Arginine and manganese supplementation on the immune competence of broilers immune stimulated with vaccine against *Salmonella* Enteritidis

Alvaro Mario Burin Junior ,[*,1] Nelson Luis Mello Fernandes,[†] Alessandra Snak,[†] Alba Fireman,[‡] Daiane Horn,[†] and Jovanir Inês Muller Fernandes[†]

[*]*Departamento de Zootecnia, Universidade de São Paulo - Escola Superior de Agricultura "Luiz de Queiroz" (USP-ESALQ), Piracicaba, São Paulo, 13.418-900 USP/ESALQ, SP, Brazil;* [†]*Departamento de Zootecnia, Universidade Federal do Paraná (UFPR) – Setor Palotina, Palotina, Paraná, 85.950-000 UFPR, Palotina, PR, Brazil; and* [‡]*Zinpro Corporation, Eden Prairie, Minnesota, 55344 Eden Prairie, MN, USA (Zinpro)*

ABSTRACT This experiment was conducted to evaluate the combined effects of manganese-amino acid complex and arginine supplementation on the immune competence of broilers. On the day of hatch 640 male Cobb 500 broiler chicks assigned to two study groups (immune stimulate and non-stimulated). A 2 × 2 factorial arrangement of treatments was used with two manganese sources ($MnSO_4$ or manganese-amino acid complex - MnAA) and two digestible Arg:Lys ratios (1.12 or 1.20). The treatments are: IM (80 ppm $MnSO_4$); MnAA (40 ppm $MnSO_4$ + 40 ppm MnAA); IM+Arg: 80 ppm $MnSO_4$+ L-Arg (Arg:DigLys 1.20); MnAA+Arg: 40 ppm $MnSO_4$ + 40 ppm MnAA + L-Arg (Arg:Lys 1.20). For treatments 1 and 2, the digestible Arg:Lys ratio was 1.12, considered normal for corn-soybean meal-based diets. Birds in the immune stimulated group received a dose of *Salmonella* Enteritidis vaccine. For growth performance and lymphoid organ development, no significant results were observed. Non-stimulated birds fed diets with Arg supplementation had higher percentage of mucosal T helper, T helper and T cytotoxic, compared to the normal Arg:Lys ratio (1.12). In the immune stimulated birds, broilers fed exclusive IM diet had a higher amount of T helper, T cytotoxic, activated T cytotoxic, and APC cells compared to broilers fed MnAA. The inorganic Mn diets, resulted in higher humoral antibody level (increased IgM levels) only when associated with supplementation of L-Arg. However, the use of an associated Mn source, could support high levels of IgM in commercial levels of Arg. No differences were observed to macrophage phagocytic activity analyses.

Key words: Nutrition, broiler, immunity, *salmonella*

2019 Poultry Science 98:2160–2168
http://dx.doi.org/10.3382/ps/pey570

INTRODUCTION

Economic loss due to enteric diseases is an important problem in the animal food industry, in spite of powerful health technologies such as all-in/all-out management, sanitation, biosecurity, vaccines, and others (Hardy, 2002). These losses may be worsened by the ban of the use of antibiotics as growth promoters in farm animals in the European Union in 2006 (Castanon, 2007), and the continuous pressure for reducing the use in the United States and Brazil.

One way to improve immune response and avian health is through the use of nutritional supplements (Kidd, 2004). Chickens do not produce citrulline in the intestine that is a precursor of arginine (**Arg**) (Kidd et al., 2001), which makes it an essential amino acid for broilers (Wu et al., 2010). Hence, studies have postulated that the serum level of Arg is influenced by its concentration in the diet and also protein breakdown in catabolic conditions (Kwak et al., 1999, 2001). In addition, studies show that arginine-deficient diets led to poor development of lymphoid organs (Kwak et al., 1999), which impacts broiler immune competence. It has been shown that Arg supplementation modulates or enhances humoral and cellular immune response (Jahanian, 2009; Munir et al., 2009), improves nitric oxide (**NO**) production (Wu et al., 2010), and attenuates inflammation (Tan et al., 2014). Arginine is degraded into ornithine, which is a precursor of polyamines, related to lymphocytes proliferation (Fernandes and Murakami, 2010). NO may enhance non-specific immunity by non-specifically killing pathogens such as bacteria, fungi, parasites, and tumor cells (Li et al., 2009). Studies show that immunity is supported by the addition of Arg to the diet due to the enhanced release of NO from macrophages (Lieboldt et al., 2016; Webel et al., 1998), which is an important component of the macrophage defense against *Salmonella* (Xie et al.,

Received May 16, 2018.
Accepted December 5, 2018.
[1]Corresponding author: amburin.jr@usp.br

1994). Another important function is that NO regulates the synthesis and secretion of some immune cytokines, such as tumor necrosis factor (**TNF**), prostaglandin E2, interleukin, and interferon (**IFN**), which leads to a more wide influence on the immune function (Guo et al., 2015). Furthermore, research has shown Arg effects on the weight of lymphoid organs (Kwak et al., 1999), the ratio of heterophils/lymphocytes and changes to the proportion of T cell subpopulations (Lee et al., 2002; Tan et al., 2014).

Manganese (**Mn**) has been identified as an important element in supporting normal immune functions in broiler chickens (Kidd, 2004), as it interacts with heterophils and macrophages through plasma membrane cells that are involved in immune response (Hurley and Keen, 1987). Son et al. (2007), reported that Mn supplementation increased Natural Killer cell cytolytic capacity and macrophage cytotoxicity against tumor cells compared to control. They concluded that immune responsiveness after supplementation with magnesium (**Mg**) and Mn presented positive effects; however, the exact explanation of how the minerals modulate the immune response was not completely clarified. Additionally, Mn is a cofactor of arginase, a metallo-enzyme that converts Arg into ornithine (and urea) (Wu and Morris, 1998). As Arg is a precursor of NO, through the nitric oxide synthase (**NOS**) enzyme, these enzymes compete for the same substrate (Wu et al., 2010). There is a complex pathway of Arg degradation; therefore, its dietary supplementation is needed to ensure both the health of the animal and the function of the body in physiological or pathological conditions.

