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Running title: Effect of herbs on natural in turkeys

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## Effect of some medicinal herbs on natural humoral immunity in turkeys

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**ABSTRACT:** The infectious diseases causes devastating economic losses in poultry industry. It is important to boost factors of natural immunity to improve defense to infectious diseases in birds. The experimental groups (105 turkey poults) allocated to 7 treatment groups, 3 replicates per treatment and 5 birds per replicate with initial body weight  $49, 78 \pm 0, 2$  g) received basal diet plus 1% dry herbs or 0.01% essential oils supplements obtained from the herbs (Chamomilla; Rosmary; Lavender; Oregano; Thyme; St. John's Wort). Blood serum lysozyme concentrations, alternative pathway of complement activation (APCA) and betalysin activities were determined. The highest blood lysozyme concentrations were determined among the control group and groups supplemented with dry herbs - rosemary, oregano, and thyme. APCA activity was the highest in thyme-supplemented group followed by the non-supplemented one. Betalysin activities were the highest in groups receiving lavender, thyme, and oregano, while the lowest levels were found out in chamomile-supplemented birds. In groups treated with essential oils (EO), blood serum lysozyme concentration was the highest in the group treated with rosemary EO, followed by control group, while the lowest activity was established in birds that received EO from Saint John's wort. The highest APCA activity was found in the lavender-treated group, and the lowest - in rosemary-treated turkeys. The highest betalysin activity was found among the groups treated with thyme, oregano and Saint John's wort, and lowest activity was determined in the control group. It could be concluded that studied herbs possess an important immunomodulating potential in turkeys, which could improve their health and consequently, their productive performance.

**Keywords:** Betalysin, Complement, Herbs, Lysozyme, Turkeys.

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## INTRODUCTION

For the last few decades, the ban on using antibiotics in livestock husbandry and poultry farming in particular, has increased the interest to biotechnological and natural products with the purpose to improve birds' productivity, health, quality and safety of produce (Liu et al., 2011). Medicinal plants constitute a new class of growth promoters which recently has gained importance in food industry for production of functional foods. Improved antioxidant status of live animals along with increased oxidative stability of raw meat are considered useful for both consumers and processing industry. Research has been focused mainly on the effect of medicinal and aromatic plants on mortality, stress hormones, blood, and muscle metabolism, and even the immune system function of domestic animals. It is reported that tea (Tang et al., 2000), rosemary (Sevim et al., 2020) and lavender contain high concentrations of antioxidants and applied in various in vitro system models (Dorman et al., 2000) reduce oxidation of muscle fat in chickens. Rasouli et al. (2020) treated chicken broilers with different doses of water extract of salvia (*Salvia officinalis* L.) and found dose dependent enhancement of the immunity response of broilers. They also reported for bactericidal effect of sage extract against *Escherichia coli*. Similar results for positive effect of salvia on immune system in chickens reported Farhadi et al. (2020). At a global scale, numerous studies on effects of herbs and herbal products on various production traits in broiler chickens are published (Ocak et al., 2008; Moorthy et al., 2009; Ali, 2014; Mohamed, 2015). Wallace et al., (2010) demonstrated that plant extracts and different phytobiotics from leaves, roots, tubers or fruits of herbs, spices and other plants were excellent growth promoters in poultry farming. Some researchers reported chamomile effects on specific immunity of broiler chickens (Abdoul-Latif et al., 2011; Roby, 2013; Munir et al., 2014; Stanojevic et al., 2016). Dehkordi et al. (2009) provided evidence for a better antimicrobial effect of natural rosemary extract against *Listeria monocytogenes* if combined with unheated or heat-treated lysozyme at a low pH = 5. Matouskova et al. (2016) reported that encapsulated extracts of various herbs, including rosemary, had a marked antibacterial effect, which is increased in the presence of lysozyme. Mohamed et al. (2020) deliberates the different practical applications of a few medical herbs to improve the health state of poultry particularly as thermoregulatory and immunomodulatory agents. Yousefi et al. (2020) reported very de-

