Kafkas Univ Vet Fak Derg
25 (3): 379-385, 2019
DOI: 10.9775/kvfd.2018.21021

Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Influence of Anticoccidials on Oxidative Stress, Production Performance and Faecal Oocyst Counts in Broiler Chickens Infected with *Eimeria* Species

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Article Code: KVFD-2018-21021 Received: 24.09.2018 Accepted: 31.12.2018 Published Online: 31.12.2018

How to Cite This Article

Pajić M, Aleksić N, Vejnović B, Polaček V, Novakov N, Andrić DO, Stanimirović Z: Influence of anticoccidials on oxidative stress, production performance and faecal oocyst counts in broiler chickens infected with *Eimeria* species. *Kafkas Univ Vet Fak Derg,* 25 (3): 379-385, 2019.
DOI: 10.9775/kvfd.2018.21021

Abstract

The influence of certain anticoccidial drugs on oxidative stress in broiler chickens infected with *Eimeria* species was assessed. There were two untreated (uninfected and infected), and three groups infected and treated with anticoccidials. The first treated group (Ro) was given robenidine, the 2nd a herbal anticoccidial (Herb) and the 3rd the combination of robenidine and the herbal anticoccidial (Ro+Herb). All infected groups were on day 14 challenged with oral inoculation of oocysts. The activities of catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST), and the concentration of malondialdehyde (MDA) were estimated in blood taken on days 21 and 40. The oocyst numbers were calculated per gram, and chicken body weight and feed conversion ratio (FCR) measured. The activities of CAT, GST and the level of MDA were significantly lower ($P<0.05$), whilst the activity of SOD was higher in infected chickens treated with anticoccidials ($P<0.05$) in comparison to those untreated. The most prominent change in the parameters of oxidative stress was recorded in the Ro+Herb group. In chickens treated with anticoccidials body weight was significantly higher ($P<0.05$), and the FCR and the oocyst counts significantly lower ($P<0.05$) than in untreated chickens. Oocyst counts were lower in the Ro and Ro+Herb groups than in the Herb group. Our study demonstrated that both anticoccidial substances exerted antioxidant and anticoccidial effects.

Keywords: *Broilers, ROS, Coccidia, Robenidine, Herbal anticoccidial*

Antikoksidiyal Maddelerin *Eimeria* Türleri İle Enfekte Etlik Piliçlerde Oksidatif Stres, Üretim Performansı ve Dışkı Oosit Sayıları Üzerine Etkisi

Öz

Eimeria türleri ile enfekte Broilerlerde bazı antikoksidiyal ilaçların oksidatif stress üzerine etkisi araştırılmıştır. Çalışmada, enfekte edilmeyen ve enfekte edilen olmak üzere iki, ayrıca enfekte edilerek uygulama yapılan üç grup vardı. Uygulama yapılan gruplardan ilkine robenidin (Ro), ikincisine bitkisel antikoksidiyal (Herb) ve üçüncüsüne robenidin ile birlikte bitkisel antikoksidiyal (Ro+Herb) uygulandı. Tüm enfekte edilen gruplara 14. günde oral oosit inookulasyonu yapıldı. Çalışmanın 21 ve 40. günlerinde alınan kan örneklerinde katalaz (CAT), süperoksit dismutaz (SOD) ve glutatyon S-transferaz (GST) aktiviteleri ile malondialdehit (MDA) konsantrasyonu incelendi. Her bir gramdaki oosit sayıları hesaplandı ve tavukların vücut ağırlıkları ile yem konversiyon oranları ölçüldü. Uygulama yapılmayan grupla karşılaştırıldığında antikoksidiyal uygulanan gruplarda CAT, GST aktivitileri ve MDA seviyeleri anlamlı olarak daha düşük ($P<0.05$), SOD aktivitesi ise daha yüksekti ($P<0.05$). Oksidatif stress parametrelerindeki en belirgin değişim Ro+Herb grubunda kayıt edildi. Antikoksidiyal uygulanan etlik piliçlerde vücut ağırlığı uygulanmayanlara göre anlamlı olarak daha yüksek ($P<0.05$), yem konversiyon oranı ile oosit sayıları ise daha düşüktü ($P<0.05$). Ro ve Ro+Herb gruplarında oosit sayıları Herb grubundan daha düşüktü. Çalışmamız, kullanılan her iki antikoksidiyal maddenin de antioksidan ve antikoksidiyal etkileri olduğunu göztermiştir.