We hypothesized that as some organic mineral sources have been reported as a more bioavailable and with higher efficacy compared to inorganic sources (Swiatkiewicz et al., 2014), more ornithine will be formed by the Arg breakdown through arginase, boosting the lymphocyte proliferation and supplementing with Arg enough substrate will be present to guarantee satisfactory NO production through NOS, modulating the immune system of broilers. No other study has assessed the immune modulation by the combination of Mn and Arg. The objective of this study was to evaluate the combined effects of amino acid-complexed Mn and Arg supplementation on macrophage phagocytic activity, cellular and humoral immunity, and weight of lymphocytes organ of broilers exposed to *Salmonella* Enteritidis as an immune stimulator.

MATERIAL AND METHODS

All animal protocols were approved by the Ethics Committee on the Use of Animals at Federal University of Paraná under protocol n° 51/2014.

Birds and Diets

On the day of hatch, 640 male, Cobb 500 broiler chicks were weighed and randomly assigned into two study groups (immune stimulated and non-stimulated). A 2 × 2 factorial arrangement of treatments was used (2 Mn sources x 2 Arg:Lys ratio). Thus, there were four dietary treatments with eight replicates in each treatment with 10 birds in each replicate. The treatments consisted of: IM: inorganic Mn (80 ppm Mn from $MnSO_4$); MnAA: partial substitution of inorganic Mn by Mn-amino acid complex (40 ppm Mn from $MnSO_4$ + 40 ppm Mn from MnAA); IM+Arg: 80 ppm Mn from $MnSO_4$+ L-Arg (Arg:DigLys 1.20); MnAA+Arg: 40 ppm Mn from $MnSO_4$ + 40 ppm Mn from Mn-amino acid complex + L-Arg (Arg:Lys 1.20).

For treatments IM and IM+MnAA, the digestible arginine:lysine ratio was 1.12, considered normal for typical corn-soybean meal based diets, which corresponds to 1.48% digestible Arg. For the treatments IM+Arg and MnAA+Arg, the 1.20 Arg:Lys ratio was obtained by the addition of 1 kg of L-Arg per ton of feed, resulting in 1.58% digestible Arg.

The organic Mn used is formed by one metal ion bound to one amino acid ion, described by the Association of American Feed Control Officials as a complex of a soluble metal salt with an amino acid. The product Availa®Mn was supplied by Zinpro Corporation (Eden Prairie, MN, USA).

The birds were kept in wired 4-level battery cages (80 cm x 55 cm) from 1 to 28 D of age, in an environmentally controlled room (exhaust fans, heaters and air conditioning). From the 1st until the 7th day, cages were lined with wood shavings to help maintain thermal comfort to the chicks. Diets were fed in mash form and birds were allowed *ad libitum* access to feed and water, with 24 h lighting schedule throughout the study. The basal diets were formulated to meet the nutrient requirements according to the Brazilian recommendation Tables of chickens from 1 to 28 D. The formula and calculated levels are presented on Table 1.

Vaccine Against Salmonella enteritidis*: Immune Stimulation of Chicks*

Bio-Enteritidis (a inactivated vaccination containing samples of PT4, PT4a, PT6a, PT7, PT7a and PT9; (Biovet Laboratories, Vargem Grande Paulista, Brazil) administered via intramuscular (breast muscle) at the dose of 0.5 mL/chick, was used to provide a cellular and humoral immune cells multiplication. The vaccination was performed at the ninth day for 320 broiler chicks only from the immune stimulated group.

Growth Performance, Lymphoid Organ Development and Mortality

The growth performance (body weight, body weight gain and feed intake) of the birds was evaluated for the whole experimental period (1 to 21 D) by weighing birds and leftover feed by replicate. Mortality was recorded daily, and feed conversion rate was calculated considering the weight of dead birds.

Table 1. Composition and calculated nutritional levels of the experimental diets.

	Starter (1 to 28 D)			
Ingredients, g/kg	IM	MnAA	IM+Arg	MnAA+Arg
Corn (CP 7.5%)	515.6	515.6	515.6	515.6
Soybean Meal (CP 46%)	419.0	419.0	419.0	419.0
Soybean Oil	24.0	24.0	24.0	24.0
Dicalcium Phosphate	15.2	15.2	15.2	15.2
Limestone	9.8	9.8	9.8	9.8
Salt	3.6	3.6	3.6	3.6
Methionine (DL, 98%)	3.8	3.8	3.8	3.8
L-Lysine-HCl (70%)	2.5	2.5	2.5	2.5
L-Threonine (98%)	0.7	0.7	0.7	0.7
Inert[1]	1.3	1.0	0.3	0.0
Sodium Bicarbonate	1.3	1.3	1.3	1.3
Choline Chloride	0.3	0.3	0.3	0.3
Salinomycin	0.5	0.5	0.5	0.5
Enramycin	0.1	0.1	0.1	0.1
Vitamin Premix[2]	1.0	1.0	1.0	1.0
Mineral Premix[2]	1.0	1.0	1.0	1.0
Phytase	0.1	0.1	0.1	0.1
$MnSO_4$ (26%)	0.15	-	0.15	-
Availa Mn (8%)	-	0.5	-	0.5
L-Arg (99%)	-	-	1.0	1.0
Total	1000.0	1000.0	1000.0	1000.0
Calculated Levels				
ME (Kcal/Kg)	2959	2959	2959	2959
CP (g/kg)	240.0	240.0	240.0	240.0
Calcium (g/kg)	10.4	10.4	10.4	10.4
Available Phosphorus (g/kg)	5.2	5.2	5.2	5.2
Lysine dig. (g/kg)	13.2	13.2	13.2	13.2
Arginine dig (g/kg)	14.8	14.8	15.8	15.8
Methionine+Cystine dig (g/kg)	10.1	10.1	10.1	10.1
Arginine:Lysine	1.12	1.12	1.20	1.20
Mn (mg/kg)	80	80	80	80

[1]Kaolin was used as an inert ingredient and was removed from the respective diets as Availa Mn (8% Mn), $MnSO_4$ (26.0% Mn) and/or L-Arginine (99.0% Arg) were added.

