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Abstract

This study was conducted to determine the optimal standard ileal digestible (SID) arginine (Arg) to SID lysine (Lys) ratio in broilers under cyclic heat stress. Additionally this study tested whether citrulline (Cit) can replace Arg under cyclic heat stress, based on the report that a large amount of Arg is metabolized in the liver while Cit can by-pass metabolism in the liver. A total of 360, one-day-old Arbor Acres broiler chickens with initial body weight of 34.50 ± 0.87 g were placed in 24 pens. The 24 pens were randomly assigned to four dietary treatments with six replicates of fifteen broiler chickens. Treatments were as follows: 1) NC (SID Arg : Lys =0.95), 2) PC (SID Arg : Lys=1.05), 3) Arg1.15 (SID Arg : Lys =1.15), 4) Arg1.25 (SID Arg : Lys =1.25), 5) Cit33 (supplementation of Cit at 33% of Arg supplementation in Arg1.15, 6) Cit50 (supplementation of Cit at 50% of Arg in Arg1.15). The Arg1.25 group had the highest BW on 32 days and BWG during the overall period (*p*<0.05) than the NC groups. However, there was no significant difference (*p*>0.05) on day 32 BW and BWG during the overall period in Arg supplemented groups (Arg1.15 and Arg1.25) and Arg replacement with Cit groups (Cit33 and Cit50). Arg1.25 and Cit33 groups had higher villus height (VH) in the duodenum, jejunum and ileum than the NC groups. Moreover, the Arg1.25 group had the lowest crypt depth (CD) in the jejunum and ileum than the NC group, while there was no significant difference (*p*>0.05) between Arg supplementation and Arg replacement with Cit groups. Arg1.25 group had the highest arginase activity in the liver and total nitric oxide synthase (NOS) and arginase activity in the kidney than other treatments, but no statistical difference was observed (*p*>0.05) in arginase in the liver among treatments. Collectively the results ascertain that Cit can effectively replace a certain part of dietary arginine in broiler diets.

Keywords (3 to 6): Arginine, Broiler chickens, Citrulline, Growth performance, Heat stress

Introduction

Broilers are susceptible to high temperatures since they have feathers on the body, the absence of sweat glands, and a high metabolic rate, and thus heat stress (HS) significantly affects well-being and production [1]. Actually, broilers exposed to HS exhibit reduced growth performance, lower nutrient digestibility, increased production of free radicals in the body, and higher mortality rates compared to those rared in thermoneutral temperatures [2-3]. Morevoer, climate change have been increasing and globally is contributing to a rise in HS in poultry production, which could increase impaired production of broilers around the world [4]. Certain functional AAs can influence metabolic pathways, thereby promoting overall health, including growth and survival in animals [5].

Arginine (Arg) among them is the fifth limiting AA in broiler chickens, and a precursor of Citrulline (Cit), ornithine, and nitric oxide (NO) [6]. Arg plays a role in immune and metabolic pathways, and supplementing the diet with Arg has been shown to improve feed efficiency, growth performance, and the immune system [7]. Arg has also been given attention in relation to HS. NO is an active effector of vasodilation [6]. Increased NO production can decrease body temperature by improved blood flow and oxidative stress by elevating the activity of superoxide dismutase (SOD) [8-10]. Ornithine, along with its downstream metabolites such as polyamines, has been shown to be critical in the process of tissue recovery [11-12]. These mechanisms could help to minimize the negative impacts of HS. Various conditions such as diets and environments considerably affect arginine requirement of broilers [13]. Moreover, the dietary Arg requirements for modern broilers need to be optimized due to superior genetic potential for protein deposition and the antagonism between Arg and lysine (Lys) [14].

Cit and Arg are both metabolic intermediates in the urea cycle. Cit is a non-protein AA and acts as a catalyst in the formation of Arg, which in turn leads to the production of NO [15-17]. It is important to note that Cit can be recycled to Arg and have Arg sparing effects [18]. Actually, previous studies have reported that dietary Cit supplementation to Arg-deficient feed improved gut health by increasing the circulation rate of Arg and NO in tissues [19-20]. Likewise, in recent studies related to mono-gastric animals, dietary Cit supplementation decreased pre-weaning mortality rate of piglets, and increased intestinal morphology villus height (VH) and improved feed efficiency of broilers under HS condition (about increased 8°C and 9°C compared to normal condition) [17, 21]. Chowdhury et al.

[22] additionally reported that supplementing Cit lowered body temperature in HS conditions (about increased 5°C compared to normal condition). Therefore, the objective of this study was to determine the optimum requirement of Arg and Cit for broilers under cyclic HS. The hypotheses tested in this experiment were (1) optimum SID Arg:Lys ratio for broilers will be greater than the current recommendations of 105% under cyclic HS; (2) Cit can replace Arg when supplemented in Arg deficient broiler diet; and (3) Cit inclusion at 33% or 50% of Arg will be sufficient to maintain optimum growth of broilers, as Cit is not metabolized in the liver as Arg does.

