Research Article

# Cytokines and Performance Analysis of Broiler Chickens Challenged with Avian Pathogenic *E.coli* After Administration of Various Immune modulatory Agents

**Ahmed H. Abed[1], Ahmed Orabi[2*], Wafaa Hassan[3], , Enas Hammad[4] , Ghada Gaber[5]**

*[1]Bacteriology, Mycology and Immunology Department, Faculty of Veterinary Medicine, Beni-Suef University, Beni-Suef, Egypt, 62511; [2]Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, 11221; [3]Chef Researcher of Poultry Diseases and The Director of Reference Lab for Veterinary Quality Control of Poultry Production, Egypt; [4]Researcher in Poultry Diseases Department, Mansoura Lab Animal Health Research Institute, Arc, Egypt; [5]Research Assistant of Reference Lab for Veterinary Quality Control of Poultry Production, Egypt.*

**Abstract** | Avian colisepticemia is the most common bacterial disease in poultry, and it costs the industry a lot of money. This study shows that some immune modulatory substances such as lectins, probiotics, and essential oils have a unique immune-potentiating effect on broiler chickens' immunity challenged with *E. coli*. One hundred and twenty broiler chicks were separated into eight equal groups in an in vitro experiment (15 birds for each group). A negative control group was the first group. There were 3 groupings were given immune modulatory agents (lectins, probiotic, and essential oil) for five days without being challenged; Immune modulatory agents were given to the remaining three groups, who were also infected with *E. coli* $O_{78,}$ and one group act as an *E. coli* $O_{78}$ challenged group. The obtained results showed improvement of the growth performance of the three groups administrated immune modulatory agents without challenge. The level of IL-4 gene expression was increased, and it effectively reduced INFG, and IL-6 mRNA gens expression.

**Keywords** | Broiler chickens, Cytokines, *E. coli,* Essential oils, Lectins, Performance, Probiotics.

**Received** | July 05, 2022; **Accepted** | August 15, 2022; **Published** | October 20, 2022
***Correspondence** | Ahmed Orabi, Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, 11221; **Email:** orabi.vet@cu.edu.eg
**Citation** | Abed AH, Orabi A, Hassan W, Hammad E, Gaber G (2022). Cytokines and performance analysis of broiler chickens challenged with avian pathogenic *E.coli* after administration of various immune modulatory agents. Adv. Anim. Vet. Sci. 10(11): 2438-2446.
**DOI** | http://dx.doi.org/10.17582/journal.aavs/2022/10.11.2438.2446
**ISSN (Online)** | 2307-8316



## INTRODUCTION

Avian colibacillosis caused by enterotoxigenic *E. coli* is a serious infectious disease occurring in different types of chickens (He et al., 2014) as estimated to cause a great hazard in the global poultry industry (Wade and Keyburn, 2015). The discovery of 8-13 virulence genes in highly pathogenic *E. coli* isolates or 5-8 virulence genes in intermediate pathogenic *E. coli* isolates is attributed to Avian pathogenic *E.coli* virulence (Wang et al., 2015). Several virulence genes were investigated in Egypt, they differed depending on the geographic location. Pathogenic strains were observed to consistently carry the virulence gene pattern of fimH, fimA (fimbriae gene cluster), and papC(pilus associated with pyelonephritis) in a few in vivo assays, iutA(ferric aerobactin outer membrane receptor gene), and tsh (temperature sensitive hemagglutinin), which were linked to fatality in one-day-old chicks (Ali et al., 2019). The increasing prevalence of APEC that is antibiotic resistant poses a zoonotic danger in poor economies (Abdallah et al., 2015; El-Shazly et al., 2017). To prevent *E. coli* infection in chicken, alternatives such as probiotics,