tailed information on the application of lavender extract (LE) in carps before and after stress. Rostami et al. (2012) reported that oily extracts (OE) from lavender (*Lavandula officinalis*) and lemon balm (*Melissa officinalis*) exhibited high-efficient bactericidal activity against some bacteria. Lillehoj et al. (2011) and Hashemipour et al. (2013) investigated the effect of phytochemicals carvacrol, cinnamaldehyde and Capsicum oleoresin, on translational regulation of genes associated with immunology, physiology, and metabolism in an in vivo model of coccidiosis in chickens. The results provided clear evidence that isolated phytoderivatives had immunostimulating properties in chickens. Boskovic et al. (2015) investigated antibacterial effects of oregano and thyme essential oils (EOs) on *Salmonella Enteritidis*, *Salmonella Thyphimurium*, *Staphylococcus aureus*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus cereus* and found that EOs exhibited antibacterial activity against all tested microorganisms. Antimicrobial properties of thyme were discussed also by Rota et al. (2008). Ji-ang et al. (2012) investigated the effect of methanolic extract from of Saint John's wort on specific humoral immune response in chickens vaccinated against various avian influenza strains. Landy et al. (2012) carried out an interesting experiment for investigation of effects of using dried ground aerial parts from of Saint John's wort as alternative of nutritional antibiotics in poultry farming.

The lack of information about the effects of Saint John's wort (*Hypericum perforatum*), lavender (*Lavandula angustifolia*), chamomile (*Matricaria chamomilla*), thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*) and rosemary (*Rosmarinus officinalis*) on natural humoral immunity in turkeys was the incentive for this research. The aim of the current study was to evaluate blood serum concentrations of lysozyme, activity of the alternative pathway of complement activation (APCA) and betalysin activity in turkeys, whose rations were supplemented with either 1% dry herbs or 0.01% herbal essential oils.

## MATERIALS AND METHODS

### Experimental Design

Turkeys were reared in the poultry farm of the Agricultural Institute, Stara Zagora. A total of 105, 1 day-old of age female turkey poults were weighed individually and were randomly allocated to 7 treatment groups (3 replicates per treatment and 5 birds per replicate with initial body weight 49, 78 ±

0, 2 g) until 126 day old, namely control (C) and six experimentals. The treatment of turkey poults starts from the 1st day and lasts until 126 days of age. Control group turkeys received basal diet without herbs (nutritional program NRC was used). The composition of the basal diet is presented in Table 1. All diets were in mashed form. The experimental groups received basal diet plus 1% supplemented with dry herbs (see table 2) or 0, 01% essential oils (Eos) (see table 3) obtained from the same herbs (*Matricaria chamomilla*; *Rosmarinus officinalis*; *Lavandula angustifolia*; *Origanum vulgare*; *Thymus vulgaris*; *Hypericum perforatum*). We use commercial Eos (Nature energie LTD, Bulgaria; ALTEYA ORGANICS LTD, Bulgaria). Each group was placed to a clean floor pen, in a brooder ring for the first seven days (5 birds in ring), with equal floor space, one feeder, one drinker and one heating lamp for each ring. After day 7, the rings were removed and the turkeys were reared together as a group. Birds had *ad libitum* access to feed and water and lighting was provided continuous. The experiments were conducted within standard ethical norms and no birds were subjected to undue stress. The minimum requirements for the protection and welfare of experimental animals and the requirements for facilities for their use, keeping and /or supply are set out in Ordinance № 20 of 1.11.2012 on the minimum requirements for protection and welfare of experimental animals and the requirements for sites for use (8.1.2018), breeding and/or delivery, which transposes Directive 2010/63/EU.

### Assay methods

At the end of the fattening at 18 weeks of age, blood samples for analyses were collected from *v. subcutanea ulnaris* from 6 turkeys from each group

to assay some parameters of humoral innate immunity (totally 42 blood samples). Serum lysozyme concentrations were determined by method of Lie et al. (1985 see Fig. 1), alternative pathway of complement activation (APCA) was evaluated by method of Sotirov (1986) and betalysin activity were assessed by method of Buharin et al. (1977). All these methods were described in detail in our previous publication (Bozakova et al., 2020).

### Statistical analysis

Data were processed by one-way analysis of variance (ANOVA) with the fixed effect model using Data analysis tool pack, Microsoft Excel 2016, Microsoft Corporation Ltd. at a level of significance  $P < 0.05$ .

### RESULTS

The results from Table 2 demonstrate that the highest lysozyme concentrations were those of control group and groups treated with rosemary, oregano, and thyme. Levels following lavender and chamomile treatments were lower, yet between-group differences were insignificant. The alternative pathway of complement activation (APCA) exhibited the highest activity in the group that received thyme ( $P < 0.001$ ), followed by the control group. Relatively lower activities were observed in birds supplemented with oregano ( $P < 0.01$ ) and rosemary ( $P < 0.001$ ). The results showed that tested herbs had a considerable effect on the activity of this important element of innate immunity in turkeys. Data for betalysin activities showed maximum values in the groups treated with lavender, oregano, and thyme ( $P < 0.001$ ), and lowest activity in chamomile-treated birds. Obviously, this parameter was also influenced significantly by tested medicinal herbs.

