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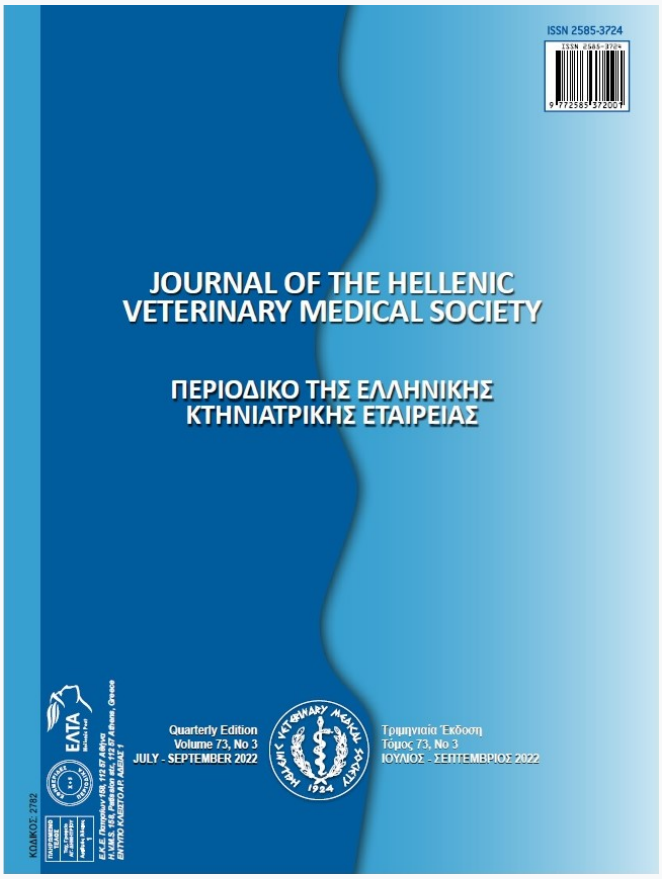

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Research article
Ερευνητικό άρθρο

Effects of dietary aspirin supplementation on liver enzymes, immune response, cecum microflora and fatty acids profile in breast meat of broiler chickens

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ABSTRACT: The present study was performed to investigate the effect of dietary aspirin (A) on broilers. A completely randomized design using 120 one-day-old male broiler chicks Ross 308, three levels of aspirin supplementation (0, 50 and 100 mg/kg) was used in 4 replicates, each including 10 chicks, during 42 days. The effects of different levels of aspirin, added to a basal diet, on blood plasma parameters, liver enzymes, immune system, cecal micro-flora, and fatty acids profile of breast muscle of chicks were investigated. Data analysis was performed by SAS statistical software and the comparison of the means with Duncan's test. The results showed that, the effects of aspirin on blood parameters, liver enzymes of broilers (except of alkaline phosphatase which significantly reduced), humoral immune system in response to antigen injection as sheep red blood cell (SRBC), antibody titer against Newcastle and influenza virus were not significant (P≥0.05). The highest percentage of neutrophils and eosinophils was related to the A100 treatment group. Also, aspirin treatments increased numerically the levels of unsaturated fatty acids and decreased saturated fatty acids in breast meat. Moreover aspirin led to a reduction in the population of Escherichia coli. So, based on the results of the present study, the use of 100 mg/kg aspirin in the diet of broilers is recommendable.

Keywords: aspirin, broilers, blood parameters, fatty acids profile, immune system

