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# Effects of arginine and phytogenic additive supplementation on performance and health of brown-egg layers

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**ABSTRACT** - This study was performed to evaluate the effects of the association of different digestible arginine and phytogenic additive dietary levels on performance and health status of brown-egg layers. In this study, a total of 504 33-week-old Hisex Brown layers were distributed into a completely randomized experimental design to a 4 × 3 factorial arrangement (dietary digestible arginine levels: 880, 968, 1056, or 1144 mg/kg of feed × phytogenic additive levels: 0, 100, and 200 mg/kg of feed) with six replicate cages of seven birds per cage. The phytogenic additive was composed of extracts of *Baccharis dracunculifolia* (40%), *Astragalus membranaceus* lipopolysaccharides (20%), cinnamon, and grape seed (20%). Feed intake was reduced when diets containing 1056 mg of arginine were supplemented with 100 or 200 mg phytogenic additive per kg. Feed conversion ratio was improved when diets were supplemented with 100 mg of phytogenic additive or with 1056 mg of arginine per kg of feed. Egg mass was increased when diets were supplemented with 1056 mg arginine per kg of feed. Arginine supplementation quadratically increased albumen percentage and reduced yolk percentage. Higher arginine and phytogenic additive levels reduced heterophyl:lymphocyte ratio and blood uric acid, total cholesterol, very-low density lipoprotein, and triglyceride levels. Dietary supplementation of 100 mg of phytogenic additive associated with high arginine levels increased nitric oxide production by peritoneal macrophages and 1056 mg of arginine increased antibodies titers against Newcastle disease virus. Blood and intestinal malonaldehyde levels were reduced when 200 mg of the phytogenic additive was added. Dietary supplementation of 968 mg of arginine or 100 mg of a phytogenic additive (40% *Baccharis dracunculifolia*, 20% *Astragalus membranaceus*, 20% cinnamon, and 20% grape seed extracts) per kilogram of diet improves the feed conversion ratio and associated inclusion of 1144 mg of arginine and 100 mg of phytogenic additive per kilogram of diet improves immune responses and health status of brown-egg layers.

Key Words: aminoacids, Arg, laying hens, plant extract

## Introduction

Phytogenic additives consist of plant dry extracts and essential oils and may have antibacterial action, improve diet digestibility, and act as antioxidants, enhancing poultry performance and health as well as the quality of poultry meat and eggs (Zhao et al., 2011; Akdemir et al., 2012). Some of the phytogenic additives used in poultry feeds are grape seed extract, *Astragalus membranaceous* extract, and cinnamon extract, which active principles boost the antioxidant system and stimulate the immune system of birds, specially humoral and cell-mediated immunity, thereby contributing to enhance dietary nutrient utilization (Fascina et al., 2012; Chamorro et al., 2013), which may lead to an increased uptake of fat (Fascina et al., 2012). The plant *Baccharis dracunculifolia* is native of South America and presents immunomodulating, antiulcerogenic, antimicrobial, and anti-inflammatory properties, in addition to being the main botanical source for the production of green propolis by bees (Lemos et al., 2007).

Supplementing arginine (Arg) above the requirements has been proposed in poultry diets to fight free radicals, stimulate the immune system, and increase egg production (Basiouni et al., 2006). Arginine also participates in the synthesis of creatine, polyamines, proline, and nitric oxide (NO). Birds are not able to synthesize arginine

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from ornithine, because they lack the enzymes carbamyl phosphatase synthetase and ornithine carbamyltransferase and depend exclusively on dietary arginine to meet their requirement; however, an excess of dietary aminoacids may lead to an increased uric acid production (Corzo et al., 2005).

Under field conditions, the stress caused by health, environmental, and management challenges increases the metabolic requirements of the immune system and for the maintenance of physiological functions. The use of phytogenic additives derived from a single plant source may not be sufficient to recover the metabolic homeostasis of challenged birds. However, studies have shown that the combined use of several plant extracts improve bird immune status, antioxidant status, diet digestibility, and pathogen elimination, consequently promoting better live performance (Akdemir et al., 2012). In addition to phytogenic additives, the supplementation of essential amino acids, such as arginine, above the requirements, allow birds to maintain adequate health status, thereby improving their performance (Fouad et al., 2013).

Considering that some phytogenic additives may lead to an increased immune response of birds and high dietary arginine levels may stimulate immune system, we hypothesize that supplementation of different levels of these feed additives may lead to a synergetic response on the performance and health status of birds. Given the lack of information about the interaction between the association of feed additives and layer nutrition, mainly with arginine supplementation above layer requirements, the objective of the present study was to evaluate the effect of the association of different digestible arginine levels with different levels of a phytogenic additive consisting of cinnamon, grape seed, *Astralagus membranaceus*, and *Baccharis dracunculifolia* extracts added to diet on the performance and health status of brown layers.

## Material and Methods

The experiment was carried out in Botucatu, SP, Brazil, and approved by the Ethics Committee on Animal Use (144/2012-CEUA). The city is located under geographical coordinates: Latitude −22.8904, Longitude −48.4553, 22° 53' 25" S, 48° 27' 19" W.

Five hundred and four Hisex Brown layers, with 33 weeks of age at the beginning of the experiment, were evaluated until 49 weeks of age during two production cycles of eight weeks each (a total of 16 weeks). From the hatch to the initiation of dietary treatments at 33 weeks of age, birds were fed a common diet based on corn and soybean without phytogenic additive or arginine supplementation. A completely randomized experimental design in a 4 × 3 factorial arrangement (dietary digestible arginine levels × phytogenic additive levels), with 12 dietary treatments with six replicates of seven birds each was applied. Treatments consisted of diets with four digestible arginine levels (880, 968, 1056, or 1144 mg Arg/kg feed) and inclusion of 0, 100, or 200 mg of a mixture of plant extracts (40% alcoholic extract of *Baccharis dracunculifolia*, 20% dry extract of *Astragalus membranaceus* lipopolysaccharides, 20% dry grape seed extract, and 20% dry cinnamon extract) per kg of diet. The detailed analyzed composition of active compounds of the phytogenic additives used are: *Baccharis dracunculifolia* containing 25% of baccharin, grape seed extract containing 75% of proanthocyanidins, composed mainly by resveratrol, and cinnamon extract with 10% of cinnamaldehyde and other polyphenols – all analyzed values.

Hens were housed in battery cages (100 cm long, 45 cm wide, 40 cm high) equipped with trough feeders and nipple drinkers. Hens were individually vaccinated against the Newcastle disease virus (NDV; La Sota strain) by eye drop at 40 weeks of age. During the entire experimental period, birds were subjected to the same feeding management, with water *ad libitum* and feed supplied twice daily. Eggs were collected once daily and feed intake was monitored weekly. A photoperiod of 17 h of light per day was applied. All parameters were evaluated based on cumulative data during the two production cycles (experimental weeks 41 and 49).

Experimental diets were based on corn and soybean meal and formulated to meet or exceed nutritional requirements of brown layers during their first egg production cycle (Table 1), according to the National Research Council (NRC, 1994), Rostagno et al. (2011), and the Hisex Brown management manual.

The following performance parameters were evaluated at the end of each production cycle: feed intake (g), egg production (%), egg mass (g), and feed conversion ratio (g of feed/g of eggs). External (eggshell percentage and egg specific gravity) and internal (albumen and yolk percentage and Haugh Unit) egg quality traits were evaluated in two eggs per experimental unit (12 eggs per dietary treatment), collected during the last three days of each production cycle.

At the end of each cycle, a hen with body weight closest to the average body weight of its experimental unit was sacrificed after 2 h of feed fasting. Spleen, thymus, and bursa were collected, weighed, and their weight relative to body weight was calculated.