**Table 1.** Composition of the basal diet

Growing period	Crude protein (%)	Metabolic energy (kcal/kg)
Starter 1 from 1 to 21 day- old turkey	28	2800
Starter 2- from 22 to 42 day- old turkey	26	2900
Starter 3 - from 43 to 63 day- old turkey	24	3000
Grower 1 from 56 to 84 day old turkey	22	3100
Grower 2 - from 85 to 100 day old turkey	19	3250
Finisher from 101 to 126 day old turkey	17	3350

**Table 2.** Effect of some dry medical herbs on lysozyme concentrations, APCA and betalysin activity in turkeys

Groups Dry herbs (1%)	Investigated traits		
	Lysozyme (mg/L)	APCA (CH50)	Beta-lysin (%)
Control	4,29 ± 0,46	716,05 ± 8,84	8,94 ± 1,2
Matricaria chamomilla	3,77 ± 0,28	712,35 ± 40,41	7,02 ± 0,9
Rosmarinus officinalis	4,29 ± 1,09	634,47 ± 19,53	11,79 ± 1,2
Lavandula angustifolia	3,44 ± 0,89	707,41 ± 11,63	17,04 ± 1,7
Origanum vulgare	4,38 ± 0,62	661,42 ± 28,06	16,39 ± 0,5
Thymus vulgaris	4,29 ± 0,46	718,94 ± 20,07***	15,42 ± 0,3
Hypericum perforatum	3,46 ± 0,44	573,6 ± 12,19	18,05 ± 1,5***

P < 0,001 - the superscripts within a column indicate statistically significant differences among the groups

**Table 3.** Effect of essential extracts obtained from some medical herbs on lysozyme concentrations, APCA and beta-lysin activity in turkeys

Groups Essential extracts from herbs (0,01%)	Investigated traits		
	Lysozyme (mg/L)	APCA (CH50)	Beta-lysin (%)
Control	4,29 ± 0,46***	716,05 ± 8,84	8,94 ± 1,2
Matricaria chamomilla	3,7 ± 0,58	612,92 ± 12,85	16,22 ± 5,6
Rosmarinus officinalis	5,45 ± 2,46	592,24 ± 7,37	15,57 ± 0,8
Lavandula angustifolia	3,49 ± 0,57	779,28 ± 18,84***	14,79 ± 2,02
Origanum vulgare	4,44 ± 0,66	667,86 ± 9,4	19,31 ± 1,1
Thymus vulgaris	3,39 ± 0,35	676,54 ± 14,72	19,79 ± 2,3***
Hypericum perforatum	2,29 ± 0,28	601,67 ± 22,11	19,04 ± 1,8

\*\*\* - P < 0,001 - the superscripts within a column indicate statistically significant differences among the groups

Table 3 present the results from supplementation of turkeys with essential oils of the same herbs. The highest serum lysozyme was observed in the group treated with rosemary essential oil, followed by untreated group, and the lowest level of lysozyme was that of Saint John's wort-treated birds. Statistically significant differences were found only between controls and Saint John's wort-treated turkeys (P<0.001). Although the highest mean value was found in the group treated with rosemary essential oil, it was not statistically significantly compared to that of the Saint John's wort group due to the presence of a single individual with exceptionally high serum lysozyme concentration (17.656 mg/L). The highest APCA activity was observed in lavender-supplemented turkeys (P<0.001), whereas the lowest in rosemary-treated birds. With regards to betalysin, the highest activity was found in the groups supplemented with thyme, oregano, and Saint John's wort essential oils (P<0.001), and the lowest one - in control group.