Materials and Methods

Experimental animal and design

A total of 360, one-day-old Arbor Acres broiler chickens (Cherrybro Co., Eumseong, Korea) with an initial body weight of 34.50 ± 0.87 g were placed in 24 pens (173 cm width, 63 cm depth and 55 cm height). The 24 pens were randomly assigned to four dietary treatments with six replicates of fifteen broiler chickens. Treatments were as follows: 1) NC (SID Arg : Lys =0.95), 2) PC (SID Arg : Lys=1.05), 3) Arg1.15 (SID Arg : Lys =1.15), 4) Arg1.25 (SID Arg : Lys =1.25), 5) Cit33 (supplementation of Cit at 33% of Arg in Arg1.15), Cit50 (supplementation of Cit at 50% of Arg in Arg1.15). All diets were formulated to meet or exceed the National Research Council [23] (Table 1 - 4). All broiler chickens were allowed to consume feed and water ad libitum. Each pen was equipped with two nipple drinkers connected to a common water supply line. The experiment period was divided into three phases: the starter phase (0 to 7 days of age), the grower phase (8 to 21 days of age) and the finishing phase (22 to 32 days of age). The lighting period was a continuous schedule with lightning intensities of 50 lux from 0 to 7 d of age, and 20 lux from 8 to 32 d of age for broiler. The experiment environment was controlled under at 33 ± 1 ℃ and 50 % relative humidity, and then temperature was reduced by 2 ℃ every week until 24 ℃ on day 25. The broilers were challenged with cyclic HS between 22 to 32 days of age. The room temperature was performed every day during the same period (from 9:00 to 18:00 h) to ensure consistency in the design. The temperature was gradually increased from 24 ± 1 ℃ to 30 ± 1 ℃ over 30 min and this temperature was maintained for the next 8 h before returning to 24 ± 1 ℃.

Growth performance

All birds and leftover feed in the cages were weighed at each time point to determine the body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) on 0, 7, 21 and 32 days. Mortality was recorded as it occurred. The BWG was calculated as the BW of the previous time point was subtracted from the BW of the current time point. FI was calculated by subtracting the remaining feed amount from the initial feed amount, and FCR was calculated by dividing FI by BWG. Adjusted FCR at 1.5 kg BW was calculated as follows: FCR at 1.5 kg BW = FCR – (average BW-1.5) × 0.3. Production index was calculated depending on the following formula: Production index = {(Average body weight (kg) × livability) / (Duration of the period (day) × FCR)} × 100.

Nutrient digestibility

Four broilers per pen were randomly selected to collect ileal digesta from Merkel's diverticulum to the ileocecal junction). The digesta samples were pooled by pen in plastic bags and stored in a freeze dryer for further analysis. Diets and freeze-dried ileal digesta were ground using a coffee grinder before further analysis. A 0.2% chromium dioxide was included in the diets as an indigestible marker and analyzed to estimate the apparent ileal digestibility (AID) of dry matter (DM), crude protein (CP), gross energy (GE) and AA. The GE was determined using a calorimeter (model 6400, Parr Instrument Company, Moline, IL, USA). DM and CP were analyzed according to the methods described in AOAC Method 930.15 and Method 990.03 [24] and AA was analyzed using high performance liquid chromatography (HPLC) (SHIMADZU, Model LC-10AT, Shimadzu Corp., Kyoto, Japan) followed with AOAC Method 982.30E (a, b, c) [24]. In order to calculate AID, the concentrations of the maker, and AA, DM and CP in diets and digesta were used, as shown below.

AID % = 100 – [100 × (Cr_2O_3 in diet/ Cr_2O_3 in ileal digesta) × (nutrient in ileal digesta/nutrient in diet)].

Relative organ weight and carcass trait

Six broilers per each treatment were euthanized on 32 days of age by an intravenous injection of pentobarbital, with cervical dislocation to confirm death. After broilers were euthanized, the abdomen area was opened to excise and weigh the carcass, gastrointestinal tract (gizzard, stomach, duodenum, jejunum, and ileum), liver, spleen, and heart. Carcass yields were calculated relative to the live BW. The weights of the gastrointestinal tract and other organs were recorded and then calculated using the following formula: Relative organ weight (g/kg) = organ weight (g)/live BW (kg).

Intestinal morphology

The intestinal segments ileum (midpoint from Meckel's diverticulum to the ileocecal junction), jejunum (midpoint from the pancreatic duct to Meckel's diverticulum), and duodenum (the midsection of the ascendant loop) were collected on 32 days and were rinsed with cold PBS and fixed with 4% paraformaldehyde. Then, the tissues were embedded with paraffin and sectioned at a thickness of 3 to 5 μm. Next, the slides were stained with hematoxylin and eosin (H&E). Five slides were prepared for each sample (from the central region of the sample), and images were captured using a light microscope (OLYMPUS DP71, BX50F-3, Olympus Optical Co. Ltd., Tokyo, Japan). The distance between the top of the villus to the villus-crypt junction was measured as VH, while the distance from the villus-crypt junction down to the bottom of the crypt was measured as CD. Three measurements were taken per slide and the average was obtained for analysis. The VH to CD ratio (VCR) was computed per observation.

Blood profile

Blood samples (2 mL each) were collected from the wing vein of broilers (one broiler per cage) using vacuum tubes containing K_3EDTA (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) at 32 days of age. Concentrations of total protein (TP), blood urea nitrogen (BUN), and cortisol were determined using an automatic biochemical analyzer (Hitachi, Tokyo, Japan). Concentration of NO (MyBioSource: MBS263050), Cit (MyBioSource: MBS2601045) and Arg (Assay Genie: CHEB0588) were determined using commercial ELISA kits (MyBioSource, San Diego, CA, USA; Assay Genie, Dublin, Ireland).

Enzyme activity

Six broilers per treatment were selected to analyze the enzyme activity of Arg and NO in the liver and kidney at 32 days of age. Enzymes involved in Arg metabolism including endothelial nitric oxide synthase (Intron Biotechnology, Korea: 21023) and arginase synthase (MyBioSource: MBS746934) were determined using chicken ELISA kits. The assay was measured at 450 nm using a microplate reader (Elx808, Bio-Tek, Winooski, Vermont, USA) and the standard curve was used to compute the sample concentration.

