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THE INFLUENCE OF ORGANIC AND INORGANIC Fe SUPPLEMENTATION ON RED BLOOD PICTURE, IMMUNE RESPONSE AND QUANTITY OF IRON IN ORGANS OF BROILER CHICKENS

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The aim of this study was to investigate the influence of organic and inorganic Fe supplementation on red blood picture, immune response and quantity of iron in organs of broiler chickens. The trial was conducted on 200 "Arbor Acres" chickens randomly alloted in four equal groups. Birds from all groups were fed standard broiler feed, supplemented with 40 mg/kg of Fe originating from different sources: Group I ($FeSO_4$), Group II (Fe bounded to yeast), Group III (ferrous ascorbate) and Group IV (iron chelate). From each group, 10 birds were sacrificed on 21st, 35th and 42nd day and the following parameters were measured: erythrocyte count, hematocrite value, hemoglobin concentration, concentration of nonheme iron in spleen, liver and bone marrow (femur), degree of cutaneous hypersensitivity to PHA and titers of antibodies to Gumboro virus following vaccination. Addition of organic iron supplements resulted in increased erythrocyte count, hemoglobin concentration and hematocrite value on the 21st day. Different iron forms did not change the concentration of nonheme iron in the liver on the 21st and 42nd day. On the 35th day, the group supplemented with ferrous ascorbate had lower liver iron concentration. Also, concentrations of nonheme iron in the spleen were lower in groups supplemented with organic iron forms. The concentration of iron in the bone marrow decreased with age and the lowest values were recorded in the ferrous ascorbate supplemented group. The degree of cutaneous hypersensitivity to PHA was higher in groups supplemented with organic iron forms on the 21st and 35th day. Titers of anti-Gumboro antibodies were higher in the group supplemented with iron helate on the 35th day, but later (day 42) no significant differences were observed among groups.

Key words: broilers, iron, red blood picture, immune response

INTRODUCTION

Iron is an essential element in all living organism, important for oxygen transport, mitochondrial respiratory chain and cell proliferation. Considering the fact that microelements take part in all biochemical processes, it is required to add them to food in intensive production. Lately, particularly when we consider the microelements, the market offers a wide palette of products that differ from each other in their bioavailability. Forms of iron used as supplements in diets both for humans and domestic animals, can be grouped as: soluble in water (ferrous sulphate), soluble in diluted acids (ferrous fumarate, feric saharate) and non-soluble in water or diluted acids, such as ferric pyrophosphate, ferric orthophosphate and elemental iron. Microelements organically bounded with amino-acids and proteins, so called chelates, are considered to have better bioavailability than inorganic salts, and are widely used in intensive cattle production (Veum *et al.*, 1995; Kegley *et al.*, 2002; Creech *et al.*, 2004). Henry and Miller (1995) showed that iron chelates have a relative bioavailability up to 125-185% compared to ferrous sulphate. Yu *et al.* (2000) used iron chelate in pig's diet and found increased levels of iron, as well as a higher total iron binding capacity in the serum. In the liver and spleen the levels of nonheme iron were also increased. It is believed that while passing through the gastrointestinal tract, chelated microelements are protected from the influence of the microenvironmental inhibitors with respect to inorganic salts (Lyons, 1994). Metals bound to amino acids are practically without electrical charge, so they do not react to changes in pH while passing through the digestive tract. Ashmead *et al.* (1985) found that amino acids and dipeptides have the role of transporters through the enterocyte membrane. Due to this way of resorption, the mechanism of homeostatic control on the enterocyte level is avoided. However, recent studies favour the statement that the resorption of iron in the form of chelate is also regulated at the enterocyte level by the same mechanism as the resorption of iron from ferrous sulphate (Bovell-Benjamin *et al.*, 2000). Mazariegos *et al.* (2004) performed experiments on Caco-2 cells and concluded that iron from chelates is resorbed like nonheme iron. Hurrell (2002), who summarized the results on bioavailability of different sources of iron, proved that iron helates have been used for several years, but there are no reliable results on their bioavailability. The author explains this with the argument that manufacturers finance the experiments with these supplements, so the results are often contradictory and unreliable.