Anahtar sözcükler: *Broiler, ROS, Koksidiya, Robenidin, Bitkisel antikoksidiyal*

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INTRODUCTION

Coccidiosis is one of the economically most important diseases threatening intensive broiler production. The annual world losses are estimated to reach 2.3 billion euros [1]. Like some other pathogens [2-5], coccidia may also cause oxidative stress [2,6,7]. Reactive oxygen species (ROS) react spontaneously, targeting membrane lipids. The oxidative destruction of unsaturated fatty acids causes cell membrane damages, its decreased function and increases its permeability. These damages lead to a series of reactions which result in permanent consequences on chicken health, or even death [2,6].

Modern broiler production renders fattening almost unimaginable without anticoccidials. To avoid residues in meat, synthetic and ionophore anticoccidials are withdrawn 3-7 days before slaughter [8]. By contrast, herbal anticoccidials are administered until the end of the fattening period [9]. Leaving no residues, herbal medicines in the prevention of certain animal diseases have been arousing interest [10,11].

This research was aimed at the investigation into the influence of certain anticoccidials on oxidative stress, production performance and faecal oocyst counts in broiler chickens infected with *Eimeria*. Thus, the activities of antioxidative enzymes (catalase (CAT), superoxide dismutase (SOD) and glutathione S-transferase (GST)) and the concentrations of malondialdehyde (MDA) were measured, and feed conversion ratio (FCR) and shed oocyst counts were assessed.

MATERIAL and METHODS

The research was conducted on 250 clinically healthy, one-day-old, unsexed broiler chickens (Ross 308 hybrid), average body weight of 39.6±1.5 g. They were randomly placed into 25 boxes, each containing 10 birds. Five boxes chosen at random were assigned to each experimental group: two control and three treated groups. Thus, each group had five replicates of 10 birds, which were kept in a separate place, unable to maintain any mutual contact. All chickens were housed on deep litter, except the negative control, which was kept in a box with meshed floor. The chickens were fed on standard commercial feed. The control groups were not treated with anticoccidials. The negative control remained uninfected, whilst the positive was infected on day 14 [12]. The Ro group was administered robenidine. The Ro+Herb group received in the first two weeks robenidine, which was followed by the administration of the herbal anticoccidial from day 15 until slaughter. The Herb group was given the herbal anticoccidial during the whole fattening period. All treated groups were infected on day 14 in the same way as the positive control.

The experiment was done at the Institute for animal husbandry (Belgrade), approved of by its Ethical committee (Decision no. 323-07-2340/2017-05) and performed in accordance with the recommendations of the European Commission (Directive 2010/63 EEC) [13] and the law on animal welfare [14].

The synthetic anticoccidial robenidine (Robenz® 66G, Zoetis Ltd) was mixed in feed (450-550 g/t), given to chickens from day 1 and withdrawn 5 days before slaughter.

The phytogenic feed additive (Herbakoks, Essentico DOO, Kula, Serbia), a mixture of essential oils (mainly derived from *Thymus vulgaris*, *Origanum vulgare* and *Coriandrum sativum*), organic acid salts, dextrose, sodium chloride was applied as recommended by the manufacturer. The details of the products' recipe are proprietary.

The oocysts of *Eimeria* species were obtained from naturally infected farm chickens, isolated by flotation and preserved in 2.5% potassium dichromate solution [15]. The experimental chickens were orally infected with sporulated oocysts: 1.5 mL of suspension containing $5x10^5$ sporulated oocysts administered with a disposable syringe.

Venous blood was taken on days 21 and 40 in heparinized tubes (BD Vacutainer®) and centrifuged to separate the plasma. The red blood cells were rinsed three times in physiological saline solution and stored at -20°C until analysis.

