ARTICLE INFORMATION	Fill in information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Effects of arginine and guanidinoacetic acid supplementation with or without phenylalanine on performance and ascites susceptibility in cold-stressed broilers fed canola meal-based diet
Running Title (within 10 words)	Reduced adverse effects of canola meal feeding to cold-stressed broilers
Author	Negin Delfani[1], Mohsen Daneshyar[1], Parviz Farhoomand[1], Younes Ali Alijoo[1], Sina Payvastegan[1], Gholamreza Najafi[2]
Affiliation	[1]Department of Animal Science, Faculty of Agricultural Sciences, Urmia University, PO Box 165, Urmia, Iran [2]Department of Basic Sciences, Faculty of Veterinary Medicine, Urmia University, PO Box 165, Urmia, Iran
ORCID (for more information, please visit https://orcid.org)	Negin Delfani (https://orcid.org/0000-0002-4199-3104) Mohsen Daneshyar (https://orcid.org/0000-0003-1854-210X) Parviz Farhoomand (https://orcid.org/0000-0002-9572-0486) Younes Ali Alijoo (https://orcid.org/0000-0001-7012-2407) Sina Payvastegan (https://orcid.org/0000-0003-1965-9027) Gholamreza Najafi (https://orcid.org/0000-0002-1085-6043)
Competing interests	No potential conflict of interest relevant to this article was reported.
Funding sources State funding sources (grants, funding sources, equipment, and supplies). Include the name and number of the grant if available.	Not applicable.
Acknowledgements	The authors appreciate Vatankhah A. M (Department of Drug Applied Research, Faculty of Medical Sciences, Tabriz University, Tabriz, Iran) for the help in the determination of blood parameters and greatly acknowledge the representative of Evonik Degussa in Iran for supplying GAA and NIR analysis.
Availability of data and material	Upon a reasonable request, the datasets of this study can be available from the corresponding author.
Authors' contributions Please specify the authors' role using this form.	Conceptualization: Delfani N., Daneshyar M, and Payvastegan S. Data curation: Daneshyar M. and Farhoomand P. Formal analysis: Daneshyar M, and Payvastegan S. Methodology: Delfani N, Najafi G, Alijoo YA., and Najafi Gh. Software: Payvastegan S. Validation: Daneshyar M, Farhoomand P, and Alijoo YA. Investigation: Delfani N and Payvastegan S. Writing the original draft: Delfani N and Payvastegan S. Writing, review, and editing: Delfani N. Daneshyar M, Farhoomand P., Payvastegan S., Alijoo YA., and Najafi Gh.
Ethics approval and consent to participate	The experiment was performed at the Research Farm of the Urmia University of Iran. The Animal Care and Use Committee of Urmia University reviewed and approved the protocol for this study.

The corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in the information in each box below
First name, middle initial, last name	Mohsen Daneshyar
Email address – This is where your proofs will be sent	m.daneshyar@urmia.ac.ir

Secondary email address	daneshyar_mohsen@yahoo.com
Address	Department of Animal Science, Faculty of Agricultural Science, Urmia University, PO Box 165, Urmia, Iran
Cell phone number	00989141402759
Office phone number	00984431942341
Fax number	00984432759558

ABSTRACT

In order to evaluate the effects of ARG sources (ARG and GAA) and PHE supplementation on performance, susceptibility to ascites, intestinal morphology, and nutrient digestibility in the cold-stressed broilers fed a canola meal (CM)-based diet, a 2×2 factorial experiment with four treatments was conducted. The dietary treatments included CM-based diet + 2.57 g/kg ARG, CM-based diet + 2.57 g/kg ARG + 1.5 g/kg PHE, CM-based diet + 1.8 g/kg GAA and CM-based diet + 1.8 g/kg GAA + 1.5 g/kg PHE. The corn-CM diet without supplementation was used as a negative control (NC) group in the fifth treatment that excluded the factorial arrangement. The results showed that adding ARG to diets without PHE supplement increased ($p < 0.05$) feed intake. Also, birds fed diets containing ARG had higher ($p < 0.05$) body weight gain (BWG) compared to those fed GAA added diets. Supplementation of PHE improved ($p < 0.05$) the FCR compared to groups fed diets without added PHE. Further, ARG addition increased ($p < 0.05$) plasma nitric oxide (NO) concentration, carcass, breast and leg yields, duodenal, jejunal and ileal villus height (VH) to crypt depth (CD, and dry matter digestibility, while decreasing ($p < 0.05$) ascites mortality and right ventricle (RV) to total ventricle (TV) ratio compared to GAA added groups. Supplementation of PHE also declined susceptibility to ascites by reducing ($p < 0.01$) RV to TV ratio while increasing ($p < 0.05$) plasma NO level. The digestibility of ether extract also increased ($p < 0.05$) in broilers fed GAA supplemented diets versus those fed ARG added diets. The findings suggested that ARG may improve BWG and lower ascites incidence in broilers fed a diet based on CM under cold stress because of its antihypertensive effects. Moreover, the findings of this study demonstrated the importance of including PHE formulation in ARG-deficient diets to attenuate the adverse effects of cold stress on broilers. It was also concluded that GAA could be efficaciously used in cold-stressed broilers fed an ARG-deficient diet.

Keywords: Arginine, Ascites susceptibility, Canola meal, Guanidinoacetic acid, Performance, Phenylalanine

INTRODUCTION

Ascites, also known as pulmonary hypertension syndrome, has been observed in fast-growing broilers throughout the world [1]. Rapid growth and low ambient temperature increase the metabolic rate, which results in a high oxygen requirement [2]. A disparity between oxygen demand and oxygen supply leads to hypoxemia [3]. Daneshyar et al. [4] demonstrated that exposure to cold temperature increased the right ventricle to total ventricle ratio (RV/TV) and the ascites mortality of broilers. Some studies have also reported that dietary protein quality and amino acid composition affect the incidence of ascites syndrome in broilers [5, 6]. The oil industry in Iran is dependent on canola since it yields three times as much oil per acre as soybean [7]. The increasing canola production provides an opportunity to use the leftover by-product called canola meal (CM) as a poultry feed ingredient.

Ascites susceptibility in broilers seems to be related to some essential amino acids. The CM contains less lysine, arginine (ARG), and phenylalanine (PHE) but more sulfur-containing amino acids compared to soybean meal, which is considered the best vegetable protein source for broilers [5, 6, 8]. The digestibility of ARG in CM is also much lower than that of soybean meal [5, 6]. Additionally, a portion of the ARG in CM might be needed for metabolic processes related to tannic acid excretion [9].

ARG is the biological precursor of nitric oxide (NO) in virtually all cell types [9]. NO acts as a potent vasodilator that directly relaxes vascular smooth muscle and modulates or inhibits the release of vasoconstrictors such as serotonin and endothelin-1 [6]. Attenuating the initiation of pulmonary hypertension syndrome is an essential function for ARG and NO in broiler chickens [9]. It has been suggested that the levels of required ARG for proper growth are insufficient for macrophages and pulmonary vascular epithelium to achieve maximum NO production [10]. Furthermore, increasing dietary CM inclusion may not provide sufficient ARG for pulmonary vascular endothelial NO production [5, 6]. Newkirk and Classen [11] showed that replacing high levels of CM in broiler diets increases the incidence of chronic heart failure. In addition, birds may require more ARG because of increased oxygen consumption when they are exposed to extremely low temperatures [12]. Previous studies confirmed that providing extra ARG in the diets of broiler chickens alleviated the adverse effect of cold stress on performance, gut development, and ascites mortality [12, 13].

There is no economically available source of ARG [14]. A feed additive that can be applied instead of ARG for other metabolic functions could help alleviate formulation limits regarding ARG supply. Guanidinoacetic acid (GAA), a compound formed from ARG and glycine, is the natural precursor of creatine in the vertebrate body [12]. Creatine synthesis represents a considerable proportion of whole-body ARG usage [14]. Thus, providing GAA via commercially available supplements is one strategy to make ARG available for other metabolic functions, including NO synthesis. Dilger et al. [12] found that GAA could efficiently replace dietary ARG in ARG-deficient diets for young chicks.

The bioavailability of tetrahydrobiopterin as a redox-sensitive cofactor is critical for NO synthesis by NO synthase [15]. Tetrahydrobiopterin is de novo synthesized from guanosine triphosphate by guanosine triphosphate cyclohydrolase I action [16]. According to available evidence, PHE increases the expression and activity of GTP cyclohydrolase I, thereby increasing the availability of tetrahydrobiopterin for NO synthesis [17]. The PHE content

of CM is about two-thirds that of soybean meal [8]. Increased oxidative degradation of tetrahydrobiopterin is also one reason for its decreased bioavailability and uncoupling of NO synthase in endothelial cells [15]. Oxidative stress is one of the factors behind pulmonary hypertension. Reactive oxygen species (ROS) may contribute to pulmonary hypertension because they promote vasoconstriction, smooth muscle cell proliferation, and vascular remodeling [18] and may trigger heart failure and pulmonary hypertension in broilers under hypoxia and oxidative stress [19].

