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Original

Leucogram of meat quails inoculated *in ovo* with conjugated linoleic acid and lauric acid

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ABSTRACT

Objective. Analyze the leukogram of meat-type quail inoculated *in ovo* with conjugated linoleic acid (CLA) and lauric acid (LA) while exposed to a sanitary challenge. **Material and methods**. The drinking water was placed seven days before the arrival of the quails and it was only refilled throughout the experiment. The treatments were applied on 480 fertile eggs in the 7th day of incubation: Negative control: not perforated; positive control: injected with the diluent (corn oil; CO); CLA120: 120 mg CLA /CO; CLA240: 240 mg CLA /CO; LA60: 60 mg LA /CO and LA90: 90 mg LA /CO. A completely randomized design with four replications was used. Total leukocytes and the differential leukocyte count were evaluated at 21 and 36 days of age. **Results**. No statistically effects (p>0.05) of any inoculations were observed in the parameters evaluated. We observed an average of 10927.1±2933.6 cell/µl, 61.6 ± 7.31%, 32.0 ± 7.13% and 0.57 ± 0.21 for leukocyte count, lymphocyte, heterophile and heterophile/lymphocyte ratio, respectively, at 21 days. At 36 days, we observed an overall average of 13291.7 ± 3559.0 cell/µl, 65.0 ± 9.29%, 29.3 ± 9.93% and 0.50 ± 0.24, for those same variables, respectively. **Conclusions**. The *in ovo* inoculation levels of CLA and LA did not affect the leucogram of meat-type quails raised in sanitary challenge.

Keywords: Hatching eggs; immune syste; nutrition; white blood cells (*Source: MeSH, NLM*).

RESUMEN

Objetivo. Evaluar el leucograma de codornices de engorde inoculadas *in* ovo del ácido linoleico conjugado (CLA) y ácido laurico (AL) mientras están expuestas a un desafío sanitario. **Material y métodos.** El agua de bebida fue ofrecida siente días antes de la llegada de las codornices y solo fue reabastecida, durante toda la fase del experimento. Los tratamientos fueron distribuidos en 480 huevos fértiles, en el 7° día de incubación: Control negativo: sin perforación, control positivo: inyectado con diluyente (aceite de maíz; AM; CLA 120: 120 mg CLA/AM; CLA240: 240 mg CLA /AM; AL 60: 60 mg AL /AM y AL90: 90 mg AL /AM. Fue utilizado delineamiento completamente al azar

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con cuatro repeticiones. Los leucocitos totales y el conteo diferencia de leucocitos fueron evaluados a los 21 y 36 días de edad. **Resultados.** Las inoculaciones, no presentaron efecto significativo (p>0.05) en los parámetros evaluados. Se observó una media de 10927.1 ± 2933.6 células/µl, 61.6 ± 7.31%, 32.0 ± 7.13% e 0.57 ± 0.21 para conteo total de leucocitos, linfocitos, heterofilos y la relación heterófilos/linfocitos, respectivamente, a los 21 días. A los 36 días, observamos una media general de 13291.7 ± 3559.0 células/µl, 65.0 ± 9.29%, 29.3±9.93% y 0.50 ± 0.24, para esas mismas variables, respectivamente. **Conclusiones**. Los niveles de inoculación *in* ovo de CLA e AL no afectaron el leucograma de codornices de engorde criadas en desafío sanitario.

Palabras clave: Glóbulos blancos; incubación de huevos; nutrición; sistema imunológicción (*Fuente: MeSH, NLM*).

INTRODUCTION

Adequate nutrition of birds is important even before birth, since it is possible that embryos have reduced availability of nutrients due to the intense genetic selection of current lineages (1). According to Gonçalves et al (2) the low reserve of nutrients in the final third of the incubation of eggs and during hatching, combined with the poor animal performance in the first days of life because of the limited digestive functions after hatching, can negatively impact the birth and survival of the birds.