Five milliliters of blood were collected by ulnar vein puncture of one bird per experimental unit to obtain plasma

for the analyses of total cholesterol (CHO), high-density lipoprotein (HDL), very-low density lipoprotein (VLDL), triglyceride (TG), glucose, uric acid, and creatinine-kinase (CK) levels using commercial kits (LaborLab®, São Paulo, Brazil). Levels were determined using an automated biochemical analyzer (BS 200, Mindray®, Shenzhen, China). Blood VLDL levels were determined by dividing TG values by 5.

One milliliter of blood of one hen per experimental unit was collected by ulnar vein puncture with a syringe with heparin for heterophil:lymphocyte (H:L) ratio determination. Differential leukocyte count was performed under an optical microscope at 100X magnification and 200 leukocytes were counted to determine H:L ratio.

Five milliliters of blood were collected by ulnar vein puncture of two hens per experimental unit to determine NDV antibody titers, using an immunoenzymatic assay (ELISA) from a commercial kit (IDEXX NDV Ab Test for chickens. IDEXX, Westbrook, Maine, United States).

One bird per experimental unit was intradermally inoculated on days 21 and 42 with 0.1 mL phytohemagglutinin (Cultilab®, Campinas, Brazil) in the third and the fourth interdigital spaces of the right foot and the same amount of saline solution was inoculated in the left foot. Skin thickness was measured before and 24 h after inoculation using a digital pachymeter. The results were obtained by the difference among measurements made at different times in mm.

Table 1 - Composition and nutritional levels of the experimental diets (as-fed basis)

| Item | Digestible arginine level (mg/kg) | | | |
|---|---|---|---|---|
| | 880 | 968 | 1056 | 1144 |
| Corn | 55.002 | 55.212 | 56.050 | 54.912 |
| Soybean meal (45%) | 14.480 | 19.460 | 23.300 | 23.455 |
| Wheat bran | 7.785 | 5.200 | 2.760 | 3.800 |
| Corn gluten meal (60%) | 9.000 | 6.120 | 3.800 | 3.300 |
| Soybean oil | 1.225 | 1.681 | 1.820 | 2.180 |
| Limestone | 9.650 | 9.634 | 9.615 | 9.625 |
| Dicalcium phosphate | 1.215 | 1.210 | 1.215 | 1.195 |
| Sodium chloride | 0.250 | 0.250 | 0.250 | 0.250 |
| Sodium bicarbonate | 0.310 | 0.310 | 0.310 | 0.310 |
| L-lysine HCl (78.4%) | 0.362 | 0.248 | 0.162 | 0.160 |
| DL-methionine (99%) | 0.131 | 0.150 | 0.168 | 0.177 |
| L-arginine (99%) | 0.000 | 0.000 | 0.030 | 0.116 |
| L-tryptophan (98%) | 0.022 | 0.005 | 0.000 | 0.000 |
| Choline chloride | 0.020 | 0.020 | 0.020 | 0.020 |
| Inert material | 0.200 | 0.200 | 0.200 | 0.200 |
| Potassium chloride | 0.048 | 0.000 | 0.000 | 0.000 |
| Premix[1] | 0.300 | 0.300 | 0.300 | 0.300 |
| Calculated analysis | | | | |
| Metabolizable energy (kcal/kg) | 2,820 | 2,820 | 2,820 | 2,820 |
| Crude protein (%) | 18.00 | 18.00 | 18.00 | 18.00 |
| Ca (%) | 4.000 | 4.001 | 4.000 | 4.000 |
| Available P (%) | 0.320 | 0.321 | 0.321 | 0.320 |
| K (%) | 0.541 | 0.578 | 0.622 | 0.632 |
| Na (%) | 0.200 | 0.200 | 0.201 | 0.201 |
| Cl (%) | 0.193 | 0.198 | 0.198 | 0.198 |
| Linoleic acid (%) | 1.250 | 2.288 | 2.348 | 2.532 |
| Digestible lysine (%) | 0.880 | 0.880 | 0.880 | 0.880 |
| Digestible arginine (%) | 0.880 | 0.968 | 1.056 | 1.144 |
| Digestible methionine (%) | 0.426 | 0.429 | 0.434 | 0.437 |
| Digestible methionine + cysteine (%) | 0.691 | 0.690 | 0.690 | 0.690 |
| Digestible threonine (%) | 0.577 | 0.591 | 0.600 | 0.594 |
| Digestible tryptophan (%) | 0.174 | 0.173 | 0.179 | 0.180 |
| Digestible glycine + serine (%) | 1.358 | 1.411 | 1.448 | 1.436 |
| Digestible valine (%) | 0.748 | 0.757 | 0.476 | 0.751 |
| Digestible isoleucine (%) | 0.654 | 0.672 | 0.683 | 0.675 |
| Digestible leucine (%) | 1.945 | 1.789 | 1.661 | 1.612 |
| Digestible histidine (%) | 0.411 | 0.424 | 0.433 | 0.430 |
| Digestible phenylalanine (%) | 0.876 | 0.865 | 0.854 | 0.839 |
| Digestible phenylalanine + tyrosine (%) | 1.545 | 1.511 | 1.480 | 0.562 |

[1] Vitamin and mineral supplement provided the following, per kilogram of diet: vitamin A (retinyl acetate), 6,999.99 IU; vitamin D3, 2,000 IU (cholecaciferol); vitamin K3, 1.60 mg; vitamin E (DL-α-tocopherol acetate), 5 IU; vitamin B2, 3 mg; vitamin B12, 8 mcg; pantothenic acid 3.50 mg; niacin, 20 mg; Se, 0.20 mg; Cu, 8 mg; Mn, 69.9 mg; Zn, 50 mg; I, 1.2 mg; Fe, 50 mg.

One bird per experimental unit was intraperitoneally inoculated with 1 mL of Sephadex solution (Sephadex G-50 Fine, Sigma Aldrich, at 3% in saline solution at 0.9%) per 200 g of body weight to attract macrophages to the abdominal cavity. Birds were sacrificed 48 h after inoculation and 20 mL of RPMI-1640 medium (Sigma-Aldrich) was injected in the abdominal cavity, which was then massaged to release the cells and for the collection of the abdominal liquid. The number of cells in each sample was determined by counting in a Neubauer chamber. Cell viability was determined by the technique of exclusion using Tripan Blue staining, which stains only non-viable cells, for the determination of $H_2O_2$ and NO production.

Hydrogen peroxide release by the macrophages was quantified using the method based on f phenol red oxidation by peroxidase-dependent $H_2O$, as described by Russo et al. (1989). Sample aliquots containing abdominal cavity cells of one bird per experimental unit were used for the $H_2O_2$ production test. Samples were suspended in 1 mL phenol red to standardize the concentration to $2 \times 10^6$ cells/mL and then plated (96-well plates). Plates were placed in an oven at 5% $CO_2$ and 41 °C for 1 h, after which 10 µL sodium hydroxide (1M) were added to stop the reaction. Plates were read in an ELISA reader (TP-Reader, ThermoPlate, China) using a 630-nm wavelength filter. Hydrogen peroxide production (in nmols) was calculated according to a standard curve using absorbance values obtained for the dilutions of 1:10, 1:40, 1:80, and 1:160. Results were expressed in nmol of $H_2O_2$, using regression equations based on the standard curve.

Nitric oxide production by abdominal macrophages was determined by the colorimetric method, based on Griess reaction (Green et al., 1982), combining 100 µL of the test sample supernatant with 100 µL of Griess reagent (Need 0.1% and sulfanilamide at 1.0% in $H_3PO_4$ at 5.0%). Readings were performed in ELISA microreader at 540 nm wavelength. Results were expressed in µmols of NO/$2 \times 10^6$ cells and compared with the optical density of the standard nitrogen dioxide curve.