The activities of CAT [16] and SOD [17] were analysed in the hemolisates and expressed in units/g of haemoglobin. GST activity (mmol of GSH-CDNB conjugate formed/ min/mg of haemoglobin) was determined [18]. The MDA levels (nmol MDA/g of haemoglobin) were estimated spectrophotometrically [19].

Haemoglobin concentrations were estimated as described in Tentori and Salvati [20]. All biochemical analyses were done simultaneously in triplicate for each sample using the Biobase UV/VIS spectrophotometer.

The chickens were measured individually at the beginning and at the end of the experiment. Body weight gain, feed consumption and FCR were calculated pen wise.

For FCR and oocyst counts each pen was the experimental unit, and for the biochemical and body weight assessment it was each animal. Given that the data on FCR were heterogeneous, the groups were compared using Kruskal-Wallis ANOVA followed by Dunn's multiple-comparison test. Data on oocyst counts were heterogeneous, and the transformation $\log_{10}$ (value+1) was applied to all data. Data on biochemical analyses and live body weight were normally distributed (Shapiro-Wilk's test, P>0.05), and along with oocyst yields compared using the two-way ANOVA with repeated measures in one factor followed by Tukey´s test. All analyses were performed with GraphPad Prism 6 (GraphPad, USA).

RESULTS

Superoxide dismutase activities in the blood of 21- and 40-day-old chickens are shown in *Fig. 1*. A significant decrease in its activities were detected in the positive control in comparison to the negative one (P<0.05) at both time points. The activities of SOD were significantly higher in infected chickens treated with both anticoccidials in comparison with the infected but untreated broilers (P<0.05). The differences in SOD activities between the treated groups at both time points did not differ significanty (P>0.05).

The activities of the antioxidative enzyme CAT in 21- and 40-day-old chickens in all experimental groups are presented in *Fig. 2*. In infected untreated broilers the activities were significantly increased in comparison to uninfected ones (P<0.05). By comparison with the infected but not treated group, a significant decrease was noticed in the CAT activity in groups treated with anticoccidials (P<0.05) in both 21- and 40-day-old broilers. At the first time point the activity of CAT in the Herb group did not differ significantly from the one in the Ro group. However, at the second time point the herbal anticoccidial led to a significant decrease in the

