM	SBM+ 0.6 g/kg GAA	SBM+ 1.2 g/kg GAA	SBM+ 2.4 g/kg GAA	CM	CM+ 0.6 g/kg GAA	CM+ 1.2 g/kg GAA	CM+ 2.4 g/kg GAA	
Duodenum									
Villus height	1.38^{b}	1.62^{a}	1.63^{a}	1.58^{a}	1.27^{b}	1.63^{a}	1.32^{b}	1.31^{b}	0.059
Villus width	0.106^{abc}	0.126^{a}	0.119^{ab}	0.120^{ab}	0.101^{bc}	0.117^{ab}	0.092^{c}	0.110^{abc}	0.0065
Crypt depth	0.138^{c}	0.162^{ab}	0.146^{bc}	0.159^{ab}	0.139^{c}	0.162^{ab}	0.169^{a}	0.167^{a}	0.0056
Villus surface area (mm^2)	0.476^{b}	0.642^{a}	0.608^{a}	0.597^{a}	0.411^{b}	0.608^{a}	0.385^{b}	0.453^{b}	0.0388
Villus height/crypt depth	10.29^{ab}	10.10^{ab}	11.27^{a}	10.17^{ab}	9.15^{bc}	10.10^{ab}	7.82^{c}	7.92^{c}	0.45
Jejenum									
Villus height	1.39^{c}	1.62^{a}	1.48^{abc}	1.41^{bc}	1.13^{e}	1.21^{de}	1.57^{ab}	1.34^{cd}	0.053
Villus width	0.118^{a}	0.124^{a}	0.104^{abc}	0.112^{ab}	0.085^{c}	0.095^{bc}	0.121^{a}	0.109 ^{ab}	0.0066
Crypt depth	0.154^{bc}	0.179^{a}	0.137^{d}	0.158^{b}	0.100^{e}	0.141^{cd}	0.148^{bcd}	0.150^{bcd}	0.0049
Surface area (mm^2)	0.519^{bc}	0.645^{a}	0.500^{bcd}	0.506^{bc}	0.308^{e}	0.374^{de}	0.598^{ab}	0.463^{cd}	0.0426
Villus height/crypt depth	9.07^{b}	9.19^{b}	11.01^{a}	9.05^{b}	11.44^{a}	8.61^{b}	10.68^{a}	9.14^{b}	0.463
Ileum									
Villus height	1.24^{bc}	1.48^{a}	1.32^{b}	1.24^{bc}	0.91^{e}	1.02^{de}	1.11^{cd}	1.03^{de}	0.050
Villus width	0.093^{bc}	0.113^{a}	0.116^{a}	0.101^{ab}	0.079^{c}	0.089^{bc}	0.095^{bc}	0.092^{bc}	0.0058
Crypt depth	0.138^{bc}	0.167^{a}	0.151^{b}	0.140^{bc}	0.093^{e}	0.117^{d}	0.133^{cd}	0.131^{cd}	0.0056
Surface area (mm^2)	0.370^{bc}	0.531^{a}	0.489^{a}	0.400^{b}	0.224^{d}	0.293^{cd}	0.338^{bc}	0.301^{cd}	0.0312
Villus height/crypt depth	9.03^{ab}	9.08^{ab}	9.15^{ab}	9.06^{ab}	10.11^{a}	9.23^{ab}	8.46^{ab}	7.88^{b}	0.561

Table 8: Effects of GAA supplementation to Soybean meal or Canola meal-based diets on broiler gut morphometry[1].

[1] Each mean represents values from 15 replicates.