Statistical analysis

Growth performance, nutrient digestibility, blood profiles, intestinal morphology, relative organ weight and carcass trait, and enzyme activity were statistically analyzed using JMP 16.0 (SAS Institute Inc., Cary, NC). One-way ANOVA was conducted and Tukey HSD test was used to separate the means. Variability in the data was expressed as the pooled standard error, $P<0.05$ was considered statistically significant, and $0.05 \leq P<0.10$ was considered statistically tendency.

Results

Growth performance

There was no significant difference ($p>0.05$) on BW on 7 and 21 days, and FI among treatments (Table 5). Arg deficiency (NC) caused ($p<0.05$) a decrease in BW at 32 days of age, and BWG during the overall period compared to other treatments. The Arg1.25 group had the highest BW at 32 days of age and BWG during the overall period ($p<0.05$) than the NC group. However, there was no significant difference ($p>0.05$) in BW at 32 days of age and BWG during the overall period in Arg-supplemented groups (Arg1.15 and Arg1.25) and Arg replacement with Cit groups (Cit33 and Cit50). As for FCR and PI, there was no significant difference ($p>0.05$) in FCR in each period except for the overall period among treatments. Arg deficiency increased ($p<0.05$) FCR during the overall period and FCR at 1.5kg, while Arg deficiency decreased ($p<0.05$) PI compared to other treatments. The ARG1.25 group had the lowest FCR during the overall period and FCR at 1.5 kg ($p<0.05$) while Arg 1.25 had the highest PI ($p<0.05$) compared to NC and PC groups. However, there was no significant difference ($p>0.05$) in FCR during the overall period, FCR at 1.5 kg and PI in Arg supplemented groups (Arg1.15 and Arg1.25) and Arg replacement with Cit groups (Cit33 and Cit50).

Nutrient digestibility

There was no significant difference (*p* >0.05) in DM and GE digestibility among treatments (Table 6). Arg deficiency caused (*p* <0.05) the reduction of CP digestibility compared to other treatments. Direct Arg supplementation (Arg1.15 and Arg1.25) and Arg replacement with Cit (Cit33 and Cit50) improved (*p* <0.05) CP digestibility compared to the NC group, while there was no significant difference among Arg and Cit supplemented groups. As for SID of AAs, there was no significant difference (*p* >0.05) except for Arg, Lys, Met and Cys among treatments (Table 7). Arg deficiency decreased (*p* <0.05) the digestibility of Arg, Lys, Met and Cys compared to other treatments. Direct Arg supplementation (Arg1.15 and Arg1.25) and Arg replacement with Cit (Cit33 and Cit50) improved (*p* <0.05) the digestibility of Arg and Lys compared to the NC group.

Blood profile

There was no significant difference (*p*>0.05) in TP, BUN, cortisol and Arg concentrations in the blood among treatments (Table 8). Arg deficiency decreased (*p* <0.05) NO and Cit concentration in the blood, while Arg supplementation and Arg replacement with Cit improved (*p* <0.05) the production of NO and Cit in the blood.

Intestinal morphology

As for duodenal morphology, Arg deficiency reduced (*p* <0.05) VH and VCR compared to other treatments (Table 9). Furthermore, Arg deficiency induced (*p* <0.05) poor morphology such as a decrease in VH and VCR, and an increase in CD of the jejunum and ileum compared to other treatments. Arg1.25 and Cit33 groups had higher VH and VCR in the duodenum, jejunum and ileum than the NC group. Moreover, Arg1.25 group had the lowest CD in the jejunum and ileum than the NC group, while there was no significant difference (*p*>0.05) between Arg supplementation and Arg replacement with Cit groups.

Organ weight

There was no significant difference in the relative weight of the gizzard, bursa of Fabricius and small intestine among treatments (Table 10). Although there was a significant difference (*p* <0.05) in the relative weight of the liver and spleen among treatments, no statistical difference was observed (p >0.05) among Arg deficiency, and Arg and Cit supplementation groups.

Carcass weight

There was no significant difference (p>0.05) in carcass weight among treatments (Table 11).

Enzyme activity

There was no significant difference (p>0.05) in total NOS in the liver among treatments (Table 12). Arg1.25 group had the highest arginase in the liver and total NOS and arginase in the kidney than other treatments, but no statistical difference was observed (p>0.05) in arginase in the liver among Arg supplementation and Arg replacement with Cit.

Discussion

This study was conducted to investigate the effects of Arg supplementation and replacing Arg with Cit in broiler chickens under cyclic heat stress. The results demonstrated that Arg deficiency (NC) negatively impacted growth performance. However, the groups that received Arg and Cit supplementation showed an improvement in growth performance counteracting the negative effects caused by Arg deficiency. These findings are consistent with previous studies that have shown impaired growth in birds fed Arg-deficient diets, which can be alleviated by supplementing with Arg [25-28]. Additionally, Abdulkarimi et al. [29] reported that dietary supplementation of Arg beyond 20% of the NRC recommendation (Arg: Lys=1.26) resulted in improved BWG and FCR. The authors suggested that polyamines, which are derived from Arg, may possess anabolic properties that enhance protein synthesis and uptake of AA by cells [30]. One of our hypotheses was that the Arg requirement for optimum growth would be higher than the current recommendation of 105% of Lys under HS. The result of the present study supports the hypothesis as the optimum growth of brids was achieved at 125% of Lys, which is around 20% higher than the recommendation. It has been well documented that under HS birds use part of arginine for production of NO that increases blood flow for radiation of internal heat [31]. Given that the part of arginine is used for response to HS the requirement of Arg for optimal protein deposition is increased.