The level of malonaldehyde (MDA) in the small intestine was determined in one bird per experimental unit at weeks 41 and 49, using the modified technique described by Madsen et al. (1998). Ten-g fractions of small intestine samples were homogenized for 1 min in 50 mL trichloroacetic acid (7.5%) in a mixer (Ultra-Turrax, IKA®, Staufen, Germany). Samples were then filtered and 5-mL aliquots were removed and mixed with 5 mL of a solution of 2-thiobarbituric acid (0.020 mol/L). The solution was placed in water bath (100 °C) for 10 min. Absorbance was measured at 532 nm wavelength under a spectrophotometer (FeNto 600 Plus®, FENTO Indústria e Comércio de Instrumentos, São Paulo, São Paulo, Brazil). Levels of MDA were evaluated in duplicate and expressed in mg MDA/kg of intestinal tissue. Measurements were based on a standard curve (0.1-6 nmol/L concentration range) with 1,1,3,3-tetraethoxypropane.

Blood MDA was determined according to the spectrophotometric method described by Buege and Aust (1978) and Paya et al. (1992), which quantifies the complex formed by the reaction of two thiobarbituric acid molecules with one MDA molecule, yielding a pink chromogen that is quantified at 532 wavelength. Lipid peroxidation results were expressed in nmol of MDA/mL of blood, according to a standard MDA curve determined in a spectrophotometer (FeNto 600 Plus®, FENTO Indústria e Comércio de Instrumentos, São Paulo, SP, Brazil).

Data were subjected to ANOVA using the General Linear Model procedure of SAS statistical package (Statistical Analysis System, version 9.2) for completely randomized designs. Cage was the experimental unit for performance and egg quality analysis and hen for other data analysis. When significant, differences of least square means were compared by Tukey test ($P<0.05$). Analyses of regression of the arginine factor were not performed when the results were not significant ($P>0.05$) or $R^2<0.70$, according to the PROC REG of SAS. Antibody titers against NDV were $\log_2$ transformed for statistical analysis.

## Results

Egg production (%) was not affected by dietary supplementation of arginine or of phytogenic additive ($P>0.05$). There was an interaction between arginine and phytogenic additive for feed intake at week 41. Including phytogenic additive (100 or 200 mg/kg) to diet containing 1056 mg Arg/kg of feed reduced feed intake.

Regardless of the phytogenic additive inclusion, the dietary supplementation of arginine promoted a quadratic increase in egg weight (data not presented) and egg mass up to 1072 and 1061 mg/kg inclusion levels, respectively ($EW_{week\ 41} = -7.56967 + 0.1276x - 0.00005949x^2$, $R^2 = 0.98$; $EM_{week\ 41} = -64.1787 + 0.23265x - 0.00010961x^2$, $R^2 = 0.98$) (Table 2). Diets containing the phytogenic additive reduced feed intake and feed conversion ratio as measured at the end of the experimental period (week 49). Arginine supplementation promoted a quadratic improvement in egg weight (data not shown), egg mass, and feed conversion ratio up to the levels of 1076, 1048, and 1048 mg, respectively ($EW_{week\ 49} = -15.17945 + 0.14315x - 0.00006649x^2$,

$R^2 = 0.98$; $EM_{week\ 49} = -75.23478 + 0.25771 - 0.00012291x^2$, $R^2 = 0.88$; $FCR_{week\ 49} = 8.60119 - 0.01264x + 0.00000603x^2$, $R^2 = 0.81$).

Albumen percentage had a quadratic increase up to 1016 mg of Arg/kg of feed ($Albumen_{week\ 41} = 26.25999 + 0.07893x - 0.00003885x^2$, $R^2 = 0.99$) (Table 3). Digestible arginine level of 1144 mg/kg of feed reduced eggshell percentage. The phytogenic additive increased Haugh unit values at the end of the experimental period. Yolk percentage was quadratically reduced up to 987 mg of Arg/kg feed ($yolk_{week\ 49} = 53.53506 - 0.05616x + 0.00002997x^2$, $R^2 = 0.76$) and eggshell percentage linearly decreased as dietary arginine level increased ($ES_{week\ 49} = 10.98136 - 0.00091289x$, $R^2 = 0.92$).

There was no effect of dietary treatments on the relative weight of the immune system organs of layers fed different arginine and phytogenic additive levels as evaluated at weeks 41 and 49 (Table 4).

There was an interaction between arginine and phytogenic additive for H:L ratio and uric acid levels measured at weeks 41 and 49, respectively (Table 5). Hens fed diets with 1144 mg of arginine and 200 mg of phytogenic additive per kg of feed presented lower H:L ratio than those not fed the phytogenic additive. The H:L ratio was quadratically reduced as arginine levels increased in diets without phytogenic additive up to 972.67 mg of Arg/kg of feed ($H:L_{week\ 41\ PA0} = 12.89816 - 0.02385x + 0.00001226x^2$, $R^2 = 0.89$). Hens fed diet with 1056 mg of arginine and 200 mg of phytogenic additive per kg presented the lowest uric acid concentration.

There was an interaction between arginine and phytogenic additive for CHO and VLDL blood levels at week 49 (Table 6). Hens fed diets containing 200 mg of phytogenic additive per kg of feed presented lower CHO and VLDL levels when diets were formulated to contain 1056 mg of arginine and 880 mg of arginine per kg of feed, respectively. Hens fed the diets containing 880 mg of arginine presented lower triglyceride levels than those fed 1144 mg of Arg/kg of feed.

On week 41, cutaneous basophil hypersensitivity (CBH) response quadratically increased with increasing arginine

Table 2 - Performance of layers fed different digestible arginine (Arg, mg/kg) and phytogenic additive (PA, mg/kg) levels

| Arginine | PA | Egg production (%) | | Feed intake (g/hen/day) | | Egg mass (g/hen/day) | | FCR (g/g) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 87.19 | 89.12 | 113.96ab | 121.46 | 55.41 | 56.36 | 2.35 | 2.33 |
| | 100 | 88.55 | 88.72 | 107.99b | 113.19 | 55.52 | 55.37 | 1.94 | 2.10 |
| | 200 | 90.40 | 92.07 | 110.91b | 116.24 | 56.18 | 57.01 | 1.98 | 2.05 |
| 968 | 0 | 89.12 | 90.97 | 112.28ab | 116.21 | 56.79 | 58.49 | 1.99 | 2.02 |
| | 100 | 89.12 | 91.61 | 114.01ab | 118.33 | 59.88 | 61.06 | 1.89 | 1.95 |
| | 200 | 90.17 | 90.20 | 111.21b | 115.36 | 58.04 | 59.34 | 1.90 | 1.95 |
| 1056 | 0 | 92.40 | 93.27 | 120.29a | 125.04 | 60.10 | 59.91 | 1.96 | 2.11 |
| | 100 | 87.47 | 89.84 | 109.19b | 115.83 | 57.52 | 58.54 | 1.86 | 1.98 |
| | 200 | 90.79 | 91.89 | 110.47b | 117.07 | 58.52 | 59.11 | 1.88 | 2.00 |
| 1144 | 0 | 91.05 | 91.16 | 113.25ab | 118.01 | 58.58 | 58.42 | 1.94 | 2.07 |
| | 100 | 90.51 | 91.89 | 111.79ab | 116.05 | 58.26 | 59.75 | 1.92 | 1.96 |
| | 200 | 91.78 | 92.10 | 113.62ab | 119.40 | 58.87 | 58.51 | 1.91 | 2.15 |
| Arginine | | | | | | | | | |
| 880 | | 88.71 | 89.97 | 110.95 | 116.96 | 55.70 | 56.25 | 2.09 | 2.16 |
| 968 | | 89.47 | 90.93 | 112.50 | 116.63 | 58.24 | 59.63 | 1.93 | 1.97 |
| 1056 | | 90.22 | 91.67 | 113.32 | 119.31 | 58.71 | 59.19 | 1.90 | 2.03 |
| 1144 | | 91.11 | 91.72 | 112.89 | 117.82 | 58.57 | 58.89 | 1.92 | 2.06 |
| PA | | | | | | | | | |
| 0 | | 89.94 | 91.13 | 114.94 | 120.18a | 57.72 | 58.29 | 2.06 | 2.13a |
| 100 | | 88.91 | 90.51 | 110.75 | 115.85b | 57.79 | 58.68 | 1.90 | 1.99b |
| 200 | | 90.79 | 91.57 | 111.55 | 117.01ab | 57.90 | 58.49 | 1.91 | 2.04ab |
| SEM | | 0.436 | 0.334 | 0.630 | 0.659 | 0.396 | 0.345 | 0.035 | 0.023 |
| P-value | | | | | | | | | |
| Arginine | | 0.242 | 0.198 | 0.485 | 0.408 | 0.024 | 0.002 | 0.210 | 0.037 |
| PA | | 0.212 | 0.414 | 0.009 | 0.014 | 0.981 | 0.883 | 0.129 | 0.047 |
| Arg × PA | | 0.480 | 0.167 | 0.032 | 0.066 | 0.564 | 0.510 | 0.710 | 0.406 |