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INTRODUCTION

The Salicylate compounds belong to the group of nonsteroidal anti-inflammatory drugs (Nowak 2014). Salicylates such as sodium salicylate (SS) and acetylsalicylic acid (ASA) are used in poultry industry, due to their immune system effects, analgesic properties, and anti-inflammatory activities (Whiting et al. 2007). However, although insufficient information on tolerance levels and side effects of salicylates in poultry is available, but Poźniak et al (2010) showed that addition of 100 and 200 mg/kg aspirin or sodium salicylate (SS) in the diet of broilers were well tolerated and showed no side effects. Acetylsalicylic acid (ASA), which is the acetylated form of salicylic acid, (Richard 2007) is the active ingredient in aspirin (Wu et al. 2016). In addition, aspirin is involved in lowering cholesterol and triglycerides of blood and meat and plays a role in improving immune functions and antioxidant enzymes in birds (Alagawanyet al. 2017). Aspirin inhibits the biosynthesis of prostaglandins, therefore, may be able to regulate the hypothalamus (Truong et al. 2016). In other words, aspirin inhibits the enzyme cyclooxygenase, thereby inhibiting the synthesis of prostaglandins. Consequently, aspirin inhibits the release of arachidonic acid from the sn-2 position of phospholipids by altering the activity of phospholipase A, thereby inhibiting the synthesis of prostaglandins and leukotrienes and by also inhibiting thromboxane A2 synthesis also prevents platelet aggregation. In addition, aspirin reduces the serum LDL-cholesterol by increasing the number of LDL-cholesterol receptors in different cells (Valkoet al. 2005; Madamanchi et al. 2005)..Rokade et al. (2017) also reached to similar findings by adding 250-500 mg/kg of acetylsalicylic acid to the diet of broilers which resulted in a significant reduction in corticosteroids and serum cholesterol ($P<0.001$). Aspartate transaminase and alanine transaminase increased significantly as an effect of aspirin supplementation. Madamanchiet al. (2005) stated that improper environmental conditions due to increased free radicals cause oxidation and destruction of biological cells, so it can cause several disorders in intestinal tissue (Ocaket al. 2008; Sahinet al. 2013).

Since blood metabolites and fatty acids play a direct role in human cardiovascular disease, the findings of using aspirin in broilers may also be used in medical investigations.

Due to the conflicting results on the effect of antioxidant compounds on the mentioned factors and since there are limited results regarding the use of this compound in the broiler growing period, the present experiment was performed to evaluate different doses of aspirin dietary supplementation on the components of blood plasma, liver enzymes, the immune system, the microbial flora of the cecum, and the profile of fatty acids in breast meat in broilers.

MATERIALS AND METHODS

This study was conducted at a broiler farm in Masal, Iran. The experiment was performed with 120 one-day-old male chickens of commercial Ross 308 strain, with a mean weight of 45±2 g, in a completely randomized design with 3 treatments and 4 replicates and 10 chickens per pen for 42 days. The studied treatments were treatment 1 (A0mg/kg), treatment 2 (A50mg/kg) and treatment 3 (A100mg/kg) which were used in combination with the basal diet. The diets were ground and adjusted according to the table of nutritional needs of poultry containing the minimum recommended nutrients in the Ross 308 feed guide (Manual. 2012) (Table 1). The chickens were grown in 1 m × 1 m cages on a culture of cellulose rolls for 42 days. The temperature in the breeding hall was 33 degrees Celsius in the first week and then gradually decreased to 23 degrees Celsius on the 18th day of breeding and then remained constant until the end of the period. Environmental conditions were similar for all the chicks and included 23 hours of exposure and one hour of darkness, with room humidity of 65 to 70%. Access to water and food during the growing period was free. In addition, birds were vaccinated against infectious bronchitis (10th day of age), Newcastle disease (4th, 21st and 35th days of age) and infectious Bursal disease (12nd day of age). All vaccines were obtained from the Razi Vaccine and Serum Institute (Karaj, Iran).