The technique of *in ovo* nutrition is an alternative that aims at improving the early supply of nutrients and reducing mortality rates of quails to ensure that birds are more resistance during their productive life. Among the nutrients used *in ovo* nutrition, we can highlight amino acids, carbohydrates, prebiotics, and fatty acids such as conjugated linoleic acid (CLA) and lauric acid (LA). According to Mehr et al (3), CLA is important in the modulation of inflammation and immune responses of broilers, and can act on the white blood cell count. In addition, LA has anti-inflammatory and antimicrobial properties against athogenic microorganisms, which improves the immune system response at different stages of production such as embryonic, post-hatch, and growth (4).

According to Almeida Filho (5), immunological stress is one of the main limitations in the intensive production of poultry. Thus, birds need an efficient immune system to shield them against infectious agents that can cause diseases. Proper nutrition is essential to ensure bird health, since energy and nutrients are necessary for the formation of cells and other substances involved in the immune system (6). In this sense, Aguiar (7) and Almeida Filho (5) argue that the leukogram analysis, which is low cost and easily implemented, can be used to evaluate the immunity of quails facing a sanitary challenge since it indicates the responsiveness of the defense cells in the body.

It is necessary to evaluate the cells involved in the immune system of quails because most of the published works refer to broiler chicken and the few existing publications with quails and are conflicting (discrepant).

Based on that, we hypothesized that *in ovo* inoculation with CLA and LA fatty acids would promote a positive effect on the immune system of meat-type quails raised in poor sanitary conditions. The objective of this study was to analyze the effect of *in ovo* inoculation of CLA and LA in the leukogram of meat-type quails at the initial and growing phases under sanitary challenge conditions.

MATERIAL AND METHODS

Study location. The experiment was carried out from May to September 2018, in the facilities of the Department of Animal Science of the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), Diamantina, Minas Gerais, Brazil, with geographical coordinates 18° 10 'south latitude, 43° 30' west longitude and altitude of 1387 m.

Four hundred and eighty fertile quail eggs were incubated. The eggs were obtained from a 29-week-old batch of the European lineage of "Fujikura quail farm", located in Suzano, São Paulo, Brazil. The incubator used was of the brand COPEMARQ, model Labo 13, set at 37 °C and 70% relative humidity.

The treatments evaluated were NC: negative control – eggs not perforated and not inoculated;

PC: positive control – eggs inoculated with the diluent 0.050 mL (corn oil; CO); CLA120: eggs inoculated with 120 mg CLA/0.50 mL CO; CLA240: eggs inoculated with 240 mg CLA / 0.50 mL CO; LA60: eggs inoculated with 60 mg LA/0.50 mL CO; and LA90: eggs inoculated with 90 mg LA / 0.50 mL CO.

The *in ovo* inoculation was performed on the seventh day of incubation. It consisted of removing all eggs from the incubator, including the NC group, for an average of 30 minutes per tray, which was the time taken to inoculate all eggs in each tray. This ensured that all treatments were subjected to the same amount of time out of the incubator. The room was completely closed during the inoculation and heating lamps were used to reduce the thermal stress suffered by the embryo outside the incubator.

The inoculation surface was disinfected with a 70% ethanol solution. Then, disposable syringes of 1 mL capacity were used to apply 0.05 mL of the solutions (treatments) in the albumen region through the wide edge of the egg, approximately 3 mm below the eggshell (preliminary tests).

For CLA inoculations, the product Lipo-6 CLA, commercial brand Nutrex Research, with a concentration of 1000 mg was used, and for the supplementation of LA, extra virgin coconut oil of commercial origin was used, with the packaging of 200 mL, belonging to the brand Copra Coco.

All eggs were transferred to the hatchers at the day 15 of incubation, where they remained until hatching for another 2 or 3 days. After the birds were born (17th and 18th day of incubation), 240 non-sexed quails were distributed in a completely randomized design based on the inoculation protocol with four repetitions containing 10 quails per repetition. The quails were housed in metallic cages (60 width x 60 length x 35 height, cm), with a trough type feeder and a small conical water fountain. The cages were previously lined with newspaper to avoid injury to the birds' limbs. Incandescent lamps of 100 and 60 watts were used for heating, so the quails remained in thermal comfort throughout the experiment.