FCR - feed conversion ratio; SEM - standard error of mean.
a,b - Means followed by different letters within the same column are statistically different ($P<0.05$).
Each mean represents six cages per treatment (with seven birds per cage).
Egg mass week $41_{Arg} = -64.1787 + 0.23265x - 0.00010961x^2$; $R^2 = 0.98$ (inflection point = 1061 mg/kg).
Egg mass week $49_{Arg} = -75.23478 + 0.25771 - 0.00012291x^2$; $R^2 = 0.88$ (inflection point = 1048 mg/kg).
Feed conversion ratio week $49_{Arg} = 8.60119 - 0.01264x + 0.00000603x^2$; $R^2 = 0.81$ (inflection point = 1048 mg/kg).

levels up to 1051 mg (CBH week 41Arg = −8.65689 + 0.01758x – 0.00000836x$^2$; $R^2$ = 0.99) (Table 7). There was an interaction between arginine and phytogenic additive for CBH evaluated at week 49. Hens fed diets with 200 mg of phytogenic additive and 968 mg of arginine per kg of diet presented stronger CBH than those fed 880 mg of Arg/kg of feed. Hens fed 100 mg of phytogenic additive/kg of diet presented a linear increase in CBH as arginine dietary level increased (CBH week $49_{100PA}$ = −0.6424 + 0.00087614x; $R^2$ = 0.93).

Antibody titers against NDV quadratically increased with increasing arginine levels up to 1055 mg of arginine (NDV week 49Arg = −1.27634 + 0.00994x – 0.00000471x$^2$; $R^2$ = 0.96). Hens fed diets with 1144 mg of arginine and 100 mg of phytogenic additive per kg presented higher NO production by peritoneal macrophages compared with those fed 0 mg and 200 mg of phytogenic additive per kg of feed. When diets contained 100 mg of phytogenic additive per kg, NO production linearly increased with increasing arginine dietary levels (NO week $49_{100PA}$ = −69.85412 + 0.10078x; $R^2$ = 0.86).

Hens fed diets with 200 mg of phytogenic additive per kg had lower intestinal MDA values at week 41 (Table 8). Supplementation of 1056 mg of arginine and 100 mg of phytogenic additive per kg of diet reduced MDA levels when compared with diets containing 0 mg or 200 mg of phytogenic additive measured at the end of the experimental period (week 49). There was no effect of arginine and phytogenic additive levels on blood MDA levels at week 41. However, at week 49, supplementation of 200 mg of phytogenic additive per kg of feed reduced blood MDA levels.

## Discussion

The present study showed that the dietary inclusion of a phytogenic additive, consisting of a mixture of 40% *Baccharis dracunculifolia*, 20% *Astragalus membranaceus*,

Table 3 - Egg quality parameters of layers fed different digestible arginine (Arg, mg/kg) and phytogenic additive (PA, mg/kg) levels

| Arginine | PA | Egg specific gravity (g/L) | | Haugh Unit | | Eggshell (%) | | Yolk (%) | | Albumen (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 1.096 | 1.097 | 87.34 | 87.53 | 10.29 | 10.27 | 24.04 | 24.58 | 65.64 | 65.30 |
| | 100 | 1.096 | 1.102 | 88.16 | 88.97 | 10.11 | 10.12 | 24.45 | 24.91 | 65.38 | 64.91 |
| | 200 | 1.097 | 1.097 | 84.84 | 86.59 | 10.06 | 10.10 | 24.18 | 24.73 | 65.76 | 65.35 |
| 968 | 0 | 1.096 | 1.097 | 86.04 | 86.57 | 9.94 | 10.13 | 23.64 | 24.30 | 66.42 | 65.65 |
| | 100 | 1.097 | 1.096 | 87.70 | 89.04 | 10.09 | 10.08 | 23.17 | 23.96 | 66.73 | 66.07 |
| | 200 | 1.096 | 1.102 | 87.14 | 87.50 | 10.05 | 10.17 | 23.94 | 24.30 | 66.01 | 65.45 |
| 1056 | 0 | 1.095 | 1.095 | 84.48 | 85.29 | 9.87 | 9.87 | 23.98 | 24.54 | 66.21 | 65.93 |
| | 100 | 1.097 | 1.096 | 88.11 | 89.11 | 10.07 | 10.09 | 23.75 | 24.87 | 66.18 | 65.40 |
| | 200 | 1.096 | 1.096 | 85.66 | 87.46 | 9.95 | 9.98 | 23.88 | 24.52 | 66.18 | 65.65 |
| 1144 | 0 | 1.095 | 1.095 | 86.64 | 86.53 | 9.95 | 10.04 | 24.14 | 24.60 | 65.91 | 65.38 |
| | 100 | 1.096 | 1.096 | 87.06 | 88.32 | 9.96 | 10.03 | 24.53 | 25.16 | 65.51 | 65.01 |
| | 200 | 1.094 | 1.094 | 85.07 | 86.31 | 9.74 | 9.79 | 24.34 | 25.00 | 65.90 | 65.31 |
| Arginine | | | | | | | | | | | |
| 880 | | 1.096 | 1.098 | 86.78 | 87.69 | 10.15a | 10.16 | 24.22 | 24.74 | 65.59 | 65.19 |
| 968 | | 1.096 | 1.096 | 86.96 | 87.70 | 10.03ab | 10.12 | 23.58 | 24.19 | 66.39 | 65.72 |
| 1056 | | 1.096 | 1.095 | 86.08 | 87.29 | 9.96ab | 9.98 | 23.87 | 24.64 | 66.19 | 65.66 |
| 1144 | | 1.095 | 1.095 | 86.26 | 87.05 | 9.89b | 9.96 | 24.33 | 24.92 | 65.77 | 65.23 |
| PA | | | | | | | | | | | |
| 0 | | 1.096 | 1.095 | 86.12 | 86.48b | 10.01 | 10.08 | 23.95 | 24.41 | 66.04 | 65.56 |
| 100 | | 1.096 | 1.097 | 87.76 | 88.86a | 10.06 | 10.08 | 23.97 | 24.72 | 65.95 | 65.35 |
| 200 | | 1.096 | 1.096 | 85.68 | 86.96ab | 9.95 | 10.01 | 24.08 | 24.64 | 65.96 | 65.44 |
| SEM | | 0.001 | 0.001 | 0.385 | 0.334 | 0.033 | 0.029 | 0.108 | 0.093 | 0.109 | 0.093 |
| P-value | | | | | | | | | | | |
| Arginine | | 0.176 | 0.143 | 0.835 | 0.872 | 0.036 | 0.024 | 0.066 | 0.044 | 0.046 | 0.085 |
| PA | | 0.332 | 0.316 | 0.079 | 0.011 | 0.383 | 0.525 | 0.861 | 0.627 | 0.928 | 0.626 |
| Arg × PA | | 0.721 | 0.678 | 0.703 | 0.877 | 0.490 | 0.283 | 0.784 | 0.836 | 0.811 | 0.635 |