In feed formulations, alternative vegetable protein meals (e.g., CM) other than the soybean meal are preferred if they are less expensive [7]. On the other hand, more information is needed on the use of CM as a replacement for soybean meal on the susceptibility to ascites in broilers. Additionally, insufficient knowledge is available on the effects of CM-based diets supplemented with ARG and GAA, alone or in combination with PHE, on broiler chickens under ascites inducing cold ambient conditions. Therefore, the current study aimed to determine the effects of the supplementation of a CM-based diet with ARG and GAA with or without added PHE on performance, susceptibility to ascites, carcass traits, internal organ weights, plasma parameters, intestinal morphology, and apparent nutrient digestibility in cold-stressed broiler chickens.

MATERIAL AND METHODS

Animal care

The experiment was performed at the Research Farm of the Urmia University of Iran. The Animal Care and Use Committee of Urmia University reviewed and approved the protocol for this study (Approval Number 2074/PD/3).

Treatments and bird husbandry

In total, 450 one-day-old Hubbard male broiler chicks were purchased from a local hatchery and were individually weighed upon arrival. The chicks were divided into 30 pens based on their initial body weight (BW: 40.31 ± 2.11 g). A 2 × 2 factorial trial involving four treatments was conducted to investigate the effects of ARG sources (ARG and GAA) and PHE, and their interaction effects in CM–based diets. The dietary treatments included 1) corn-CM based diet + 2.57 g/kg ARG (L-ARG, Merck Millipore Co., Darmstadt, Hesse, Germany), 2) corn-CM based diet + 2.57 g/kg ARG + 1.5 g/kg PHE (L-PHE, Merck Millipore Co., Darmstadt, Hesse, Germany), 3) corn-CM based diet + 1.8 g/kg GAA (CreAMINO® , Evonik Degussa Gmbh, Essen, Germany) and 4) corn-CM based diet + 1.8 g/kg GAA + 1.5 g/kg PHE. The corn-CM diet without supplementation was used as the negative control (NC) group in the fifth treatment. A NC group that was not included in the factorial arrangement. Treatments were repeated with six-floor pens, each housing fifteen chicks. The purity of CreAMINO® was 96%, and the molecular weight of GAA was 117.11 g/mol, providing 14.76 mol of GAA in each 1.8 g/kg CreAMINO® . ARG is supplemented as a pure source (100%) at isomolar quantity relative to GAA through multiplying 14.76 mol into its molecular weight (174.2 g/mol).

Cold stress was used to trigger ascites in this study and was provided according to the method of Varmaghany et al. [20]. For this purpose, all groups were presented to a temperature of 32 ˚C at one-day old, with stepwise decreases to 25 ± 1 ˚C, 20 ± 1 ˚C, and 15 ± 1 ˚C on d 7, 14, and 21, respectively. At this point, a temperature of 15 ± 1 ˚C was kept up until the end of the experiment. The room temperature was controlled by a thermostat and thermometer located at three different points in the house and was recorded every five hours. Wood shavings were employed as bedding

materials. Feed as mash and drinking water were provided ad libitum throughout the experimental period. The light regimen was continuous for the first week and then was shortened to 23 hours of light. A 38-d experiment was conducted on starter (d 0 to 10), grower (d 11 to 22), and finisher (d 23 to 38) stages.

Diets

Prior to the trial, the applied corn, CM and gluten for formulating the experimental diets were analyzed for dry matter (DM), apparent metabolizable energy, crude fiber (CF), acid detergent fiber, neutral detergent fiber, crude protein (CP), and standard ileal digestible amino acid contents by near-infrared reflectance spectroscopy (NIRS, Paya Amin Mehr, Tehran, Iran). The data on the CP and amino acid composition of CM are presented in Table 1. All diets were formulated according to Hubbard nutrient specifications for macro and micro nutrients for starter, grower, and finisher periods (Table 2). The experimental diets were also formulated to meet the amino acid requirements based on Hubbard's recommendations of standardized ileal digestible amino acids for broilers. The samples of mixed diets were analyzed for CP and amino acid contents. To determine the CP of the diets, nitrogen was determined by the Kjeldahl procedure using the factor 6.25 to convert nitrogen into CP. For amino acid analyses, the samples of each diet were hydrolyzed in 6 N HCl for 24 h at 110 ˚C under an atmosphere of nitrogen. For methionine and cysteine, performic acid oxidation was performed before acid hydrolysis. The hydrolysate samples were analyzed for amino acid content through ion-exchange chromatography (LKB Biochrom 4141, UK).

Data collection and sampling

Feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) were calculated for the whole period of the study. The mortality was recorded once observed to adjust FCR data. Mortalities were considered ascitic by the fluid accumulation in the abdominal cavity and pericardium [20], and the RV/TV ratio was higher than 0.25 [12]. On day 38, three birds randomly selected from each replicate pen (18 birds/treatment) were euthanized by carbon dioxide asphyxiation to evaluate carcass characteristics. All viscera were immediately removed, and then the weights of carcass, breast, leg, heart, liver, pancreas, gizzard, lung, bursa of Fabricius, spleen, and abdominal fat were obtained using a digital scale. All data regarding carcass parts and organ weights were expressed as a percentage of live BW. After determining the whole weight of the heart, the right ventricle was dissected away from the left ventricle and the septum and separately weighed to compute the RV to TV ratio.

Intestinal morphology

At the end of the experiment (38 d), the same eighteen chickens from each treatment (three birds per replicate) were also selected for small intestinal status evaluations. After eviscerating and rinsing, the small intestine was collected, weighed, and expressed as a percentage of live BW. The three segments of the small intestine (duodenum, jejunum, and ileum) were also excised, weighed, and calculated in relation to live BW. For histomorphometry, the intestinal segment samples (approximately 2 cm in length) of the duodenum, jejunum, and ileum were excised and flushed with 0.9% saline to remove the contents. The segments of the intestine were taken from the loop of the duodenum, the midpoint between the bile duct entry and Meckel's diverticulum (jejunum), and midway between Meckel's diverticulum and the ileocecal junction (ileum). All samples were fixed in 10% buffered formalin for histological evaluation. Following fixation, the samples were trimmed, cleared, dehydrated, and embedded in paraffin. Serial

sections were cut at 7 μm by the use of a microtome (Microm, HM 335) and placed on glass slides. After deparaffinization in xylene, the sections were rehydrated in graded ethanol solutions, stained with hematoxylin and eosin, and examined under a light microscope. There were three cross-sections per sample (54 cross-sections for each treatment) and ten measurements per cross-section (540 measurements per treatment). Intestinal morphological measurements included villus height (VH) and crypt depth (CD) in each segment. VH was measured from the tip of the villus to the top of the lamina propria, and CD was calculated from the villus-crypt axis to the tip of the muscularis mucosa. Then, the ratio of VC to CD was estimated by dividing the VH by the CD.

Plasma parameters

On the morning of day 38, blood samples (5 ml) were collected from the same three birds in each pen by venipuncture (wing vein). Heparin-containing glass tubes were used to collect the samples. Blood samples were centrifuged at 3500 × *g* for 10 min at 4 ˚C to obtain plasma. The obtained heparinized plasma samples were stored at -20 C until further analyses of glucose, albumin, total protein, uric acid, blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK) by commercial enzymatic kits (Pars Azmoon Kits, Pars Azmoon Inc., Tehran, Iran) using a plasma autoanalyzer (Abbott Laboratories, Illinois, US). Plasma samples were also employed to determine total antioxidant capacity (TAC), malondialdehyde (MDA), and NO levels. Plasma TAC and MDA levels were measured using Zellbio kits (ZellBio GmbH Co., Ulm, Germany) by the colorimetric method as described by the manufacturer. Moreover, plasma NO was measured by the total NO assay kit (NatrixTM, West Azerbaijan, Iran) according to the manufacturer's instructions.

Digestibility assay

At the end of the experiment (day 38), five birds from each pen were randomly selected and euthanized by CO_2 asphyxiation. Ileal digesta were collected from the entire ileum (a portion of the small intestine from Meckel's diverticulum to 5 mm proximal to the ileocecal junction) by flushing with distilled water. The collected ileal samples were pooled within replicates and stored in a freezer at -20 ˚C until processing. The frozen digesta samples were thawed, lyophilized, and ground using an electric coffee grinder to make finely ground samples while avoiding significant loss. All diets and ileal digesta samples were analyzed for DM, CP, ether extract (EE), CF, gross energy (GE), and titanium (Ti). The samples were placed in a dry oven (105 ˚C) for 24 hours to determine the amount of DM. Crude protein was calculated by converting nitrogen to CP using the factor 6.25, which was determined by the Kjeldahl procedure. According to previous research, the ether extract was determined by the Soxtec extraction procedure (Method 920.39) [21]. CF and GE were also determined by the Association of Official Analytical Chemists method 962.09 [21] and using a bomb calorimeter (Parr 6200 bomb calorimeter; Parr Instruments Co., Moline, IL.) standardized with benzoic acid, respectively. Ti dioxide was included in the feed at 5 g/kg as an indigestible marker. Diet and digesta Ti concentrations were determined in duplicate in accordance with the procedures described in previous research [22]. The apparent ileal digestibility of nutrients and GE were computed using the following formula:

$$\text{Digestibility of diet component (\%)} = \frac{[(Component/Ti)\ diet - (Component/Ti)\ ileal]}{(Component/Ti)\ diet}$$

where (Component/Ti) diet = Ratio of component to Ti in the diet, and (Component/Ti) ileal = Ratio of component to Ti in the ileal digesta.