To produce a sanitary challenge, drinking water was placed in the cages seven days before the arrival of the quails and it was only refilled throughout the study period, without cleaning the water fountain. Additionally, excreta were not removed from floor linings and excreta collection trays. This was done for all treatments evaluated in this study and it simulated sanitary conditions observed under practical conditions in farms that do not follow an adequate cleaning protocol.

The experiment consisted of an initial (1 to 21 days of age) and growth (22 to 36 days) phase. The temperature and relative humidity were monitored daily using thermometers attached to the cage. The average humidity as well as minimum and maximum temperatures in the initial phase were 66.1%, 27.8°C, and 30.8°C, respectively. In the growth phase, the average humidity, minimum temperature, and maximum temperature were 72.8%, 22.5°C, and 25.2°C, respectively.

The feed provided was formulated based on corn and soybean meal, according to the recommendations of Silva and costa (8). The rations were isoenergetic and isoproteic. They were formulated for initial and growth phases (Table 1). Feed and water were provided *ad libitum* throughout the experimental period.

Laboratory methods. Leukogram was assessed by analyzing the blood cells of birds at 21 and 36 days of age. Blood samples were collected through the jugular vein of two birds (experimental unit) per repetition and eight birds per treatment, with a total of 48 birds being sampled per stage (initial and growth). Blood samples were placed in vials containing EDTA-anticoagulant diluted 200 times in Natt-Herrick diluent (20 µl of blood: 1,980 µl of the diluent).

Within 12 hours after blood sample collection, the total number of leukocytes were counted using optical microscopy under 400x magnification and using the modified Neubauer chamber. Differential leukocyte count was done on blood smears stained using the May-Grünwald-Giemsa method and analyzed in the optical microscope with a 1000X magnification. Next, the differential leukocyte counts were converted to percentage. An average per experimental unit was calculated for the variables total count of leukocytes (LEUK; cell/µl), lymphocytes (LYMPH; %), heterophiles (HETER; %), and the heterophile to lymphocyte ratio (HETER/LYMPH) before statistical analysis.

Table 1. Ingredients of the diets fed to meat-type quails in the initial (1 to 21 days) and growth phases (22 to 36 days).

Ingredients	Initial	Growth
Ground corn (7.92%)	49.6157	54.8315
Soybean meal (45%)	42.8111	38.4348
Soy oil	3.0763	4.0230
Limestone	1.2768	1.0579
Dicalcium phosphate	1.0318	0.7937
Salt	0.3748	0.3232
Minerals[1]	0.0500	0.0500
Vitamins[2]	0.0500	0.0500
L-Lysine HCL (78%)	0.2340	0.0000
L-Valine (98%)	0.0162	0.0000
L-Isoleucine (98.5%)	0.3212	0.1637
DL-Methionine (98%)	0.4228	0.2131
L-Threonine (99%)	0.2740	0.0490
Antioxidant[3]	0.0100	0.0100
Total	100.00	100.00
Calculated Composition		
Metabolizable Energy (Kcal/Kg)	2900.00	3000.00
Calcium (%)	0.85	0.70
Available Phosphorus (%)	0.32	0.27
Sodium (%)	0.17	0.15
Crude Protein (%)	25.00	22.00
Digestible Arginine (%)	1.92	1.52
Digestible Isoleucine (%)	1.14	0.9
Digestible Lysine (%)	1.37	1.08
Digestible Methionine + Cystine (%)	1.04	0.80
Digestible Threonine (%)	1.04	0.78
Digestible Tryptophan (%)	0.27	0.24
Digestible Valine (%)	1.01	0.92

[1]Composition/kg of product: Copper: 2500.00 mg; Choline: 27.00 mg; Iron: 12.5 mg; Iodine: 250.00 mg; Manganese: 7.5 mg; Methionine: 130.00 g; Selenium: 20.00 mg; Sodium: 120.00 g; Zinc: 4500.00 mg. [2]Composition/kg of product: Folic acid: 175.00 mg; Nicotinic acid: 28000.00 mg; Pantothenic acid: 2500.00 mg; Zinc Bacitracin: 5100.00 mg; BHA: 500.00 mg; BHT: 500.00 mg; Biotin: 12.50 mg; Vitamin A: 500,000.00 UI; Vitamin B1: 150.00 mg; Vitamin B12: 2500.00 mg; Vitamin B2: 800.00 mg; Vitamin B6: 250.00 mg; Vitamin D3: 170,000.00 UI; Vitamin E: 2100.00 UI; Vitamin K3: 400.00 mg; Salinomycin: 12500.00 mg/kg.[3]Butilated hydroxytoluene.