SEM - standard error of the mean.
a,b - Means followed by different letters within the same column are statistically different (P<0.05).
Each mean represents two eggs per cage (12 eggs per dietary treatment).
Eggshell percentage week $49_{Arg}$ = 10.98136 – 0.00091289x; $R^2$ = 0.92.
Yolk percentage week $49_{Arg}$ = 53.53506 – 0.05616x + 0.00002997x$^2$; $R^2$ = 0.76 (inflection point = 987 mg/kg).
Albumen percentage week $41_{Arg}$ = 26.25999 + 0.07893x – 0.00003885x$^2$; $R^2$ = 0.99 (inflection point = 1016 mg/kg).

20% cinnamon, and 20% grape seed extracts, together with the supplementation of 1056 mg arginine per kg of diet, which exceeds brown layer nutritional requirements, improved the performance by reducing feed intake and therefore, increasing feed efficiency. Zhao et al. (2011) demonstrated that plant compounds, such as cinnamaldehyde, catechins, cinnamic acid, terpenes, and resveratrol, stimulate the release of pancreatic enzymes, increase nutrient utilization, and mobilize a higher amount of amino acids for deposition in the eggs, resulting in better feed conversion ratio and lower feed intake, without affecting egg production, as observed in the present study. Despite not determined in the present study, higher pancreatic and intestinal enzyme secretion and activity were observed in broilers (Jamroz et al., 2005) fed diets supplemented with phytogenic additives, resulting in better performance and lower nitrogen excretion in the environment.

In addition to improving production performance, adequate essential amino acid supplementation promotes better internal and external egg quality (Novak et al., 2004; Silva et al., 2012). This effect was observed in the present study, in which egg mass increased with arginine supplementation and presented a quadratic behavior up to 1048 mg of digestible arginine addition per kg of feed. Because calcium deposition in the eggshell does not depend on protein supplementation, the higher egg mass and egg weight observed resulted in a linear decrease in eggshell percentage, but did not change egg specific gravity. These results are different from findings of previous studies, which obtained higher egg weight as egg specific gravity decreased in broiler breeders (Silva et al., 2012) and in low-production layers (Basiouni et al., 2006).

In the present study, yolk percentage was quadratically reduced up to 987 mg Arg/kg, indicating that the observed egg mass increase was due to higher albumen deposition and not to yolk deposition. Literature studies indicate that, as commercial layers and breeders age, egg mass increases due to higher albumen or yolk deposition and eggshell becomes thinner (Roberts, 2004). However, at the same age, albumen and yolk deposition are apparently more dependent on nutrient supply, i.e., protein (amino acids) and lipid (fatty acids) supply, respectively. The results obtained with arginine supplementation (higher albumen

Table 4 - Relative weight of the immune system organs of layers fed different digestible arginine (Arg, mg/kg) and phytogenic additive (PA, mg/kg) levels

| Arginine | PA | Spleen (%) | | Thymus (%) | | Bursa (%) | |
|---|---|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 0.094 | 0.104 | 0.217 | 0.240 | 0.010 | 0.008 |
| | 100 | 0.105 | 0.103 | 0.207 | 0.278 | 0.010 | 0.010 |
| | 200 | 0.109 | 0.114 | 0.234 | 0.318 | 0.012 | 0.012 |
| 968 | 0 | 0.114 | 0.103 | 0.269 | 0.300 | 0.009 | 0.009 |
| | 100 | 0.115 | 0.112 | 0.258 | 0.263 | 0.016 | 0.009 |
| | 200 | 0.123 | 0.105 | 0.266 | 0.265 | 0.008 | 0.008 |
| 1056 | 0 | 0.096 | 0.112 | 0.227 | 0.210 | 0.011 | 0.008 |
| | 100 | 0.113 | 0.090 | 0.222 | 0.262 | 0.013 | 0.007 |
| | 200 | 0.100 | 0.109 | 0.226 | 0.316 | 0.012 | 0.011 |
| 1144 | 0 | 0.104 | 0.096 | 0.282 | 0.270 | 0.011 | 0.009 |
| | 100 | 0.088 | 0.112 | 0.265 | 0.311 | 0.010 | 0.010 |
| | 200 | 0.109 | 0.095 | 0.259 | 0.266 | 0.010 | 0.008 |
| Arginine | | | | | | | |
| 880 | | 0.103 | 0.107 | 0.219 | 0.279 | 0.011 | 0.010 |
| 968 | | 0.117 | 0.107 | 0.264 | 0.276 | 0.011 | 0.008 |
| 1056 | | 0.103 | 0.104 | 0.225 | 0.263 | 0.012 | 0.009 |
| 1144 | | 0.100 | 0.101 | 0.269 | 0.283 | 0.010 | 0.009 |
| PA | | | | | | | |
| 0 | | 0.102 | 0.104 | 0.249 | 0.256 | 0.010 | 0.008 |
| 100 | | 0.105 | 0.104 | 0.238 | 0.279 | 0.012 | 0.009 |
| 200 | | 0.110 | 0.106 | 0.247 | 0.291 | 0.011 | 0.010 |
| SEM | | 0.002 | 0.003 | 0.007 | 0.008 | 0.001 | 0.001 |
| P-value | | | | | | | |
| Arginine | | 0.076 | 0.809 | 0.057 | 0.822 | 0.792 | 0.617 |
| PA | | 0.435 | 0.916 | 0.816 | 0.199 | 0.270 | 0.587 |
| Arg × PA | | 0.473 | 0.281 | 0.992 | 0.130 | 0.238 | 0.40 |

SEM - standard error of the mean.
Each mean represents one bird per cage (six birds per dietary treatment).

percentage and lower yolk and eggshell percentages) are consistent with the findings of Novak et al. (2004), who also observed that 44 to 63-week-old layers fed 900 mg lysine/kg also produced heavier eggs with higher albumen percentage and lower yolk percentage.

At the end of the experimental period, at week 49, Haugh units, which indicate albumen quality, increased when 100 mg of the phytogenic additive was added per kg of diet. The use of plant extracts with antioxidant and antibacterial activity, such as those included in the phytogenic additive used (grape seed, *Astragalus membranaceus*, cinnamon, and *Baccharis dracunculifolia*) may improve internal egg quality, as previously shown by Bozkurt et al. (2012), who added a mixture of oregano, basil, sage, myrtle, and fennel essential oils with citrus peel to layer diets. However, regardless of the action of the phytogenic additive, arginine supplementation did not affect albumen quality, as measured by Haugh unit.