[2]The premix provided the following per kg of diet: retinol, 9,000 IU; cholecalciferol, 4,000 IU; tocopherol, 30 IU; mandeione, 3.0 mg; thiamine, 2.0 mg; riboflavin, 7.0 mg; pyridoxine, 4.0 mg; cyanocobalamin, 15 mcg; niacin, 0.05 g; pantothenic acid, 0.012 g, folic acid, 3.0 mg; biotin 0.20 mg, BHT, 0.10 mg. Manganese, 40.0 mg; zinc, 80.5 mg; iron, 39.90 mg; copper, 10 mg; iodine, 0.71 mg; selenium, 0.30 mg.

At 21 D old, 8 birds from each treatment for both groups (non-stimulated and immune stimulated) were selected randomly, submitted to electrical stunning and then slaughtered by severing the jugular vein. The lymphatic organs (spleen, thymus and bursa) were removed immediately, carefully stripped of adhering connective tissue and individually weighed. Relative organ weights were calculated as percentage of body weight. The upper 3 lobes of the thymus from the left side of the neck were collected.

By the end of the trial, the percentage of mortality was calculated using the following formula: number of dead birds/number of birds on day one $\times$ 100.

Flow Cytometry Analyses

Seven days after the vaccination (16 D of age), blood samples were obtained by heart puncture from one random bird from each pen (immune stimulated and non-stimulated birds). These samples were collected with the aid of heparinized vacutainer tubes (Becton Dickenson Co., Franklin Lakes, NJ, USA) and kept at 2 to 8°C. Then the samples were submitted to flow cytometer analyses in a FACSCalibur® (Becton Dickinson Co., Franklin Lakes, NJ, USA). A no-lysis-no-wash protocol was used for flow cytometry. Briefly, 50 μl of whole blood was stained with 1 μl (concentration was fluorophore-dependant) of primary antibodies (diluted in 10 μl) for 30 min at room temperature (antibodies shown on Table 2). Paraformaldehyde at 2.3% was used to fix the sample; it was added at 30 μl/sample and was kept for 30 min at room temperature. Following fixation, samples were diluted to 2 ml in PBS/1% BSA. The samples were then read in a FACSCalibur (BD). All samples were stained for CD45 (clone panleucocyte marker), which is SPRD-coupled (red, read at FL-3). $CD45^{bright+}$ cells were gated and then analysed for expression of other markers. Small debris were excluded from analysis. Anti-CD4, anti-CD8 and anti-Kul-1 were FITC-coupled (green, read at FL-1); anti-TCR Vβ1, anti-CD28 and anti-MHC class II were PE-labelled (yellow, read at FL-2). At least 10,000 leucocytes were counted. Single-colour controls were used for diminishing spectral overlaps. All fluorophores were excited with an argon laser.

Table 2. Different subpopulations of immune cells and their antibodies assessed by flow cytometry.

Lymphocyte subpopulations	Cell names	Antibodies
Mucosa T helper lymphocyte	$CD4^+TCRv\beta1^+$	CD4 (clone CT-4)/TCRvβ1 (clone TCR-2)/CD45
T helper lymphocyte	$CD4^+TCRv\beta1^-$	CD4/TCRvβ1/CD45
T cytotoxic lymphocytes	$CD8^+CD28^+$	CD8α (clone CT-8)/CD28 (clone AV-7)/CD45(clone LT40)
Activated T cytotoxic lymphocytes	$CD8^+CD28^-$	CD8α/CD28/CD45
Suppressor monocytes	Kul^+MHCII^-	Kul-1 (clone Kul01)/MHCII (clone 2G11)/CD45
APC	Kul^-MHCII^+	Kul-1/MHCII/CD45

ELISA Analyses of Immunoglobulins

Serum samples were collected from one random bird per experimental unit (n = 8) in immune stimulated and non-stimulated groups, and used to measure the concentrations of IgM and IgY isotypes, 19 D after the day of stimuli (28 D old). Indirect ELISA (Enzyme Linked Immunosorbent Assay) was used, in which the antigens were extracted from the same *Salmonella* vaccine used to immune stimulate the birds, and quantified by Bradford. After quantification, it was diluted in carbonate buffer with final concentration of 1 μg of protein in 100 μl. Then, this solution was added to each assay plate well and incubated at 4 to 8°C for 16 h to allow protein adsorption in the well. The plate was then washed 3 times with a wash solution, when 100 μl the 1:400 serum:incubation buffer were added and the plate submitted to incubation at 37°C for 1 h and then washed 3 times with wash solution. Then, 100 μl of block solution was added, with the aim to block areas where the antibody did not adhere, incubated at 37°C for 1 h and then washed 3 times with wash solution. It used IgM and IgY conjugates (Sigma) in 1:15,000 and 1:30,000 conjugate:incubation buffer ratio, respectively, and incubated for 1 h at 37°C. After that it was added OPD (o-phenylenediamine dihydrochloride) substrate and then the plate was incubated in a dark room for 40 min at room temperature. Stop solution (Sulfuric Acid 5%) were added before reading the plate. Absorbance was assessed using a 495 nm wavelength.

Macrophage Phagocytic Activity

At 19 D of age, 8 birds per treatment, 1 per pen (only for the non-stimulated group) were inoculated with intra-abdominal injection of Sephadex G-50® (Sigma) at 3%, (1.0 mL/100 g of live weight) to migrate macrophages to abdominal cavity (Qureshi et al., 1986; Konjufca, 2004). For this procedure, G-14 intravascular catheters were used. After 48 h, the birds were submitted to electrical stunning then euthanized by cervical dislocation. The abdominal cavity was opened slightly to perform the abdominal liquid collection with PBS (phosphate buffer solution). This step was conducted in an extremely clean environment, with the aid of sterile equipment. Previously, the abdominal liquid collection was prepared the saline solution of *Escherichia coli* (ATCC 8739). The bacterial concentration was determined spectrophotometrically using a standard curve at a reference wavelength of 625 nm. A stock solution (1×10^7 CFU/mL) was prepared by diluting in sterile PBS (pH 7.2). With the aid of a Neubauer chamber, macrophages were counted to make sure we were keeping the desired macrophage:bacteria ratio (1:10). The culture plates with 15 mm cover slips in the bottom, with the macrophage and bacteria solutions were incubated at 37°C for 1 h. After that, the cover slips were removed from the culture plate, allowed to dry at room temperature and then stained with Panotico. On each slide 300 macrophages and the numbers of these cells with enclosed bacteria were counted. Phagocytic activity was calculated from the number of macrophages containing enclosed bacteria divided by the total number of macrophages counted. Slides that did not contain at least 300 macrophages were not considered for statistical analyses.

