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The Effect of *Jordanian* Herbs on Gut Microflora, and Immunity of Broiler Chickens

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Abstract

*The study aimed to examine and compare the influence of adding synthetic antioxidant butylated hydroxyanisole (BHA), with two herbal medical plants (Oregano germander) on broiler gut microflora, and its immunity. One hundred and forty broilers were raised for three weeks and were distributed into five treatment groups. The prepared dietary treatments were included (w/w):**1**). Control (without supplement) **2**). Germander (GER; inclusion level 1.5 %) **3**). Oregano (ORE; inclusion level 2.5 %) **4**). Combination of GER and ORE (CM; inclusion levels 1.5 and 2.5 %, respectively)**5**). BHA (0.02 %). The highest total number of ileal lactobacilli counts was found in broilers fed CM dietary treatment. On the other hand, their disgust samples (i.e. in CM) showed the lowest total count of Escherichia coli compared to other treatments. Among different dietary treatments, CM treatment showed the best positive effect on broiler immune system response, where it showed the highest Bursa Fabricius relative weight, and IgG values. Overall, the results showed that such dietary treatment (i.e. Combination of GER and ORE)positively enhanced gut microflora, and bird's immunity response.*

Keywords: *Germander, Gut microflora, Ideal microflora, Oregano essential oil, Broilers.*

1. Introduction

Many feed supplements are widely used in poultry diets to enhance their growth performance and enhancing their health status (Senay et al., 2019; Gopi et al., 2014; Hassan et al., 2018). Many researchers investigated the effect of adding different plant-based supplements on poultry growth performance, feed efficiency, immunity, antioxidant defense systems, and the quality of their products (Al-Rabadi et al., 2020; Al-hijazeen, 2019; Al-hijazeen et al., 2022 AL-Rawashdeh et al., 2022; Ri et al., 2017; Surai et al., 2019). Furthermore, several reasons encourage animal production scientists toward this approach; however, replacing natural with synthetic anti-oxidant/antimicrobial supplements from poultry diets was their major goal (Senay et al., 2019; Al-Hijazeen, 2019). It is well known that using synthetic additives (synthetic antioxidants and antibiotics) in animal diets became

unfavorable due to their direct and indirect side effect on human health (Gopi et al., 2014; Mohammadi Gheisar and Kim, 2018). In addition, these additives reduce poultry disease resistance (Agyare et al., 2018) for several bacterial pathogenic strains (Gopi et al., 2014; Mohammadi Gheisar and Kim, 2018). Furthermore, it had been reported that the presence of these additives in the poultry diet may be adsorbed by the animal tissues (lean meat), and so indirectly affect humans (Senay et al., 2019; Jiang and Xiong, 2016; Mohammadi Gheisar and Kim, 2018; Abbdool et al., 2017).

Nowadays, all scientists are interested in finding natural and safe alternatives, especially in the poultry feed industry (Gopi et al., 2014; Hassan et al., 2018; Mohammadi Gheisar and Kim, 2018). One of the most suggested dietary alternatives is using phytobiotics (e.g., dried medicinal plant leaves, and their extracts) (Aroche et al., 2018; Windisch et al., 2008). These plants material composed of many phenolic compounds which positively improve broiler chickens' performances, feed efficiency, nutrient digestibility, and immune function (Aroche et al., 2018; Mohammadi Gheisar and Kim, 2018; Giannenas et al., 2004; Platel and Srinivasan, (2004); Jamroz et al., 2006).

For instance, oregano, sage, green tea, and varieties of plants resources showed a positive effect on overall animal performance; enhance animal growth, health status, and final carcass quality (Botsoglou et al., 2003a, b; Attia et al., 2017; Peng et al., 2016; Ri et al., 2017; Giannenas et al. 2005). It had been reported that adding these plants supplement (as dried plants or extracts) may improve poultry feed digestibility, feed efficiency, and thus their growth performance (Jamroz et al., 2003; Botsoglou et al., 2003a; Giannenas et al. 2005; Windisch et al., 2008). The improvement in poultry performance was also connected to the positive morphological changes, increasing in digestive enzyme activity which enhances its nutrient digestibility (Li et al., 2012; Lee et al., 2003; Jang et al., 2004; Jang et al., 2007). However, it always depends on many factors such as plant components, combination used, and animal response. These beneficial effects are highly dependent on their antioxidant and antibacterial properties especially when animals have stressed conditions (Al-Hijazeen, 2019; Al-Bandak, 2007; Gopi et al., 2014; Al Bahtiti, 2012; Jaradat, 2015). In addition, it enhances the blood immune system by improving animal reducing capacity, antioxidant defense system, and the formation of free radicals at the cellular level (Park et al., 2015; Gheisar and Kim, 2018). Animal tissues may also uptake certain compounds found in herbal plants oil (extract) like the vitamin E absorption mechanism (Leal et al., 2018; Lui et al., 1995; Li et al., 2009; Botsoglou et al., 2002; 2003a; 2003b).