Statistical analyses

After the NC group was excluded, the data were analyzed as a completely randomized design with a 2 × 2 factorial arrangement of treatments using the General Linear Models procedures of SAS (version 9.1, SAS Institute, Cary, NC, USA). The following statistical model was used for the factorial analysis of the four treatments:

$Y_{ijk} = \mu + A_i + P_j + (A \times P)_{ij} + e_{ijk}$

In this model, Y_{ijk} is the dependent variable, μ is the overall mean, A_i is the main effect of ARG sources (i= ARG or GAA), P_j is the main effect of PHE supplementation (j= with or without PHE), $(A \times P)_{ij}$ is the interaction effect of ARG sources and PHE supplementation and e_{ijk} is the random error. Also, data from five groups, including the NC group, were analyzed as a completely randomized design to determine differences between NC and the other four groups, as well as the NC group and GAA supplemented groups. All data were tested for normality using the univariate procedure of SAS before analysis of variance. Although actual means were presented, a log transformation was used for mortality data to obtain a normal distribution. The treatment means compared by the PDIFF option with adjustment for the Tukey test in SAS when a significant effect was detected. Differences were considered significant at $p < 0.05$.

RESULTS

Performance and susceptibility to ascites

The results on FI, BWG, and FCR in the whole period of the study are provided in Table 3. The interaction between ARG sources and PHE supplementation was observed for FI ($p < 0.05$), showing that addition of ARG to diets without PHE supplement increased FI, but not in diets containing PHE. Supplementation of ARG also significantly ($p < 0.05$) improved BWG compared to GAA supplemented groups, but similar ($p > 0.05$) BWG was observed between groups fed diets supplemented with and without PHE. Supplementation with PHE reduced ($p < 0.05$) FCR in comparison to the bird fed diets without PHE, while the birds received either ARG or GAA added diets did not differ ($p > 0.05$). There were no interaction effects ($p > 0.05$) between ARG sources and PHE supplementation on BWG and FCR. Orthogonal contrast comparison also indicated that broilers receiving diets supplemented with ARG sources with or without supplemental PHE presented higher ($p < 0.05$) BWG and lower ($p < 0.05$) FCR compared with broilers fed the NC diet. In addition, the orthogonal contrast of GAA supplemented diets versus NC was significant ($p < 0.05$) for BWG and FCR. However, FI were similar ($p > 0.05$) among all experimental groups.

Table 3 presents the effects of dietary ARG and GAA supplementation with or without added PHE on ascites susceptibility. Broilers fed ARG supplemented diets had higher ($p < 0.05$) plasma NO level and lower ($p < 0.05$) RV to TV ratio and ascites mortality compared to those fed GAA added diets. Supplementing with PHE also increased ($p < 0.05$) NO concentration and reduced ($p < 0.05$) RV to TV ratio compared to groups did not receive PHE supplement. The supplementation of ARG sources and PHE did not significantly ($p > 0.05$) affect heart weight as a percentage of

BW. Moreover, NO level, RV to TV ratio, heart weight, and ascites mortality were not affected ($p > 0.05$) by the interaction between ARG sources and PHE supplementation.

Based on orthogonal contrast analysis, the plasma NO level of broilers fed diets supplemented with ARG and GAA alone or in combination with PHE and also GAA supplemented groups were higher ($p < 0.05$) than those fed NC diet. As compared to other experimental groups, broilers fed the NC diet had also higher ($p < 0.05$) RV to TV ratio, heart relative weight, and ascites mortality. Furthermore, the orthogonal contrast between GAA supplemented diets and the NC group was significant ($p < 0.05$) for heart relative weight and RV to TV ratio.

Carcass parameters and internal organ weights

Table 4 summarizes the carcass parameters and internal organ weights of broilers fed experimental diets at 38 d. The addition of ARG significantly ($p < 0.05$) increased the carcass, breast and yields compared to the GAA supplemental groups. A lower ($p < 0.05$) carcass yield was also observed in broilers fed PHE added diets compared with birds fed diet without PHE. However, breast and leg yield values were similar ($p > 0.05$) among PHE supplemental groups and who did not receive PHE supplement. Birds fed the GAA supplemented diets exhibited significant ($p < 0.05$) decline in liver weight compared to the ARG containing groups. Supplementation of experimental diets with PHE significantly ($p < 0.05$) increased the relative weights of the pancreas and bursa in comparison to other dietary treatments without supplemental PHE. Nevertheless, the supplementation of ARG or GAA with or without supplemental PHE did not significantly ($p > 0.05$) affect gizzard, abdominal fat and spleen relative weights. Moreover, no interaction ($p > 0.05$) effects were observed between ARG sources supplements and PHE supplements for all carcass traits or internal organ weights.

Orthogonal contrast analysis also revealed that carcass, breast, leg and pancreas relative weights were lower ($p < 0.05$) and liver and abdominal fat relative weights were greater ($p < 0.05$) in NC group compared with those fed the other diets. Gizzard, Spleen bursa relative weights, however, were comparable ($p > 0.05$) among all experimental groups. Moreover, carcass and leg yields of broilers supplemented by GAA were higher ($p < 0.05$) and liver and abdominal fat relative weights were lower ($p < 0.05$) than that for NC group.

Intestinal morphology

Table 5 lists the measured relative weights and morphometric parameters of intestinal segments at the end of the experiment. The current study found no significant ($p > 0.05$) differences in the relative weights of the small intestine, duodenum, jejunum, and ileum between broilers fed various diets. Supplementation of ARG did not affect CD or VH ($p > 0.05$) in the duodenum, however, a higher ($p < 0.05$) ratio of VH to CD was detected in comparison with GAA supplementation. In the jejunum, although ARG supplementation had no ($p > 0.05$) effect on CD, higher ($p < 0.05$) VH and VH to CD ratio were found compared to GAA supplemented groups. Ileal VH and VH to CD ratio increased ($p < 0.05$) and CD decreased ($p < 0.05$) following addition of ARG compared to GAA supplementation. Neither the addition of PHE nor its interaction with ARG sources supplementation caused significant ($p > 0.05$) effects on duodenal, jejunal, and ileal morphometric parameters.

Likewise, the orthogonal comparison analysis results showed that NC-fed broilers had greater ($p < 0.05$) relative weights of the duodenum, jejunum, and small intestine compared to the other four treatments and GAA supplemented groups. However, there was no significant ($p > 0.05$) difference in the relative weight of the ileum between experimental groups. For each of the three parts of the small intestine, the VH and VH to CD ratio were higher ($p < 0.05$) in the groups fed diets supplemented with ARG sources and PHE than in the group fed a NC diet. Also, the CD in the duodenum and ileum of the NC group was higher ($p < 0.05$) compared to the other four dietary treatments, but not ($p > 0.05$) in the jejunum. Dietary supplementation of GAA increased ($p < 0.05$) duodenal, jejunal and ileal VH to CD ratio compared to NC group. Likewise, the VH in jejunum and ileum were higher ($p < 0.05$) in GAA supplemented diets compared with the NC group, while the CD in the duodenum was lower ($p < 0.05$).

Plasma parameters

Table 6 presents the measured concentration of plasma parameters at the end of the experiments at 38 d. The results revealed that the supplementation of ARG significantly ($p < 0.05$) decreased plasma concentrations of AST, LDH, uric acid, and creatinine compared with the GAA supplemented groups. Conversely, broilers fed ARG had higher ($p < 0.05$) plasma BUN levels than those fed GAA. Moreover, adding GAA to diet that contained PHE supplement increased ($p < 0.05$) plasma glucose concentration. Nonetheless, the plasma levels of ALT, CK, TAC, MDA, albumin and total protein were not affected ($p > 0.05$) by dietary treatments.

Contrast analyses indicated no significant ($p > 0.05$) differences between NC and other four treatments for plasma ALT, CK, total protein and albumin concentrations. However, in the NC group, the levels of AST, LDH, uric acid, and MDA were higher ($p < 0.05$) and the levels of creatinine, and TAC were lower ($p < 0.05$) than those in the other four experimental groups. There were also no significant ($p > 0.05$) differences between the groups fed diets containing GAA and the NC group regarding ALT, CK, and albumin. The NC group, on the other hand, had greater ($p < 0.05$) levels of AST, LDH, uric acid, total protein and MDA and lower ($p < 0.05$) levels of creatinine and TAC than the GAA supplemented groups.

Digestibility assay

Data on the effects of ARG and GAA supplementation alone or in combination with PHE on the apparent ileal digestibility of DM, CP, EE, CF and GE are summarized in Table 7. The results demonstrated that the ileal digestibility of DM in birds fed diets supplemented with ARG was greater ($p < 0.05$) than the values for birds fed diets containing GAA. The groups fed GAA had also higher ($p < 0.05$) ileal EE digestibility in comparison with the ARG-fed groups. However, the ileal digestibility values of GE, CP and CF did not differ ($p > 0.05$) among the treatment groups. The orthogonal comparison analysis also revealed that the ileal digestibility of DM, GE, CP, EE and CF in the NC group were lower ($p < 0.05$) than those of the groups receiving ARG, GAA, ARG plus PHE and GAA plus PHE supplements. Further, GAA supplementation also increased ($p < 0.05$) the ileal digestibility of CP, EE and GE in comparison to the NC group.