## DISCUSSION

Matouskova et al. (2016) reported that encapsulat-

ed extract of various herbs, including chamomile had a marked antibacterial effect that is increased in the presence of lysozyme. Our results confirmed that dry chamomile and chamomile essential oil possessed immunomodulating properties on blood serum lysozyme in turkeys (Tables 1 and 2). Primo et al. (2018) let us know that lysozymes are enzymes that break down the bacterial cell wall and disrupt the bacterial life cycle by cleaving the linkage between the N-acetylglucosamine and N-acetylmuramylpentapeptide carbohydrates. So, adding medical herbs to diet of turkeys will increasing the serum lysozyme concentrations and will improve their resistance to infectious diseases. This fact explains the similarities between our results and these one obtained by Matouskova et al. (2016). Other researchers reported chamomile effects on specific immunity of broiler chickens (Abdoul-Latif et al., 2011; Roby, 2013; Munir et al., 2014; Stanojevic et al., 2016) which, in some instances are contradictory. For instance, Mahmmod (2013) reported that chamomile did not influence antibody titers against Newcastle disease, and 4 years later reported the exact opposite results (Mahmmod et al., 2017). In

the available literature, no research studies were found on the effects of dried chamomile and chamomile essential oils on APCA activity. It is acknowledged that APCA is the primary humoral means for control of viruses, virus-infected cells, Gram-negative bacteria, cancer cells etc. (Sotirov et al., 1998; Andonova et al., 2001; Goundasheva et al., 2002; Yotova et al., 2004; Bozakova et al., 2018). Matouskova et al. (2016) reported that encapsulated extracts of various herbs, including rosemary, had a marked antibacterial effect, which is increased in the presence of lysozyme. Our results confirmed that dried rosemary and especially rosemary essential oil exerted an immunomodulating effects on serum lysozyme concentrations in turkeys (Tables 1 and 2). This correspondence in the results can be explained again by the information published by Primo et al. (2018). Ayoub et al. (2019) have investigated the effects of dried and ground leaves from moringa (*Moringa oleifera*), rosemary (*Rosmarinus officinalis*) and curcuma (*Curcuma longa*) on immune parameters of Nile tilapia before and after infection with *Aeromonas hydrophila*. The results showed that serum concentrations of albumin, globulins and total protein were statistically significantly higher compared to those in the control group. Also, serum lysozyme and respiratory activity were statistically significantly higher in treated groups than in control fish. Shokrollahi et al. (2015) investigated the effect of rosemary extract (*Rosmarinus officinalis*) on weight, haematological parameters, and cell-mediated immune response in newborn goat kids. The results confirmed that rosemary essential oil added to the milk of kids exerted a positive effect on their immunity. Franciosini et al. (2016) established positive effect of aqueous extracts of oregano (*Origanum vulgare L.*) and rosemary (*Rosmarinus officinalis L.*) on immune functions and intestinal microbial population of broiler chickens.

Yousefi et al. (2020) reported that application of lavender extract (LE) increased total white blood cell counts, plasma globulins, APCA activity, serum lysozyme concentrations in carps before and after stress.

Hashemipour et al. (2013) evaluated the effect of a phytogenic product containing a mixture of equal parts of thymol and carvacrol applied at 4 dose rates (0, 60, 100, and 200 mg/kg) to broiler chickens. The authors concluded that combination thymol and carvacrol improved immune response of broilers. Haghghi et al. (2018) and Ali et al. (2018) investigated the effect of oregano extract on total serum protein, albumin and

globulins, respiratory activity, phagocytic activity and serum lysozyme in rainbow and reported improved growth performance, increased serum lysozyme concentrations, increased total antibodies and better survival rate.

Borugă et al. (2014) analyzed the chemical composition and antimicrobial properties of essential oil isolated from thyme (*Thymus vulgaris*), cultivated in Romania and found that thyme essential oil had an antimicrobial effect and could be used as source of natural antiseptic substances. Chun et al. (2001) have carried out a unique experiment that demonstrated anticomplementary activity (inhibition of complement system activation) of a polysaccharide isolated from thyme leaves (*Thymus vulgaris L.*). The isolated polysaccharide has inactivated both pathways of complement activation - the classical and the alternative. The analysis of our results on APCA activity in turkeys treated with thyme (dried and essential oil) presented in Tables 1 and 2 show that APCA in turkeys treated with dried thyme was the highest (718.94 CH50), whereas birds treated with essential thyme oil - average (676.54 CH50) with statistically significant differences ( $P < 0.05$ ). The results suggested that possibly, thyme essential oil contained a small amount of the polysaccharide reported by Chun et al. (2001).