Cit is a metabolite of Arg [32]. Previous studies have shown that Cit can be transported to the kidney and other tissues where conditions for arginine synthesis are favorable without passing through the liver and intestine [19-20]. On the other hand, Arg after absorption is transported to the liver where significant amounts are metabolized, partly through increased arginase activity. Our study found no significant difference in growth performance between groups that received direct Arg supplementation and those that had partial Arg replacement with Cit groups. These results are consistent with those reported by Uyanga et al. [33], who found no significant difference in growth performance between the group supplemented with Arg and a group that had full Arg replacement with Cit. While the partial replacement of Arg with Cit did not significantly improve growth performance compared to the Arg supplemented group in our study, Cit supplementation at 33% of supplemented Arg did maintain growth similar to that of the group receiving direct Arg supplementation. These findings could be indirectly support our hypothesis that Cit inclusion at 33% or 50% of Arg will be sufficient to maintain optimum growth of broilers, as Cit is not metabolized in the liver as Arg is.

The small intestine plays a vital role in digestion and nutrient absorption, making it one of the most important digestive organs. Intestinal morphology is particularly important for assessing intestinal health as it affects nutrient absorption, gut immunity, and gut barrier function [34]. In this study, the groups supplemented with direct Arg showed improved VH and CD in the duodenum, jejunum, and ileum. However, the relative weight of the small intestine was not affected. These findings are consistent with a previous study by Murakami et al. [35], which found that Arg supplementation did not impact the weight and length of the small intestine, but did improve intestinal morphology by increasing VH and decreasing CD in broilers. Similarly, Castro et al. [36] and Zhang et al. [34] reported that VH and CD in the small intestine increased with additional Arg supplementation in diets. Arg supplementation has also been shown to improve intestinal morphology after an inflammatory injury caused by *Clostridium perfringens* and *Eimeria* spp. [37-39].

This tendency was also observed in nutrient digestibility during this study. We found that adding additional Arg improved the digestibility of CP and amino acids, particularly Lys, Met, Arg, and Cys, when compared to Arg-deficient diets. Previous research has reported a positive correlation between increased nutrient absorption and gut morphology in relation to Arg [29, 40].

Arg plays an essential role in intestinal physiology [41]. It serves as a precursor of polyamines and can be considered a nutritional agent in promoting intestinal mucosal growth. Arg speeds up the mitotic process in the villus-crypt area, consequently increasing the number of villus cells [42]. Moreover, Arg can enhance the growth of intestinal cells by activating the mechanistic target of rapamycin (mTOR), toll-like receptor 4 (TLR 4), and promoting nitric oxide (NO) production [29, 42-43].

Arginase is an important enzyme that breaks down Arginine into urea and ornithine [7]. In broilers, the activity of kidney and liver arginase plays a crucial role in controlling the metabolism of Arg because it is necessary for the degradation of Arg [33]. NOS, also known as catalyzing enzymes, is responsible for converting Arg into NO. Previous studies have shown that supplementation of Arg increased the activity of arginase in the kidney and liver [33, 44-45].

In line with our objectives, our study has shown that supplementation with Arg increased the activity of arginase and NOS in the liver and kidney. Arg is converted by NOS into Cit and NO, and by arginases into L-ornithine for polyamine biosynthesis [46]. Interestingly, we also observed increased production of NO in the blood when Arg and Cit were added to the diets. It is worth noting that replacing Arg with Cit did not result in any changes in intestinal morphology, nutrient digestibility, or NO production in the blood, compared to direct Arg supplementation in this study. This finding is consistent with a previous study which found that a complete replacement of Arg with Cit did not affect the concentrations of Arg and NO in the plasma [33]. These positive results from Cit supplementation may be attributed to the improved availability of Arg through Cit supplementation. HS has negative effects on poultry performance by causing physiological changes such as hyperthermia, oxidative stress, and systemic inflammation [31, 47-48]. In this study, we exposed broiler chickens to HS conditions and observed significant differences in growth performance, intestinal morphology, and nutrient digestibility among the treatment groups. Chowdhury [49] reported that an increase in blood Cit concentration is considered a biomarker of HS, as it is elevated during short periods of HS. Additionally, Luiking et al. [50] found that NO concentration in the blood can alleviate HS damage by improving vascular tone and blood flow in the smooth muscle.

We observed a significant difference in NO and Cit concentrations in the blood between the NC group and the Arg and Cit supplemented groups in this study. However, there was no significant difference in NO and Cit concentrations in the blood between the Arg and Cit supplemented groups in this study. These findings are in agreement with a previous study that reported that full Arg replacement with Cit did not affect NO concentration compared to the Arg supplementation group [33].

CONCLUSION

All things taken together, additional Arg supplementation improved NO production in the blood, which enhanced growth performance, gut morphology and nutrient digestibility. Also, Cit supplementation maintain growth, gut morphology, nutrient digestibility and even NO production in the blood similar with Arg supplementation, and thus Cit can replace with arginine in broiler's diet. Therefore, this study would be useful to prove the requirement of optimal Arg and Cit levels in poultry feeding programs.