Statistical Analyzes

The results are reported as means with standard errors and all data were analyzed as a completely randomized design using the General Linear Models procedure of SAS 9.4 (SAS Institute, Cary, NC). Data were subjected to two-way ANOVA in a 2 × 2 factorial arrangement, where Mn source and arginine:lysine ratio were the main effects. Previously, the data were tested to outliers, normal distribution of residuals and homogeneity of variances, using the PROC UNIVARIATE function and Levene option of SAS, respectively. The immune stimulated and non-stimulated birds were separated to perform the analyses. Means were compared by pdiff option.

RESULTS

Growth Performance, Lymphoid Organ Development, and Mortality

The growth performance results from 1 to 21 D are presented in Table 3. No differences due to the main effects (Mn source and Arg supplementation) or interaction of Mn source and Arg:Lys ratio was observed, regardless of the presence of the vaccination. Data of the lymphoid organ relative weight (Table 4), also shown no statistical difference either in non-stimulated or immune stimulated birds. For the whole experimental period the percentage of dead birds was 1.88%. The

Table 3. Growth performance of broilers fed diets with different Mn sources and Arg:Lys ratio from 1 to 21 D.

	Mn[3]		Arg:Lys			*P* Value		
Items, g	IM	MnAA	1.12	1.20	SEM	Mn	Arg:Lys	Mn∗Arg:Lys
Unchallenged[1]								
BW	774.88	780.08	779.75	775.21	8.95	0.684	0.722	0.136
BWG	694.55	708.25	704.48	698.33	8.13	0.243	0.597	0.264
FI	1065.64	1092.86	1080.74	1077.77	12.65	0.139	0.869	0.807
FCR	1.535	1.544	1.535	1.544	0.015	0.682	0.665	0.269
Challenged[2]								
BW	765.61	770.50	758.92	777.19	10.58	0.746	0.232	0.988
BWG	687.83	697.37	688.51	696.70	11.89	0.574	0.630	0.950
FI	1066.81	1076.37	1075.46	1067.73	10.29	0.517	0.599	0.752
FCR	1.557	1.546	1.566	1.537	0.023	0.738	0.373	0.860

[1,2]Groups of unchallenged and challenged birds with their means ± SEM were analyzed separately for the two main effects (Mn and Arg:Lys). N = 8 replicates of 10 birds each per treatment combination.

[3]IM: 80 ppm of inorganic Mn; MnAA: 40 ppm of inorganic Mn + 40 ppm of Mn from Mn-amino acid complex. BW: Body weight (g); BWG: Body Weight gain (g); FI: Feed Intake (g); FCR: Feed Conversion Ratio (g:g).

Table 4. Relative weight of organs of broilers fed diets with different Mn sources and Arg:Lys ratio.

	Mn[4]		Arg:Lys			*P* Value		
Items, %[1]	IM	MnAA	1.12	1.20	SEM	Mn	Arg:Lys	Mn∗Arg:Lys
Unchallenged[2]								
Spleen	0.120	0.127	0.125	0.122	0.008	0.540	0.769	0.362
Bursa	0.175	0.172	0.178	0.169	0.012	0.876	0.602	0.513
Thymus	0.091	0.084	0.089	0.086	0.005	0.426	0.676	0.924
Challenged[3]								
Spleen	0.120	0.124	0.126	0.118	0.008	0.740	0.488	0.155
Bursa	0.150	0.136	0.135	0.151	0.011	0.422	0.347	0.314
Thymus	0.086	0.081	0.085	0.082	0.004	0.443	0.674	0.975

[1]Results expressed as a percentage of the body weight.

[2,3]Groups of unchallenged and challenged birds with their means ± SEM were analyzed separately for the two two main effects (Mn and Arg:Lys). N = 8 replicates of 1 bird each per treatment combination.

[4]IM: 80 ppm of inorganic Mn; MnAA: 40 ppm of inorganic Mn + 40 ppm of Mn from Mn-amino acid complex.

mortality was similarly distributed among the four treatments.

Flow Cytometry Analyses

There was no interaction between the main effects (Mn source and Arg:Lys ratio) for non-stimulated or immune stimulated birds. However, non-stimulated birds fed diets with higher Arg:Lys ratio had higher percentage ($P < 0.05$) of mucosa T helper ($CD4^+TCRv\beta1^+$), T helper ($CD4^+TCRv\beta1^-$) and T cytotoxic ($CD8^+CD28^+$), compared to the normal Arg:Lys ratio (1.12) (Figure 1). No differences were observed for activated T cytotoxic lymphocytes, suppressor monocytes or APC. The Mn sources tested did not influence any cell subpopulation assessed.

Interestingly, this response pattern (increased cell counting) for Arg supplementation compared to the basal level (Arg:Lys 1.12), was not found within the SE immune-stimulated birds. In turn, in immune stimulated birds, the main significant effect was for Mn source. Broilers fed exclusive inorganic Mn diet had a higher number of cells ($P < 0.05$) compared to associated sources for T helper ($CD4^+TCRv\beta1^-$), T cytotoxic ($CD8^+CD28^+$), activated T cytotoxic ($CD8^+CD28^-$), and APC (Kul^-MHCII^+). For suppressor monocytes (Kul^+MHCII), this pattern has changed, which birds fed MnAA had a higher amount ($P < 0.05$) compared to birds fed inorganic Mn. We also observed an increase of suppressors monocytes (Kul^+MHCII^-) when Arg:Lys ratio was increased, in comparison to non-supplemented diets (Figure 2).

ELISA Analyses of Immunoglobulins

Indirect ELISA was performed for humoral immune response assessment for both groups: non-stimulated and immune stimulated. For the non-vaccinated group, there was no difference for IgM or IgY. In the immune stimulated group, the interaction may be seen for IgM, showed in Figure 3. The supplementation of diets with MnAA resulted in increased IgM levels when birds were fed the basal Arg:Lys ratio, but not when fed increased Arg:Lys ratio ($P < 0.05$).