Carcass and internal organ parameters

The impact of GAA supplementation to SBM or CM-based diets on carcass characteristics of broilers at 42 d of age has been shown in table 5. Adding GAA had not significant effect on abdominal fat and thigh yield in neither SBM nor CM groups. The carcass yield was not affected by diet protein source (SBM or CM) or adding GAA (0.6 and 1.2g/kg diet; $P > 0.05$) but adding 2.4g/kg GAA to the CM diets decreased this parameter, dramatically. In contrast, breast yield increased in SBM group by adding GAA (0.6 and 2.4g/kg diet; $P < 0.05$). As can see in table 6, the weight (% of BW) of liver and heart were decreased with inclusion of GAA at level 1.2g/kg for CM diets ($P < 0.05$), also right ventricle to total ventricle ratio (RV: TV) reduced by adding 1.2 and 2.4g GAA to the CM group. According to results of current study, adding GAA to the broilers feed had no impact of spleen weight (% of BW; $P > 0.05$). About the relative weight of Bursa, GAA supplementation had not impact on this parameter in CM diets, while for SBM diets 0.6g and 2.4gAA per kg diet reduced and accreted bursa weight respectively despite this changes were not significant compare with control group but were significant with each other (0.045 *vs.* 0.081% of BW for 0.6 and 2.4g/kg GAA).

Blood and plasma parameters

Augmenting of GAA to the CM (like SBM)diets in cold stress and hypobaric condition reduced blood Malondialdehyde (MDA), Uric acid (UA), hematocrit, and Heterophil/lymphocyteat 42 d of age (table 7), however, some of these difference in parameters were not significant ($P > 0.05$). On the other hand, plasma NO and creatine increased with doubling the GAA dosage in diets, dramatically. In this study GAA had no effect on plasma urea ($P > 0.05$).

Assessment of intestinal morphology

Morphological measurements in different segments of the small intestine are presented in table 6. Enhancing the diets (SBM or CM) with GAA supplement intensified the villus height, crypt depth and villus surface area of duodenum and the differences were more significant in SBM treatments. Also, supplementation of 0.6g/kg GAA in SBM diets increased the villus height, crypt depth and surface area of jejenum as well as ileum, but in higher dose of GAA (1.2 or 2.4g/kg diet) this significant improvement disappeared. On the other hand, for CM based diets we observed same improvement stream in 1.2g GAA per kg diet. The villus width of duodenum and jejenum was not affected by adding GAA in SBM groups, while GAA promoted the villus width of ileum in SBM diet group, significantly. In the CM treatments, inclusion of GAA (1.2 or 2.4g/kg diet) increased the villus width of jejenum however, has no effect on other parts of small intestine significantly.

Discussion

Growth Performance and ascites mortality

In present study, despite we considered the nutrition specifications for ROSS 308 (target live weight 2.50 - 3.00 kg) recommendations, the broiler performance was fairly poor compared with the estimated weight of breed in its catalog, because the birds were exposed to high altitude (2500m above sea level) as well as cold temperatures. Such high altitude reduces approximately 2% in oxygen availability for every 1,000-m increase in altitude from 20.95% at sea level [16,17].

In this trial, while feed intake in CM based diet was influenced by supplementation of 2.4g GAA per kg diet, WG was not improved, result FCR was deteriorated by 2.4g/kg GAA supplementation to CM control diet. Previous studies indicated an improvement in FCR (28, 8, 30, 13, and 1) or WG (28, 8, and 1) by supplementation of GAA to broiler diets. Despite it was reported that GAA usage as a performance-enhancing agent [36], recent studies (9, 1) showed adding GAA at level 1.2g/kg diet reduced FI of birds grown in normal temperature, with no effect of GAA under cold temperature, as well as our results for birds grown in high altitude and cold condition with SBM diets, also the WG and FCR did not significantly differ compared to other treatments, in both studies. In contrast, birds fed on CM diets supplemented with the higher level of GAA (2.4g/kg) increased FI but deteriorated the FCR under cold temperature and hypobaric hypoxia for the whole of present study period. As table 4 shows, supplementation high level of GAA had no effect on FI or FCR of birds in SBM based diet, so we cannot say GAA caused this effect only. Previous study on CM indicated that substitution of CM for SBM caused a reduction in WG and FI and resulted in impaired FCR [22] as well as our findings.