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Tables

Table 1. Ingredient composition of experimental diets (Phase 1/day 1–7)

Ingredient / TRT	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**
CORN	57.50	57.50	57.50	57.50	57.50	57.50
SBM (46%CP)	25.15	25.15	25.15	25.15	25.15	25.15
DDGS	6.00	6.00	6.00	6.00	6.00	6.00
Animal Fats	2.20	2.20	2.20	2.20	2.20	2.20
Animal Protein	6.25	6.25	6.25	6.25	6.25	6.25
Limestone	0.60	0.60	0.60	0.60	0.60	0.60
MDCP	0.40	0.40	0.40	0.40	0.40	0.40
SALT-Proc	0.20	0.20	0.20	0.20	0.20	0.20
Vit premix[a]	0.12	0.12	0.12	0.12	0.12	0.12
Min premix[a]	0.20	0.20	0.20	0.20	0.20	0.20
Liq-Choline	0.27	0.27	0.27	0.27	0.27	0.27
Sand	0.05	0.11	0.11		0.11	0.11
Glycine	0.36	0.16	0.02		0.21	0.16
DL-MET 99%	0.30	0.30	0.30	0.30	0.30	0.30
L-Lys-SO_4(55%)	0.40	0.40	0.40	0.40	0.40	0.40
Arginine 98%		0.14	0.28	0.41		
Citrulline					0.09	0.14
Calculated nutrient value						
ME, Kcal/kg	3030	3030	3030	3030	3030	3030
C protein	22.36	22.41	22.52	22.76	22.39	22.39
Total Ca	0.92	0.92	0.92	0.92	0.92	0.92
Total P	0.69	0.69	0.69	0.69	0.69	0.69
Total Na	0.20	0.20	0.20	0.20	0.20	0.20
Total Cl	0.30	0.30	0.30	0.30	0.30	0.30
SID Lys	1.35	1.35	1.35	1.35	1.35	1.35
SID TSAA	0.85	0.85	0.85	0.85	0.85	0.85
SID Arg	1.28	1.41	1.55	1.68	1.28	1.28

[a]Supplied per kilogram diet: vitamin A (retinyl acetate), 9,000 IU; vitamin D3, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 48 mg; vitamin K, 3 mg; thiamin, 1.8 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin B12, 0.012 mg; niacin, 42 mg; folic acid, 1.2 mg; biotin, 0.24 mg; pantothenic acid, 12 mg; manganese, 120 mg; zinc 100 mg; iron, 80 mg; copper, 20 mg; iodine, 2 mg; selenium, 0.3 mg; cobalt, 0.5 mg

DDGS, Dried distiller's grains with soluble; MDCP, Mono-dicalcium phosphate; SID, standardized ileal digestibility; TSAA, total sulfur amino acid; Lys, lysine; Arg, Arginine; MET, methionine; P, phosphorus; ME, metabolizable energy

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

Table 2. Ingredient composition of experimental diets (Phase 2/day 8–14)

TRT \ Ingredient	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**
CORN	59.50	59.50	59.50	59.50	59.50	59.50
SBM (46%CP)	24.00	24.00	24.00	24.00	24.00	24.00
DDGS	5.00	5.00	5.00	5.00	5.00	5.00
Animal Fats	2.20	2.20	2.20	2.20	2.20	2.20
Animal Protein	6.25	6.25	6.25	6.25	6.25	6.25
Limestone	0.60	0.60	0.60	0.60	0.60	0.60
MDCP	0.40	0.40	0.40	0.40	0.40	0.40
SALT-Proc	0.20	0.20	0.20	0.20	0.20	0.20
Vit-PX[a]	0.12	0.12	0.12	0.12	0.12	0.12
Min-PX[a]	0.20	0.20	0.20	0.20	0.20	0.20
Liq-Choline	0.27	0.27	0.27	0.27	0.27	0.27
Sand	0.20	0.20	0.20	0.20	0.20	0.20
Glycine	0.40	0.27	0.14		0.31	0.27
DL-MET 99%	0.28	0.28	0.28	0.28	0.28	0.28
L-Lys-SO4(55%)	0.38	0.38	0.38	0.38	0.38	0.38
Arginine 98%		0.13	0.26	0.40		
Citrulline					0.09	0.13
Calculated nutrient value						
ME, Kcal/kg	3050	3050	3050	3050	3050	3050
C protein	21.73	21.84	21.95	22.07	21.82	21.82
Total Ca	0.92	0.92	0.92	0.92	0.92	0.92
Total P	0.69	0.69	0.69	0.68	0.69	0.69
Total Na	0.19	0.19	0.19	0.19	0.20	0.20
Total Cl	0.30	0.30	0.30	0.30	0.30	0.30
SID Lys	1.30	1.30	1.30	1.30	1.30	1.30
SID TSAA	0.82	0.82	0.82	0.82	0.82	0.82
SID Arg	1.24	1.36	1.49	1.63	1.24	1.24

[a]Supplied per kilogram diet: vitamin A (retinyl acetate), 9,000 IU; vitamin D3, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 48 mg; vitamin K, 3 mg; thiamin, 1.8 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin B12, 0.012 mg; niacin, 42 mg; folic acid, 1.2 mg; biotin, 0.24 mg; pantothenic acid, 12 mg; manganese, 120 mg; zinc 100 mg; iron, 80 mg; copper, 20 mg; iodine, 2 mg; selenium, 0.3 mg; cobalt, 0.5 mg

DDGS, Dried distiller's grains with soluble; MDCP, Mono-dicalcium phosphate; SID, standardized ileal digestibility; TSAA, total sulfur amino acid; Lys, lysine; Arg, Arginine; MET, methionine; P, phosphorus; ME, metabolizable energy