DISCUSSION

This study did not include a control group raised under normal conditions. The feeding of CM-based diets and the cold stress induction method used in the current study have been shown to induce ascites in previous studies [5, 6, 20]. In addition, high ratio of RV to TV and ascites mortality in the NC group indicate ascites induction in the current study. Using 2.57 g/kg ARG without adding PHE in the CM-based diet resulted in higher FI compared to the other treatments, which is in line with the results of Wang et al. [23], Kodambashi Emami et al. [12], and Castro et al. [24]. Wang et al. [23] found that dietary ARG could regulate appetite by converting ARG to NO. Neuropeptides such as neuropeptide Y were reported to increase FI through the NO pathway [23]. CM contains a limited amount of digestible ARG [5]. Amino acid requirements may also increase under suboptimal conditions such as cold stress. According to Castro et al. [24], dietary ARG deficiency decreases appetite, which may explain why cold-stressed broilers fed a diet supplemented with 2.57 g/kg ARG had a higher FI compared to those fed diets without supplemental ARG in the current study.

Nonetheless, adding ARG with PHE decreased FI compared to the ARG supplemented group. Keene and Austic [25] concluded that PHE was anorexic in leghorn chicks. The exact cause of reducing FI by introducing PHE is unknown. Still, it can be due to the function of PHE as a precursor to tyrosine synthesis and thus catecholamines secretions. Katayama et al. [26] demonstrated a dose-dependent decrease in FI following the intracerebroventricular injection of norepinephrine in chickens. Another reason for decreased FI following the supplementation of PHE, according to Lartey and Austic [27], is probably the negative effect of excess PHE on serotonin secretion. FI is thought to be influenced by serotonin, which is produced by serotonergic neurons in the brainstem from dietary tryptophan [28]. Excess PHE may reduce the transport of other amino acids across the blood-brain barrier, especially tryptophan, lowering serotonin levels [29]. In addition, it has been suggested that some amino-acid-sensing receptors, like the calcium-sensing receptor in the gastrointestinal tract, trigger satiety by sensing amino-acids released from protein digestion [30]. The PHE is the most potent amino-acid activator of the calcium-sensing receptor [31]. Alamshah et al. [30] found that PHE reduced food intake in rats and suggested that PHE anorexic properties are mediated through the calcium-sensing receptor. In chickens, the calcium-sensing receptors exhibit similar properties to those in mammals [32]. It is noteworthy that adding PHE to the ARG-containing diet reduced FI, but did not affect FI in the GAA-containing diet, suggesting an interaction between ARG and PHE supplements. Increased plasma NO level could enhance feed intake [23], however, high levels of NO in broilers fed ARG plus PHE are likely to adversely affect feeding pattern.

Supplementation of ARG led to a significant rise in BWG, which is in agreement with the findings of previous studies [33, 34]. Several factors may contribute to this issue, including higher FI in the ARG-fed group, supply excess ARG demand caused by cold stress exposure, and CM-based diet feeding, enhancing protein synthesis, reducing protein degradation in skeletal muscle [34], and involving ARG in creatine, proline, glutamine, and polyamines synthesis [6, 12].

Likewise, the present study's findings showed that broilers' FCR decreased by adding PHE supplements. To the best of our knowledge, the information related to the effects of PHE supplementation on performance in broilers is scanty.

Nevertheless, the lower FCR of broiler following PHE addition may be caused by reducing FI but not BWG in PHE supplemented groups. Moreover, it is believed that the amount of amino acid necessary to improve FCR is higher than the other performance objectives, although the exact cause is unknown [35], which may play a role in improving FCR in broilers fed diets containing PHE supplement versus those fed diets without added PHE. The orthogonal contrast analysis also revealed that experimental groups fed diets supplemented with ARG and GAA with or without PHE and also GAA supplemented groups had better FCR and BWG than NC group. Supplementation of GAA has demonstrated improved FCR [35; 36; 37; 38] and BWG [14; 39] in broiler chickens as a result of its sparing effect on ARG.

The addition of ARG in CM-based diets increased the concentrations of plasma NO in broilers raised under cold stress. Physiological NO levels produced by pulmonary endothelial cells mediates the marked reduction in pulmonary vascular resistance when cardiac output represents an increase [13]. Synthesis of NO is limited in birds feeding on low ARG diets such as a CM-based diet [5, 40]. Furthermore, exposure to stressful conditions raises the need for all nutrients such as ARG. An increased plasma concentration of NO in broilers receiving supplemental ARG can be attributed to rises in available ARG for NO synthesis. Other studies have also revealed that the addition of ARG to the diets induced increases in the plasma NO concentrations of broilers raised under cold stress [13] or reared at high altitudes [6, 41].

Moreover, birds fed with PHE added diets had also higher plasma NO levels compared to those fed with diets without PHE. NO synthases require a surprisingly rich selection of cofactors to perform the conversion of ARG to citrulline and NO [15, 17]. The depletion of intracellular tetrahydrobiopterin levels reduces NO production [15]. Supplementation of PHE greatly enhanced the synthesis of biopterins, but ARG had no effect in this regard [17]. Therefore, it appears that PHE-containing diets have a higher availability of tetrahydrobiopterin for NO synthase activity.

Cardiac output is elevated to provide more oxygen to the tissues in response to cold stress conditions [6]. Consequently, the right side of the heart has to work harder to push blood through the lungs at high pressure. In broilers, the increased workload of the heart is reflected in the higher heart weight and RV wall thickness [6]. It was observed that the RV to TV ratio is closely related to the incidence of ascites [4]. The RV to TV ratio greater than 0.25 to 0.30 was considered an index of ascites in broiler chickens [20]. Reduced availability of NO impairs cardiovascular function [42]. Limited bioavailability of tetrahydrobiopterin also causes uncoupling of the NO synthase, thus leading to superoxide formation instead of NO [43]. Increased NO synthesis could counteract hypoxic contraction of the pulmonary arteries [12], which may alleviate workload and pressure on the RV. A higher RV to TV ratio indicates impaired endothelium-dependent vasodilation mediated by NO [13]. In the current study, significant lower RV to TV ratios in ARG and PHE supplemented groups were associated with a reduced pumping activity for greater oxygenation due to elevated plasma NO level. In accordance with our result, Khajali et al. [6] reported that supplementation of ARG decreased the RV to TV ratio of broiler chickens raised at high altitudes. In a rodent model of hypertension, Heikal et al. [42] also showed that dietary PHE supplementation increased vascular tetrahydrobiopterin and NO levels, and improved vascular relaxation.

The dietary supplementation of ARG alleviated ascites mortality in the present study, which may be attributed to the higher plasma NO concentration in these birds when compared to those fed a GAA added diet. The impairment of NO-mediated endothelium-dependent vasodilation is likely to play an important role in the pathogenesis of pulmonary hypertension [13]. Increasing the NO plasma level counteracts pulmonary vascular constriction and resistance by inducing the relaxation of the vascular smooth muscle [12]. Similarly, other studies such as Khodambashi Emami et al. [12], Tan et al. [13], and Sharifi et al. [41] reported the effect of ARG on lowering ascites mortality.

Orthogonal contrast analysis of broilers fed the NC diet showed lower concentration of NO than those fed the other experimental diets as well as those fed the GAA supplemented diets. Few studies evaluated the effect of GAA on NO production in ARG-deficient diets. Wu and Meininger [44] found that animals fed insufficient ARG had impaired NO synthesis by constitutive and induced NO synthases. The increase in plasma NO of GAA supplemented groups was probably due to ARG being spared from serving as a precursor for creatine synthesis. Thus, it was available for other uses throughout the body, including NO synthesis [35, 45]. In studies conducted by Ale Saheb Fosoul et al. [40], Ahmadipour et al. [46], and Raei et al. [47], increased NO concentrations were reported following GAA supplementation. According to an orthogonal contrast analysis, feeding experimental diets containing ARG and GAA with or without PHE also decreased RV to TV ratio, relative heart weight, and ascites mortality, possibly due to the higher plasma NO levels of birds fed these diets.

Broilers fed ARG supplemented diets had higher percentage yields of the carcass, breast, and leg meat, which is in line with findings of Khajali et al. [6], Kodambashi Emami et al. [12], Ale Saheb Fosoul et al. [40], and Ebrahimi et al. [48]. The insulin-like growth-I factor is known to cause multiple anabolic effects on skeletal muscle metabolism, including satellite cell proliferation [49]. In the study by Xu et al. [34], the plasma insulin-like growth factor-I and growth hormone levels increased as the dietary ARG level increased from 0 to 1.8% in broiler chickens. It was also found that ARG increased protein synthesis, while decreasing protein degradation through upregulating the gene expression of the target of the rapamycin signaling pathway, as well as suppressing the expression of cathepsin B and 20S proteasome [50]. Additionally, ARG increases NO production, thus relaxing blood vessels and increasing blood flow. The increase in blood flow allows muscles to receive more nutrients.

Nevertheless, the present study has found that adding PHE reduced carcass yield, which could be explained by the fact that excess PHE interferes with the transport of other amino acids, particularly tryptophan, from the blood-brain barrier and reduces serotonin production [28]. The neuromodulator serotonin is involved in a variety of physiological processes such as feeding behavior [51]. Also, oversupply of one amino acid may interfere with the absorption of other amino acids, leading to a nutritional imbalance or deficiency [52]. The reduction in FI observed in the current study in response to PHE supplementation could affect the intake of essential amino acids and may disrupt the amino balance required for tissue protein synthesis. There is no doubt that further studies are required to clarify the issue. Tamimie and Pscheidt [52] and Franco et al. [29] found that excess levels of PHE reduced BWG of broiler chickens, but in the present study only a numerical decrease in BWG was observed.