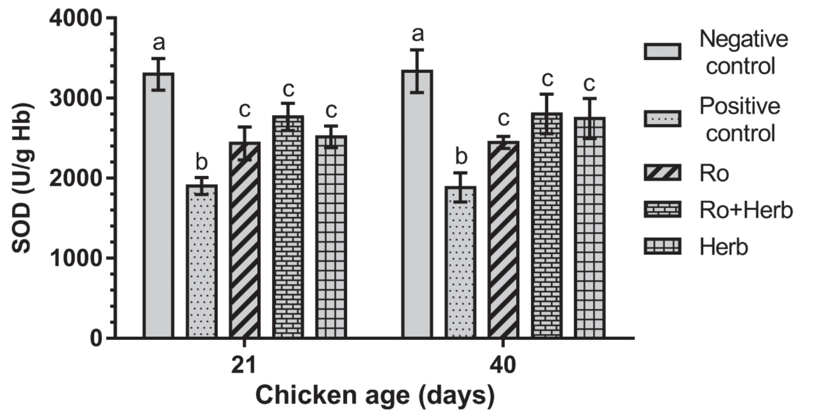

Fig 1. SOD activities in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

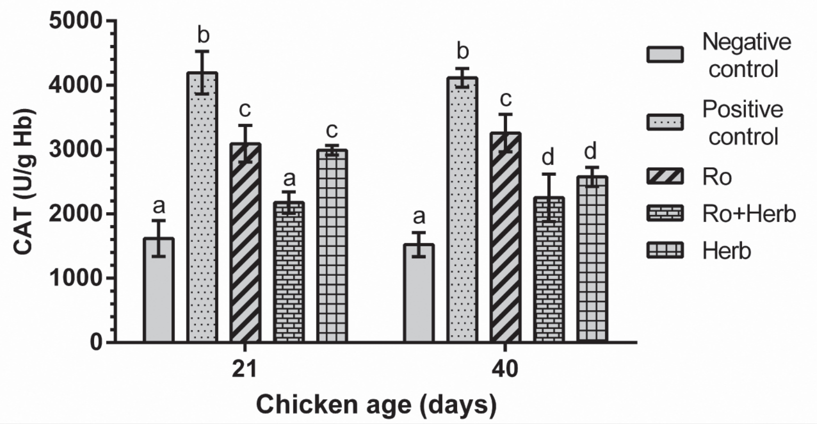

Fig 2. CAT activities in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

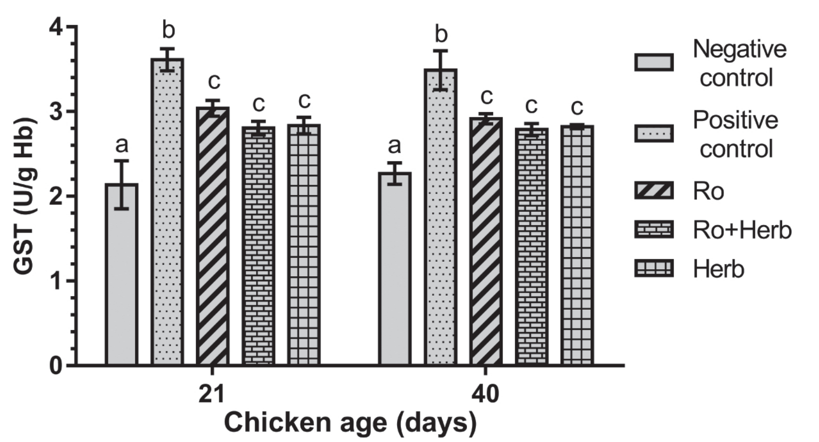

Fig 3. GST activities in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

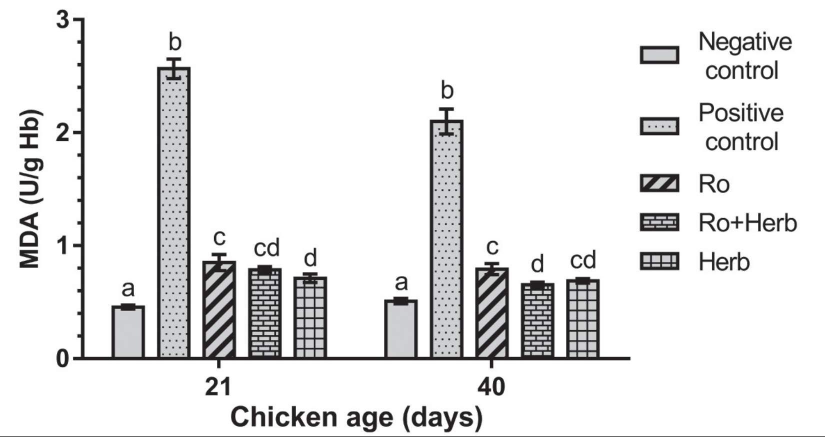

Fig 4. Concentration MDA in blood in 21- and 40-day-old chickens (mean ± SD). Different letters in chickens of the same age indicate significant differences between the groups (P<0.05). Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

Table 1. *Body weight and FCR in control and treated chicken groups*

Groups	Initial Body Weight (g) Mean±SD	Final Body Weight (g) Mean±SD	FCR Median (IQR)
Negative control	39.84±1.18[a]	2821.22±531.82[a]	1.61 (1.45-1.85)[a]
Positive control	39.87±0.99[a]	1858.68±533.93[b]	2.76 (2.10-3.62)[b]
Ro	39.67±1.06[a]	2618.70±494.79[ac]	1.69 (1.57-2.08)[ac]
Ro+Herb	39.62±0.91[a]	2440.35±391.35[c]	1.91 (1.76-2.07)[c]
Herb	39.72±0.96[a]	2523.00±521.94[cd]	1.82 (1.60-2.28)[ac]

Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine+herbal anticoccidial
[a-d]Values within a column with different superscripts are significantly different (P<0.05)

CAT activity in comparison to robenidine (P<0.05). The most pronounced decrease of CAT activity among treated groups was observed in Ro+Herb group in 21-day-old broilers (P<0.05).