Considering all the contradictions related to the usage of chelate forms of microelements in the diet for both humans and animals, we thought it was interesting to investigate the influence of these forms of iron on hematological parameters, immune response and iron quantity in organs of broilers.

MATERIALS AND METHODS

Experimental animals

The experiment was performed on 200 "Arbor Acres" chickens divided in four equal groups. The diet for broilers was formulated in accordance to NRC

recommendations (1994) and supplemented with 40 mg/kg of Fe originating from different sources: Group I ($FeSO_4$), Group II (Fe bound to yeast), Group III (ferrous ascorbate) and Group IV (iron chelate, BioPlex®, Alltech inc).

Blood and tissue samples

Blood samples were taken by cardiac puncture from 10 birds in each group, on the 21st, 35th and 42nd day in the amount of 3-5 mL. Number of erythrocytes (Er), hematocrite value (HCT), hemoglobin concentration (Hb) and titer of antibodies specific for Gumboro virus, were determined by standard laboratory procedures using the automatic hematological analyzer Arcus Diatron, Gmbh Wien, Austria. Broilers were sacrificed by decapitation, and samples of liver, spleen and bone marrow were taken in order to determine the quantity of nonheme iron.

Titers of antibodies specific for Gumboro virus were determined by ELISA (Aidexx, Russia). Content of nonheme iron in liver, spleen and bone marrow samples was determined using the colorimetric method with bathophenantroline (Cook, 1980).

Cutaneous basophil hypersensitivity reaction (CBHR): Test for cutaneous hypersensitivity was performed on 20th, 34th and 41st day of the experiment using intradermal application of 0.1 mL of the solution containing 100 μg of phytohemagglutinin (INEP, Zemun). The solution of phytohemagglutinin (PHA) was applied between the third and fourth digit of the left foot, and at the same time, between the third and fourth digit of the right foot PBS was applied (phosphate buffered solution, pH 7.2) in the same amount. Skin tickness (ST) was measured by a cutimeter before application (ST 0) and 24 hours later (ST 24). The magnitude of the reaction was calculated as the difference in skin ticcness between the sites of PHA and PBS application according to the following formulas:

$$\Delta PHA = ST\ 0^h - ST\ 24^h$$
$$\Delta PBS = ST\ 0^h - ST\ 24^h$$
$$CBHR = \Delta PHA - \Delta PBS$$

RESULTS AND DISCUSSION

During the experiment, the erythrocyte count in the blood of the experimental animals was within physiological limits (Feldman, 2000). The slight increase in the erythrocyte count concurrent with the increase in age of the experimental individuals, is also in accordance with literature data (Rusov *et al.*, 1976). The experimental group treated with ferrous ascorbate, had 9.21% more erythrocytes on the 21st day of the experiment, compared with the control group (ferrous sulphate), which is statistically significant at the level of $p<0.05$ (Figure 1).

Similar result were observed by Lim *et al.* (2000) with ascorbic acid given to fish. The increase of the erythrocyte count in broilers from this group was probably the consequence of a better bioavailability of ferrous ascorbate, compared to other forms of iron. Authors enlisted several mechanisms for the positive influence of ascorbic acid on iron resorption. Lynch and Cook (1980) found that ascorbic

acid facilitates the resorption of iron forming a chelate with ferric iron in the acid environment. This chelate is soluble and suitable for resorption in the alkaline environment of the duodenum. By reducing the trivalent food iron into a divalent form, ascorbic acid increas resorption (Hallberg *et al.*, 1980).