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

Table 3. Ingredient composition of experimental diets (Phase 3/day 15–21)

Ingredient / TRT	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**
CORN	60.80	60.80	60.80	60.80	60.80	60.80
SBM (46%CP)	22.85	22.85	22.85	22.85	22.85	22.85
DDGS	5.00	5.00	5.00	5.00	5.00	5.00
Animal Fats	2.20	2.20	2.20	2.20	2.20	2.20
Animal Protein	6.25	6.25	6.25	6.25	6.25	6.25
Limestone	0.60	0.60	0.60	0.60	0.60	0.60
MDCP	0.40	0.40	0.40	0.40	0.40	0.40
SALT-Proc	0.20	0.20	0.20	0.20	0.20	0.20
Vit-PX[a]	0.12	0.12	0.12	0.12	0.12	0.12
Min-PX[a]	0.20	0.20	0.20	0.20	0.20	0.20
Liq-Choline	0.27	0.27	0.27	0.27	0.27	0.27
Sand	0.10	0.17	0.17	0.17	0.17	0.17
Glycine	0.45	0.26	0.19		0.32	0.28
DL-MET 99%	0.19	0.19	0.19	0.19	0.19	0.19
L-Lys-SO4(55%)	0.37	0.37	0.37	0.37	0.37	0.37
Arginine 98%		0.12	0.19	0.38		
Citrulline					0.06	0.10
Calculated nutrient value						
ME, Kcal/kg	3060	3060	3060	3060	3060	3060
C protein	21.30	21.32	21.43	21.54	21.31	21.30
Total Ca	0.91	0.91	0.91	0.91	0.91	0.91
Total P	0.68	0.68	0.68	0.68	0.68	0.68
Total Na	0.19	0.19	0.19	0.19	0.19	0.19
Total Cl	0.28	0.28	0.28	0.28	0.28	0.28
SID Lys	1.26	1.26	1.26	1.26	1.26	1.26
SID TSAA	0.72	0.72	0.72	0.72	0.72	0.72
SID Arg	1.21	1.32	1.45	1.58	1.21	1.21

[a]Supplied per kilogram diet: vitamin A (retinyl acetate), 9,000 IU; vitamin D3, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 48 mg; vitamin K, 3 mg; thiamin, 1.8 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin B12, 0.012 mg; niacin, 42 mg; folic acid, 1.2 mg; biotin, 0.24 mg; pantothenic acid, 12 mg; manganese, 120 mg; zinc 100 mg; iron, 80 mg; copper, 20 mg; iodine, 2 mg; selenium, 0.3 mg; cobalt, 0.5 mg

DDGS, Dried distiller's grains with soluble; MDCP, Mono-dicalcium phosphate; SID, standardized ileal digestibility; TSAA, total sulfur amino acid; Lys, lysine; Arg, Arginine; MET, methionine; P, phosphorus; ME, metabolizable energy

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

Table 4. Ingredient composition of experimental diets (Phase 4/day 22–32)

Ingredient / TRT	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**
CORN	62.46	62.46	62.46	62.46	62.46	62.46
SBM (46%CP)	21.15	21.15	21.15	21.15	21.15	21.15
DDGS	5.00	5.00	5.00	5.00	5.00	5.00
Animal Fats	2.20	2.20	2.20	2.20	2.20	2.20
Animal Protein	6.25	6.25	6.25	6.25	6.25	6.25
Limestone	0.60	0.60	0.60	0.60	0.60	0.60
MDCP	0.40	0.40	0.40	0.40	0.40	0.40
SALT-Proc	0.20	0.20	0.20	0.20	0.20	0.20
Vit-PX[a]	0.12	0.12	0.12	0.12	0.12	0.12
Min-PX[a]	0.20	0.20	0.20	0.20	0.20	0.20
Liq-Choline	0.27	0.27	0.27	0.27	0.27	0.27
Sand	0.25	0.25	0.25	0.25	0.25	0.25
Glycine	0.36	0.25	0.13		0.29	0.24
DL-MET 99%	0.19	0.19	0.19	0.19	0.19	0.19
L-Lys-SO4(55%)	0.35	0.35	0.35	0.35	0.35	0.35
Arginine 98%		0.11	0.23	0.36		
Citrulline					0.07	0.12
Calculated nutrient value						
ME, Kcal/kg	3070	3070	3070	3070	3070	3070
C protein	20.52	20.62	20.72	20.83	20.61	20.60
Total Ca	0.91	0.91	0.91	0.91	0.91	0.91
Total P	0.69	0.69	0.69	0.69	0.69	0.69
Total Na	0.19	0.19	0.19	0.19	0.19	0.19
Total Cl	0.29	0.29	0.29	0.29	0.29	0.29
SID Lys	1.21	1.21	1.21	1.21	1.21	1.21
SID TSAA	0.71	0.71	0.71	0.71	0.71	0.71
SID Arg	1.16	1.27	1.38	1.51	1.16	1.16

[a]Supplied per kilogram diet: vitamin A (retinyl acetate), 9,000 IU; vitamin D3, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 48 mg; vitamin K, 3 mg; thiamin, 1.8 mg; riboflavin, 6 mg; pyridoxine, 3 mg; vitamin B12, 0.012 mg; niacin, 42 mg; folic acid, 1.2 mg; biotin, 0.24 mg; pantothenic acid, 12 mg; manganese, 120 mg; zinc 100 mg; iron, 80 mg; copper, 20 mg; iodine, 2 mg; selenium, 0.3 mg; cobalt, 0.5 mg