The obtained result showed that dietary GAA supplementation led to a significant reduction in the proportional weight of the liver. Avian lipogenesis occurs primarily in the liver, and a decrease in liver proportion could be indicative of

reduced lipogenesis, due to the inhibitory effects of GAA [40, 41]. Similarly, Mousavi et al [36], Ale Saheb Fosoul et al. [40] and Ahmadipour et al [46] also found that supplementing broiler diets with GAA decreased liver weight. The supplementation of ARG also inhibits the hepatic lipogenesis [41, 48, 53], however, GAA appears to provide a more potent effect than ARG in this regard. Besides having a spare effect on ARG, GAA may be able to spare glycine. It has been shown that glycine can lower lipogenic enzyme activity while increasing lipase activity [54].

Another finding in the current study was that dietary PHE increased relative bursa weight. No report is available about effect of PHE supplementation on the weight of lymphatic organs in chickens. Bursa is unique primary lymphoid organ that plays a crucial role in B cell differentiation and immunoglobulin production [55]. The development status of bursa is usually evaluated by its relative weight [56]. Therefore, the increase bursa weight as a result of adding PHE may have a beneficial effect on the immune system. In broiler chickens, relative lung weight and lung volume were significantly lower than in native and layer chickens [57]. Relative lung weight and volume also decreased with age in broiler chicks, which coincided with the appearance of ascites [57]. However, ARG and PHE, which lowered ascites susceptibility, decreased the relative lung weights in our study. This may be due to higher concentration of NO in broilers given ARG and PHE, which attenuates the effects of cold stress and hence does not cause the lungs to expand in response to stress. Dietary supplementation of CM-based diets with PHE increased the relative weight of pancreases. Steinert et al. [58] concluded that aromatic amino acids could act as the potent secretagogues of cholecystokinin. Wu et al. [59] also reported the trophic effects of exogenous cholecystokinin on the pancreas in rats.

Additionally, orthogonal contrast analysis showed broiler chickens fed NC diet had lower carcass, leg, and breast yields but had higher abdominal fat and liver weight compared with those fed GAA supplemented diets or other experimental groups. According to studies performed by Ale Saheb Fosoul et al. [40], Michiels et al. [45] and Ahmadipour et al. [46], GAA supplementation improved carcass, leg, and breast yields. DeGroot et al. [14] found an increase in breast muscle phosphocreatine (i.e., ATP ratio, phosphocreatine, and total creatine concentrations) in response to dietary GAA supplementation at 0.06 and 0.12%. Thus, GAA supplementation in an ARG-deficient diet may lead to improved energy homeostasis in muscle cells, resulting in increased broiler carcass, leg and breast yields. Another reason for the increased carcass, leg and breast yields in broilers fed GAA-containing diets is likely the ARG sparing effect of GAA, suggesting that more ARG would be available for pathways involved in protein synthesis [35, 60]. Likewise, Ebrahimi et al. [48] and Fouad et al. [53] observed reductions in abdominal fat relative weights due to ARG supplementation. Ale Saheb Fosoul et al. [40] also found that supplementing broiler diets with GAA decreased abdominal fat content. The decreases in percentage of abdominal fat and the relative weight of the liver are probably due to the inhibitory effects of ARG and GAA on lipogenesis [40, 41, 48].

Cold stress affects the function and morphology of the small intestine in broilers [61]. The small intestine increases in size as it works hard in digestion [62]. The ratio of VH to CD is an important indicator of intestinal digestion and absorption capacity [40]. The longer villi enable birds to utilize their feed better, which leads to better health [63]. On the other hand, deeper crypts indicate that tissue regeneration processes rapidly occur, particularly when pathogens or their toxins are present [63]. The present study results revealed that ARG supplementation increased the VH to CD ratio of all small intestine segments. Oso et al. [64] reported a linear and quadratic increase in duodenal and ileal VH by supplementing ARG to growing turkey diets. In another study, Kodambashi Emami et al. [12] observed an increase

in the jejunal VH to CD ratio by feeding 0.86 and 1.72 g/kg ARG in broilers grown at cold temperatures. Abdulkarimi et al. [65] also demonstrated that ARG supplementation increased the VH to CD ratio in the duodenum and jejunum of broilers reared under a cold environment.

Increased VH and VH to CD ratio indicated that intestinal epithelial cell proliferation had accelerated, reflecting the probable occurrence of rapid protein synthesis [66]. Improvements in the small intestinal morphometric changes in cold-stressed birds fed a CM-based diet are likely due to four reasons. First, ARG is involved in producing polyamines, which are essential for cell division, and it has been shown that a deficiency or lack of polyamines inhibits intestinal cell proliferation [40]. In addition, a previous study confirmed that ARG and its product NO enhanced protein synthesis while reducing protein degradation in broiler intestinal cells by activating the target of cell-signaling rapamycin pathway gene expression [50]. Further, it is known that ARG has a secretagogue function that stimulates the release of pituitary and gastrointestinal hormones, resulting in an increase in protein synthesis and mucosal growth in the small intestine [67]. Finally, Gao et al. [67] identified the role of ARG in promoting gut health by modulating intestinal immune and barrier functions. It is noteworthy that the improvement of small intestinal morphology may cause an increase in the BWG of cold-stressed broilers fed a CM-based diet supplemented with ARG in the present study.

Supplementation with ARG sources and PHE influenced the morphometric characteristics and various segments relative weights in the small intestine. In the current trial, broilers fed diets supplemented with GAA had a greater VH to CD ratio and lower small intestine, duodenum and jejunum relative weights compared to NC group. The increase in the VH to CD ratio agrees with the result of Kodambashi Emami et al. [12], indicating that GAA supplementation at 1.2 g/kg increased the VH to CD ratio in cold-stressed birds. Additionally, Ren et al. [68] found that duodenal, jejunal, and ileal VH to CD ratios increased linearly and quadratically by increasing the GAA supplementation level from 0.4 to 1.2 g/kg in broiler chickens. A notable portion of the observed changes in the jejunal tissue of birds on fed diets containing GAA may be attributed to the sparing effect of GAA in reducing the ARG need for creatine synthesis and thus increasing the availability of ARG for other metabolic pathways. Additionally, upon absorption from the small intestine, the GAA included in the diet will be converted to creatine. Glover et al. [69] found that dietary creatine supplementation promotes intestinal epithelial restitution and ameliorates mucosal inflammation through increasing epithelial cell energy. Moreover, GAA could increase energy supply for cell accretion and protein synthesis by other energy-related metabolites such as phosphocreatine and ATP [68].

The current study observed that the addition of ARG decreased LDH and AST levels at 38 d. These findings are in line with the findings of Emadi et al. [70], demonstrating that dietary ARG supplementation reduced AST and LDH levels in broiler chickens challenged with infectious bursal disease vaccine. It is unclear how liver enzymes such as ALP, ALT, AST, and LDH are related to ascites incidence in broilers. ALT, AST, LDH and CK activity assays could be used to detect cell injuries. Plasma elevations in AST and ALT enzymes represent leakage from damaged hepatocytes and are a reliable indicator of liver injury [71]. AST was also released into the bloodstream by the damaged heart [72]. LDH is commonly present in high concentrations in the liver, heart, erythrocytes, skeletal muscles, and kidneys. Diseases affecting these organs have been linked to significant increases in plasma LDH activity [73].

The addition of ARG also increased BUN concentrations. In line with the current finding, Ebrahimi et al. [48] and Emadi et al. [70] found that adding ARG to the diet increased the plasma levels of BUN in broiler chickens (183% of the Ross catalog value and 250% of the NRC value, respectively). Kidney arginase activity is readily upregulated when excess ARG is included in the diet, resulting in an increased rate of ARG degradation to urea and ornithine [74]. Consistent with the present result, Jahanian and Khalifeh-Gholi [33] reported that adding ARG 110% of NRC values to broiler diets decreased the plasma concentration of uric acid in broiler chickens. Uric acid is the end-product of the protein catabolism in avian species [46]. Plasma uric acid and excreta uric acid may properly evaluate the amino acid utilization and dietary protein quality in boilers [7; 75]. Reduced production of uric acid implies improved dietary protein utilization [46, 76]. Donsbough et al. [75] observed that methionine deficiency in corn-soy bean based diet increased plasma uric acid concentrations, whereas methionine supplementation decreased plasma uric acid concentration. Consequently, ARG supplementation in an ARG deficient diet for broilers under cold stress may improve the utilization of dietary protein as well as the ratio of ARG to lysine.

The plasma creatinine level was elevated by the dietary inclusion of 1.8 g/kg GAA. Similar results were observed when broilers [76] and Japanese quails [47] were fed diets supplemented with GAA. Creatinine is the end-product of skeletal muscle creatine and phosphocreatine degradation and diffuses from the muscle into the bloodstream before being excreted by the kidneys [77]. According to DeGroot et al. [14] and Michiels et al. [45], the rise in the plasma creatinine concentration in broilers fed the GAA supplement may be attributed to increases in skeletal muscle creatine and phosphocreatine contents.