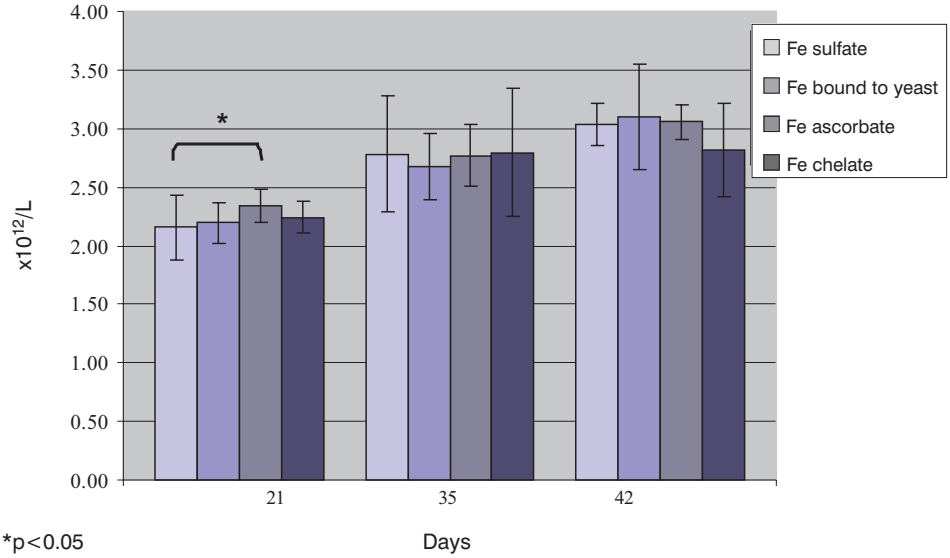


Figure 1. Erytrocite count (x10^{12}/L)

Hemoglobin concentrations in the blood of experimental broilers were within the physiological limits during the experiment and in accordance with the results of Pavkov *et al.* (1998) and Rusov *et al.* (1976). Due to the fact that about 80% of the resorbed iron incorporates into hemoglobin, the different bioavailability of iron might have an influence on hemoglobin concentration. As stated by Bothwell *et al.* (1979), the time required for the incorporation of resorbed iron into hemoglobin is about 14 days. High concentrations of iron in the plasma samples in groups III (ferrous ascorbate) and IV (iron chelate) were recorded in 21-day old chickens (data not shown here). Statistically significant increase of hemoglobin concentrations (Figure 2) in these groups on the 35th day of the experiment, relative to the control group (ferrous sulphate), could be a consequence of a better bioavailability of these forms of iron in 21-day old chickens.

These results are in accordance with the results published by Miski and Kratzer (1976), who found increased hemoglobin concentrations when ferrous ascorbate was used in broilers. Lim *et al.* (2000) studied the effects of supplementation with vitamin C in fish food, and also recorded a higher hemoglobin concentration in individuals supplemented with vitamin C.

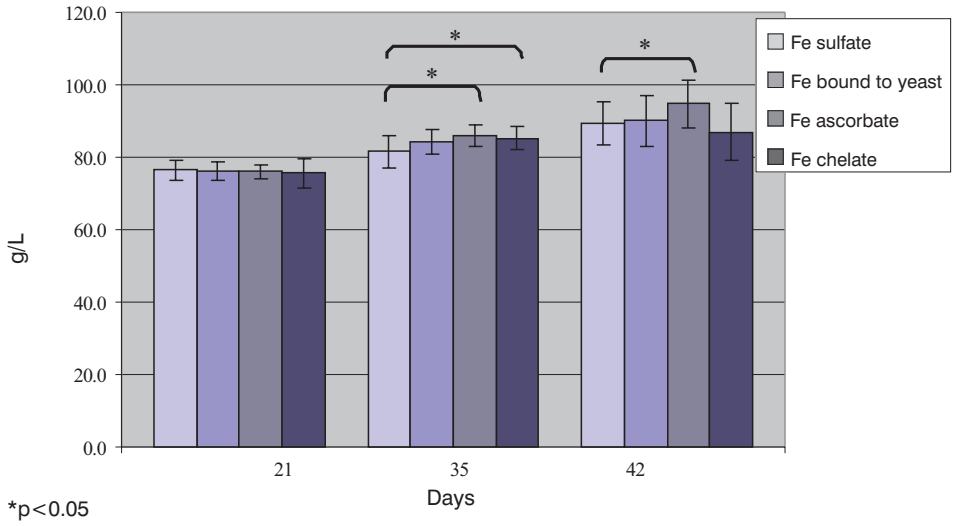


Figure 2. Hemoglobin concetrations (g/L)