Macrophage Phagocytic Activity

With the purpose to quantify the macrophage activation, resulting in phagocytic capacity of the treated birds, this analyze was performed. No differences were observed in the main effects Mn source or Arg supplementation (Figure 4).

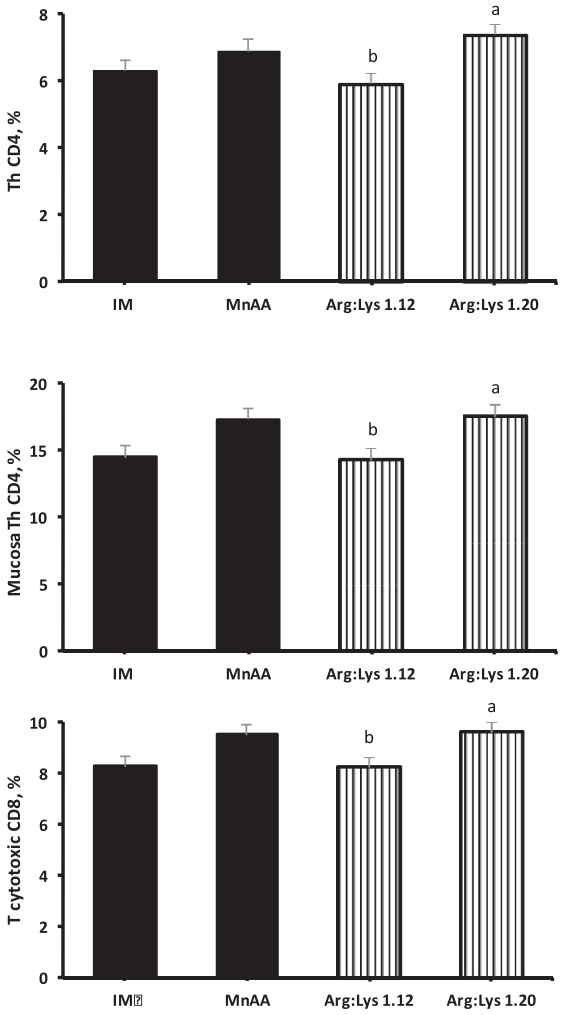

Figure 1. Data are presented as means ± SEM for sub-populations of lymphocytes CD4+ and CD8+ within the *non-stimulated birds group*. Each bar corresponds to the averaged results for each main effect. No significant interaction was observed. Different letters indicate significant differences within the main effect ($P < 0.05$). N = 8 replicates of 1 birds each per treatment combination.

DISCUSSION

This experiment aimed to assess the combined effects of Mn-amino acid complex and Arg supplementation during immune stimulus condition on cellular and humoral-adaptive immunity and phagocytic capacity of macrophages of birds (innate immunity) previously immune-stimulated by a SE vaccine.

The isolated effects of supplementation with Arg to healthy birds have been reported in the literature, showing that the maintenance of the immune response requires higher levels of Arg than used in commercial diets. However, it was observed in the present study that during immune stimuli, the increased Arg level (from 1.48 to 1.58 %) was not sufficient to activate the lymphocyte populations. Taylor and colleagues (1992) demonstrated that only the intake of high levels of dietary Arginine (2.4%) was able to increase the lymphocyte populations in birds challenged by virus. Conversely, Tan et al. (2014) reported that increasing levels

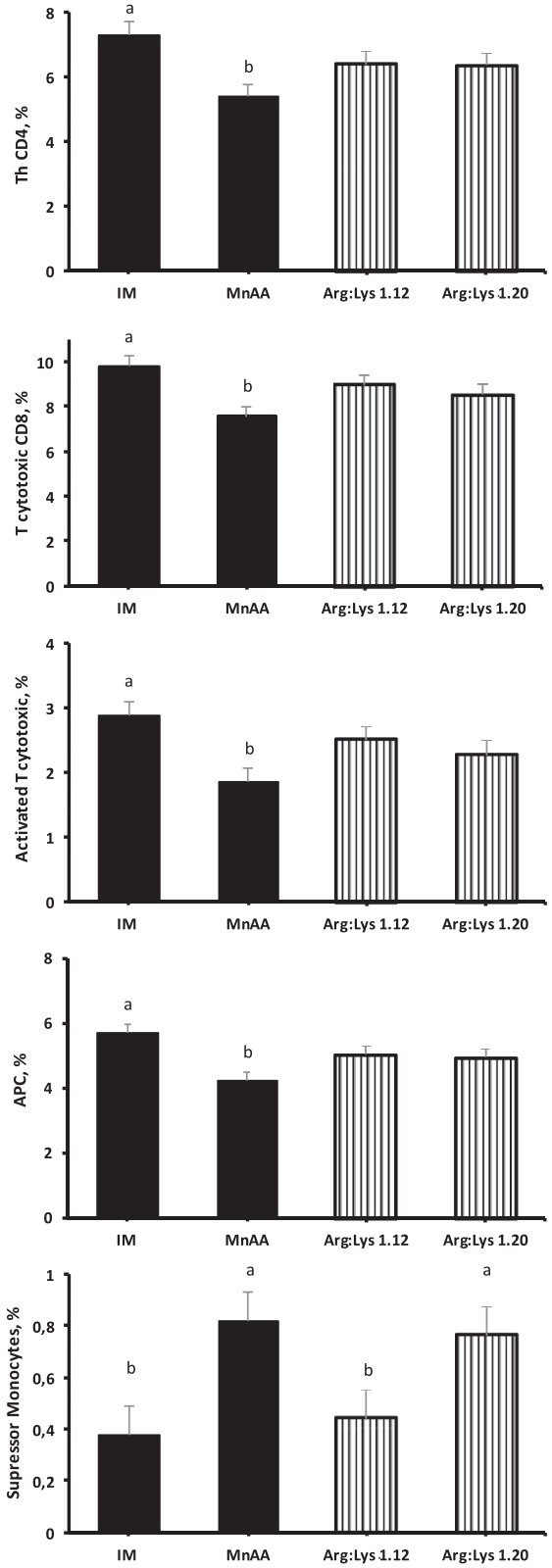

Figure 2. Data are presented as means ± SEM for sub-populations of lymphocytes CD4+ and CD8+, APC and suppressor monocytes within the *immune stimulated birds group*. Each bar corresponds to the averaged results for each main effect. No significant interaction was observed. Different letters indicate significant differences within the main effect ($P < 0.05$). N = 8 replicates of 1 birds each per treatment combination.