Blood plasma components and liver enzymes

At the end of the experiment (42 days), 2 birds from each pen with a weight near to the mean were randomly selected and 5 ml of blood was collected from the wing vein in order to measure blood parameters. The samples were integrated and maintained for 12 hours at room temperature and then centrifuged at 5000 rpm for 3 minutes (Eppendorf 5702, Germany). Then, their serum was isolated and transferred to microtubes and brought to the laboratory. After separation, the serum was kept at minus 20˚C until the time of measuring blood metabolites. The serums were defrosted at room temperature and then, glucose, tri-

Table 1. Ingredients, chemical composition, and energy of the basal diets (from 1 to 42d of age)

Ingredients (g/kg as-fed)	Starter diet (1st-10th days of age)	Grower diet (11st-24th days ofage)	Finisher diet (25th-42nd days of age)
Corn	47.03	59.60	65.99
Wheat	5.58	5.00	5.00
Soybean meal (44% crude protein)	29.02	16.15	10.28
Corn gluten	10.00	11.48	11.50
soy oil	3.50	3.40	3.09
Limestone	1.45	1.23	1.00
Di-calcium phosphate	1.95	1.80	1.83
Salt	0.20	0.20	0.20
Vitamin and mineral supplements[1]	0.50	0.50	0.50
DL-methionine	0.52	0.58	0.57
L-lysine hydrochloride	0.25	0.06	0.04
Calculated compounds			
Metabolizable energy (kcal / kg)	2950	3000	3050
Crude protein (%)	22	20	19
Lysine (%)	1.3	1.2	1.1
Methionine (%)	0.56	0.54	0.52
Met+Cys (%)	0.92	0.90	0.88
Calcium (%)	1.04	0.95	0.92
Available phosphorus	0.52	0.47	0.41

1. The amount of vitamins and minerals per kg of the final diet:vitamin A, 9000 IU; vitamin D3, 3000 IU; vitamin E, 18 IU; vitamin K3, 3 mg; vitamin B1(Thiamine), 1/8 mg;vitamin B2(Riboflavin),6 mg; vitamin B6(Pyridoxine),3 mg; vitamin B12(Cyanocobalamin), 0/012 mg;vitamin B3(Niacin),30 mg; vitamin B9(Folic acid),1 mg;vitamin H3(Biotin),0/24mg;vitamin B5(Pantothenic acid),10 mg; 500 mg;Choline,100 mg;Mn,100 mg;Zinc,80 mg;Iron,10 mg; Cu,1 mg;I,0/2 mg;Selenio

glycerides, cholesterol, total protein, albumin, globulin, creatine kinase, lactate dehydrogenase, VLDL (Very-low-density lipoprotein), HDL (high-density lipoprotein), LDL (Low-density lipoprotein), alanine aminotransferase and alkaline phosphatase were measured. These metrics were tested with Pars Azmoon commercial kits and measured by the autoanalyzer (Hitachi 917, Japan) based on the method of Golrokhet al. (2016).

Immune responses

To investigate humoral immunity, the broiler chickens were immunized against SLBC by Lerner method (Lerner et al. 1971). To prepare an SRBC injecting suspension, blood samples were collected from 3 sheeps and poured into a glass containing EDTA. The globules were washed three times in PBS saline phosphate buffer and at the end 2% SRBC suspension was prepared in PBS. All the above steps were performed under sterile conditions. SRBC injection was performed on 28 and 36 days of age to 2 birds for each pen injecting 0.1 cc of the above mentioned solution to the wing vein. The blood samples were collected 7 and 14 days after the first and second injections, on days 35 and 42 (Gore and Qureshi 1997). Then, the antibody levels of the samples against SRBC were measured by hemagglutination method. To measure the antibody titer, special plates for microhemagglutination V were prepared. Van derzipp method was used to measure the total antibody. According to this method, to measure total anti-SRBC, 50ul of serum sample was mixed with 50µl Phosphate-buffered saline (PBS) inside the microtiter plate and then serial dilutions from 1:2 to 256:1 were prepared from serum. In the next step, 50ul was added to each well from 2% SRBC suspension and then placed at room temperature for 4 to 5 hours. The titers were expressed based on Log_2 of the highest dilution showing complete agglutination (Pourhosseinet al. 2015). In order to investigate the Newcastle disease (NDV) and influenza on 28 and 42 days of age, two birds from each pen were selected for blood samples and then the samples were merged and then to evaluate the serum levels of Newcastle and influenza, hemagglutination inhibition (HI) test was performed on the samples according to OIE standard (Office international des epizooties). 96-well microplates were used for the experiment. First, 25 microliters of PBS (Pripheral Blood Smear) were poured into all the wells, then 25 microliters of bird serum were poured into the first well and its dilution was performed until the last well. In the next step, 25 microliters of Newcastle and influenza antigens were