Jiang et al. (2012) investigated the effect of methanolic extract from of Saint John's wort on specific humoral immune response in chickens vaccinated against various avian influenza strains. They found that the application of the extract as dietary supplement during the immunization period enhanced the effect from vaccination against avian influenza. Landy et al. (2012) carried out an interesting experiment for investigation of effects of using dried ground aerial parts from of Saint John's wort as alternative of nutritional antibiotics in poultry farming. It was observed that this herb improved feed conversion, increased antibody titer against avian influenza, decreased blood cholesterol concentration compared to chickens treated with a nutritional antibiotic (flavophospholipol). Other researchers (Shang et al., 2012) attempted to treat chickens infected with Gumboro disease virus (IBDV BC-6/85) by applying Saint John's wort extract. According to the results, Saint John's wort extract applied at 1330 and 667.9 mg/kg resulted in statistically significant therapeutic response and improvement of immune functions of infected chickens. Mohammadi et al. (2020) treated Nile tilapia (*Oreochromis niloticus*) juveniles with

extracts of *Hypericum perforatum* (HP), *Origanum vulgare* (ORG), and *Melissa officinalis* (MOF) and found that ORG 0.5 % and MOF 0.5 % groups showed a significant increase in serum total protein, and alternative complement activity (ACH50). Serum lysozyme levels were also increased in all groups fed on phytogetic diets. The MOF 0.5 % group showed the highest activities of skin mucus lysozyme and ACH50. ORG 0.5 % and MOF 0.5 % groups showed the highest protection levels of fish following challenge with pathogenic *Aeromonas hydrophila*. The authors concluded that both ORG and MOF extract at a 0.5 % level can effectively improve the growth, health, and immune status of Nile tilapia juveniles.

Some authors try to explain the mechanisms of immunostimulation of medicinal plants. For example according to Kumar et al. (2012) the main substances of medical plants are alkaloids, polysaccharides, cannabinoids, triterpenoid saponins, glycosides. Mentioned substances which acts on immune system by different ways for example increasing of serum immunoglobulin levels, neutrophil adhesion, phagocytosis, total number of T-helper and T-suppressor cells, activation of macrophages, inhibition of C3 convertase of the classical complement pathway, induction of cytokine (TNF- $\alpha$ , IFN- $\gamma$ ), lymphoid cells stimulation, cellular immune function enhancement and nonspecific cellular immune system effect, increase immunoglobulin production, nonspecific immunity mediators and natural killer cell numbers. Sharma et al. (2017) in their review also showed that alkaloids, flavonoids, terpenoids, polysaccharides, lactones, and glycoside products possessed immunomodulatory properties. For example, the root extract of *Astragalus membranaceus* was found to lower IL-6 in in vitro human model (IL-6 is inflammatory and impending deterioration marker). Garlic (*Allium sativum*) is found to lower IL-1 and IL-6, TNF, IL-8 acting as anti-inflammatory inhibitor and boosting effect on IL-10 which is an antagonist to pro-inflammatory cytokines. Megna et al. (2012) tested the effect of Echinacea, Rhodiola and Ginseng on the immune system of athletes especially in endurance sports in relation

to exercise and reported that *Echinacea purpurea* is stimulating on all cytokines as to the root, while the leaf has an immunosuppressive action. *Rhodiola rosea* stimulates all the cell lines of the immune system like Echinacea. Trinh et al. (2020) investigated the effect of herbal formulation KM1608 (alcohol extract from *Saussurea lappa*, *Terminalia chebula*, and *Zingiber officinale*) on RAW264.7 murine macrophages and showed that KM1608 stimulates the expression of immune cytokines (interferon (IFN) - $\alpha$ ,  $\beta$ , IL-1 $\beta$ , IL-6, IL-10 in macrophages. Bozakova et al. (2018) reported that preparation Immunobeta (CHEMIFARMA S.p.A., Animal nutrition products, Italy) which consist  $\beta$ -glucans and mannan oligosaccharides (they are produced by bacteria, yeast, fungi, and many plants) enhanced serum lysozyme concentrations, the activity of alternative pathway of complement activation and betalysine, IgM and IgG immunoglobulins in layer hens. Immunomodulatory effect of  $\beta$ -glucans and mannan oligosaccharides is based on activation of neutrophils, B cells, T cells, and natural killer cells. They also enhance cytotoxic activity and inflammatory cytokines of primary macrophages and RAW264.7 cell lines (Kim et al., 2011).

## CONCLUSION

Based on obtained results, it could be concluded that studied herbs possess an important immunomodulating potential in turkeys, which could improve their health and consequently, their productive performance.

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## CONFLICT OF INTEREST

All authors declare that there is no conflict of interest and disclose that we have not any financial and personal relationships with other people or organizations that might inappropriately influence or bias our work.

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