lectins, and essential oils are being developed (Projahn et al. 2018; Jiang et al. 2019)., a result of their antibacterial properties as a substitute for antibiotic resistance pathogens recent research has focused on the medicinal and preventive applications of lectins (Iordache et al., 2015). A novel lectins isolated from the mushroom's fruiting bodies demonstrated antibacterial action against *E. coli* (Jiang et al. 2019). Furthermore, lectins had an immune modulatory effect via causing macrophages and tumour necrosis factor (TNF) to become active, promoting the expression of IL-2 and IFN genes, and therefore upregulating the T-helper-1 cell population (She et al., 1998). Probiotics are live, non-pathogenic microbial feed supplements that provide health advantages to the host and are thought to be a viable option for preventing and treating bacterial illnesses (Gareau et al., 2010). *Lactobacillus acidophilus* has also been shown to improve immune function by boosting both innate and adaptive immune responses (Liu et al., 2010). *Lactobacillus acidophilus* is a potent inducer of T-helper 1 (Th1) cytokines, such as interleukin (IL-12) and interferon (IFN), according to some in vitro research (Gackowska et al., 2006; Zeuthen et al., 2006). Essential oils (EO) have been gaining popularity as a result of consumer preference for natural products (Brenes and Roura, 2010). Additionally, Improvements in cellular and humoral immunity have been linked to 5EO supplementation in hens (Lee et al., 2011; Awaad et al., 2014) as well as regulate the host immunity gene expression (Kim et al., 2010). The two isomers thymol and carvacrol, which are also essential components of frequently used herbs like thyme and oregano, are among the thousands of EO constituents have strong antibacterial properties (Bassole and Juliani, 2012). Thymol and carvacrol have been shown in experiments to block pro-inflammatory cytokines, decrease inflammatory cell recruitment, and reduce oxidative damage (Riella et al., 2012; Guimaraes et al., 2012). In the current study, the influence of administrated lectins, probiotic and essential oils on the health and immunity of diseased broiler chickens infected with avian pathogenic *E. coli* was studied.

## MATERIALS AND METHODS

### Ethical approval

The Faculty of Veterinary Medicine, Beni-suef University, Egypt, followed Animal Research Ethics Guidelines for conducting this work with Ethical approval number. (021-195)

### Experimental chickens

A commercial hatchery provided 120 one-day-old commercial broiler chicks. The birds were raised in separate rooms in metal cages and were1lit continuously and fed antibiotic-free traditional broiler feeds at will.

### Escherichia *coli* Challenge Isolate

One strain of *E. coli* O78 was isolated and identified from a broiler with respiratory illness. broiler hens. Congo-red binding assay and virulence gene identification indicated that the bacteria were pathogenic (Ali et al., 2019). McFarland's barium sulphate standard solution was used to adjust the bacterial suspension to include $10^8$ CFU/mL- (Nagano et al., 2012). Subcutaneously, each APEC strain in 0.5 mL ($10^8$ CFU/mL). was used in the challenge (Abd El-Mawgoud et al., 2020).

### Administrated lectins, probiotic and essential oils

A commercial product called Lector50®, manufactured by Microbiotech INT. INC. in the USA, including 15,000 mg of lectin, 5000 mg of xylitol, 15,000 mg of fructo-oligosaccharide, and 30,000 mg/liter of NaCl, was employed. Probac® contains mixed types of *Lactobacillus* spp. and essential oil obtained from sigma Aldrich.

### Experiment Design

A total of 120 broiler chicks were separated into eight groups of equal number (15 birds for each group). A negative control group was the first group. Immune modulatory agents such as lectins (0.5 mL/L drinking water), probiotic 0.25 g/L in water supply) and 0.5 mL of an essential oil in drinking water) were given to three groups, separately for five days without being challenged, and the other group acted as the E. coli O78 challenged control. Immune modulatory agents were given to the remaining three groups, which were also infected with *E. coli* $O_{78}$ (Table 1).

### Evaluation of the Performance Parameters

Each group's average body weight was measured on a weekly basis. By dividing the total amount of feed consumed by the increase in the total mass of the hens in each group, conversion ratios (FCR) for each group were estimated. (Coneglian et al., 2010).

### The Clinical signs and post mortem lesions

The clinical symptoms were documented in accordance with Nagano et al. (2012), and the postmortem lesions were documented in accordance with Rawiwet and Chansiripornchai (2009).