added to all of the wells. Then the microplate was put on the mechanical shaker for 1 minute and the microplate was then placed at 25˚C for 30 min. In the next step, 25 microliters of 1% red blood cells were added to all the wells, and the microplate was again placed on a mechanical shaker for 15 seconds, and then the microplate was placed at 25˚C for 30 minutes and the results were recorded. A 4-unit antigen (Pasouk, Iran) was used to perform the HI test. The titers were diluted based on $\log_2$. The red blood cell 1% was also obtained from SPF (Sun Protection Fector) chicks. On the day 42, two birds from each pen were selected for blood samples to count the total number of white blood cells and their differential counts, and their blood was transferred to the tubes containing anticoagulants after fusion. Determining the blood cells was performed by staining, cell differentiating and eye counting with optical microscope (Seidavi et al. 2014). In addition to examining the effect of aspirin on the immune system, at the end of the experiment, 2 birds per replicate with a weight near to the mean were slaughtered. The weights of spleen, bursa of Fabricius and thymus were measured using a digital scale with an accuracy of 0.01g (A&D GF-300 digital scale balance (310 gr × 0.001 gr, A&D Weighing Design and Manufacture, San Jose, CA)) (Shabani et al. 2015).

Microbial flora

To investigate cecalmicroflora, two birds of each treatment were slaughtered on day 42 and after opening the abdominal cavity, the right and left cecum were separated with sterile scissors and the contents were discharged into sterile microtubules and were maintained until microbial culture to examine the *Escherichia coli* population at 20˚C (Dibajiet al. 2014). To dilute the samples, successive dilution (1 to 10 ratio) in distilled water was used being autoclaved at a pressure of 120atm. One gram of each frozen sample was added after defrosting to 9 ml of distilled water to form a series of dilutions from 10^{-1} to 10^{-6}. Then 300 µl of each of the dilution series, 10^{-3}, 10^{-4}, and 10^{-5} was taken and inoculated on the plates containing the culture medium and spread completely on the culture surface by the loop. The cultivation was performed next to the flame and under the laboratory hood. The samples inoculated at 37˚C for 24 hours for the growth of Escherichia coli bacteria in the culture medium EMB (Eosin methylene blue Agar) were placed in incubation (Jang et al. 2007). Then, counting the colonies formed in the most suitable dilution (10^{-4}) was performed to determine the CFU (Colony Forming Units). After the counting, the number of colonies on each culture medium was multiplied in the inverse of dilution. Due to the magnitude of the numbers obtained from the bacterial count, in order to facilitate the calculations, the base-10 logarithm of the numbers was calculated and then used to analyze the data (Dibajiet al. 2014).