Orthogonal contrast results showed that experimental treatments and groups supplemented with GAA had lower plasma concentrations of AST and LDH compared with the NC group, which can be attributed to improved antioxidant status in those treatments. Evidence indicates that the physiological concentrations of ARG and NO have antioxidant properties. Izadi Yazdanabadi et al. [78] found that ARG supplementation increased TAC concentrations while decreasing the MDA level in the plasma of broiler chickens. ARG has been shown to have vigorous radical scavenging activity against oxygen radicals [79]. Furthermore, ARG plays a role in NADPH regulation, which is required for glutathione production from glutathione disulfide. This may improve the body's antioxidant status by increasing the glutathione to glutathione disulfide ratio and lowering lipid peroxidation [80]. NO is also considered a superoxide radical scavenger responsible for inactivating superoxide radicals [79]. Our findings suggest that a higher level of ARG is needed for the antioxidant defense system to reduce the susceptibility of body tissues and cells to lipid peroxidation in cold-stressed broiler chickens fed with a CM-based diet.

There is limited information about the effects of exogenous GAA on the oxidant-antioxidant system in chickens. GAA has been observed to have an antioxidant effect, either directly or indirectly [60]. Ahmadipour et al. [46] and Raei et al. [47] indicated that the optimal level of the GAA exerts antioxidant activity, whereas high levels may serve as a prooxidant. Additionally, Amiri et al. [81] concluded that adding 0.6 g/kg GAA significantly improved antioxidant status in broilers fed low-protein diets under heat stress. It has also been found that creatine, the end-product of GAA, can act as an antioxidant [82]. Despite the above-mentioned reasons, GAA could also improve the antioxidant status in cold-stressed chickens by increasing ARG availability for other metabolic pathways, including antioxidant

protection. The GAA may also have a sparing effect on glycine, thus allowing for more glycine to be provided for glutathione synthesis.

Oxidative stress is one of the main causes of ascites in broilers exposed to cold temperatures [66]. Excessive production of reactive oxygen species may contribute to the destruction of pulmonary vascular endothelium and increase its membrane permeability. Endothelial cell damage increases pulmonary vascular resistance, leading to pulmonary hypertension [9, 84]. The improvement in antioxidant status may also be a contributing factor in reducing the ascites mortality of birds fed ARG, ARG plus PHE, GAA and GAA plus PHE added diets compared to birds fed NC diets in the present study. Regarding uric acid, Ahmadipour et al. [46] also observed decreased plasma uric acid levels in broiler chickens when 0.5-2 g/kg of GAA were added to the diets. Interestingly, GAA have sparing effects on ARG and therefore can serve as a source of ARG in ARG deficient diets and improve the utilization of amino acids.

In agreement with the findings of this study, Oso et al. [64] found that increasing ARG supplementation from 0 to 1 g/kg resulted in linear increases in apparent digestibility of DM in grower turkeys. Castro et al. [85] also revealed that supplemental ARG quadratically increased the ileal digestibility of DM in broiler chickens. The current study results showed that dietary ARG supplementation improved broiler absorption capacity by increasing the VH to CD ratio in the duodenum, jejunum, and ileum. Moreover, Gao et al. [86] demonstrated that *in ovo* injection of ARG increased digestive and absorption capacity in 21-day-old broiler chickens by enhancing gastrointestinal hormone secretions, digestive enzyme activities, and the mRNA expression of Jejunal sensing receptors and nutrient transporter. Based on the findings of Zhang et al. [87] on the ileal microbiota population, dietary ARG supplementation also reduced clostridium perfringens colonization and mucosal damage in broiler chickens. According to the above-mentioned results, factors contributing to improved DM ileal digestibility following ARG supplementation may include an increase in digestion and absorption capacity, modulating gut microbiota, and facilitation of gastrointestinal hormone release.

There are a few studies on the effect of GAA supplementation on nutrient digestibility in broiler chickens. For instance, Raei et al. [47] found that dietary supplementation of GAA at all tested levels (0.6, 1.2, and 1.8 g/kg) increased the digestibility of DM, CP, EE, and Ash in laying Japanese quails. However, in the present study, only EE digestibility improved following GAA supplementation. Dietary GAA supplementation can also spare glycine from endogenous creatine synthesis. Glycine is the major component of bile salts (glycocholic acid), accounting for 90% of the secreted AA in the bile juice [88]. Bile acids are synthesized in the liver by cholesterol and then conjugated with glycine or taurine in liver peroxisomes [88]. In the present study, supplementing GAA might have increased the availability of glycine in other metabolic pathways, including the synthesis of bile salts.

According to the results of the orthogonal contrast analysis, feeding diets supplemented with ARG and GAA with or without PHE enhanced the ileal digestibility of DM, CP, EE, CF and GE compared with the NC group. The results of Oso et al [64] in turkeys and Castro et al [85] in broilers indicated that ARG supplementation improved energy, CP and DM digestibility. Ale Saheb Fosou et al. [76] reported that supplemental GAA enhanced dietary net energy for production in broiler chickens. A study by Mousavi et al. [36] also found that GAA supplementation resulted in improved energy efficiency of broiler chickens. The stimulating effect of PHE on cholecystokinin may increase

pancreatic secretions and improve nutrient digestion. Aromatic amino acids PHE and tryptophan are the most effective in promoting cholecystokinin secretion [89]. No studies have been conducted regarding the effect of PHE on nutrient digestibility in broilers, so further studies are needed. The increase in digestibility of CF in broilers fed ARG or GAA with or without PHE or in broilers fed diets supplemented with GAA compared to the NC group may be due to the effects of these supplements on the microbial population of the gut. Several studies have demonstrated the impact of ARG on the microbial population [87, 90, 91]. Currently, no data are available relating to the response of the gut microbial population to PHE or GAA supplementation in broiler chickens.

CONCLUSION

In general, the findings indicated that adding ARG to CM-based diets improved BWG, carcass traits, digestibility of DM, and small intestinal digestive capacity while decreasing ascites susceptibility in cold-stressed broiler chickens. Feeding CM-based diets containing PHE supplement improved FCR, increased plasma NO level, and reduced RV to TV ratio in cold-stressed broiler chickens. Consequently, PHE supplementation may also be beneficial in alleviating the adverse effects of cold-stress induction and high levels of CM substitution in broilers, along with ARG and GAA treatments. Furthermore, orthogonal contrast results showed that GAA supplementation could be helpful in cold-stressed broilers fed ARG deficient practical diets, as evidenced by a reduction in FCR and improvements in carcass yield, TAC, small intestine digestion capacity, and digestibility of CP, EE, and GE.

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Table 1. DM, AME, CF, ADF, NDF, CP and standardized ileal digestible amino acid contents (%) of CM sample via NIRS (DM basis)

Item	**CM**
DM	92.92
AME (kcal/kg)	1870
CF	13.00
ADF	19.50
NDF	29.20
CP (N×6.25)	38.94
Arginine	2.06
Lysine	1.42
Methionine	0.58
Cysteine	0.77
Methionine + cysteine	1.34
Threonine	1.04
Tryptophan	0.41
Valine	1.40
Phenylalanine	1.21
Leucine	2.00
Isoleucine	1.12
Histidine	0.84

DM: Dry matter; AME: Apparent metabolizable energy; CF: Crude fiber; ADF; Acid detergent fiber; NDF: Neutral detergent fiber; CP: Crude protein; CM: Canola meal; NIRS: Near-infrared reflectance spectroscopy.

Table 2. Composition of basal diets and calculated and analyzed composition (as-fed basis)

Ingredients (%)	Starter (d 0 to 10)	Grower (d 11 to 22)	Finisher (d 23 to 38)
Corn	54.10	56.05	56.19
Canola meal (38.94 % CP)	30.00	30.00	30.00
Soybean oil	3.51	4.46	6.09
Dicalcium phosphate	2.61	2.28	1.93
Calcium carbonate	0.60	0.79	0.72
Gluten (80% CP)	6.53	4.10	2.98
Mineral premix*	0.25	0.25	0.25
Vitamin premix**	0.25	0.25	0.25
Sodium chloride	0.13	0.21	0.21
L-lysine HCl	0.83	0.63	0.54
DL-methionine	0.13	0.15	0.11
L-threonine	0.21	0.20	0.17
Potassium carbonate	0.49	0.40	0.32
Sodium bicarbonate	0.36	0.24	0.24
Titanium dioxide	0	0	0.50
Calculated composition (%)			
Metabolizable energy (Kcal/kg)	3000	3050	3150
Crude protein	22	20	19
Crude fiber	5.21	5.22	5.20
Calcium	1.00	1.00	0.90
Available phosphorus	0.50	0.45	0.40
Digestible arginine	0.92	0.88	0.86
Digestible lysine	1.23	1.06	0.98
Digestible methionine + Cysteine	0.90	0.85	0.78
Digestible threonine	0.78	0.72	0.67
Digestible phenylalanine	0.79	0.70	0.66
Na + K - Cl (mEq/kg)	213.18	197.11	189.53
Analyzed composition (%)			
Crude protein	21.94	19.97	18.96
Crude fiber	5.23	5.22	5.21
Total arginine	1.04	1.00	0.98
Total lysine	1.35	1.17	1.09
Total methionine + cysteine	1.02	0.97	0.90
Total threonine	0.91	0.85	0.80
Total phenylalanine	0.88	0.79	0.75

*Provided the following per kilogram of diet: $MnSO_4 \bullet H_2O$, 60 mg; $FeSO_4 \bullet 7H_2O$, 80 mg; ZnO, 51.74 mg; $CuSO_4 \bullet 5H_2O$, 8 mg; iodized NaCl,0.8 mg; Na_2SeO_3, 0.2 mg.