### Cytokines assay using qRT- PCR;

RNA extraction from spleen samples was applied according to Yuan et al. (2006) by using RNeasy Mini Kit, and the reaction was prepared by using specific primes for IL-4, IL-6, and IFNG (Table 2). Amplification curves and ct values were determined by the stratagene MX3005P software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group

**Table 1:** Experimental design

| Groups | infection with *E. coli* $O_{78}$ | Lector 0.5ml/L in the drinking water for 5days at age of 25 days | Probiotic 1ml/L in the drinking water for 5days at age of 25 days | Essential oil 0.5ml/L in the drinking water for 5days at age of 25 days |
|---|---|---|---|---|
| 1.Negative control | - | - | - | - |
| 2.Positive control | + | - | - | - |
| 3.gp. administrated lector without challenge | - | + | - | - |
| 4.gp. administrated probac without challenge | - | - | + | - |
| 5.gp. administrated E. O without challenge | - | - | - | + |
| 6.gp. administrated lector with challenge | + | + | - | - |
| 7.gp. administrated probac with challenge | + | - | + | - |
| 8.gp. administrated E. O. with challenge | + | - | - | + |

**Table 2:** Primers used

| Gene | Primer sequence (5'-3') | Reference |
|---|---|---|
| IL6 | GCTCGCCGGCTTCGA | Suzuki *et al.*, 2009 |
| | GGTAGGTCTGAAAGGCGAACAG | |
| | (FAM) AGGAGAAATGCCTGACGAAGCTCTCCA (TAMRA) | |
| IL4 | AACATGCGTCAGCTCCTGAAT | |
| | TCTGCTAGGAACTTCTCCATTGAA | |
| | (FAM) AGCAGCACCTCCCTCAAGGCACC (TAMRA) | |
| 28SrRNA | GGCGAAGCCAGAGGAAACT | |
| | GACGACCGATTTGCACGTC | |
| | (FAM) AGGACCGCTACGGACCTCCACCA (TAMRA) | |
| IFN-G | AAACAACCTTCCTGATGGCGT | Markowski-Grimsrud and Schat, 2003 |
| | CTGGATTCTCAAGTCGTTCATCG | |
| | (FAM) TGAAAGATATCATGGACCTGGCCAAGCTC (TAMRA) | |

**Table 3:** Zoo-technical results of the experimental groups

| Group | Clinical signs | Postmortem lesions | Mortality rates | Re-isolation rates |
|---|---|---|---|---|
| Negative Control | None | none | none | None |
| Positive Control *E. coli* $O_{78}$ | Respiratory signs-off food- dullness depression- brownish diarrhea-reluctance to walk | Pericarditis-air sacculitis-Cellulitis- | 40% | 80% |
| Gr. administrated lectin without challenge | None | None | none | None |
| Gr. administrated probiotic without challenge | None | None | none | None |
| Gr. administrated E.O without challenge | None | none | 13.3% | None |

| | | | | |
|---|---|---|---|---|
| Gr. administrated lectins with challenge | Brownish diarrhea-dullness-off food -un ability to walk | Cellulitis-congestion of liver and spleen - | 6.6% | 40% |
| Gr. administrated probiotic with challenge | Dullness-depression-off food- diarrhea | Turbid air sac-pericarditis | 10% | 40% |
| Gr. administrated E. O. with challenge | Reluctance to walk-brownish diarrhea-off food-rales | Ascites- cellulitis-mild pericarditis and air saculitis | 13.3% | 60% |