There are very few literature data about the quantity of nonheme iron in the liver of chickens. A number of authors studied the quantity of total iron in this organ. As iron accumulates in the liver by bounding to the molecule of ferritin the quantity of nonheme iron in the liver might be a better indicator of iron status in the organism, than is the quantity of total iron in the liver. Underwood and Suttle (1999) reported iron values within the range of 1.79 - 2.68 μmol/g in poultry liver tissue, as a reference value for the quantity of total iron in the liver. In our experiment, the quantity of nonheme iron in the liver was 1.25-1.77 μmol/g on the 21st day, 1.60-3.21 μmol/g on the 35th day, and 2.31-2.84 μmol/g on the last day of the experiment. The increase in the quantity of iron in the liver with time (Figure 3), i.e. with the age of the individuals, coincide with the results of Crissey *et al.* (2000), who obtained similar results in an experiment on European starlings (*Sturnus vulgaris*). Authors treated experimental birds with different quantities of inorganic iron, and its concentrations in the liver were similar 8 weeks later.

When rats were fed with the chelate form of iron for five weeks, lower levels of total iron in the liver were detected, compared to the animals supplemented with ferrous sulphate, but a significant difference was not proved (Oliveira *et al.* 1995). Appel (2001) also reported lower values of nonheme iron in the liver of rats, when the organically bounded iron was used. These results are similar to our findings.

Lower levels of iron in the liver of 21-day and 35-day old experimental chickens treated with ferrous ascorbate, are in accordance with the results reported by Lim *et al.* (2000), who supplemented with vitamin C fish food. Results of Premkumar and Bowlus (2003) on 21-days old mice are similar to the results obtained at the end of our trial. Levels of nonheme iron in the liver of individuals

fed with ferrous ascorbate were higher than the quantity of iron in the liver of individuals fed ferrous sulphate, but statistically significant differences were not proved.

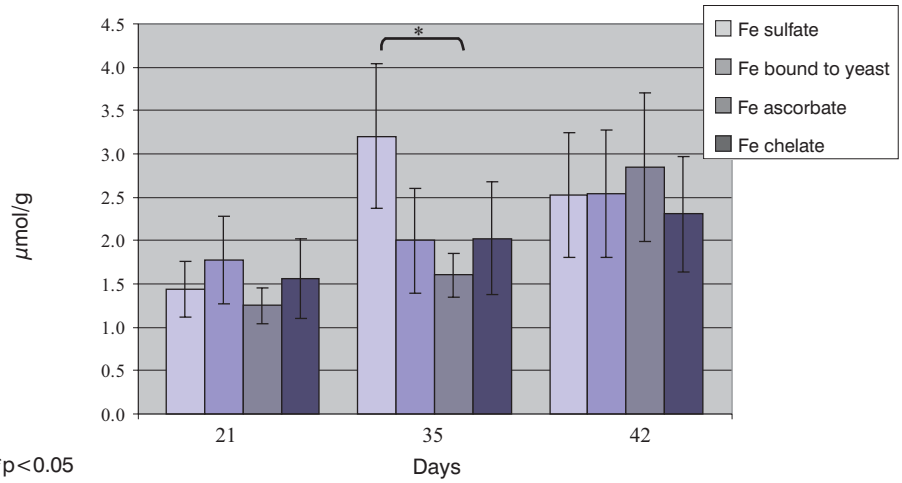


Figure 3. Concentrations of nonheme iron in the liver (µmol/g)

There are no literature reference values for the level of nonheme iron in the spleen of birds. Theurl *et al.* (2005) found that the level of iron in the spleen of rats is somewhat higher than its level in the liver. Results of Appel (2001) show that levels of iron in the liver and spleen, change with the increase of iron levels in the

