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Effect of *Amphora coffeaeformis* and *Star anise* as Dietary Supplementson the Immunity and Growth Performance of Broiler Chickens

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ABSTRACT

The present study was designed to evaluate the impacts of daily dietssupplemented with *Amphora coffeaeformi*s and *Star anise* on growth performance and immunity of Cobb broiler chickens. *Amphora coffeaeformi*s is considered a potent free radical scavenger due to the presence of β-carotene and fucoxanthin, which are used widely as food additives. *Star Anise* has a natural antioxidant, which can also be used for the chemo-prevention of disease occurring due to oxidative deterioration. A total of 270 broiler chickens were divided into three groups, each with three replicates of 30 birds. The control group (G1) was given the basal diet, the *Amphora* group (G2) received *Amphora* in a dose of 1g/Kg in the ration, and the *Star anise* group (G3) received *Star anise* in a dose of 2g/Kg in the ration. The results indicated that *Amphora* and *Star anise* significantly improved the final body weight, weight gain, and feed conversion ratio, total white blood cells count, phagocytic activity percentage, phagocytosis index in plasma, IgM, IgG, and A/G ratio in serum. In addition, *Amphora* and *Star anise* significantly increased mRNA expression of hepatic growth hormone gene, insulin-like growth factor-1 (IGF-1) genes (IGF1), and mRNA expression of splenic interferon-gamma (INF-γ) and Interleukin 12 (IL-12p35) genes from broiler chickens, compared to the control group. In conclusion, the use of fed additives, such as *Amphora coffeaeformis*and *Star anise* in the diet of broiler chickens for 35 days was sufficient to improve broiler growth performance and could modulate their immunity.

Keywords: *Amorpha coffeaeformis*, Broiler chickens, Diet supplementation, Growth performance, Immunity, *Star anise*

INTRODUCTION

Global food production is facing a greater challenge than ever before. Protein in particular is one of the most important nutrients in animal and human life (FAO, 2018).

Broiler production as a source of protein has become an important issue in many growing countries. However, the recent increase in the costs of traditional feed was once a major contributor to net returns from the poultry business. Feed accounted for 70-80 percent of the total costs of poultry production (Bolu and Balogun, 2004). It was found that reducing feed costs using cheaper and more unconventional feed was once a required problem for commercial poultry production (Bhatta and Sharma, 2001). The feed additives are a collection of nutrients and non-nutrients compounds that help to increase the competence of feed usage and thus reduce the high feed costs. In recent years, these additives have played an important role as feed supplements for many purposes in poultry production (Zhang et al., 2009).

Microalgae were identified as microscopic, unicellular, and photosynthetic organisms and can grow in saline and freshwater which provide a rich supply of nutrients and biologically active compounds, such as proteins, amino acids, polyunsaturated fatty acids, microelements, vitamins, antioxidants called carotenoids, which have long records of human application as a food (Belotti et al., 2014). Microalgae of exclusive species could be properly involved in poultry diets, which can affect advisably on birds' health, performance, and comfort of chickens' meat and egg (Abdelnour et al., 2019).

Microalgae were considered to be an adequate supply of a large number of metabolites that were suitable for animal feed. These metabolites included proteins,

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carbohydrates, fats, vitamins, and minerals (Andrade et al., 2018).

Currently, microalgae are receiving more attention in the market as nutraceuticals and fitness meals. Several microalgae, such as Spirulina, Chlorella, and *Amphora* are grown commercially for the production of algal products such as β-carotene, lutein, and phycocyanin (Hirata et al., 2000). *Amphora* is a major genus of diatoms of marine and freshwater origin (Parnell and Trevor, 2007).

Amphora coffeaeformis is one of the most common alkaline fresh ecosystems and brackish-water localities (Bhosleac et al., 1993). *Amphoracoffeaformis* has strong antioxidant activity against lipid peroxidation (Sugiharto et al., 2018), and is rich in polyunsaturated fatty acids (PUFAs), especially Docosahexaenoic (DHA), Eicosapentaenoic (EPA), and α-linolenic acid (Lee et al., 2009).

The microalga was a source of bioactive compounds including *Amphora coffeaeformis*, especially the carotenoids, sulfated polysaccharides, polyunsaturated fatty acids, α-tocopherol, especially β-glucans, in addition to vitamins C and E (El-Sayed et al., 2018)

Several studies on diatoms such as Amphora showed the possibility of their extract to use in both protective and antioxidant agents (Mekkawy et al., 2020), except for their antibacterial (Ayoub et al., 2019), antiviral (Abdel-Wahab, 2018), anti-inflammatory factors (Lauritano et al., 2016) and their dietary supplements (Selvaraj et al., 2013).