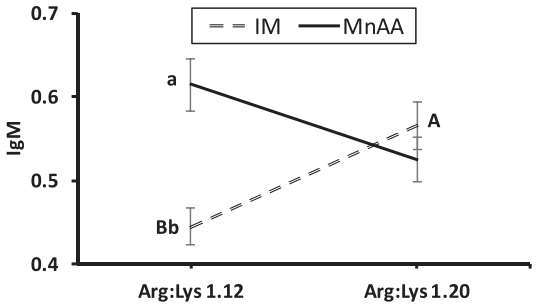

Figure 3. Data are presented as means ± SEM for IgM concentration within the *immune stimulated birds group*. Each point corresponds to the averaged results for each treatment combination. Different uppercase letters indicate significant differences within each Mn source and lowercase letters indicate significant differences within the Arg:Lys ratio ($P < 0.05$).

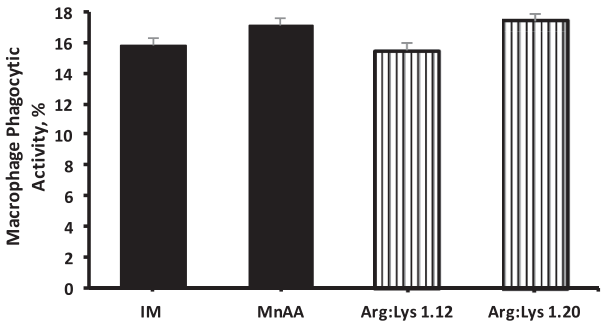

Figure 4. Data are presented as means ± SEM for percentage of macrophage phagocytic activity for unchallenged broilers fed diets with different Mn sources and Arg supplementation. Each point corresponds to the averaged results for each treatment combination. N = 8 replicates of 1 bird each per treatment combination in the *non-stimulated birds group*.

of Arg suppressed the percentages of both splenic and circulating B cells. They attribute the responses to an increase in H_2O_2 production in *in vitro* macrophages, that indicates a boost of innate immune response that plays an important role in the defense against various infections (Seifert et al., 2011).

It is understood that during lymphocyte activation, there is a clonal expansion generating effector and specific memory lymphocytes. For immune stimulated birds, there was smaller T cytotoxic lymphocytes (CD8+) percentage when birds were fed MnAA diets, compared to birds fed IM; despite this, the dietary supplementation with MnAA associated with a higher level of Arg, resulted in a higher production of antibodies (IgM, Figure 3). This demonstrates that birds fed MnAA could express an adaptive cellular response to vaccine stimulus in this period in a shorter, but more efficiently than birds fed inorganic Mn.

Salmonella killed vaccine generates cellular response, mainly by eliciting T cells, specifically Th1 cells (Babu et al., 2003; Chaudhari et al., 2012), which plays an important role on the clearance of *Salmonella* infection in poultry (Withanage et al., 2005). Additionally, oil adjuvant knowingly presents the capacity of, also, elicit a predominant Th1 response (Brunner et al., 2010), which promotes the generation of cytotoxic T cells, markedly stimulating humoral as well as cellular responses (Radosevic et al., 2008). Therefore, adjuvants can achieve qualitative modulation of the immune response and promote types of immunity not effectively generated by the non-adjuvanted antigens.

On the other hand, an increase of suppressors monocytes was observed when MnAA was added to the basal diet. Despite characterization of suppressor monocytes has not been complete in this study, myeloid cells that do not express MHCII can be considered suppressive (Peranzoni et al, 2010, Dilek et al., 2012).

It is well understood that circulating monocytes can block the function of lymphocytes, and are therefore named supressors. During inflammation, macrophages act as antigen-producing cells, enhancing the release of pro-inflammatory cytokines IL-1, IL-6, IL-12, TNF-α, and IFN_y chemokines. They also produce reactive oxygen species such as superoxide anion, hydroxyl radical, hydrogen peroxide, and reactive nitrogen intermediates, which can contribute to the oxidative instability of the body. The aim of this process is to remove the inducer stimuli of the innate immune response, which is interspecific, decrease feed intake and induce various metabolic changes such as muscle catabolism to obtain substrates necessary for the immune system and hepatic metabolism leading to reduction of the nutrients available for growth and development. Rather, an acquired humoral immune response that has higher specificity, requires less metabolic changes and therefore, is more efficient. In this work, immune stimulated birds and supplemented with MnAA commercial Arg:Lys ratio showed higher level of IgM immunoglobulins, despite of smaller T cytotoxic lymphocytes (CD8+) percentage observed when birds were fed MnAA diets, compared to birds fed IM.

Mn has been identified as an important element in supporting normal immune functions in broiler chickens (Kidd, 2004), as it interacts with neutrophils and macrophages through plasma membrane cells that are involved in immune response (Hurley and Keen, 1987). Furthermore, Mn is a component of the superoxide dismutase metallo-enzyme, which converts superoxide peroxide ion, free radical that glutathione peroxidase enzyme eliminates during the process of phagocytosis.

Phagocytosis is an innate immune reaction for the clearance of bacterial infections of animals, which plays an indispensable role in the defense against various infections (Seifert et al., 2011). Macrophages are a key arm of the innate immune defense system in intracellular bacterial killing (Ibuki et al., 2011). The antimicrobial activities of macrophages are due to the generation of reactive oxygen species and reactive N species, such as H_2O_2 and NO, which are important metabolites of Arg (Bronte and Zanovello, 2005). An *in vitro* study in channel catfish suggests that supplementation of Arg to cell-culture media enhances the phagocytic activity of head kidney macrophages (Pohlenz et al., 2012). As in

the present study, no significant influence was observed in the study published by Tan et al. (2014). On the contrary, Sung et al. (1991), found that NO production of macrophages was increased by a local concentration of Arg.