For instance, oregano oil (*Origanuimsyriacum* L.) extraction was analyzed by different laboratories and procedures (Ibrahim et al., 2012a; b). Its extracts are composed of various polyphenols which consider being responsible for its antioxidant activity (Giannenas et al., 2013). Volatile compounds such as *carvacrol* and *thymol* are the major antioxidant compounds in oregano oil Kosar et al., 2003; Al-Bandak, 2007; Ibrahim *et al*., 2012a; Kokkini, 1997). Another example is germander plant (*TeucriumPolium* L.) extract has many poly-phenolic compounds that may be useful to treat illness and prevent many human diseases (Jaradat, 2015; Mahmoudi and Nosratpour, 2013) Germander essential oil and its extracts (*wild* Jordanian: *Teucriumpolium* (Ja'adeh) were consist unique phenolic compounds that showed a strong antioxidant and radical scavenging activities (Djilas et al., 2006). *Teucrium. Polium* L. (Wild Jordanian) composition is extensively analyzed, and well documented. Jaradat (2015) summarized 50 years of scientific knowledge related to germander phytochemical composition into different categories. Furthermore, the essential oil and extracts of both plants (i.e. oregano and germander) showed significant antimicrobial effects against several pathogenic bacterial strains (Jaradat, 2015; Alhijazeen, 2018; Mohammadi Gheisar and Kim, 2018). The antimicrobial mode of action of these plants' additives was varying and inconsistent. This effect is due to differences in their functional hydroxyl or alkyl group location (Yang et al., 2015). Researchers suggested that the antimicrobial activity of these plants derived was due to their direct influence on the microflora cell membrane, ion exchange and internal protein synthesis (Burt, 2004; Ultee et al., 2002).

Both medicinal plants (Oregano and germander) are available in the wild of the south Jordanian region (Al Bahiti, 2012; Ibrahim et al., 2012a; Afifi et al., 2009) with high levels of phenolic compounds (Ibrahim et al., 2012a; Acar and Goldstein, 1996). However, many aspects could affect their potency as antioxidant/antimicrobial agents. For example, their storage condition, cultivation

period, harvesting time, and plant variety (Burt, 2004; Badi, et al., 2004; Ibrahim et al., 2012a; Afifi et al., 2009). Both *Teucrium polium* L. and *Origanuim syriacum* L. are characterized by their unique volatiles and composition compared to other plant species (Ibrahim et al., 2012a; Jaradat. 2015). These alternatives are highly recommended due to many reasons, which promote the use of it as poultry feed additives (Gheisar and Kim, 2018). A little investigation had been done on their combined effect (Oregano and germander) on the broiler immune system, and their effect on gut microflora. The main purpose of this study was to examine the influence of supplementing dietary oregano and germander herbal plants alone or in combination; on the immunity and gut microflora in broiler chicken and to compare the influence of these dietary herbs with the synthetic antioxidant (BHA).

2. Materials And Methods

Birds and Diet Formulation

One hundred and forty broilers were raised for three weeks (during the growing stage; from 21 to 42 days old). The five dietary treatments of 140 broilers were randomly assigned (each treatment include 7 replicates and 4 broilers per replicate). The formulated dietary treatments were: **1**). Without supplement (control);**2**). Level of 1.5 % Germander (GER);**3**). Level of 2.5 % Oregano (ORE);**4**) Combination of GER and ORE (CM, levels of 1.5 and 2.5 %, respectively);**5**). *butylated hydroxyanisole* BHA (level of 0.02 %). All dietary treatments were prepared to meet nutrient specifications suggested by poultry NRC (1994).