**Table 4:** Results of the performance parameters in the experimental groups

| Groups | BWT (g) | Feed intake(g) | BWT gain(g) | FCR |
|---|---|---|---|---|
| Negative Control | 14250.33 | 7870.24 | 5500.32 | 1.430.22 |
| Positive control | 9650.45 | 4000.42 | 1500.43 | 2.670.45 |
| Gr. administrated lectins without challenge | 14800.22 | 7980.23 | 6300.24 | 1.260.26 |
| Gr. administrated Probiotic without challenge | 15500.33 | 8200.43 | 6870.44 | 1.1942 |
| Gr. administrated E.O. without challenge | 14500.35 | 7300.42 | 5700.43 | 1.280.34 |
| Gr. administrated lectins with challenge | 10200.33 | 4500.45 | 1650.48 | 2.730.34 |
| Gr. administrated Probiotic with challenge | 11700.22 | 5750.43 | 3200.34 | 1.80.52 |
| Gr. administrated E.O. with challenge | 9800.44 | 4150.45 | 1450.52 | 2.860.56 |

E.O.: essential oil

**Table 5:** Results of cytokines analysis in the experimental groups

| Group ID | $IL_4$ | | $IL_6$ | | INFG | |
|---|---|---|---|---|---|---|
| | CT | Fold change | CT | Fold change | CT | Fold change |
| Negative Control | 22.15 1.2 | -- | 21.30 1.4 | -- | 22.04 2.4 | -- |
| Positive control | 25.56 2.1 | 0.19140.32 | 18.99 1.4 | 10.05611.3 | 20.18 2.4 | 7.38711.8 |
| Gr. administrated lectins without challenge | 20.29 2.3 | 8.33970.25 | 24.79 1.5 | 0.20450.33 | 24.88 2.5 | 0.32140.45 |
| Gr. administrated Probiotic without challenge | 20.71 2.2 | 4.80650.38 | 23.45 1.6 | 0.39980.42 | 23.71 3.2 | 0.55860.34 |
| Gr. administrated E.O. without challenge | 21.69 1.6 | 4.09110.34 | 23.12 2.2 | 0.84380.34 | 24.11 3.2 | 0.71080.45 |
| Gr. administrated lectin with challenge | 22.95 1.7 | 0.83620.22 | 20.18 1.5 | 3.16021.3 | 21.52 2.4 | 2.08781.5 |
| Gr. administrated Probiotic with challenge | 23.34 1.5 | 0.68110.23 | 19.19 1.4 | 6.69002.4 | 20.64 3.2 | 4.09542.4 |
| Gr. administrated E.O. with challenge | 23.99 1.6 | 0.42930.24 | 19.07 2.3 | 7.19002.3 | 20.33 2.4 | 5.01413.1 |

according to the "ΔΔCt" method stated by Yuan *et al.*, 2006 using the following ratio: $(2^{-ct})$.

Whereas ΔΔCt = ΔCtreference - ΔCttarget

$\Delta Ct\ target = Ct\ control - Ct\ treatment$ and $\Delta Ct\ reference = Ct\ control - Ct\ treatment$

E: efficiency of amplification.

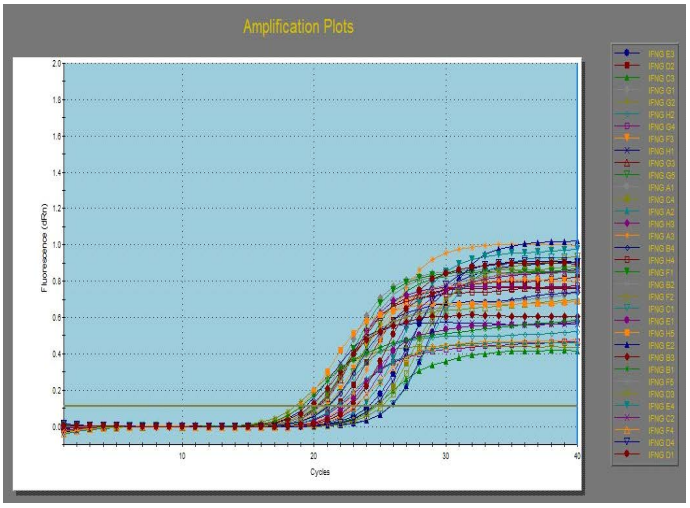
## Re isolation of the avian pathogenic *E. coli*

Ten grams of solid sample from heart, and liver in 90 ml normal saline was prepared, followed by enrichment for 24 h incubation at 37°C in non-differential broth such as nutrient broth, according to Irwin et al. (2010). This technique allows *E. coli* to multiply before being streaked on EMB agar and biochemically identified.