It is necessary to consider that a competition among arginase and nitric oxide synthetases for Arginine might happen. As a common substrate, it could lead to a reduction of the synthesis of NO, the main cytotoxic mediator of immune-activated effector cells and one of the most important regulating molecules of the immune system. This could lead to a major impairment to the bird immune capacity, leaving it more exposed to common challenges present in modern poultry production, as *Salmonella* or *Clostridium*.

Broilers from non-stimulated group were subjected to analyses of macrophage phagocytic activity with 19 days old. The percentage is provided by a ratio among the number of active macrophage divided by total macrophages counted × 100. There was no prejudice on macrophage phagocytic activity for the treatments assessed. As previously stated, NO is an important component of macrophage defense and activation, besides its capacity of modulate cytokines release (Xie et al., 1994; Guo et al., 2015). As Arg is a common substrate of both NOS and arginase enzymes, Arg supplementation was able to keep macrophage activation capacity, even though arginine was being converted into ornithine and urea to be used as precursors for bone mineralization.

No differences were obtained in the present study regarding the effect of immune stimulation, Mn source or arginine supplementation on absolute or relative weight of organs. The size and development of lymphatic organs are directly correlated with the health status of animals (Abdukalykova and Ruiz-feria, 2006). Conflicting results have been obtained in recent years about Arg supplementation/deficiency and development and size of organs related to the immune system. Authors have reported that feeding chickens with a diet that contains insufficient Arg level decreases the relative weight of bursa (Kwak et al., 1999; Konashi et al., 2000). However, the diet supplemented with more than optimum Arg level increased the relative weight of spleen rather than cloacal bursa (Kwak et al., 1999). In other studies, it has been reported that the level of Arg in the diet did not affect the relative weights of spleen and bursa of Fabricius in broilers (Kidd et al., 2001; Abdukalykova and Ruiz-feria, 2006; Cengiz and Küçükersan, 2010). Bulbul et al. (2014) reported that the relative weight of bursa of Fabricius decreased in Arg-deficient birds during grower and finisher phases. This is in agreement with Kwak et al. (1999) and Konashi et al. (2000). Furthermore, the relative weight of spleen increased when Arg was supplemented to the diet at starter phase (Kwak et al., 1999).

In recent times, Mn has become an element of increasing concern because of extremely rapid growth of broiler chickens, which exerts additional stress on the skeletal structure. So, there is a need to reassess the responses of broiler chicken to higher levels of Mn supplementation with emphasis on performance, mineral uptake by the tissues and immune competence.

It is difficult to identify the potential association of Mn and Arg toward a greater immune competence as just isolated main effects were observed for most of the immune assessments. However, dietary supplementation with MnAA resulted in improved modulation of immunogenicity and better immune stimulus to produce humoral antibodies (IgM). Birds fed MnAA could express a cellular response to vaccine stimulus in this period more efficiently than birds fed inorganic Mn. Higher levels of Mn or more bioavailable sources may be needed to optimize the immune competence and improve immune response in broiler chicken. Increasing levels of MnAA in association with arginine supplementation should be evaluated for better understanding of the contribution of the Mn in the activity of arginase.

REFERENCES

Abdukalykova, S., and C. A. Ruiz-Feria. 2006. Arginine and vitamin E improve the cellular and humoral immune response of broiler chickens. Int. J. Poult. Sci. 5:121–127.

Babu, U., M. Scott, M. J. Myers, M. Okamura, D. Gaines, H. F. Yancy, H. Lillehoj, R. A. Heckert, and R. B. Raybourne 2003. Effects of live attenuated and killed salmonella vaccine on T-Lymphocyte mediated immunity in laying hens. Vet. Immun. Immunopat. 91:39–44.

Bronte, V., and P. Zanovello. 2005. Regulation of immune responses by L-arginine metabolism. Nat. Rev. Immunol. 5:641–654.

Brunner, R., E. Jensen-Jarolin, and I. Pali-Schöll 2010. The ABC of clinical and experimental adjuvants: A brief overview. Immunol. Lett. 128:29–35.

Bulbul, T., V. Ozdemir, A. Bulbul, and E. Ulutas. 2014. The effect of dietary L-arginine intake on the level of antibody titer, the relative organ weight and colon motility in broilers. Pol. J. Vet. Sci. 17:113–121.

Castanon, J. I. R. 2007. History of the use of antibiotic as growth promoters in european poultry feeds. Poult. Sci. 86:2466–2471.

Cengiz, Ö., and S. Küçükersan. 2010. Effects of graded contents of arginin supplementation on growth performance, haematological parameters, and immune system in broilers. Rev. Med. Vet. 161:409–417.

Chaudhari, A. A., C. V. Jawale, S. W. Kim, and J. H. Lee. 2012. Construction of a salmonella gallinarum ghost as a novel inactivated vaccine candidate and its protective efficacy against fowl typhoid in chickens. Vet. Res. 43:1–11.

Dilek, N., R. Vuillefroy de Silly, G. Blancho, and B. Vanhove. 2012. Myeloid-derived suppressor cells: mechanisms of action and recent advances in their role in transplant tolerance. Front. Immun. 3:208.

Fernandes, J. I. M., and A. E. Murakami. 2010. Arginine metabolism in uricotelic species. Acta Sci. Anim. Sci. 32:357–366.

Guo, Y. W., B. L. Shi, S. M. Yan, Y. Q. Xu, J. L. Li, and T. Y. Li. 2015. Effects of arginine on cytokines and nitric oxide synthesis in broilers. J. Anim. Plant. Sci. 25:366–371.

Hardy, B. 2002. The issue of antibiotic use in the livestock industry: what have we learned? Anim. Biotechnol. 13:129–147.

Hurley, L. S., and C. L. Keen. 1987. Manganese. Pages 185–223 in Trace Elements in Human and Animal Nutrition. W. Mertz, ed. Acad. Press, Orlando, Fla.

Ibuki, M., J. Kovacs-nolan, K. Fukui, H. Kanatani, and Y. Mine. 2011. β 1-4 mannobiose enhances Salmonella-killing activity and activates innate immune responses in chicken macrophages. Vet. Immunol. Immunopathol. 139:289–295.