Statistical analysis. Statistical analyses were performed for each age separately and always adopting a significance level of 5%. A Poisson regression model was initially evaluated to analyze the LEUK. However, the assumption of equality between mean and variance, which is inherent of this methodology and was tested using the dispersion test, was not met for both ages. Thus, a negative binomial regression model was used in the analysis instead.

The ANOVA was used to analyze the remaining variables. The normality, heteroscedasticity, and independence assumptions were evaluated using the tests of Shapiro, Bartlett, and Durbin-Watson, respectively. The assumptions were not met for both LYMPH and HETER/LYMPH at 36 days of age. In these cases, the non-parametric test of Kruskal-Wallis was used instead.

Ethics and biosafety committee. Number 026/2017 of the Ethics Committee on the Use of Animals of the UFVJM.

RESULTS

It was not observed a statistically significant ($p>0.05$) effect of *in ovo* inoculation of CLA or LA on LEUK, LYMPH, HETER, and HETER/LYMPH in the blood of meat-type quail at 21 days of age (Table 2). The overall averages ± standard deviation of 10927.1±2933.6 cell/µl, 61.6±7.31%, 32.0±7.13% and 0.57±0.21 for LEUK, LYMPH, HETER, and HETER/LYMPH, respectively, were observed in this age.

Table 2. Mean total leukocyte (LEUK), lymphocyte (LYMPH), heterophile (HETER) and heterophile to lymphocyte ratio (HETER/LYMPH) in the blood of meat-type quail at 21 days of age after being *in ovo* inoculated with conjugated linoleic acid (CLA) and lauric acid (LA).

Item	Treatment[1]						Mean	SD[2]	P -value
	NC	PC	CLA 120	CLA240	LA60	LA90			
LEUK (cell/µl)	8437	11250	10125	13437	9812	12500	10927.1	2933.6	0.16
LYMPH (%)	62.8	64.0	65.5	54.2	59.2	64.0	61.6	7.31	0.26
HETER (%)	31.0	30.5	27.5	38.8	34.8	29.8	32.0	7.13	0.27
HETER/ LYMPH	0.53	0.49	0.45	0.79	0.64	0.50	0.57	0.21	0.18

[1]NC: Negative control; PC: Positive control; CLA 120: 120 mg of CLA/50 mL of CO; CLA 240: 240 mg of CLA/50 mL of CO; LA 60: 60 mg of LA /50 mL of CO; LA 90: 90 mg of LA/ 50 mL of CO.
[2]SD = Standard deviation

Likewise, *in ovo* inoculations with CLA and LA did not affect (p>0.05) the LEUK, LYMPH, HETER, and HETER/LYMPH in the blood of meat-type quail at 36 days of age (Table 3). In this age, we observed the overall averages of 13291.7 ± 3559.0 cell/µl, 65.0% ± 9.29%, 29.3% ± 9.93% and 0.50 ± 0.24 for LEUK, LYMPH, HETER and HETER/LYMPH, respectively.

Table 3. Mean total leukocyte (LEUK), lymphocyte (LYMPH), heterophile (HETER) and heterophile to lymphocyte ratio (HETER/LYMPH) in the blood of meat-type quail at 36 days of age after being *in ovo* inoculated with conjugated linoleic acid (CLA) and lauric acid (LA).