DDGS, Dried distiller's grains with soluble; MDCP, Mono-dicalcium phosphate; SID, standardized ileal digestibility; TSAA, total sulfur amino acid; Lys, lysine; Arg, Arginine; MET, methionine; P, phosphorus; ME, metabolizable energy

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

Table 5. Effects of dietary arginine or citrulline on growth performance in broiler

Items	NC	PC	Arg1.15	Arg1.25	Cit33	Cit50	SEM	*p*-value
BW, g								
D0	34.50	34.44	34.56	34.50	34.28	34.33	0.439	0.997
D7	127.39	136.06	138.28	133.28	126.61	124.00	4.388	0.162
D21	913.56	902.17	930.56	972.17	929.78	903.89	18.872	0.125
D32	1658.50^{b}	1673.72^{ab}	1729.67^{ab}	1788.72^{a}	1726.17^{ab}	1671.94^{ab}	29.621	0.035
BWG, g								
D 0 to 7	92.89	101.61	103.72	98.78	92.33	89.67	4.490	0.192
D 7 to 21	786.17	766.11	792.28	838.89	803.17	779.89	16.425	0.067
D 21 to 32	744.94	771.56	799.11	816.56	796.39	768.06	32.462	0.672
D 0 to 32	1624.00^{b}	1639.28^{ab}	1695.11^{ab}	1754.22^{a}	1691.89^{ab}	1637.61^{ab}	28.430	0.018
FI, g								
D 0 to 7	110.77	114.48	122.23	114.61	112.30	111.62	6.371	0.826
D 7 to 21	1056.80	1059.65	1056.94	1085.28	1034.26	1027.69	26.44	0.698
D 21 to 32	1413.32	1389.52	1318.09	1297.02	1371.02	1354.94	30.33	0.097
D 0 to 32	2580.88	2563.66	2497.26	2496.91	2517.57	2494.25	39.77	0.489
FCR								
D 0 to 7	1.22	1.16	1.21	1.19	1.26	1.27	0.032	0.196
D 7 to 21	1.36	1.39	1.35	1.31	1.30	1.33	0.026	0.169
D 21 to 32	1.93	1.85	1.69	1.61	1.76	1.80	0.073	0.063
D 0 to 32	1.60^{a}	1.58^{a}	1.49^{ab}	1.44^{b}	1.51^{ab}	1.54^{ab}	0.028	0.002
FCR at 1.5kg	1.56^{a}	1.53^{a}	1.42^{ab}	1.35^{b}	1.44^{ab}	1.48^{ab}	0.033	0.002
PI	329.43^{b}	337.52^{b}	371.23^{ab}	395.96^{a}	366.04^{ab}	346.01^{b}	10.803	0.001

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio; PI, production index

SEM, stand error of means

[a, b] Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 6. Effects of dietary arginine or citrulline on nutrient apparent ileal digestibility

Items, %	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**	**SEM**	***p*-value**
DM	67.64	67.91	68.09	68.15	67.90	67.78	0.158	0.241
CP	77.62^{c}	78.50^{bc}	79.35^{ab}	80.61^{a}	79.62^{ab}	79.36^{ab}	0.351	<0.001
GE	72.71	72.66	72.83	72.98	72.97	72.63	0.256	0.872

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

DM, dry matter; CP, crude protein; GE, gross energy; SEM, stand error of means

$^{a-c}$ Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 7. Effects of dietary arginine or citrulline on amino acid apparent ileal digestibility

Items, %	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**	**SEM**	***p*-value**
EAA								
Arginine	86.33^{c}	87.10^{bc}	88.09^{abc}	89.45^{a}	88.46^{ab}	88.92^{ab}	0.457	<0.001
Histidine	85.54	86.38	87.63	87.29	88.41	88.01	1.184	0.548
Isoleucine	86.78	86.73	87.10	87.45	86.94	87.09	0.637	0.973
Leucine	86.94	86.98	87.14	87.34	87.39	87.62	0.212	0.208
Lysine	86.49^{c}	86.91^{bc}	87.88^{ab}	88.90^{a}	88.40^{a}	88.19^{ab}	0.316	<0.001
Methionine	84.77^{b}	85.59^{ab}	86.60^{ab}	88.90^{a}	87.84^{ab}	88.64^{a}	0.789	0.003
Phenylalanine	88.16	88.40	87.94	88.46	88.09	88.03	0.155	0.142
Threonine	87.73	87.70	87.81	87.78	87.72	87.92	0.109	0.752
Valine	88.88	89.27	88.86	88.73	88.83	88.80	0.283	0.801
Tryptophan	86.34	87.15	87.45	87.49	88.25	87.10	0.777	0.671
NEAA								
Alanine	87.98	86.99	87.42	88.41	88.35	87.93	0.438	0.200
Aspartic	88.81	89.12	89.28	89.58	88.87	89.19	0.304	0.513
Cystine	87.18^{b}	88.41^{ab}	88.59^{ab}	89.30^{a}	88.90^{ab}	88.34^{ab}	0.404	0.021
Glycine	88.38	88.29	88.85	89.23	88.48	88.95	0.404	0.530
Glutamic acid	88.19	88.65	88.07	88.59	88.19	87.98	0.194	0.102
Proline	87.24	87.36	87.38	87.15	88.04	87.34	0.305	0.385
Serine	86.38	86.51	86.71	87.15	86.23	86.56	0.379	0.624
Tyrosine	87.24	87.10	86.67	87.34	86.19	87.32	0.940	0.944