Dietary addition of phytogenic additives with immunostimulating action (Rajput et al., 2013) or the supplementation of arginine above the nutritional requirements (Ruiz-Feria and Abdukalykova, 2009) promotes the development of immune system organs of broilers. However, in the present study, no effect of phytogenic additive and/or arginine supplementation was detected on the relative weights of the spleen, thymus, or bursa of the evaluated layers. The physiological ages of broilers and commercial layers in production are very different, which may explain the differences in organ development responses, particularly of the immune organs. Therefore, the immune organs of physiologically mature chickens (layers in egg production) may respond differently to the supplementation of phytogenic additives and arginine compared with growing broiler chickens. Development of the immune system organs of 18-week-old pullets did not respond to dietary arginine levels as reported by Lieboldt et al. (2016).

Supplementation of phytogenic additives and arginine may promote stronger immune responses. Among other tools for the evaluation of the immune system, leukocyte

Table 5 - Heterophil:lymphocyte ratio (H:L), uric acid (mg/dL), creatinine kinase (IU/L), and glucose (mg/dL) blood levels of layers fed different digestible arginine (Arg, mg/kg) and phytogenic additive (PA, mg/kg) levels

| Arginine | PA | H:L | | Uric acid | | Creatinine kinase | | Glucose | |
|---|---|---|---|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 0.36ab | 0.38 | 5.02 | 5.56ab | 683.62 | 569.43 | 191.95 | 225.44 |
| | 100 | 0.51ab | 0.42 | 5.72 | 5.32ab | 951.90 | 692.52 | 200.36 | 225.77 |
| | 200 | 0.35ab | 0.34 | 5.64 | 6.01ab | 692.55 | 750.82 | 200.14 | 222.32 |
| 968 | 0 | 0.36ab | 0.39 | 5.68 | 6.01ab | 738.60 | 564.87 | 197.57 | 230.97 |
| | 100 | 0.38ab | 0.49 | 6.05 | 6.25ab | 740.85 | 728.20 | 185.54 | 232.34 |
| | 200 | 0.39ab | 0.44 | 5.21 | 5.55ab | 675.98 | 606.18 | 189.57 | 225.42 |
| 1056 | 0 | 0.33ab | 0.36 | 5.50 | 5.38ab | 678.19 | 676.09 | 192.48 | 227.29 |
| | 100 | 0.44ab | 0.35 | 4.62 | 6.59a | 607.81 | 622.04 | 197.27 | 227.82 |
| | 200 | 0.47ab | 0.28 | 5.54 | 3.86b | 676.62 | 709.47 | 202.40 | 224.48 |
| 1144 | 0 | 0.72a | 0.36 | 5.54 | 5.59ab | 824.12 | 889.03 | 192.77 | 228.63 |
| | 100 | 0.39ab | 0.39 | 5.27 | 5.20ab | 788.49 | 715.68 | 191.67 | 229.13 |
| | 200 | 0.26b | 0.41 | 5.58 | 6.50ab | 587.02 | 663.99 | 193.76 | 226.61 |
| Arginine | | | | | | | | | |
| 880 | | 0.41 | 0.38 | 5.46 | 5.61 | 776.02 | 669.65 | 197.48a | 224.64 |
| 968 | | 0.38 | 0.44 | 5.65 | 5.94 | 718.48 | 633.08 | 190.89b | 229.58 |
| 1056 | | 0.42 | 0.34 | 5.22 | 5.20 | 654.21 | 669.20 | 197.38ab | 226.53 |
| 1144 | | 0.44 | 0.39 | 5.46 | 5.74 | 733.21 | 753.70 | 192.73ab | 228.19 |
| PA | | | | | | | | | |
| 0 | | 0.44 | 0.37 | 5.38 | 5.60 | 731.13 | 665.54 | 193.69 | 227.93 |
| 100 | | 0.43 | 0.41 | 5.41 | 5.81 | 772.26 | 689.48 | 193.71 | 228.61 |
| 200 | | 0.36 | 0.38 | 5.49 | 5.41 | 658.04 | 683.43 | 196.47 | 224.70 |
| SEM | | 0.027 | 0.022 | 0.143 | 0.175 | 29.863 | 29.491 | 1.071 | 1.168 |
| P-value | | | | | | | | | |
| Arginine | | 0.732 | 0.486 | 0.793 | 0.554 | 0.554 | 0.547 | 0.046 | 0.523 |
| PA | | 0.433 | 0.708 | 0.975 | 0.668 | 0.299 | 0.980 | 0.424 | 0.371 |
| Arg × PA | | 0.030 | 0.973 | 0.590 | 0.034 | 0.505 | 0.490 | 0.060 | 0.999 |

SEM - standard error of the mean.
a,b - Means followed by different letters within the same column are statistically different ($P<0.05$).
Each mean represents one bird per cage (six birds per dietary treatment).
Heterophil:lymphocyte ratio week $41_{PA0.0} = 12.89816 – 0.02385x + 0.00001226x^2$; $R^2 = 0.89$ (inflection point = 972.67 mg/kg).

profile helps to identify the efficiency of the action of immunostimulating agents and the effects of stressors. In the present study, layers fed 1114 mg Arg/kg and no phytogenic additive supplementation presented higher number of heterophils (data not shown), resulting in higher and statistically different H:L ratio compared with those fed the same arginine level, but also 200 mg of phytogenic additive/kg diet at week 41. This was also reported by Jahanian (2009) in broilers fed arginine above their nutritional requirements. Under stress (metabolic or other), the body of poultry reacts by stimulating heterophil production (Maxwell and Robertson, 1998), as it may be the case of the high arginine supplementation level (1144 mg/kg of feed) in the present study. The other experimental groups that were fed the same arginine level and were supplemented with the phytogenic additive presented H:L ratios considered optimal by Gross and Siegel (1993), possibly due to the immunoprotective or antioxidant action of the active principles of the plant extracts contained in the additive. At the end of the experiment (week 49), no significant differences in H:L ratios were observed among treatments, which is consistent with the reports of studies that applied similar treatments to layers (Freitas et al., 2011) and broilers (Khalaji et al., 2011). As in the present study, all treatments resulted, at the end of the experiment, in H:L ratios considered low or optimal (minimum = 0.28 and maximum = 0.49); according to Gross and Siegel (1993), it was concluded that the birds did not suffer metabolic stress and were not immune-stimulated, as there was no increase in leukocyte values. Gross and Siegel (1993) propose the following H:L ratios for poultry: 0.20, 0.50, and 0.80, which characterize low, optimal, and high stress degrees, respectively.

Although the hens fed the diets with 1056 mg arginine and 200 mg phytogenic additive/kg of diet presented the lowest uric acid level, the results obtained with the other treatments did not indicate any kidney changes that could influence their health or performance. These results are consistent with the findings of Campbell (2004), who proposed values higher than 15 mg/dL as an indication of renal changes, and with studies evaluating phytogenic additives for layers that did not find any effect on uric acid levels (Yalçin et al., 2012). Blood glucose levels were not influenced by the treatments and indicated that the hens were not subjected to long periods of stress, because the levels