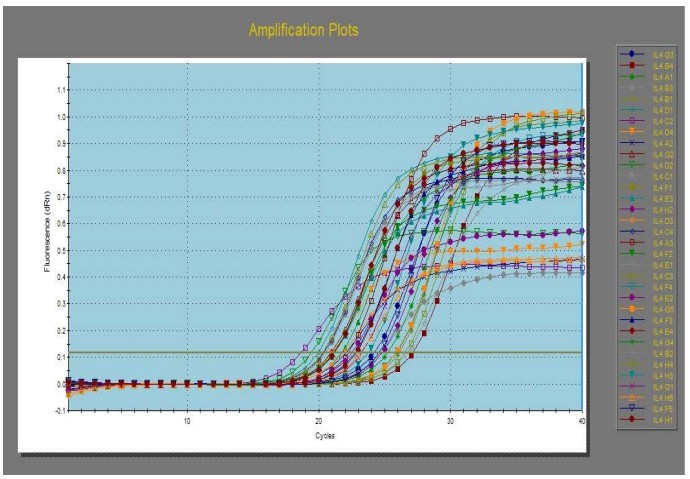
## Statistical analysis

The results were presented as means± SE. All given parameters were compared between the control group and the experimental groups using the one way ANOVA with fixed effects of the factors using statistica 6.0 (Start Soft INC.). Differences were considered significant at $p<0.05$.

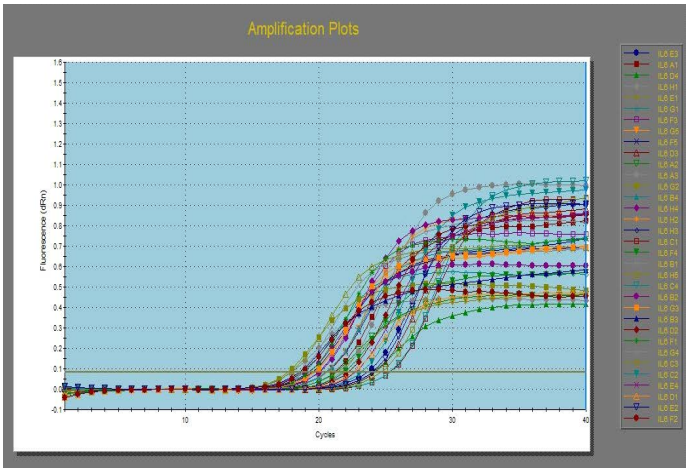
**RT-PCR plots showing fold change amplification for different cytokines indices of different experimental groups using specific probe**



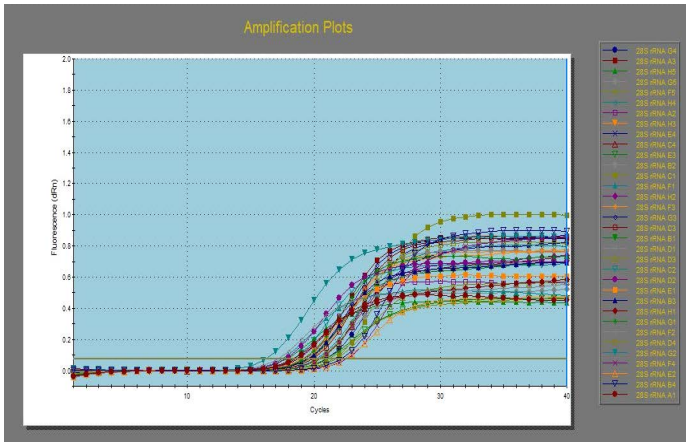

a-**Interferon gamma**




**b-Interleukin 4**




**c-Interleukin6**




d-**28SrRNA**

## RESULTS

### The clinical symptoms, mortality rates and re isolation rates

All of the administered groups had significantly reduced clinical symptoms, post mortem lesions, and reisolation rates than the challenge control group (Table 3).