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit2, supplementation of Cit at 50% of Arg in Arg1.15

EAA, essential amino acid; NEAA, non-essential amino acid; SEM, stand error of means

$^{a\text{-}c}$ Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 8. Effects of dietary arginine or citrulline on blood profile in broiler

Items	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**	**SEM**	***p*-value**
TP, (g/dL)	3.10	3.30	3.25	2.97	3.22	3.13	0.081	0.081
BUN, (mg/dL)	2.17	1.83	1.83	2.17	2.33	2.17	0.175	0.266
Cortisol, (µg/dL)	0.04	0.04	0.04	0.04	0.04	0.03	0.006	0.831
Arginine, (µmol/L)	27.98	29.98	30.67	30.59	30.10	27.68	1.505	0.576
NO, (µmol/L)	2.12^{b}	3.09^{ab}	3.49^{a}	3.65^{a}	2.91^{ab}	2.36^{b}	0.248	0.001
Citrulline, (nmol/mL)	31.94^{b}	33.18^{ab}	33.87^{ab}	38.70^{a}	35.16^{ab}	32.32^{ab}	1.512	0.039

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

TP, total protein; BUN, blood urea nitrogen; NO, nitric oxide; SEM, stand error of means

$^{a, b}$ Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 9. Effects of dietary arginine or citrulline on intestinal morphology of small intestine in broiler

Items, μm	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**	**SEM**	***p*-value**
Duodenum								
VH	148.58[b]	158.74[ab]	170.76[a]	173.44[a]	167.05[a]	160.74[ab]	6.998	0.003
CD	10.88	10.67	11.56	9.64	11.09	11.85	0.593	0.162
VCR	13.96[b]	15.17[ab]	14.89[ab]	18.03[a]	15.09[ab]	14.10[b]	0.898	0.039
Jejunum								
VH	116.74[b]	139.95[a]	142.38[a]	155.46[a]	149.89[a]	134.87[ab]	5.250	<0.001
CD	18.82[a]	15.52[ab]	15.15[ab]	13.15[b]	13.77[b]	15.34[ab]	0.959	0.005
VCR	6.28[b]	9.65[ab]	9.70[ab]	11.98[a]	11.07[a]	8.84[ab]	0.839	0.001
Ileum								
VH	75.29[b]	82.51[ab]	84.12[ab]	101.17[a]	96.16[a]	84.98[ab]	4.378	0.003
CD	18.89[a]	15.82[ab]	14.79[abc]	10.61[c]	11.61[bc]	14.48[abc]	1.074	<0.001
VCR	4.00[c]	5.33[c]	5.80[bc]	9.82[a]	8.66[ab]	6.21[bc]	0.669	<0.001

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

VH, villus height; CD, crypt depth; VCR, villus to crypt ratio; SEM, stand error of means

[a-c] Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 10. Effects of dietary arginine or citrulline on internal organ weight in broiler

Items, (g/kg of live body weight)	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**	**SEM**	***p*-value**
Gizzard	21.52	22.99	18.56	21.79	24.40	21.92	1.893	0.410
Liver	22.17^{ab}	20.43^{b}	20.09^{b}	20.42^{b}	20.10^{b}	25.05^{a}	0.903	0.003
Spleen	0.76^{abc}	0.97^{a}	0.66^{bc}	0.79^{ab}	0.63^{bc}	0.50^{c}	0.065	0.001
Bursa of Fabricius	1.55	1.95	1.55	1.63	1.92	1.32	0.244	0.445
Small intestine	40.70	33.76	38.31	40.38	38.24	39.01	1.581	0.053

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

SEM, stand error of means

$^{a-c}$ Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 11. Effects of dietary arginine or citrulline on carcass weight in broiler

Items, (g/kg of live body weight)	**NC**	**PC**	**Arg1.15**	**Arg1.25**	**Cit33**	**Cit50**	**SEM**	***p*-value**
Abdominal fat	10.40	9.63	11.75	10.00	9.79	10.49	0.653	0.258
Thigh	83.69	86.91	85.71	82.07	81.56	86.74	3.172	0.740
Drumstick	92.46	93.79	91.11	94.27	87.99	90.44	3.202	0.751
Breast	218.41	211.51	213.62	215.19	208.41	213.59	6.084	0.905

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15
SEM; stand error of means

Table 12. Effects of dietary arginine or citrulline on enzyme activity

Items, (ng/mg protein)	NC	PC	Arg1.15	Arg1.25	Cit33	Cit50	SEM	*p*-value
Liver								
Total NO synthase	17.71	18.69	22.67	22.81	21.27	19.95	1.659	0.191
Arginase	2.86b	3.39b	3.99ab	5.06a	3.88ab	3.97ab	0.276	<0.001
Kidney								
Total NO synthase	15.61b	16.10b	17.69b	24.53a	16.55b	15.29b	1.150	<0.001
Arginase	2.52c	3.69b	3.68b	4.59a	3.69b	3.79b	0.138	<0.001

NC, Arg: Lys=0.95; PC, Arg: Lys=1.05; Arg1.15, Arg: Lys=1.15; Arg1.25, Arg: Lys=1.25. Arg, arginine; Lys, lysine; Cit33, supplementation of Cit at 33% of Arg in Arg1.15; Cit50, supplementation of Cit at 50% of Arg in Arg1.15

NO, nitric oxide; SEM, stand error of means

a, b Means within a column with different superscripts differ significantly ($p < 0.05$).