Item[2]	Treatment[1]						Mean	SD[3]	P-value
	NC	PC	CLA 120	CLA 240	LA60	LA90			
LEUK (cell/µl)	13500	11375	15750	12937	15875	10312	13291.7	3559.03	0.13
LYMPH (%)	70.2	63.8	59.2	61.8	63.0	72.0	65.0	9.29	0.33
HETER (%)	22.8	29.5	37.5	32.8	31.2	22.0	29.3	9.93	0.27
HETER/ LYMPH	0.34	0.50	0.71	0.56	0.52	0.33	0.50	0.24	0.25

[1]NC: Negative control; PC: Positive control; CLA 120: 120 mg of CLA/50 mL of CO; CLA 240: 240 mg of CLA/50 mL of CO; LA 60: 60 mg of LA /50 mL of CO; LA 90: 90 mg of LA/ 50 mL of CO.
[2]SD = Standard deviation

DISCUSSION

In the present study, the total and differential leukocyte count were analyzed to assess the possible effect of *in ovo* inoculation with CLA and LA fatty acids in the immune system of meat-type quails. We focused on blood cells (leukocytes) of birds under a health sanitary challenge (environment with poor sanitary conditions). Despite the beneficial effect of fatty acids in the organism of the animal reported by some authors such as Zeiger et al (9) and Shanbhag (4), no effect was found in the present study. Our results showed that the evaluated levels of CLA and LA inoculations did not have an effect in the immune system of the birds based on the LEUK, LYMPH, HETER, and HETER/LYMPH measured in the blood of meat-type quails at 21 and 36 days of age.

According to Dal'alba et al (10), the effect of the substance inoculated on the egg depends on the stage of the embryo, the species evaluated, the characteristics of the substance inoculated, the volume injected, and the application spot. In addition, other factors such as volume, concentrations, and types of solvents used to inoculate the solution can also have an influence in the effect (11). However, no recommendations are available for meat-type quails, making this a rich research field.

It is possible that the lack of effect of the *in ovo* inoculation occurred because the sanitary challenge adopted was not sufficient to trigger an immune response. In that case, no change on the number of leukocytes and, consequently, no effect of the treatments would be observed, regardless of their effectiveness.

Given the scarcity of laboratory values for LEUK of quails, some authors such as Rosa et al (12) and Stanquevis et al (13) have sought to determine reference values. However, there remain many divergences and inconsistencies between the values in the literature. This can be attributed to factors such as different species, diets, and stress levels in which quails are exposed. Thus, the control groups could be used as the reference levels to assess the effect of the CLA and LA inoculations in this study, which indicates no effect of the inoculation in LEUK of meat-type quails.

Based on the work conducted by Aguiar (7), the values established for Japanese quail lymphocytes are between 40% and 74.37%. Compared to our results, which ranged from 54.2% to 72%, the lymphocyte percentage at both 21 and 36 days are within the previously reported range. Our values of heterophiles were also within the range also close to that reported (31.75%) for *Coturnix coturnix Japonica* (14). Heterophiles can indicate the presence of infections and stress if the value is outside the recommended range for the species (15).

The HETER/LYMPH is an important indicator of animal welfare (16) since it is more sensitive to the stress level imposed to the animals. According to Carvalho et al (17), HETER/LYMPH is less variable than the LEUK, as well as LYMPH and HETER separately. The HETER/LYMPH ratio recommended for Japanese quails is between 0.52 and 0.70 (7). Compared to the results found in the present study, which ranged from 0.33 to 0.79, it possible to see that the ratio in most treatments were within the recommended range. This indicates that the sanitary challenge methodology used here was not sufficient to stress the immune system of the birds during the experimental period.

In addition to the leucogram results presented here, animal performance as well as the weight of the thymus, spleen, and Fabricius bursa, which are organs associated with the immune system, were reported elsewhere (18). However, no effect of *in ovo* inoculation of CLA and LA was observed either (18). On the other hand, Zeitz et al (19) argue that lauric and myristic fatty acid modulated the microbiota and intestinal morphology of birds, improving both nutrient absorption and utilization as well as the performance of broilers. Additionally, Martins (20) reported the beneficial effects of CLA in stimulating both passive and adaptive immunity of broiler chicks when added to the feed of the hens or in the post-hatch ration.

In conclusion, the *in ovo* inoculation of different levels of CLA and LA did not affect the leucogram of meat-type quails at 21 and 36 days of age raised in sanitary challenge.

Conflict of interests

The authors of this study declare no conflict of interest.

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