Profile of fatty acids

In order to measure the profile of fatty acids in 42 days from each treatment, one bird was slaughtered and the sample of breast meat was transferred to the laboratory. To do this, the muscle breast was first isolated, then ground and kept in a freezer at -20˚C (to protect the tissue and compound of the flesh). The fat content of the samples was extracted by the method of Folch et al. (1957). First a mixture of two strong solvents of chloroform and methanol was prepared with the ratio of 2 to 1, respectively. The amount of one gram of the mixed samples was then weighed and poured into the closed test tubes. Then 15 cc of the prepared solvent was added to it and thoroughly mixed and then kept in the refrigerator for 24 hours. After the above time, 5 cc of distilled water was added to the samples to create 3 phases in the test tube. After separating the bottom phase, which contains chloroform and dissolved fat, this part was poured into special centrifugal tubes and centrifuged at 25˚C with 300 rpm for 15 minutes until the phases of chloroform and fat were completely separated. This process was performed twice, and at the end, after final centrifugation of the bottom phase, which contained only chloroform and dissolved fat, it was separated and poured into a clean laboratory container. After the solution was poured into the test tube, the tube was placed under the laboratory hood in 70˚C water and nitrogen gas was blown on it to evaporate the chloroform. Then, 50 mg of pure extracted fat was removed and it was treated with base and acid methylation in two stages. First, the fat sample was kept for 30 minutes under the influence of 0.5M methoxide solution in methanol at 50˚C and then under the influence of chloridric acid solution in methanol (1:1 ratio) for 30 minutes at 50˚C. The methyl ester of fatty acids produced in hexane was dissolved by adding hexane to the solution. Then, by adding dry sodium sulfate to the solution and final dehydration, methyl esters of fatty acids dissolved in hexane were passed through a special filter and prepared for injection into the column of chromatographic gas device (Agilent America, 7890B

GC Series). Cl3 fatty acids were used as the internal standard. Pure nitrogen was used as the carrier gas for injection into the chromatographic gas device in a ratio of 1 to 50. The temperature schedule used for the column was such that the temperature of the oven was kept constant for 4 minutes at 100˚C. Then, it reached a temperature of 240˚C at a rate of 3˚C per minute, and was then kept at a constant temperature of 240˚C for 20 min. The temperature of injector was 225˚C and the detector temperature was 250˚C. The analysis time of each sample was 71 minutes and it should be noted that the nitrogen gas pressure inside the column was 2.2, the hydrogen gas pressure was 0.5 and the air pressure was 0.4 kg/m^2.

Statistical Analysis

All data collected during the experiment and laboratory traits were analyzed by SAS statistical software based on completely randomized design (CRD). The comparison of the means was performed with Duncan's multiple-range test at 5% statistical level.

RESULTS

Blood parameters and digestive enzymes

The results of using two different levels of aspirin on blood parameters and liver enzymes are shown in Tables 2 and 3. The results showed that the effects of two different levels of aspirin on blood parameters and liver enzymes of broilers except for alkaline phosphatase and Alanine transaminase were not significant throughout the period (P≥0.05). The lowest activity of this enzyme was at A50, but the difference was only significant compared to the control group (P<0.05).Indeed, according to the results, using the level A100 numerically led to a decrease in HDL and total protein. In addition, the use of the same level of aspirin caused the reduction of glucose concentration.

Immune system

The effects of adding different levels of aspirin in the diet on the function of the humoral immune system in response to the injection of SRBC antigen and antibody titer against Newcastle disease and influenza virus are shown in Tables 4 and 5. The results of the use of two levels of aspirin showed that there was no significant effect on the function of the humoral immune system in response to injection of SRBC antigen and antibody titer against Newcastle disease and influenza virus (P≥0.05). However, the highest percentage of neutrophils and eosinophilswere observed in the diets containing 100 mg/kg aspirin, and the lowest percentage of lymphocytes was related to the same level of aspirin. In addition, aspirin levels resulted in a significant correlation between relative weight of thymus and bursa of Fabricius (P<0.05) but did not significantly affect spleen weight (P≥0.05).

Table2. Bloodconstitutes mean (±SEM) of broilers at 42nd dayof age fed diets containing the different levels of aspirin

Aspirin (mg/kg)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	VLDL (Very low density lipoprotein) (mg/dl)	HDL Cholesterol (High Density Lipoproteins) (mg/dl)	LDL Cholesterol (Low Density Lipoproteins) (mg/dl)	HDL /LDL	Glucose (mg/dl)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
0	131.25	76.25	15.28	72.25	36.00	0.50	197.75	3.80	2.48	1.33
50	154.75	114.25	22.85	79.50	45.25	0.56	188.00	3.35	1.58	1.78
100	135.00	99.00	19.65	72.25	35.75	0.50	186.25	3.10	1.70	1.40
P-value	0.36	0.75	0.75	0.45	0.42	0.61	0.84	0.30	0.23	0.19
SEM	11.78	35.20	7.05	4.49	5.54	0.05	14.66	0.30	0.37	0.17