### The assessment of performance

performance review The average body mass of the birds across all groups was remarkably consistent. before challenge while, after the challenge, significant differences in both bodyweights and FCR were noticed (Table 4).

### Avian cytokines gene expression analysis using qRT- PCR:

**Lectins administrated group:** There was an increase of IL-4 in lectins group fold change than other groups before challenge to 8.3397, and to 0.8362 after challenge in comparison with positive control group (0.1914). While, IL-6 and INFG fold change were linearly inhibited in IL-6 from10.0561 to 0.2045 before challenge, to 3.1602 after challenge, INFG fold change from 7.3871 to 0.3214 before challenge, and to 2.0878 (Table 4).

**Probiotic administrated group:** There was an increase of IL-4 in probiotic group fold change before challenge to 4.8065, and to 0.6811 after challenge in comparison to positive control group (0.1914). While, IL-6, and INFG fold change were linearly inhibited in IL-6 from10.0561 in positive control group to 0.3998 before challenge, to 6.6900 after challenge, INFG fold change from 7.3871 to 0.5586 before challenge, and to 4.0954 after challenge.

### Essential oil administrated group

There was an increase of IL-4 in essential oil group fold change before challenge to 4.8065, and to 0.6811 after challenge in comparison with positive control group (0.1914). While, IL-6 and INFG fold change were lin-

early inhibited in IL-6, from 10.0561 in positive control group to 0.3998 before challenge, to 6.6900 after challenge, INFG fold change from 7.3871 to 0.5586 before challenge and to 4.0954 after challenge (Table 5).

## DISCUSSION

Mannose-binding lectins (MBL) are also considered a member of the PRR family. It is a soluble-type protein which can also be considered as an effector molecule. MBL binding to PAMPs on pathogen cell walls can trigger complement activation via the lectins route, which further neutralizes infections by aggregating them (denying attachment of bacteria to epithelial cells of host). MBL can also bind apoptotic cells on the phagocyte membrane via ligation (the joining of two DNA strands by a phosphate ester bond) (Nielsen et al., 1999). For organogenesis, tissue upkeep, and optimal immune system function, apoptotic cells must be eliminated. The term «acute-phase protein» refers to proteins that respond to inflammatory signals by elevating their plasma concentration by at least 25%. These proteins serve as transport proteins and have antioxidant action in addition to participating in the host's protection and adaptation. (Norup et al., 2009). Mannose-binding lectin is an inherent host defense protein that has a high affinity to initiate the complement lectins pathway via a serine protease linked to the mannose-binding lectins (MASP-2) (Zhang et al., 2017; Ulrich-Lynge et al., 2015). wide range of protections against the physiochemical actions of pathogens are provided by the MBL structure. (Takahashi, 2011). Lectins were shown to affect the levels of several cytokine mRNAs. In lectins-treated splenic lymphocytes, the expression of IL-2 and IFN-g was significantly increased. This was explained in the current study, as the group administrated lectins elevated level of IL-4 gene expression to 8.3397 before challenge, and to 0.8362 after challenge in comparison to positive control group (0.1914). While, the level of INFG and IL-6 decreased from 7.3871 to 2.0878, and from 10.0561 to 3.1602 after challenge, respectively, and that was in agreement with other study done by Park et al. (2010).