**Provided the following per kilogram of diet: retinyl acetate: 9,000 IU; cholecalciferol: 2,000 IU; dl-α-tocopheryl acetate: 12.5 IU; menadione sodium bisulfite: 1.76 mg; biotin: 0.12 mg; thiamine: 1.2 mg; riboflavin: 3.2 mg; calcium d-pantothenate: 6.4 mg; pyridoxine: 1.97 mg; nicotinic acid: 28 mg; cyanocobalamin: 0.01 mg; choline chloride: 320 mg; folic acid: 0.38 mg.

Table 3. Effects of supplementation of ARG sources and PHE levels on performance (0 to 38 d) and ascites susceptibility in cold-stressed broilers fed a CM-based diet.

Item[†]	FI (g/bird)	BWG (g/bird)	FCR	NO (μmol/l)	Heart (% of BW)	RV/TV	Ascites mortality (%)
Factorial analysis							
ARG sources							
ARG	2478.6[a]	1310.4[a]	1.89	45.55[a]	0.551	23.80[b]	10.00[b]
GAA	2343.5[b]	1238.8[b]	1.89	38.65[b]	0.572	27.03[a]	15.33[a]
SEM	27.52	21.74	0.023	0.840	0.009	0.476	1.86
PHE levels							
0 g/kg	2511.4[a]	1289.0	1.95[a]	40.49[b]	0.559	26.12[a]	13.33
1.5 g/kg	2310.7[b]	1260.2	1.84[b]	43.72[a]	0.565	24.72[b]	12.00
SEM	27.52	21.74	0.023	0.840	0.009	0.476	1.86
ARG sources × PHE levels							
ARG × 0 g/kg	2626.4[a]	1338.7	1.97	43.81	0.540	24.19	10.67
ARG × 1.5 g/kg	2330.9[b]	1282.0	1.82	47.29	0.562	23.41	9.34
GAA × 0 g/kg	2396.5[b]	1239.2	1.93	37.16	0.578	28.05	16.00
GAA × 1.5 g/kg	2290.5[b]	1238.4	1.85	40.14	0.568	26.02	14.67
SEM	38.91	30.74	0.033	1.19	0.013	0.673	2.62
p-value							
ARG sources	0.003	0.033	0.939	<.0001	0.108	<.0001	0.046
PHE levels	<.0001	0.364	0.004	0.008	0.641	0.042	0.618
ARG sources × PHE levels	0.027	0.377	0.317	0.833	0.254	0.355	0.896
Orthogonal analysis							
NC[§]	2394.7	1149.2	2.09	31.14	0.645	30.06	24.00
EG[&]	2411.1	1274.6	1.89	42.10	0.562	25.42	12.67
GAA[*]	2343.5	1238.8	1.89	38.65	0.573	27.04	15.34
p-value I[1]	0.739	0.002	0.0001	<.0001	<.0001	<.0001	0.006
p-value II[2]	0.346	0.0270	0.0003	<.0001	<.0001	0.001	0.073

[†]Means in the same column with different superscripts differ significantly ($p < 0.05$).
[§]NC: Negative control group (birds fed CM-based diets without supplements), n = 6 replicates; for each replicate, n = 15 birds.
[&]EG: Experimental groups (other four treatments fed diets with supplements), n = 24 replicates; for each replicate, n = 15 birds.
*GAA: GAA supplemented groups, n = 12 replicates; for each replicate, n = 15 birds.
[1]I: Orthogonal contrast of NC vs. EG
[2]II: Orthogonal contrast of NC vs. GAA.
SEM: Standard error of the means; ARG: Arginine; GAA: Guanidinoactic acid; PHE: Phenylalanine; FI: Feed intake; BWG: Body weight gain; FCR: Feed conversion ratio; NO: Nitric oxide; RV/TV: Right ventricle weight to total ventricle weight ratio.

Table 4. Effects of supplementation of ARG sources and PHE levels on carcass parameters (% of BW) and organ weights (% of BW) in cold-stressed broilers fed a CM-based diet at 38 d.

Item[†]	Carcass	Breast	Leg	Liver	Spleen	Bursa	Lung	Gizzard	Pancreas	Abdominal fat
Factorial analysis										
ARG sources										
ARG	63.74[a]	22.34[a]	20.17[a]	2.48[a]	0.132	0.202	0.540[b]	1.95	0.304	1.281
GAA	61.82	20.82[b]	19.51[b]	2.28[b]	0.124	0.201	0.578[a]	2.03	0.299	1.346
SEM	0.440	0.291	0.209	0.050	0.003	0.006	0.012	0.042	0.006	0.034
PHE levels										
0 g/kg	63.47[a]	21.44	19.87	2.32	0.127	0.192[b]	0.578[a]	1.96	0.279[b]	1.275
1.5 g/kg	62.09[b]	21.72	19.82	2.45	0.130	0.213[a]	0.540[b]	2.02	0.324[a]	1.351
SEM	0.440	0.291	0.209	0.050	0.003	0.006	0.012	0.042	0.006	0.034
ARG sources × PHE levels										
ARG × 0 g/kg	64.81	22.39	20.35	2.39	0.131	0.192	0.553	1.91	0.280	1.26
ARG × 1.5 g/kg	62.67	22.28	19.99	2.58	0.134	0.213	0.526	2.00	0.323	1.30
GAA × 0 g/kg	62.14	20.49	19.39	2.25	0.123	0.192	0.601	2.01	0.279	1.29
GAA × 1.5 g/kg	61.50	21.15	19.63	2.32	0.126	0.210	0.555	2.06	0.319	1.40
SEM	0.632	0.412	0.296	0.070	0.004	0.008	0.017	0.059	0.008	0.050
p-value										
ARG sources	0.003	0.0005	0.031	0.006	0.084	0.891	0.034	0.210	0.480	0.187
PHE levels	0.030	0.507	0.849	0.068	0.519	0.015	0.041	0.300	<.0001	0.123
ARG sources × PHE levels	0.236	0.355	0.312	0.380	0.988	0.851	0.596	0.667	0.550	0.545
Orthogonal analysis										
NC[§]	57.99	20.08	18.18	2.96	0.138	0.204	0.601	2.10	0.279	1.60
EG[&]	62.78	21.58	19.84	2.39	0.129	0.202	0.559	2.00	0.300	1.31
GAA[*]	61.82	20.82	19.51	2.26	0.126	0.201	0.578	2.04	0.299	1.35
p-value I[1]	<.0001	0.002	<.0001	<.0001	0.052	0.769	0.039	0.108	0.014	<.0001
p-value II[2]	<.0001	0.152	0.0003	<.0001	0.051	0.742	0.291	0.337	0.050	<.0001

[†]Means in the same column with different superscripts differ significantly ($p < 0.05$).
[§]NC: Negative control group (birds fed CM-based diets without supplements), n = 6 replicates; for each replicate, n = 15 birds.
[&]EG: Experimental groups (other four treatments fed diets with supplements), n = 24 replicates; for each replicate, n = 15 birds.
*GAA: GAA supplemented groups, n = 12 replicates; for each replicate, n = 15 birds.
[1]I: Orthogonal contrast of NC vs. EG

[2]II: Orthogonal contrast of NC vs. GAA.
SEM: Standard error of the means; ARG: Arginine; GAA: Guanidinoactic acid; PHE: Phenylalanine.

Table 5. Effects of supplementation of ARG sources and PHE levels on small intestine segment weights (% of BW) and morphometric parameters in cold-stressed broilers fed a CM-based diet at 38 d.