* Means within each column of dietary treatments with no superscript letter or at least one common superscript letter do not differ significantly (P≥0.05); SEM: Standard Error of Means

Table 3. Liver enzymes mean (±SEM) of broilers at 42nd day of age fed diets containing the different levels of aspirin

Aspirin (mg/kg)	Alkaline phosphatase (U/L)	Alanine transaminase (IU/L)	Lactate dehydrogenase (IU/L)	Creatine kinase (IU/L)
0	5048.30^{a}	239.25^{b}	4280.00	10051.00
50	3559.50^{b}	362.00^{a}	5024.30	26776.00
100	3743.30^{b}	315.25^{ab}	5334.00	18863.00
P-value	0.04	0.04	0.52	0.08
SEM	392.01	28.39	644.70	4541.99

* Means within each column of dietary treatments with no superscript letter or at least one common superscript letter do not differ significantly (P≥0.05); SEM: Standard Error of Mea

Table 4. Immune response mean (±SEM) of broilers fed diets containing the different levels of aspirin

Aspirin (mg/kg)	White blood Cells (42 day of age) ($n \times 10^3$/mL)	Neutrophils (42 day of age) (%)	Lymphocytes (42 day of age) (%)	Eosinophils (42 day of age) (%)	Antibody against Newcastle disease (28 day of age) (lg 2)	Antibody against Newcastle disease (42 day of age) (lg 2)	Antibody against avian influenza (28 day of age) (lg 2)	Antibody against avian influenza (42 day of age) (lg 2)	Antibody against sheep red blood cell (35 day of age)	Antibody against sheep red blood cell (42 day of age)
0	5250.00	14.25	80.00	5.50	4.00	6.00	2.50	4.50	5.50	6.75
50	1475.00	13.50	81.50	5.00	3.00	5.00	2.50	4.50	3.75	5.00
100	1850.00	23.25	70.50	6.25	3.25	4.00	2.75	4.75	4.50	5.75
P-value	0.11	0.20	0.17	0.87	0.49	0.13	0.77	0.77	0.05	0.07
SEM	1228.91	3.92	4.04	1.66	0.60	0.62	0.28	0.28	0.43	0.46

* Means within each column of dietary treatments with no superscript letter or at least one common superscriptletter do not differ significantly (P≥0.05); SEM: Standard Error of Means

Table 5. Immunity related organs mean (±SEM) of broilers at 42nd day of age fed diets containing the different levels of aspirin

Aspirin (mg/kg)	thymus (%)	spleen (%)	bursa of Fabricius (%)
0	0.49[a]	0.13	0.20[a]
50	0.21[c]	0.08	0.08[b]
100	0.38[b]	0.10	0.05[c]
P-value	0.0001	0.11	0.0001
SEM	0.02	0.01	0.006

* Means within each column of dietary treatments with no superscript letter or at least one common superscriptletter do not differ significantly (P≥0.05); SEM: Standard Error of Means

Microbial flora

The effect of aspirin on the population of *Escherichia coli* bacteria is shown in Table 6. The results showed that the population of *Escherichia coli* bacteria using the level A100 numerically decreased compared to the other treatments and the highest population of *Escherichia coli* bacteria was observed in the intestines of the chickens fed by A0.

Table 6. Sacrosemicroflora in broilers at 42nd day of age fed diets containing different amounts of aspirin

Aspirin (mg/kg)	*Escherichia coli* bacteria (log10 CFU/gr)
0	8.66
50	8.58
100	8.56

Profile of fatty acids in breast meat

The effect of the treatments on the percentage of fatty acids in the breast muscle tissue is shown in Table 7. The results showed that the levels of saturated fatty acids such as myristic acid, palmitic acid and stearic acid numerically decreased with increasing aspirin levels (no statistical analysis was performed on these data). The results also showed a positive effect of high levels of aspirin on the percentage of unsaturated fatty acids such as palmitoleic acid and oleic acid, and the highest increase was related to A100.