In the positive control there were significant increases in the GST activity (P<0.05) in comparison to the negative control *(Fig. 3)*. Moreover, in the groups treated with anticoccidials the average activities of this enzyme were significantly lower than that in the infected but untreated group (P<0.05). By comparison of the GST activity between the treated groups it was revealed that there were no significant differences between groups of 21-day-old and 40-day-old chickens (P>0.05).

In *Fig. 4* the average concentrations of MDA in the experimental broilers can be seen. The concentrations of this lipid oxidation marker were significantly higher in the positive control in comparison to the negative one (P<0.05), as well as comparison with infected broilers which were treated with anticoccidials (P<0.05). These changes apply for both 21- and 40-day-old broilers. As for the treated groups, in 21-day-old chickens the lowest average MDA concentration was measured in Herb group (P<0.05). However, in 40-day-old broilers MDA level was lowest in the Ro+Herb group (P<0.05). By comparison of the MDA activity between the treated groups it was revealed that there were significant differences between Ro and Herb groups in 21-day-old chickens as well as between Ro and Ro+Herb in 40-day-old chickens (P<0.05).

The average body weight and FCR were monitored throughout the experiment *(Table 1)*. In the beginning, on day 0, the average body weight was uniform in all experimental groups. However, in the end it was significantly lower in the positive control than in all others (P<0.05). FCR in the positive control was significantly higher (P<0.05) than in uninfected and infected but treated groups (P<0.05).

In uninfected broilers in faecal samples taken on days 14, 21, 28, 35 and 42 oocysts were not detected *(Fig. 5)*. In infected untreated chickens eimerian oocysts were first detected on day 21 and were continually on increase. In Ro group the oocyst counts were significantly lower than in the positive control. Similar tendency was observed in Ro+Herb group, where a reduction of oocyst counts was noticed in comparison to the positive control (P<0.05). In Herb group the numbers of oocysts per gram were higher than in chickens treated with robenidine and those which were treated with both anticoccidials, but remained significantly lower than in the positive control.

DISCUSSION

In spite of plenty of literature data which indicate that there are significant differences in the parameters of oxidative stress in chickens infected with coccidia, those obtained on broilers treated with anticoccidials are scarce. Monitoring of the parameters of oxidative stress following their application can provide useful information on the

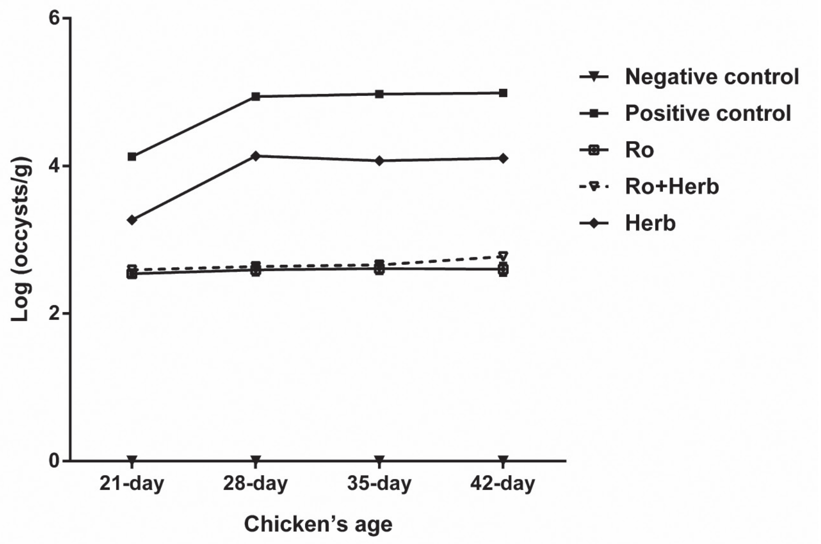

Fig 5. Faecal oocyst counts (o.p.g.) in control and treated chicken groups. Ro - group treated with robenidine, Herb - group treated with a herbal anticoccidial, Ro + Herb - group treated with robenidine + herbal anticoccidial

possible antioxidative effects of anticoccidial drugs. In the current research the synthetic anticoccidial robenidine, a herbal anticoccidial preparation, and their combination were administered to broilers infected with *Eimeria*. The results indicated that the anticoccidials applied significantly influenced the changes in the oxidative stress parameters (SOD, CAT, GST and MDA) in infected chickens.