Table 6 - Blood lipids of layers fed different digestible arginine (Arg, mg/kg) and phytogenic additive (PA, mg/kg) levels

| Arginine | PA | Total cholesterol (mg/dL) | | Triglycerides (mg/dL) | | HDL (mg/dL) | | VLDL (mg/dL) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 87.04 | 96.48ab | 935.36 | 1062.60 | 21.65 | 25.51 | 187.08 | 212.58ab |
| | 100 | 84.04 | 87.27ab | 1019.21 | 979.82 | 20.91 | 25.73 | 200.67 | 196.00ab |
| | 200 | 82.43 | 80.89ab | 911.91 | 743.34 | 27.41 | 26.16 | 182.33 | 150.33b |
| 968 | 0 | 92.59 | 87.20ab | 1051.11 | 979.47 | 23.57 | 26.94 | 210.17 | 235.10ab |
| | 100 | 95.88 | 82.26ab | 1067.57 | 894.91 | 19.93 | 30.26 | 213.50 | 178.92ab |
| | 200 | 89.58 | 109.29a | 992.43 | 1180.30 | 15.99 | 22.41 | 196.83 | 261.80a |
| 1056 | 0 | 92.41 | 93.79ab | 1035.40 | 953.09 | 22.42 | 26.63 | 207.08 | 211.42ab |
| | 100 | 90.70 | 118.07a | 950.60 | 1123.01 | 24.09 | 30.40 | 190.08 | 224.58ab |
| | 200 | 58.52 | 74.60b | 621.45 | 823.00 | 21.47 | 28.27 | 124.42 | 164.80ab |
| 1144 | 0 | 148.28 | 100.11ab | 961.03 | 1136.73 | 20.01 | 21.21 | 192.58 | 227.40ab |
| | 100 | 100.77 | 99.96ab | 1213.08 | 1275.12 | 17.56 | 24.10 | 426.08 | 229.33ab |
| | 200 | 97.30 | 104.09ab | 981.77 | 1187.96 | 19.46 | 19.78 | 208.83 | 237.60ab |
| Arginine | | | | | | | | | |
| 880 | | 84.50 | 88.64 | 955.49 | 928.58b | 23.42 | 25.82 | 190.03 | 186.30 |
| 968 | | 92.69 | 93.25 | 1037.04 | 1018.22ab | 19.83 | 26.51 | 206.83 | 222.37 |
| 1056 | | 80.54 | 95.49 | 869.15 | 966.37ab | 22.59 | 28.42 | 173.86 | 202.35 |
| 1144 | | 115.45 | 101.3 | 1051.96 | 1199.94a | 19.10 | 21.83 | 275.83 | 231.31 |
| PA | | | | | | | | | |
| 0 | | 105.08 | 94.46 | 995.73 | 1030.69 | 21.82 | 24.88 | 199.23 | 220.75 |
| 100 | | 92.85 | 96.89 | 1062.62 | 1059.22 | 20.64 | 27.48 | 257.58 | 207.21 |
| 200 | | 81.96 | 92.19 | 876.89 | 981.67 | 21.30 | 24.47 | 178.10 | 201.09 |
| SEM | | 5.928 | 3.089 | 33.181 | 33.822 | 1.020 | 0.929 | 17.609 | 6.796 |
| P-value | | | | | | | | | |
| Arginine | | 0.170 | 0.482 | 0.166 | 0.019 | 0.388 | 0.099 | 0.168 | 0.039 |
| PA | | 0.289 | 0.810 | 0.062 | 0.536 | 0.882 | 0.296 | 0.150 | 0.467 |
| Arg × PA | | 0.793 | 0.037 | 0.429 | 0.065 | 0.633 | 0.889 | 0.328 | 0.032 |

HDL - high-density lipoprotein; VLDL - very low-density lipoprotein; SEM - standard error of the mean.
a,b - Means followed by different letters within the same column are statistically different (P<0.05).
Each mean represents one bird per cage (six birds per dietary treatment).

were maintained within the range considered normal for poultry (200-500 mg/dL) (Campbell, 2004). Studies with other phytogenic additives also did not find any differences in blood glucose levels when birds were subjected to stress (Zhang et al., 2013).

The dietary inclusion of the phytogenic additive associated with the supplementation of 1056 mg of arginine reduced total CHO levels and 200 mg phytogenic additive and the basal level of arginine in the diet reduced VLDL levels. The reduction of CHO by phytogenic additives may be related to the inhibition of the enzyme HMG-CoA reductase, thereby reducing CHO biosynthesis (Ting et al., 2011). These results are consistent with studies that evaluated *A. membranaceus* (Zhang et al., 2013) extracts, hesperidin and naringin (Ting et al., 2011), and black cumin seeds (Yalçin et al., 2012). In the present study, hens fed 130% arginine presented reduced blood triglyceride levels. This result is consistent with those of Fouad et al. (2013) in broilers fed arginine above their requirements, suggesting that extra arginine supply promoted the conversion of triglycerides into glycerol and free fatty acids, thereby reducing triglyceride blood levels.

Although the CBH test indicates the degree of immune organ responsiveness, particularly of the thymus that accounts for higher T lymphocyte and basophil release in the blood stream, the obtained results were not relevant. The only exception was that arginine inclusion level was lower (968 mg/kg of feed) when 200 mg of the phytogenic additive was added to the feed. The weaker CBH observed in the present study with the dietary inclusion of the phytogenic additive associated with arginine supplementation was also reported in studies with carvacrol associated with thymol (Hashemipour et al., 2013) and arginine (Jahanian, 2009).

Antibody titers against poultry pathogens and

Table 7 - Cutaneous basophil hypersensitivity (CBH, mm) response, antibody titers against Newcastle disease virus (NDV, $log_2$), and nitric oxide (NO, µmol/2 × $10^6$ cells) and peroxide ($H_2O_2$, nmol/2 × $10^6$ cells) production by peritoneal macrophages of layers fed different digestible arginine (Arg, mg/kg) and phytogenic additive (PA, mg/kg) levels

| Arginine | PA | CBH | | NDV | | NO | | $H_2O_2$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 0.36 | 0.15bc | 3.95 | 3.79 | 9.98 | 26.42b | 0.81 | 0.86 |
| | 100 | 0.38 | 0.15bc | 3.94 | 3.90 | 13.69 | 22.18b | 0.83 | 0.84 |
| | 200 | 0.30 | 0.10c | 3.92 | 3.80 | 16.02 | 28.53b | 1.41 | 0.77 |
| 968 | 0 | 0.58 | 0.31ab | 3.96 | 3.88 | 15.09 | 24.97b | 0.97 | 0.94 |
| | 100 | 0.45 | 0.19b | 4.03 | 3.90 | 19.26 | 25.22b | 0.92 | 1.08 |
| | 200 | 0.53 | 0.35a | 3.99 | 3.99 | 13.59 | 26.01b | 0.75 | 0.78 |
| 1056 | 0 | 0.59 | 0.27ab | 3.99 | 3.94 | 11.80 | 24.26b | 0.85 | 0.96 |
| | 100 | 0.57 | 0.25ab | 4.05 | 4.03 | 13.91 | 31.49b | 0.78 | 0.85 |
| | 200 | 0.65 | 0.21b | 3.97 | 3.98 | 14.10 | 28.83b | 1.27 | 0.87 |
| 1144 | 0 | 0.40 | 0.16bc | 3.98 | 3.95 | 16.19 | 29.02b | 0.93 | 1.04 |
| | 100 | 0.57 | 0.38a | 4.00 | 3.88 | 9.60 | 49.65a | 0.84 | 1.12 |
| | 200 | 0.58 | 0.29ab | 4.01 | 3.95 | 12.83 | 21.37b | 0.93 | 0.68 |
| Arginine | | | | | | | | | |
| 880 | | 0.35 | 0.14 | 3.94 | 3.83 | 13.23 | 25.71 | 1.02 | 0.82 |
| 968 | | 0.52 | 0.28 | 3.99 | 3.92 | 15.98 | 25.40 | 0.88 | 0.94 |
| 1056 | | 0.60 | 0.24 | 4.00 | 3.98 | 13.27 | 28.19 | 0.97 | 0.89 |
| 1144 | | 0.52 | 0.28 | 4.00 | 3.93 | 12.87 | 33.35 | 0.90 | 0.95 |
| PA | | | | | | | | | |
| 0 | | 0.48 | 0.22 | 3.97 | 3.89 | 13.27 | 26.17 | 0.89 | 0.95 |
| 100 | | 0.49 | 0.24 | 4.01 | 3.93 | 14.11 | 32.13 | 0.84 | 0.97 |
| 200 | | 0.51 | 0.24 | 3.97 | 3.93 | 14.13 | 26.18 | 1.09 | 0.78 |
| SEM | | 0.022 | 0.017 | 0.013 | 0.015 | 0.984 | 1.541 | 0.054 | 0.038 |
| P-value | | | | | | | | | |
| Arginine | | <0.001 | 0.003 | 0.282 | 0.001 | 0.684 | 0.175 | 0.785 | 0.671 |
| PA | | 0.800 | 0.854 | 0.490 | 0.406 | 0.924 | 0.145 | 0.134 | 0.080 |
| Arg × PA | | 0.364 | 0.040 | 0.910 | 0.194 | 0.583 | 0.023 | 0.257 | 0.699 |