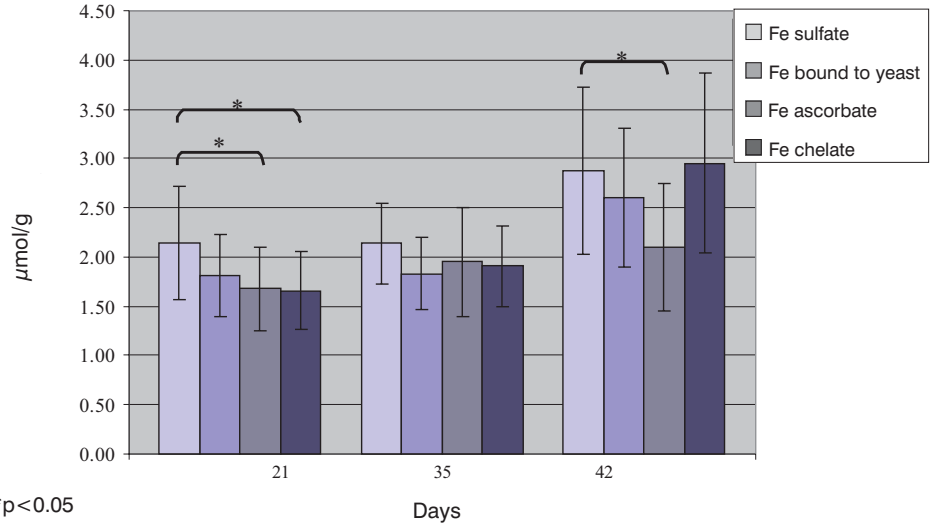


Figure 4. Concentrations of nonheme iron in the spleen (µmol/g)

food i.e. levels increase in the liver, and first increase and then decrease in the spleen.

Levels of nonheme iron in the spleen were increasing in all birds during our experiment. On the 21st and 35th day, in all experimental groups levels of nonheme iron in the spleen were lower than the control group. On the 21st day of the experiment there was a statistically significant difference between the control group and experimental groups III (ferrous ascorbate) and IV (iron helate). At the end of the trial, iron levels in the spleen of broilers from Group III (supplemented with ferrous ascorbate) were significantly lower (Figure 4). Appel (2001) also noted lower levels of nonheme iron in the spleen of 31-day and 62-day old rats, when organically bound iron was used in the feed mixture. Ahluwalia (2000) noticed that the level of iron decreased with age in both liver and spleen of rats. In the spleen values were about 4 times higher than in the liver.

In 21-day old chickens, levels of iron in the bone marrow were from 10.09 μmol/g (experimental group III) to 14.51 μmol/g (experimental group IV). Statistically significantly lower levels of nonheme iron in this organ, were detected in experimental Group III (ferrous ascorbate).