To combat pathogenic organisms, various tactics have been utilized. Antibiotic-resistant organisms and diseases have grown to be a significant public health concern due to the abuse of antibiotics by people and animals, as well as environmental changes, .Probiotics are being regarded as an extra effective technique for illness prevention and treatment alongside the development of medicines and vaccinations. Probiotic bacteria have grown in popularity as a mean of enhancing both innate and adaptive immune responses (Liu et al., 2010; Konstantinov et al., 2008). Antimicrobial activity is one of probiotics' positive qualities. This is due to a variety of causes, including lower pH levels (due to lactic acid generation), and the formation of bacteriocins with bactericidal or bacteriostatic properties (Parente and Ricciardi, 1999). The current study found that giving broilers a probiotic, helped them recover from the growth inhibition caused by *E. coli* infection. Probiotics' favorable benefits on broiler growth performance are similar with findings from earlier studies utilizing probiotics in broilers (Zulkifli et al., 2000; Wang et al., 2017). Immune regulatory peptides; known as cytokines; play a role in both innate and adaptive immune responses. They have a critical function in immunological system control (Tayal and Kalra, 2008). $Th_2$ cells generate IL-4 in response to antigenic stimulation. Up regulation of IL-4 levels has been linked to *E. coli* infection (Zhang et al., 2013), which explains the increase in the Fold Change of IL-4 from 0.1914 to 0.6811 following infection. Interferon- has been identified as a pro-inflammatory cytokine that causes immunopathology in a variety of inflammation models (Van Holten et al., 2004).

The present study showed that administration of probiotic decreases fold change of IL-6 from 10.0561 to 6.6900, and the level of fold change of INFG from 7.3871 to 4.0954, and IL-6 from 10.0561 to 6.6900, suggesting that probiotics had an anti-inflammatory effect. Finally, because of its acid and bile resistance, strong inhibitory impact against *E. coli*, and high adhesion ability, the in vitro investigation revealed the value and potentiality of probiotics.

Essential oils (EO) have been gaining popularity as a result of consumer preference for natural products (Brenes and Roura, 2010). The antibacterial activity of EO against a variety of pathogenic bacteria, including both Gram-positive and Gram-negative bacteria, has been thoroughly investigated in vitro and is well established.(Ouwehand et al., 2010; Upadhyaya et al., 2013). Supplementing the ration with EO has been demonstrated to enhance humoral and cellular immunity (Lee et al., 2011; Awaad et al., 2014). In chickens, that influence host immunity gene expression (Kim et al., 2010). Among the hundreds of compounds in essential oils, carvacrol has a strong antibacterial action (Bassole and Juliani, 2012). Carvacrol has been shown to block pro-inflammatory cytokines, reduce inflammatory cell recruitment, and reduce oxidative damage (Riella et al., 2012; Guimaraes et al., 2012) .Lipopolysaccharide-injected broiler chickens' spleens, a prior study revealed that Carvacrol may increase IL-4 gene expression while lowering cytokines such as tumor necrosis (TNF) gene expression. (Du et al., 2015). In the current study, EO elevated the level of IL-4 gene expression from 0.1914 to 0. Carvacrol may increase IL-4 gene expression while lowering cytokines such as tumor necrosis (TNF) gene expression. 4293 after challenge, and to 4.0911 before challenge, however, linearly inhibited gene expression of IL-6 from10.0561 in control positive group to 7.1900 after challenge, and to 0.8438

before challenge. Finally, the in vitro study proved the efficacy and potential of probiotics, lectins, and essential oils in the fight against *E. coli*. The results of the animal studies revealed that administering these immune modulatory agents alleviated the growth suppression induced by *E. coli* challenge, increased the growth performance, decreased *E. coli* reisolation rate, and improved the immunity in *E. coli* challenged broilers. This data suggest that probiotics, lectins, and essential oils could be employed as an alternative to antibiotics as growth promoters in broilers, especially when it comes to *E. coli* management.

## CONCLUSION

Avian pathogenic *E.coli* is a great problem in poultry, and poultry farms as it is accused of causing respiratory, and enteric lesions, with economic losses, and difficulty to be controlled. The present study provides to some extent a safe solution to manage this problems by using lectins, probiotics, and essential oils as they have immune modulatory effects, and improve performance of the birds to overcome the *E. coli* infections.

## ACKNOWLEDGMENT

The authors would like to thank the Bacteriology, Mycology and Immunology Department staff at the Faculty of Veterinary Medicine, Beni-Suef, and Cairo University for providing technical support.

## AUTHORS' CONTRIBUTION

Authors' involvement Planning the study and writing the manuscript were both responsibilities undertaken by all of the researchers. as well as approveing the final version of the article.

## CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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