SEM - standard error of the mean.
[1] Difference in thickness (mm) of the third interdigital fold before and after phytohemagglutinin inoculation or injection with NaCl solution at 0.9%.
a,b - Means followed by different letters within the same column are statistically different (P<0.05).
Each mean represents one bird per cage (six birds per dietary treatment), except for NDV (12 birds per dietary treatment).
Cutaneous basophil hypersensitivity week $41_{Arg} = -8.65689 + 0.01758x - 0.00000836x^2$; $R^2 = 0.99$ (inflection point = 1051 mg/kg).
Newcastle disease virus week $49_{Arg} = -1.27634 + 0.00994x - 0.00000471x^2$; $R^2 = 0.96$ (inflection point = 1055 mg/kg).
Cutaneous basophil hypersensitivity week $49_{100PA} = -0.6424 + 0.00087614x$; $R^2 = 0.93$.
Nitric oxide week $49_{100PA} = -69.85412 + 0.10078x$; $R^2 = 0.86$.

sensitization with sheep red blood cells are simple, direct, and efficient methods to evaluate immune system responsiveness in poultry. The development and maturation of lymphoid organs, releasing defense cells, increases antibody production. In the present study, arginine supplementation increased anti-NDV antibody titers, demonstrating the immunostimulating effect of this amino acid on lymphoid organs, with higher production of T-cells, which are the cells that present antigens, and plasma cells. Some studies have reported this effect of arginine supplementation on broilers immunized against coccidiosis (Perez-Carbajal et al., 2010) or vaccinated against NDV (Jahanian, 2009) or infectious bursal disease (Ruiz-Feria and Abdukalykova, 2009).

High arginine levels associated with the phytogenic additive increased NO production by macrophages. This demonstrates the immunostimulating effect of arginine on the cell-mediated immune response, with higher macrophage and lymphocyte production and response (Guo et al., 2015) and of the phytogenic additive, particularly of *A. membranaceus* and *B. dracunculifolia* extracts due to their proven immunostimulating and antibacterial actions (Qiu et al., 2007), resulting in higher heterophil and monocyte activity (Faix et al., 2009) and intestinal IgA production (Klasing, 2007).

Table 8 - Intestinal and blood malonaldehyde (MDA) levels of layers fed different digestible arginine (Arg) and phytogenic additive (PA) levels

| Arginine (mg/kg) | PA (mg/kg) | Intestinal MDA (mg/kg) | | Blood MDA (nmol/mL) | |
|---|---|---|---|---|---|
| | | Week 41 | Week 49 | Week 41 | Week 49 |
| 880 | 0 | 0.505 | 0.460a | 12.82 | 13.30 |
| | 100 | 0.505 | 0.393ab | 10.34 | 10.12 |
| | 200 | 0.376 | 0.241ab | 13.59 | 8.62 |
| 968 | 0 | 0.553 | 0.361ab | 11.86 | 12.00 |
| | 100 | 0.604 | 0.234ab | 9.34 | 10.80 |
| | 200 | 0.373 | 0.303ab | 13.88 | 6.83 |
| 1056 | 0 | 0.399 | 0.357ab | 14.76 | 9.15 |
| | 100 | 0.422 | 0.188b | 13.03 | 10.72 |
| | 200 | 0.297 | 0.477a | 12.70 | 5.67 |
| 1144 | 0 | 0.513 | 0.243ab | 10.85 | 11.36 |
| | 100 | 0.501 | 0.396ab | 7.69 | 10.78 |
| | 200 | 0.225 | 0.348ab | 8.63 | 11.46 |
| Arginine | | | | | |
| 880 | | 0.460 | 0.365 | 12.25 | 10.68 |
| 968 | | 0.510 | 0.300 | 11.69 | 9.82 |
| 1056 | | 0.373 | 0.340 | 13.50 | 8.51 |
| 1144 | | 0.413 | 0.329 | 9.06 | 11.20 |
| PA | | | | | |
| 0 | | 0.493a | 0.355 | 12.57 | 11.45a |
| 100 | | 0.508a | 0.303 | 10.10 | 10.59a |
| 200 | | 0.318b | 0.342 | 12.20 | 8.15b |
| SEM | | 0.033 | 0.021 | 0.629 | 0.554 |
| P-value | | | | | |
| Arginine | | 0.487 | 0.711 | 0.093 | 0.320 |
| PA | | 0.038 | 0.535 | 0.228 | 0.039 |
| Arg × PA | | 0.934 | 0.017 | 0.912 | 0.589 |

SEM - standard error of the mean.
a,b - Means followed by different letters within the same column are statistically different (P<0.05).
Each mean represents one bird per cage (six birds per dietary treatment).

The higher NO concentrations obtained in the hens fed the phytogenic additive are consistent with the findings of Gore and Qureshi (1997), who observed higher NO production by macrophages in the peritoneal cavity of broilers fed 10 IU of vitamin E *in ovo*. The authors attributed this result to the possible increased affinity of macrophage membrane receptor to stimulating agents, such as lipopolysaccharides, or to the higher NO synthesis promoted by vitamin E. This may have occurred in the present study due to the immunostimulating action of the lipopolysaccharides of *A. membranaceus* and to the action of baccharin present in *B. dracunculifolia*, increasing NO production. Other studies have previously demonstrated the immunostimulating action of those plants (Wang et al., 2015).

The dietary inclusion of the phytogenic additive reduced intestinal and blood MDA levels, showing the efficacy of its antioxidant action by reducing peroxidation and the release of free radicals. These effects are promoted by cinnamaldehyde present in cinnamon, resveratrol and proanthocyanins present in grape seed, *B. dracunculifolia* baccharin, cumaric and cafeic acids, and *A. membranaceus* polysaccharides. These results are consistent with the report of Faix et al. (2009), who also observed an antioxidant effect of cinnamon on the intestinal tissues, and with studies on the effects of other phytogenic additives on the muscle and other organs (Hashemipour et al., 2013). The blood MDA reduction results obtained in the present study were also observed with ginger extract (Zhao et al., 2011), carotenoids (Akdemir et al., 2012), *A. membranaceus* extract (Zhang et al., 2013), and carvacrol and thymol (Hashemipour et al., 2013).

## Conclusions

Dietary supplementation of 968 mg of arginine or 100 mg of a phytogenic additive (40% *Baccharis dracunculifolia*, 20% *Astragalus membranaceus*, 20% cinnamon, and 20% grape seed extracts) per kilogram of diet improves the feed conversion ratio and associated inclusion of 1144 mg of arginine and 100 mg of phytogenic additive per kilogram of diet improves immune responses and health status of brown-egg layers.

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