Generally, CM has 10% lower amino acid digestibility values and contains higher amount of crude fiber (12%) compared with SBM (3-4%) because canola hull (which is a relatively high proportion of the canola seed) stays with the meal. The side chains of the main non-starch polysaccharides of fiber in CM consisting of arabinose, galactose, and xylose residues. Other polysaccharides include cellulose, xylans, arabinoxylans, and xyloglucans, which are predominantly found in the hull fraction. The water-soluble fraction of non-starch polysaccharides tends to increase the digesta viscosity and reduce nutrient digestion and absorption, subsequently resulting in poor growth performance [27]. This increasing in bulk density of the CM diet, per se, results in reduced FI, which was observed in the present study. Furthermore, the presence of phenolic compounds including tannins with bitter flavor in CM causes less palatability and reduced FI and increases endogenous loss of amino acids [26].

Our thesis was GAA supplemented as spare Arg in Arg-deficient (CM) diets, despite some studies indicated that dietary Arg may regulate appetite in ducks [47] or broilers [9] through conversion to nitric oxide, others reported that the growth performance of broilers raised at hypobaric condition with or without cold temperature was not affected by dietary Arg supplementation compared to those fed on a control diet [20].

Yang and Denbow [50] stated that substances like neuropeptide Y and ghrelin provoke feeding through a nitric oxide pathway.

Regard to the ratio of ascites to total mortality, it is evident that ascites was the major cause of death in broiler chickens under conditions of this experiment and that the substitution of CM at the expense of SBM decreased plasma NO concentrations, result higher mortality in broiler chickens [15,22]. Mushtaq., *et al*. [32] stated that inclusion of CM at 300g/kg of broiler diets caused higher mortality, totally. Newkirk and Classen [35] demonstrated that total and ascitic mortality of broilers increased from 5.2% to 13.9% and 1.9% to 9.6%, respectively, by replacing SBM with CM between 19 and 39 days of age. They used a low-glucosinolate CM and were conducted under thermo neutral condition in a low land area. Khajali., *et al*. [22] indicated ascites mortality increased from 10.7% in broilers fed the SBM-based diet to 16.0% in those fed the CM-based diet. They suggested that lower endothelial NO synthesis aggravated the onset of pulmonary hypertension, as a possible reason for the high rate of ascites mortality induced by CM. Also, it has been shown that NO benefits the antioxidant system by eliminating reactive oxygen species (ROS) such as superoxide and hydrogen peroxide and protects cells from apoptosis [39,52].

Carcass and internal organ parameters

In this trial, the substitution of CM for SBM had no significant impact on carcass and thigh yields, however breast yields and heart weight (as a percentage of BW) were significantly reduced and increased, respectively. Khajali., *et al.* [22] reported carcass and breast yields reduced while thigh yield and heart weight increased as a result of substituting CM for SBM according to NRC [34] recommendation, however, in present trial diets were balanced according to nutrition specifications for ROSS 308 recommendations. So, we can suggest that with higher ME and CP the broilers can tolerate such a hard condition and secure the carcass yield.

Considering the lower percentage of breast (glycolytic tissues) beside the unchanged thighs (oxidative tissues) when SBM replaced with CM, can suggests that feeding CM exacerbated PHS as reflected by higher RV:TV and higher mortality from ascites. This shift to oxidative tissues and its correlation with PHS has been acknowledged [19].

Newkirk and Classen [35] demonstrated that feeding CM resulted in a linear increase in heart weight as a proportion of the BW, which is similar to the results of Khajali., *et al*. [22] and present study. It can be described that in broilers with pulmonary hypertension, the heart must pump blood through the lungs at a higher pressure, so the wall of the right ventricle undergoes characteristic work hypertrophy and higher RV:TV index. This extra pressure on the heart also is reflected in the higher heart weight in broilers fed the CM-based diet compared with those fed the SBM-based diet.

In the present experiment, the RV:TV and plasma NO levels were inversely related. When compared with birds fed the SBM-based diet, birds fed the CM diet had a higher RV:TV, indicating the development of pulmonary hypertension. Despite adding all levels of GAA ameliorate the RV:TV index, about the relative heart weight, only 1.2g GAA per kg diet reduced the value.

It has been reported, that Arg [20] or GAA [30] supplementation did not affect carcass yield, except that addition of GAA significantly reduced the relative weight of liver, which the last is same as current study while the changes in liver weights were not significant for diets based on SBM but were for CM ones. In contract, a significant increase in yields of carcass and breast was observed when GAA was added at 1 and 1.5g/kg [1]. Michiels., *et al*. [28] reported a significant effect on yield of breast when GAA was added at 0.6 and 1.2g/kg to broiler diets. Mousavi., *et al*. [30] supplemented broiler diets with 0.6g/kg GAA at different metabolizable energy levels and did not observe any difference for carcass components.