The results of this research detected lower activity of SOD in the blood of infected but untreated broilers (positive control). SOD is involved in the antioxidative defence system, the first line of defence against ROS [4]. Lower SOD activity in the blood of infected poultry than in uninfected probably results from the increased production of H_2O_2. Decreased SOD activity in broilers infected with coccidia has also been detected by some other researchers [2,21]. The administration of anticoccidials in infected chickens resulted in increased activity of SOD, most prominent in the Ro+Herb group, which means diminished the effect of oxidative stress. It is supposed that the anticoccidials prevented the inactivation of SOD by H_2O_2, which results from the dismutation of superoxide anion [22]. Bozkurt et al.[12] were the ones who also noticed significant increase in the activity of SOD in infected chickens fed with feed supplemented with the anticoccidial based on oregano oil.

Besides SOD, the activity of CAT was monitored following the treatment of infected broilers with anticoccidials. There are no published data on the influence of the combination of herbal and synthetic anticoccidials on CAT activity. In our experiment the largest increase in the activity of this enzyme was detected in the positive control, which is in accordance with the findings published by Georgieva et al.[2]. Similar results were obtained by other authors, who pointed to the increase in the activity of CAT in broilers infected with *E. acervulina* and *E. tenella* [21,23]. CAT activity is on the increase in oxidative stress, which is considered a compensatory mechanism in poultry infected with coccidia. In infected chicken groups which were administered anticoccidials in feed lower CAT activity was detected than in infected but untreated broilers. In the treated groups the largest decrease in the activity of CAT was noticed in the Ro+Herb group, which means that the combination of the two anticoccidials significantly influenced the decrease in the oxido-reduction disbalance which resulted from the response to the presence of the parasites. CAT plays an important role in preserving the cellular integrity by degrading the reactive hydrogen peroxide, which can lead to the emergence of reactive hydroxyl radical. Hydroxyl radical is highly unstable and can produce cellular damage via lipid peroxidation, and the oxidation of DNA and proteins [24].

The analysis of GST in this research detected its increased activity in infected chickens. The same phenomenon in broilers, due to some environmental factors, was described by Ismail et al.[25] GST is involved in the protection of cells from the negative effects of ROS, to which it bonds directly, covalently and renders it less reactive [26]. In this research the GST activity was lower in treated groups in comparison to the positive control, which leads to a hypothesis that the anticoccidials influenced the decrease in the substrate (ROS) production resulting in decreased activity of the detoxication enzyme. The largest decrease in the GST activity was observed in the Ro+Herb group. In the research conducted by Giannenas et al.[27] it was revealed that preparations based on fungi can increase the activity of GST and positively influence growth and feed utilization, and stimulate the secretion of digestive enzymes. Similarly, it was proven that a herbal preparation based on rosemary exerts antioxidative effects by increasing the activity of GST [28].

Malondialdehyde is produced in the process of lipid peroxidation due to the influence of ROS on the polyunsaturated lipids. Its concentrations in blood and tissues are directly proportional to cellular damages caused by ROS and is, for this reason, a useful marker in the analysis of oxidative stress [2]. The results of the current research revealed increased concentrations of MDA in infected chickens, resulting from increased lipid peroxidation. Similar data on the changes in the concentrations of MDA were reported by some other researchers [2,21,29]. Significant decrease in the levels of MDA was detected in broilers in all treated groups, which leads to conclusion that anticoccidials influenced the decrease in the production of ROS and thus led to reduced lipid peroxidation. In the research undertaken by Bozkurt et al.[12] it was found that herbal anticoccidials mitigate oxidative stress by decreasing the concentrations of MDA. Giannenas et al.[27] noticed that preparations based on fungi have anti-oxidative properties and decrease the concentrations of MDA.