Immune System Response and their Organs Index

Two measurements for each replicate were reported to evaluate both spleen and *Bursa Fabricious* relative weights (in proportion to body weight %) at the age of 42 days as a good indication about the immune system response of broiler chicken by adding these plants supplements. On the final day (day 42), blood samples were taken from the jugular vein into plain vacutainer tubes, coagulated at room temperature for 10-20 mins, and then the separated serum was stored at −20°C until further analysis. Serum immunoglobulin (IgG or IgY) was measured and analyzed using a prepared kit (Chicken IgG (IgY) ELISA Life Diagnostics, Inc., Catalog Number: IGG-5) according to the manufacturer's recommendations. Samples were incubated for 5 min at 37 °C, and the absorbance (At 450 nm/after adding stop solution/within 15 min) was recorded using a microplate reader (ELISA). The concentration of IgG was calculated depending on the absorbance of the tested samples and is derived from a standard curve.

Gut Microflora

All intestine samples (two birds per replicate treatment) were cut and collected immediately after slathering according to Ao and Kim's (2019b) protocol with minor modifications [50]. Small intestine cut samples were vacuum packaged (Baalbaki®DZ 400/2D Vacuum Packer, 14KT6013-HZ-5), and cooled at 4˚C until used. Fresh cut samples (1 g of internal digesta in duplicate) were aseptically diluted in 9 Ml of 1 % sterilized peptone broth (BiolabZrt. 1141 BUDAPEST Öv u. 43, Hungary), (10-fold dilution) then the subsequent dilutions were vortex and used directly to enumerate selected digestive bacterial. This protocol evaluated the effect of adding these plant supplements on the survival of selected bacterial populations from birds DSI (Digesta of Small Intestine) at day 42. The colony-forming units (CFU/g) of certain were evaluated for all treatment groups according to the method described by Rahimi et al (2011) and Ao et al (2019). MacConkey agar (Merk, Germany) media was prepared to enumerate viable E. *coli* at 37 ˚C for 24 hours, and MRS (Merk, Germany) agar was used to enumerate *Lactobacillus* viable count under the anaerobic condition at 37˚C for 72 hours.

Statistical Analysis: measurements were statistically examined using the procedures of a generalized linear model (Proc. GLM, SAS program, 2012). Complete randomized design (**CRD**) was used to analyze measurements. Tukey test or Tukey's Multiple Range test was used to determine whether there is a significant difference between the average values (the significance was determined at $P<0.05$.

3. Results and Discussion

Gut Microflora

Ileal microflora, its varieties, stabilization, and their content of both beneficial and pathogenic were reported as effective factors affecting overall bird's health and growth performance (Song et al., 2014; Mohammadi Gheisar and Kim, 2018). Several studies (in *vivo* antimicrobial effect) showed that adding plants derived/phytogenic feed additives (PFAs) in broiler diet were suppress the growth of many pathogens such as *Clostridium perfringens* (Mitsch et al. 2004), *Eimeria* species (Oviedo-Rondon et al., 2006; Hume et al., 2006). In Table (1), it was clearly shown that adding all feed additives suppress the growth of *E.coli* significantly compared to the control treatment. However, the CM showed the highest antibacterial effect against *E.coli* survival among the other treatments. In addition, the anti-*E.coli* growth effect of adding ORE supplements was significantly higher than adding GER and BHA alone. On the other hand, the CM showed the highest significant ($P < 0.05$) positive effect on the growth and survival of *Lactobacillus* (6.11 Log CFU/g^{-1}). Oregano (ORE) additive showed a higher significant effect in increasing *Lactobacillus* count compared to GER and BHA alone. Mohammadi Gheisar and Kim (2018) reported that adding (PFAs) to the gut of poultry and swine will decrease the total count of pathogens which causes an increase in the beneficial bacteria count (*Lactobacillus*). Adding PFAs may improve the growth of some beneficial bacteria strains in the bird's digestive system, on the other hand, it may decrease the total count of certain pathogenic bacteria (Brenes et al., 2016; Mohammadi Gheisar et al., 2015a). Viveros et al. (2011) also concluded that adding GSE positively affects the total bacterial count of beneficial bacteria and suppresses the growth of some pathogenic bacteria. Researchers explain the increase in the total number of ileal *lactobacilli* counts through the ability of these bacterial strains to utilize or metabolize phenolic compounds (nutritional substances) (Garcia-Ruiz et al., 2008; Viveros et al., 2011). Tschirch (2000) reported that carvacrol (oregano and thyme main constituent) enhanced *Lactobacillus* survival and growth rate. A similar trend was found by Mohammadi Gheisar et al. (2015a) where the essential oils blend increased the *Lactobacillus* count of broilers chickens. Furthermore, phenolic compounds can increase the shedding of microbial bacteria (*Lactobacillus*) which can improve bird's immunity (Paszkiewicz et al., 2012). Other researchers documented the anti *E.coli* effect in *vitro* by using these plant-derived additives (Jamroz et al., 2003; 2006). Similar results found that the *E.coli* (ileal *microflora*) count decreased by adding herbal extract (garlic, coneflower, thyme) in broilers feed (Skyrme, 1997; Rahimi et al., 2011). Furthermore, researchers reported that the inclusion of dietary herbal plants may improve the jejunum morphology (crypt depth and villus height) and this was reflected positively in the bird's feed efficiency and gut health (Boka et al., 2014; Yang et al., 2016; Viveros et al., 2011; Samuel et al., 2017).