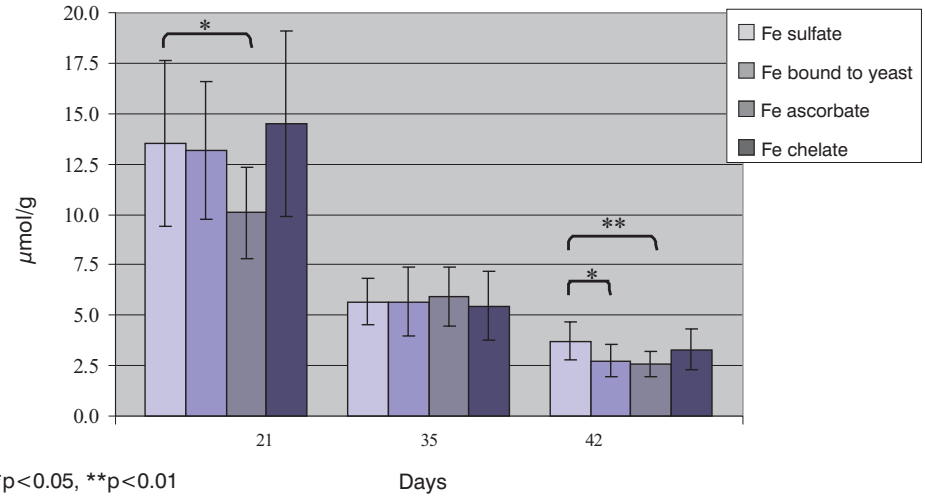


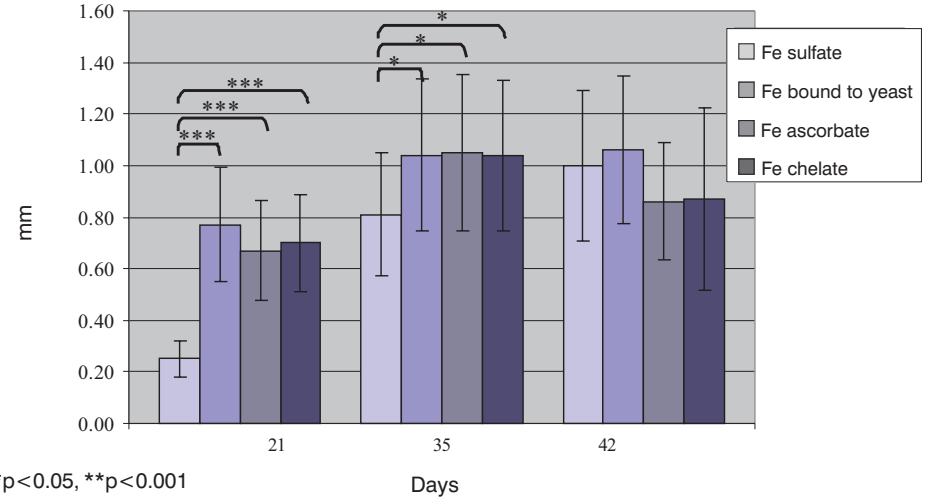
Figure 5. Concentrations of nonheme iron in the bone marrow (μmol/g)

In the next phase of the experiment, the level of iron decreased and the values were uniform (5.46-5.90 μmol/g) in all groups and no statistically significant differences between them were found. At the end of the experiment, the level of nonheme iron in the bone marrow was approximately the same as the level in the liver. Experimental groups II (yeast enriched with iron) and III (ferrous ascorbate) both had significantly lower levels of bone marrow iron.

After hatching, the marrow of long bones and vertebras is the main location where hematopoiesis occurs in birds (Feldman *et al.*, 2000). However, the marrow

of long bones is replaced by fat tissue with ageing, and the main location for hematopoiesis becomes the marrow of the vertebras and ribs. Ahluwalia (2000) also noted a decrease in iron level in the femur of rats with age of the individuals. As the level of nonheme iron in broilers' femur was measured in our trial, the results obtained in our experiment were expected.

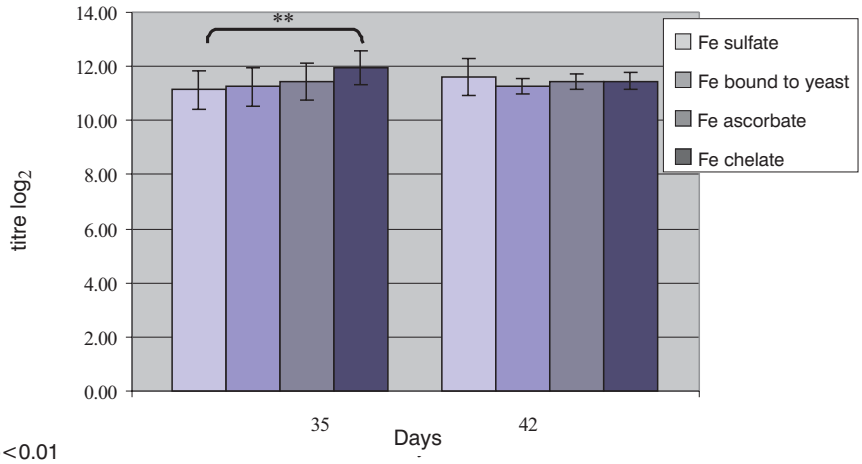
In 21-day old chickens, we were able to document significant statistical differences in cutaneous hypersensitivity to PHA, between the control group and all other experimental groups ($p<0.001$). Two weeks later these differences were still present, but at a lower degree of significance ($p<0.05$), which is in accordance with the results of Budimirović (2003). Statistically significant differences between the observed groups were not proven at the end of the trial.