The analysis of production performance of the broilers in the experiment showed that the lowest average body mass was in infected untreated chickens, which had the highest FCR. This lower weight gain can be attributed to infection with coccidia. Developing in the intestines *Eimeria* produce mucosal disruptions resulting in malabsorption and direct negative effect on growth, and facilitate infections with other pathogens [30]. It is obvious from the performance data that the broilers treated with robenidine had highest average body mass and lowest FCR. The lowest average body mass was recorded in chickens treated with both anticoccidials (Ro+Herb). Our results of increased weight gain and body weight in broilers treated with both the synthetic and herbal anticoccidials are in line with some previous data and resulted from their beneficial effects [29,31]. Positive effects on production performance resulting from the use of chemical and herbal anticoccidials have already been described [32]. Herbal anticoccidials, unlike synthetic ones, do not leave residues in broiler meat, which is why they are gaining increasing interest [33,34]. They contribute to weight gain and decrease FCR, which can be explained by increased absorption area of the intestines and better enzyme activities resulting from healing [12,35,36]. Data showed that herbal anticoccidials containing oregano, thyme, coriander, carvacrol, thymol and some other active ingredients exert anticoccidial and antioxidative effects [9,12,35].

The results obtained in this work showed that infected broilers shed significantly lower oocyst counts following the treatment with anticoccidials in comparison to those untreated. The least average number of oocysts was detected in chickens treated with robenidine, proving its satisfactory anticoccidial effect. Its mechanism of action is based on the inhibition of oxidative phosphorylation in the parasite mitochondria, which prevents their development. Chickens treated with robenidine and the herbal anticoccidial shed small numbers oocysts in comparison with the infected untreated group, which indicates that this combination of anticoccidials produced strong anticoccidial effect. This corresponds to the data obtained with the use of combinations of diclazuril and oregano essential oil [9] and, amprolium and garlic [32]. The analysis of our results obtained by spectrophotometry showed that the combination of the two anticoccidials significantly influenced the decrease in the oxidative stress, given that the values of biochemical parameters were closest to those in uninfected broilers. By this mechanism, robenidine influenced the decrease of oocyst counts in broilers, whilst the use of the herbal anticoccidial influenced the increased level of antioxidative defence, which resulted in the obtained values of oxidative stress parameters. In the current research it was proven that herbal extracts (oregano, thyme and coriander) in the tested herbal anticoccidial exert antioxidative properties. Herbal anticoccidials mainly consist of bioactive compounds such as polyphenols, kinins, flavonoids, alkaloids and polypeptides. Phenol compounds of aromatic herbs and their essential oils are potent sources of natural antioxidants. Flavonoids can act as powerful antioxidants by scavenging free radicals and stop oxidative reactions [36]. In broilers which were administered the herbal anticoccidial faecal oocysts shedding was higher than in the other two treated groups (Ro, Ro+Herb). Nevertheless, the applied herbal preparation exerted powerful anticoccidial effect.

The synthetic and herbal anticoccidials significantly influenced the parameters of oxidative stress: the activity of CAT and GST and the concentration of MDA were lower, whilst the activity of SOD was higher in treated groups than in untreated infected broilers, which points to the decrease in oxidative stress. Moreover, the anticoccidials led to the decrease in the oocysts production. Oocyst counts were lower in Ro and Ro+Herb groups in comparison to Herb group, which means that the synthetic anticoccidial alone and in combination with the herbal one resulted in better effect in the control of coccidiosis than the herbal applied exclusively. However, it was proven that the tested herbal preparation can be used in coccidiosis control and the prevention of oxidative stress. These results can help in the selection of anticoccidial drugs and influence directly the decrease in the economic losses attributed to coccidiosis.

Acknowledgements

This work was supported by the grant provided by the Ministry of Education, Science and Technological Development of the Republic of Serbia for the Projects Nos. III 46002 and TR 31071.

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