Dilger., *et al*. [8] stated that GAA might be important in poultry nutrition in order to support overall energy homeostasis of the bird; an impact which is beyond the Arg sparing effect of GAA.

Emami., *et al*. [9] demonstrated that birds reared under cold stress had higher ($P \leq 0.05$) relative spleen weight than those under normal temperature, in contrast, birds receiving a diet supplemented with 1.72g/kg Arg had lower relative spleen weight compared to other dietary treatments. However, in our study birds were exposed to cold temperature for all days of trial and relative weight of spleen was not affected by adding GAA supplementation or SBM replacement with CM.

The positive effect of Arg in reducing abdominal fat deposition in broilers [6] and quails [2] had been observed, and recently the same effect for GAA in broilers [1]. About L-Arg supplementation, it may reduce fat deposition by modulating lipogenesis, by reducing the expression of lipogenic genes or increasing the expression of genes associated with lipolysis [5,49].

Blood and plasma parameters

Complete substitution of CM for SBM caused a significant reduction in serum NO concentration. In fact, the lower content of Arg in CM group resulted in a lower serum NO level, because Arg serves as precursor for NO, as a relaxing factor, NO as a vasodilatation is responsible for regulation of blood pressure [41].

It has been reported; about 40 to 60% of the urea circulating in birds is the result of ornithine synthesis [37]. Arginine transfers a guanidino group to glycine, forming ornithine and guanidine acetate. In a next reaction, GAA is methylated by S-adenosyl methionine to form S-adenosylhomocysteine and creatine. According to mentioned pathway, despite altered urea levels were expected by the dietary replacement of SBM with CM, but urea level did not alter. A hypothesis that may explain is that the both diets were faced to dietary Arg deficiency resulted in all Arg utilized to meet the broilers' requirements.

Creatine is the only organic compound that is involved in protein metabolism and that participates in the muscular energy buffering system [23]. Michiels., *et al*. [28] stated, supplementing a diet with GAA (0.6 and 1.2g/kg) increased the creatine concentration in broiler breast meat, obviously. In this sense, GAA supplementation can be particularly important in broiler diets with fast initial growth due to their high energy requirements to supply muscle creatine [4].

Assessment of intestinal morphology

The most information on the effect of GAA supplement on intestinal morphology in poultry has addressed the role of Arg on gut morphology and function. Reported benefits of Arg supplement on morphometry of the duodenum mucosa [31] and increased villus height, width and absorptive surface area in the jejunum [20]. This increasing in villus height and total luminal villus absorptive area, results in satisfactory digestive enzyme action and higher transport of nutrients at the villus surface [43], as well as, stimulated the growth intestinal epithelial cells by up regulating gene expression of the target of rapamycin cell-signaling pathway that promoted protein synthesis and reduced protein degradation, in the intestine of chicken [51]. Results of present study showed that villus surface area in cold-stressed birds fed on a SBM or CM based diet supplemented with 0.6 or 1.2g/kg GAA was improved, similar to what Ahmadipour., *et al*. [1] observation for GAA supplementation in SBM based diets and Emami., *et al*. [9] who reported same for supplementing of 1.72g/kg Arg in diet and resulted in villus surface area improved to the extent that was similar to those grown in normal temperature.

Conclusion

As a conclusion, thanks to balanced diets according to nutrition specifications for ROSS 308 (target live weight 2.50-3.00 kg) recommendations instead of NRC [34] which has more dietary protein composition (average 1.5%) that cause marginally adequate levels of Arg for broilers grown at high altitudes, the carcass yield and feed intake had not affected by complete substitution of CM for SBM, while a concomitant decrease in serum NO concentration and a significant increase in RV/TV and mortality from ascites. The results of this study indicate that feeding CM based diets supplemented with the higher level of GAA (2.4g/kg) promote the FI of birds grown in cold temperature for the whole experimental period.

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