Table 1. Number (Log CFU/g-1Digesta) of viable Escherichia coli, Lactices inileo-cecum of broiler chickens.

Treatments	Control	Germander	Oregano	Combination	BHA	SEM
Microflora	----------	----------	**Log CFU/g^{-1}Digesta**	----------	----------	
Escherichia coli[x]	7.177^{a}	4.891^{c}	4.327^{d}	4.031^{e}	6.371^{b}	0.067
Lactics	4.362^{c}	4.777^{c}	5.505^{b}	6.112^{a}	4.355^{c}	0.104

[x-] Within a row, average values with different subscript differ significantly ($p < 0.05$).
Prepared intestinal digesta (SID) samples stored at 4 C. n=7.

Bird's Immunity Response and their Organ Index

Immunity organs relative weight (index), and IgG in blood samples of broiler fed different diets were reported (Table 2). Generally, the broiler immune system response is correlated with the immune organ size such as the spleen, thymus, and bursa (Króliczewska et al., 2017; Zhou et al., 2019). In the current study, there were no differences found among all treatments when evaluating their spleen relative weight (S. *RW*). However, a significant difference is identified on *Bursa Fabricious* relative weight (B. F*RW*) between the control and the CM treatments diets. In addition, no significant differences showed when testing B.F *RW* (%) among all treatment additives. The CM treatment showed higher B. F *RW* (%) of all birds compared to other dietary treatments which indicates a higher immunity response. However, the effect of adding various plant additives on broiler immune organs (Organ's index: such as spleen, thymus, bursa and liver) was varied among all studies done previously (Zhou et al., 2019; Ri et al., 2017; Farahat et al., 2017). This variation had been attributed to the differences between these plants derived in their effective antioxidant compounds (Abu Hafsaand

Ibrahim, 2018; Attia et al., 2017). Avian serum immunoglobulins (IgG or IgY) which are the major antibody in birds was also used to measure its immunity response (Suzuki and Lee, 2004). In this study, there was no significant difference in IgG mean level between the control diet, and the other treatment diets. In contrast, both ORE and CM treatment diets showed the highest levels (6.24 and 6.43 mg/ml, respectively). These results agreed with Hamal et al. (2006) where the normal chicken serum IgG level was in the range of 3-6 mg/ml. Several research studies reported that dietary plant-derived or their extracts in animal feed raise their serum antibody levels (Zhou et al., 2019; Farahatet al., 2017; Facina et al., 2017). Furthermore, broiler's immunity response is correlated with many factors such as type of infection, pathogens' effectiveness, and bird's genetic susceptibility factors (Oviedo-Rondon et al., 2006; Yang et al., 2016; Zahoor et al., 2018).

Table 2. The immune system response and relative weight of broilers fed different dietary supplements.

Parameter	Control	GER	ORE	CM	BHA	SEM
S. RW (%) [x]	0.114[a]	0.116[a]	0.127[a]	0.128[a]	0.122[a]	0.005
B. F RW (%)	0.195[b]	0.212[ab]	0.217[ab]	0.227[a]	0.209[ab]	0.006
IgG	5.43[a]	5.89[a]	6.24[a]	6.43[a]	5.91[a]	0.396

Within a row, average values with different subscripts differ significantly ($p < 0.05$).
S. RW: Spleen relative weight (% of body weight).
B.F RW:Bursa Fabricious relative weight (% of body weight).
IgG: mg/ml.
N=7.

4. Conclusion

Dietary supplements affected broiler gut microflora and their immunity response. Combining both herbs treatments (i.e. CM) exposed the highest effect in enhancing bird's immunity response. In addition, it (CM)exhibited the highest effect suppressing the *E. coli* growth and enhances *Lactobacillus* survival. Their positive effect on the birds' gut microflora considers a mirror in the improvements of the bird's overall health. It is concluded that adding a combination of ORE and GER could improve the overall broiler immune response and gut microflora.

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Conflict of interest:
The author declares no conflict of interest.

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