Jahanian, R. 2009. Immunological responses as affected by dietary protein and arginine concentrations in starting broiler chicks. Poult. Sci. 88:1818–1824.

Kidd, M. T. 2004. Nutritional modulation of immune function in broilers. Poult. Sci. 83:650–657.

Kidd, M. T., E. D. Peebles, S. K. Whitmarsh, J. B. Yeatman, and R. F. Wideman, 2001. Growth and immunity of broiler chicks as affected by dietary arginine. Poult. Sci. 80:1535–1542.

Konashi, S., K. Takahashi, and Y. Akiba. 2000. Effects of dietary essential amino acid deficiencies on immunological Effects of dietary essential amino acid deficiencies on immunological variables in broiler chickens. Br. J. Nutr. 83:449–456.

Konjufca, V. K. 2004. Influence of dietary vitamin E on phagocytic functions of macrophages in broilers. Poult. Sci. 83:1530–1534.

Kwak, H., R. E. Austic, and R. R. Dietert. 1999. Influence of dietary arginine concentration on lymphoid organ growth in chickens. Poult. Sci. 78:1536–1541.

Kwak, H., R. E. Austic, and R. R. Dietert. 2001. Arginine-genotype interactions and immune status. Nutr. Res. 21:1035–1044.

Lee, J. E., R. E. Austic, S. A. Naqi, K. A. Golemboski, and R. R. Dietert. 2002. Dietary arginine intake alters avian leukocyte population distribution during infectious bronchitis challenge. Poult. Sci. 81:793–798.

Li, H. Y., S. M. Yan, B. L. Shi, and X. Y. Guo. 2009. Effect of chitosan on nitric oxide content and inducible nitric oxide synthase activity in serum and expression of inducible nitric oxide synthase mrna in small intestine of broiler chickens. Asian-Australas. J. Anim. Sci. 22:1048–1053.

Lieboldt, M. A., J. Frahm, I. Halle, S. Görs, L. Schrader, S. Weigend, R. Preisinger, C. C. Metges, G. Breves, and S. Dänicke. 2016. Metabolic and clinical response to Escherichia coli lipopolysaccharide in layer pullets of different genetic backgrounds supplied with graded dietary L-arginine. Poult. Sci. 95:595–611.

Munir, K., M. A. Muneer, E. Masaoud, A. Tiwari, A. Mahmud, R. M. Chaudhry, and A. Rashid. 2009. Dietary arginine stimulates humoral and cell-mediated immunity in chickens vaccinated and challenged against hydropericardium syndrome virus. Poult. Sci. 88:1629–1638.

Peranzoni, E., S. Zilio, I. Marigo, L. Dolcetti, P. Zanovello, S. Mandruzzato, and V. Bronte. 2010. Myeloid-derived suppressor cell heterogeneity and subset definition. Curr. Opin. Immunol. 22:238–244.

Pohlenz, C., A. Buentello, W. Mwangi, and D. M. Gatlin. 2012. Arginine and glutamine supplementation to culture media improves the performance of various channel catfish immune cells. Fish Shellfish Immunol. 32:762–768.

Qureshi, M. A., R. R. Dieteri, and L. D. Bacon. 1986. Genetic variation in the recruitment and activation of chicken peritoneal macrophages. Exp. Biol. Med. 181:560–568.

Radosevic, K., A. Rodriguez, R. Mintardjo, D. Tax, K. L. Bengtsson, C. Thompson, M. Zambon, G. J. Weverling, F. Uytdehaag, and J. Goudsmit. 2008. Antibody and T-cell responses to a virosomal adjuvanted H9N2 avian influenza vaccine: impact of distinct additional adjuvants. Vaccine 26:3640–3646.

Seifert, S., C. Fritz, N. Carlini, S. W. Barth, C. M. A. P. Franz, and B. Watzl. 2011. Modulation of innate and adaptive immunity by the probiotic Bifidobacterium longum PCB133 in turkeys. Poult. Sci. 90:2275–2280.

Son, E. W., S. R. Lee, H. S. Choi, H. J. Koo, J. E. Huh, M. H. Kim, and S. Pyo. 2007. Effects of supplementation with higher levels of manganese and magnesium on immune function. Arch Pharm Res. 30:743–749.

Sung, Y. J., J. H. Hotchkiss, R. E. Austic, and R. R. Dietert. 1991. L-arginine-dependent production of a reactive nitrogen intermediate by macrophages of a uricotelic species. J. Leukoc. Biol. 50:49–56.

Swiatkiewicz, S., A. Arczewska-wlosek, and D. Józefiak. 2014. The efficacy of organic minerals in poultry nutrition: review and implications of recent studies. Worlds Poult. Sci. J. 70:475–486.

Tan, J., S. Liu, Y. Guo, T. J. Applegate, and S. D. Eicher. 2014. Dietary l-arginine supplementation attenuates lipopolysaccharide-induced inflammatory response in broiler chickens. Br. J. Nutr. 111:1394–1404.

Taylor, R. L., Jr., R. E. Austic, and R. R. Dietert. 1992. Dietary arginine influences rous sarcoma growth in a major histocompatibility b complex progressor genotype. Exp. Biol. Med. 19:38–41.

Webel, D. M., R. W. Johnson, and D. H. Baker. 1998. Lipopolysaccharide-induced reductions in food intake do not decrease the efficiency of lysine and threonine utilization for protein accretion in chickens. J. Nutr. 128:1760–1766.

Withanage, G. S. K., P. Wigle, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell 2005. Cytokine and chemokine responses associated with clearance of a primary salmonella enterica serovar typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immun. 73:5173–5182.

Wu, G., F. W. Bazer, R. C. Burghardt, G. A. Johnson, S. W. Kim, X. L. Li, M. C. Satterfield, and T. E. Spencer. 2010. Impacts of amino acid nutrition on pregnancy outcome in pigs: Mechanisms and implications for swine production1,2. J. Anim. Sci. 88:E195–E204.

Wu, G., and S. M. Morris. 1998. Arginine metabolism: nitric oxide and beyond. Biochem. J. 336:1–17.

Xie, Q., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-kappaB/Rel in induction of nitric oxide synthase. J. Biol. Chem. 269:4705–4708.