*p<0.05, **p<0.001

Figure 6. The degree of cutaneous hypersensitivity to PHA (mm)

Using ELISA, we proved significant differences between titers of anti-Gumboro antibodies in broilers from the control group (ferrous sulphate), and broilers from the group supplemented with the chelated form of iron (Figure 7). At the end of the trial (42nd day), no significant differences were observed. Titer values were from 11.25 to 11.94. As reported by Solano *et al.* (1986), a vaccine titer lower than 4.9 ($-\log_2$), obtained by ELISA, offers insufficient protection of individuals. The titer values obtained in our trial following vaccination are considered to be of good protective value. To the best of our knowledge, there are no literature data on the influence of organic iron on post-vaccinal titer in broilers. If we compare our results with the results obtained when polysaccharide complexes of microelements were used for supplementation (Budimirović, 2003), we can conclude that the usage of organic bound iron causes a slightly higher production of antibodies following vaccination.



**p<0.01

Figure 7. Titers of anti-Gumboro antibodies, (-$\log_2$)

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UTICAJ ORGANSKI I NEORGANSKI VEZANOG GVOŽĐA NA HEMATOLOŠKE PARAMETRE, IMUNSKI ODGOVOR I KOLIČINU GVOŽĐA U ORGANIMA BROJLERA

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SADRŽAJ

Cilj ovog rada je bio da se ispita uticaj organski i neorganski vezanog gvožđa na crvenu krvnu sliku, imunski odgovor i količinu gvožđa u pojedinim organima. Ogled je izveden na ukupno 200 brojlera podeljenih u četiri jednake grupe. U smeše za ishranu brojlera dodavano je gvožđe u količini od 40 mg/kg koje je poticalo iz različitih izvora: fero sulfat (ogledna grupa I), gvožđe vezano za kvasac (ogledna grupa II), fero askorbat (ogledna grupa III) i helatno gvožđe (ogledna grupa IV). Kod piladi u dobi od 21, 35 i 42 dana praćeni su sledeći parametri: broj eritrocita, koncentracija hemoglobina, količina nehemskog gvožđa u jetri, slezini i kostnoj srži, vrednosti testa kožne preosetljivosti na fitohemaglutinin (PHA) i titar specifičnih antitela u krvnoj plazmi posle vakcinacije protiv Gumboro bolesti. Kod piladi u dobi od 21 dan svi ispitivani preparati gvožđa u kojima je ono bilo organski vezano, doveli su do povećanja broja eritrocita i koncentracije hemoglobina. Različiti oblici gvožđa u ishrani živine nisu uticali na količinu deponovanog nehemskog gvožđa u jetri piladi u dobi od 21 i 42 dan. Pilad u dobi od 35 dana, koja su u hrani dobijala fero askorbat, imala su značajno manje gvožđa deponovanog u jetri. Količina nehemskog gvožđa u slezini bila je manja kod piladi koja su hranom dobijala organski vezano gvožđe. Količina nehemskog gvožđa u kostnoj srži butne kosti piladi smanjivala se sa starošću jedinki a najmanja količina nehemskog gvožđa u kostnoj srži je zabeležena u grupi koja je hranom dobijala fero askorbat. Stepen kožne reaktivnosti na PHA bio je veći kod jedinki u dobi od 21 i 35 dana suplementiranih organski vezanim gvožđem. Upotreba helatno vezanog gvožđa u ishrani brojlera dovela je do statistički značajno veće produkcije antitela protiv Gumboro virusa nakon vakcinacije, kod piladi u dobi od 35 dana. Titar antitela kod jedinki u dobi od 42 dana u svim ogledним grupama bio je ujednačen.