DISCUSSION

Blood parameters and digestive enzymes

The use of two different levels of aspirin on alkaline phosphatase was significant. Compounds that have antioxidant properties can affect blood parameters (Sahin et al. 2002). Naturally, the liver cells break down key proteins in the structure of lipoproteins, such as VLDL. This means that VLDL will not be converted to LDL which is the most important cholesterol carrier in the blood. In fact, antioxidants prevent this from happening in the liver cells. So, no oxidation of apoprotein B in VLDL means the continuation of the metabolism of lipoproteins and the formation of LDL (Krauss 2004).

Microbial flora

In fact, increasing free radicals oxidizes and destroys biological cells, so it can cause a number of disorders in intestinal tissue (Sahin et al. 2003; Ocak et al. 2008). Besides, antioxidants, including aspirin, inhibit free radicals actions, may ameleorate the problems related to the intestinal disorders caused by high-temperature and improve functional traits (Wang et al. 2008) which is almost in agreement with the

Table 7. Profile of breast fatty acids of the broilers at 42nd day of age fed diets containing different amounts of aspirin

Aspirin (mg/kg)	Myristic acid methyl ester C14:0 (%)	Palmitic acid methyl ester C16:0 (%)	Palmitoleic acid methyl ester C16:1c (%)	Stearic acid methyl ester C18:0 (%)	Oleic acid methyl ester C18:1n9c (%)	Linoleic acid methyl ester C18:2n6c (%)	Linolenic acid methyl ester C18:3n3 (%)	Cis-11,14-eicosadienoic acid methyl ester C20:2c (%)	Cis-8,11,14-eicosatrienoic acidmethyl ester c20:3n6c (%)	Cis-11,14,17-eicosatrienoic acid methyl ester C20:3 (%)
0	2.02	37.48	3.14	11.40	22.57	17.65	0.50	0.38	0.67	4.18
50	2.85	37.78	2.57	13.14	22.12	15.73	0.37	0.53	0.60	4.30
100	1.71	37.11	3.28	10.61	23.81	17.31	0.43	0.36	0.81	4.57

present results regarding microflora.

Profile of fatty acids in breast meat

According to the results of the above study, high levels of aspirin resulted in the numerically increase of the levels of cis-8,11,14-eicosatrienoic acid and cis-11,14,17-eicosatrienoic acid. The lowest cis-11,14-eicosadienoic acid was related to the same level of aspirin mentioned above. Cis-11,14-eicosatrienoic acid is produced by an enzyme delta-9 elongase from linoleic acid and can be converted to dihomo-γ-linolenic acid, arachidonic acid, sciadonic acid and other unsaturated fatty acids. Cis-11,14- eicosadienoic acid is able to adjust unsaturated fatty acids and is responsible for the response of macrophages to inflammatory stimulus. Along with other monounsaturated fatty acids, cis-11,14- eicosadienoic acid can inhibit the binding of leukotriene B_4 to the neutrophil membrane, which is part of these anti-inflammatory activities (Huang et al. 2011). According to Ghalib et al. (2011) aspirin also has this antioxidant activity; which means that aspirin can protect the cell membrane and the unsaturated fatty acids of the membrane against the oxidation of free radicals.

CONCLUSION

In general, it can be concluded that according to the present study, the use of aspirin in the diet of broilers (Ross 308), although did not influence the blood parameters measured here, but improved the immune system. Also, the use of 100 mg/kg aspirin led to an increase in unsaturated fatty acids and a decrease in saturated fatty acids in meat, which improve carcass meat quality and is beneficial to human health. Therefore, according to the results of this experiment, the use of 100 mg/kg aspirin is recommended in the diets for growing broiler chickens as an antioxidant compound.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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