Item[†]	Small intestine weight	Duodenum				Jejunum				Ileum			
		Weight	VH (μm)	CD (μm)	VH:CD	Weight	VH (μm)	CD (μm)	VH:CD	Weight	VH (μm)	CD (μm)	VH:CD
Factorial analysis													
ARG sources													
ARG	3.11	0.627	1808.2	269.1	6.76[a]	1.28	1204.2[a]	191.04	6.40[a]	1.20	825.9[a]	150.5[b]	5.56[a]
GAA	3.23	0.617	1785.7	273.3	6.57[b]	1.36	1148.7[b]	191.62	6.10[b]	1.25	803.4[b]	160.5[a]	5.08[b]
SEM	0.050	0.016	8.64	2.08	0.062	0.036	7.51	2.67	0.088	0.028	7.40	2.11	0.082
PHE levels													
0 g/kg	3.15	0.611	1789.9	269.8	6.66	1.31	1173.0	192.7	6.19	1.23	809.3	156.2	5.27
1.5 g/kg	3.19	0.633	1804.0	272.5	6.66	1.33	1179.9	189.9	6.30	1.23	820.0	154.9	5.38
SEM	0.050	0.016	8.64	2.08	0.062	0.036	7.51	2.67	0.088	0.028	7.40	2.11	0.081
ARG sources × PHE levels													
ARG × 0 g/kg	3.05	0.605	1798.4	266.6	6.78	1.26	1200.3	193.3	6.31	1.19	820.5	151.8	5.48
ARG × 1.5 g/kg	3.17	0.649	1818.0	271.6	6.74	1.30	1208.1	188.8	6.48	1.22	831.3	149.2	5.65
GAA × 0 g/kg	3.26	0.618	1781.4	273.1	6.55	1.36	1145.6	192.1	6.07	1.28	798.0	160.5	5.05
GAA × 1.5 g/kg	3.20	0.616	1790.0	273.4	6.59	1.35	1151.7	191.1	6.12	1.23	808.8	160.5	5.12
SEM	0.072	0.023	12.22	2.95	0.088	0.051	10.6	3.78	0.124	0.039	10.47	2.99	0.115
p-value													
ARG sources	0.118	0.664	0.679	0.157	0.033	0.156	<.0001	0.879	0.017	0.216	0.033	0.001	<.0001
PHE levels	0.649	0.354	0.249	0.361	0.993	0.716	0.517	0.477	0.389	0.845	0.305	0.667	0.318
ARG sources × PHE levels	0.205	0.320	0.653	0.428	0.660	0.603	0.935	0.646	0.643	0.307	0.999	0.668	0.674
Orthogonal analysis													
NC[§]	3.59	0.795	1766.7	280.8	6.31	1.50	1082.6	198.4	5.53	1.30	777.7	164.8	4.80
EG[&]	3.17	0.622	1796.9	271.2	6.67	1.32	1176.4	191.3	6.25	1.23	814.7	155.5	5.33
GAA[*]	3.23	0.617	1785.7	273.25	6.57	1.36	1148.7	191.6	6.10	1.26	803.4	160.5	5.09
p-value I[1]	<.0001	<.0001	0.028	0.003	0.0002	0.002	<.0001	0.088	<.0001	0.109	0.001	0.007	<.0001
p-value II[2]	<.0001	<.0001	0.204	0.033	0.014	0.023	<.0001	0.134	0.0001	0.336	0.041	0.249	0.041

[†]Means in the same column with different superscripts differ significantly ($p < 0.05$).
[§]NC: Negative control group (birds fed CM-based diets without supplements), n = 6 replicates; for each replicate, n = 15 birds.
[&]EG: Experimental groups (other four treatments fed diets with supplements), n = 24 replicates; for each replicate, n = 15 birds.
*GAA: GAA supplemented groups, n = 12 replicates; for each replicate, n = 15 birds.

[1]I: Orthogonal contrast of NC vs. EG
[2]II: Orthogonal contrast of NC vs. GAA.
SEM: Standard error of the means; ARG: Arginine; GAA: Guanidinoactic acid; PHE: Phenylalanine; VH: Villus height; CD: Crypt depth; VH:CD: Villus height to Crypt depth ratio.

Table 6. Effects of supplementation of ARG sources and PHE levels on plasma parameters in cold-stressed broilers fed a CM-based diet at 38 d.

Item[†]	ALT (U/L)	AST (U/L)	LDH (U/L)	CK (U/L)	TAC (mmol/ml)	MDA (mmol/ml)	BUN (mg/dl)	UA (mg/dl)	TP (g/dl)	Albumin (g/dl)	Creatinine (μmol/l)	Glucose (mg/dl)
Factorial analysis												
ARG sources												
ARG	8.05	249.73^{b}	1159.93^{b}	1596.43	1.57	1.63	1.31^{a}	3.83^{b}	3.83	1.57	3.53^{b}	240.50
GAA	8.17	267.20^{a}	1180.00^{a}	1561.33	1.51	1.71	1.10^{b}	4.34^{a}	3.67	1.54	3.80^{a}	241.30
SEM	0.214	4.83	5.68	44.40	0.041	0.038	0.028	0.111	0.071	0.038	0.085	2.91
PHE levels												
0 g/kg	7.99	254.70	1170.90	1609.20	1.52	1.67	1.19	4.02	3.79	1.61	3.62	238.70
1.5 g/kg	8.22	262.23	1169.03	1548.57	1.56	1.67	1.21	4.14	3.71	1.50	3.71	241.30
SEM	0.214	4.83	5.68	44.40	0.041	0.038	0.028	0.111	0.071	0.038	0.085	2.91
ARG sources × PHE levels												
ARG × 0 g/kg	7.86	248.40	1157.07	1620.00	1.55	1.64	1.31	3.75	3.85	1.63	3.44	243.27^{ab}
ARG × 1.5 g/kg	8.25	251.07	1162.80	1572.87	1.58	1.63	1.32	3.91	3.81	1.51	3.63	237.73^{ab}
GAA × 0 g/kg	8.13	261.00	1184.73	1598.40	1.49	1.70	1.08	4.30	3.73	1.58	3.80	234.13^{b}
GAA × 1.5 g/kg	8.20	273.40	1175.27	1524.27	1.53	1.71	1.11	4.38	3.61	1.49	3.79	248.47^{a}
SEM	0.303	6.83	8.03	62.79	0.059	0.053	0.040	0.156	0.100	0.054	0.121	4.11
p-value												
ARG sources	0.714	0.013	0.015	0.578	0.369	0.165	<.0001	0.002	0.123	0.538	0.033	0.846
PHE levels	0.454	0.275	0.817	0.338	0.500	0.976	0.609	0.438	0.409	0.053	0.458	0.289
ARG sources × PHE levels	0.603	0.479	0.348	0.830	0.950	0.902	0.808	0.799	0.667	0.712	0.426	0.019
Orthogonal analysis												
NC[§]	8.41	292.80	1200.20	1653.80	1.21	1.94	1.14	4.87	3.94	1.67	3.25	245.60
EG[&]	8.11	258.47	1169.97	1578.89	1.54	1.67	1.20	4.08	3.75	1.55	3.67	240.90
GAA[*]	8.165	267.2	1180	1561.34	1.51	1.71	1.10	4.34	3.67	1.54	3.80	241.3
p-value I[1]	0.347	<.0001	0.001	0.268	<.0001	<.0001	0.193	<.0001	0.100	0.075	0.002	0.314
p-value II[2]	0.483	0.004	0.042	0.213	0.0001	0.0003	0.373	0.008	0.040	0.629	0.0003	0.400

[†]Means in the same column with different superscripts differ significantly ($p < 0.05$).
[§]NC: Negative control group (birds fed CM-based diets without supplements), n = 6 replicates; for each replicate, n = 15 birds.
[&]EG: Experimental groups (other four treatments fed diets with supplements), n = 24 replicates; for each replicate, n = 15 birds.
*GAA: GAA supplemented groups, n = 12 replicates; for each replicate, n = 15 birds.
[1]I: Orthogonal contrast of NC vs. EG
[2]II: Orthogonal contrast of NC vs. GAA.

SEM: Standard error of the means; ARG: Arginine; GAA: Guanidinoactic acid; PHE: Phenylalanine; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LDH: Lactate Lactate dehydrogenase; CK: Creatine kinase; TAC: Total antioxidant capacity; MDA: Malondialdehyde; BUN: Blood urea nitrogen; UA; Uric acid; TP: Total protein.

Table 7. Effects of supplementation of ARG sources and PHE levels on apparent ileal digestibility of DM, CP, EE, CF, and GE in cold-stressed broilers fed a CM-based diet at 38 d.

Item[†]	DM (%)	CP (%)	EE (%)	CF (%)	GE (%)
Factorial analysis					
ARG sources					
ARG	77.42[a]	77.82	87.87[b]	23.16	74.83
GAA	76.09[b]	76.19	89.60[a]	22.84	75.24
SEM	0.346	0.634	0.510	0.263	0.667
PHE levels					
0 g/kg	76.76	76.95	88.05	22.87	75.01
1.5 g/kg	76.75	77.06	89.42	23.13	75.03
SEM	0.346	0.634	0.510	0.263	0.667
ARG sources × PHE levels					
ARG × 0 g/kg	77.56	77.49	87.08	22.96	74.94
ARG × 1.5 g/kg	77.28	78.14	88.65	23.35	74.72
GAA × 0 g/kg	75.96	76.40	89.02	22.79	75.08
GAA × 1.5 g/kg	76.22	75.99	90.18	22.91	75.40
SEM	0.489	0.896	0.721	0.372	0.943
p-value					
ARG sources	0.015	0.089	0.029	0.415	0.670
PHE levels	0.980	0.899	0.076	0.496	0.960
ARG sources × PHE levels	0.591	0.563	0.773	0.736	0.777
Orthogonal analysis					
NC[§]	74.59	73.11	86.07	21.93	70.53
EG[&]	76.76	77.01	88.73	23.00	74.04
GAA[*]	76.09	76.20	89.60	22.85	75.24
p-value I[1]	0.006	0.002	0.002	0.015	0.0003
p-value II[2]	0.064	0.018	0.0003	0.052	0.0005

[†]Means in the same column with different superscripts differ significantly ($p < 0.05$).
[§]NC: Negative control group (birds fed CM-based diets without supplements), n = 6 replicates; for each replicate, n = 15 birds.
[&]EG: Experimental groups (other four treatments fed diets with supplements), n = 24 replicates; for each replicate, n = 15 birds.
*GAA: GAA supplemented groups, n = 12 replicates; for each replicate, n = 15 birds.
[1]I: Orthogonal contrast of NC vs. EG
[2]II: Orthogonal contrast of NC vs. GAA.
SEM: Standard error of the means; ARG: Arginine; GAA: Guanidinoactic acid; PHE: Phenylalanine; DM: Dry matter; CP: Crude protein; EE: Ether extract; CF: Crude fiber; GE: Gross energy.