Amphora coffeaeformis was considered as a potent radical scavenger due to the presence of β-carotene and fucoxanthin, which are widely used as food additives in addition to the various nutraceutical applications such as pro-vitamin (Jaswir et al., 2011). The oral administration of *Amphora coffeaeformis* at three attentions (10, 20, and 30g / kg diet) in Nile tilapia (*O. niloticus*) diets led to enhance in growth performance, feed efficiency, and serum lysozyme (Ayoub et al., 2019).

Herbs are mixed into poultry diets to replace synthetic products and to stimulate or promote the efficient use of feed nutrients, which may subsequently result in faster body weight gain, higher production rates, and increased feed efficiency. In addition, active ingredients in herbs could improve digestion and stimulate the immune functions of broilers (Ghazalah and Ali, 2008; Shawky et al., 2020 a, b).

Star anise is a medium-sized evergreen tree that is native to southwest China and is also extensively cultivated in the subtropical and tropical regions of Asia (Benmalek et al., 2013; Elmasry et al., 2018). It has been shown that *star anise* promoted digestion, and has antioxidant, antibacterial, antiparasitic, antipyretic, and antifungal properties (Ertas et al., 2005; Mohammed, 2008).

However, anethole is an important compound in the *Star anise*, the other important compounds in the seeds include p-anisaldehyde, anise alcohol, acetophenone, pinene, and limonene. Seeds have excellent supplies of minerals such as calcium, iron, manganese, magnesium, zinc, potassium, and copper. This essential spice contains precise amounts of antioxidant nutritional vitamins such as vitamin C and vitamin A (Zhou et al., 2005). Shikimic acid, which is a component in *Star anise*, is one of the best components of the antiviral drug Tamiflu for combating avian influenza (Ohira et al., 2009; Borah, 2015). *Star anise is* pronounced to possess antioxidant residences (Chempakam and Balaji, 2008) as well as a tremendous anticancer (Shu et al., 2010).

MATERIALS AND METHODS

Ethical approval

The experimental design was approved by the Ethical Research Committee of the Faculty of Veterinary Medicine, the University of Sadat City, Egypt with approval number VUSC-014-2-18.

Experimental animals

The current study was conducted in the Department of Physiology, the University of Sadat City, Faculty of Veterinary Medicine, Sadat City, Egypt, on 270 Cobb broiler chickens that were one-day-old and had an average weight of 45.0 ±2.0 gram (Cobb strain) of the Misr-El-Arabia Company for Poultry.

Feed and water were supplied *ad libitum*, and synthetic lights were supplied 24 hours a day for the first 15 days to assist newly hatched chickens to commence drinking and eating. After two weeks, one hour of darkness was applied once a day. The chickens were reared at 33°C to 34°C for the first week, and then regularly decreased by 2-3°C per week until the temperature reached around 21 to 23°C, while the relative humidity was maintained around 55 to 65 percent. Chickens were reared in the poultry house, equipped with feeders, drinkers, and wood shaves used as bedding material. Strict sanitation practices were applied during some stages of the experiment. The chickens were reared under comparable environmental conditions. The experimental house was warm, dry, and free from drafts. Sparkling air provided some sort of ventilation to remove excess moisture and ammonia from the facility.

Nutrition

Chickens were feed starter rations that contained all of the necessary nutrients needed for maintenance, growth, and reproduction from day one to day 14. At the beginning of the third-week, chickens fed on the finisher ration (Table 1).

Table 1. The ingredients and chemical analysis of basal diet of broiler chickens

Ingredients and composition (%)	Starter	Finisher
Corn	55.59	61.07
Soybean meal	37.32	31.83
Soy oil	2.98	3.41
Lime stone	1.21	1.42
Dicalcium Phosphate	1.60	1.16
DL. Methionine	0.20	0.10
*Vitamin and Minerals	0.60	0.60
Sodium chloride	0.23	0.18
Sodium bicarbonate	0.27	0.23
Chemical Analysis (%)		
Metabolizable energy(ME) kcal/kg	2950	3050
Crude Protein (%)	21.20	19.16
Lysine (%)	1.14	1.01
Methionine (%)	0.50	0.39
Methionine and Cysteine (%)	1.03	0.84
Available Methionine + Cysteine (%)	0.85	0.71
Calcium (%)	0.93	0.90
Available Phosphate (%)	0.44	0.35

*Supplied per kilogram of diet: vitamin A, 1,500 IU; cholecalciferol, 200 IU; vitamin E, 10 IU; riboflavin, 3.5 mg; pantothenic acid10 mg; niacin, 30 mg; cobalamin, 10 µg; choline chloride, 1,000 mg; biotin, 0.15 mg; folic acid, 0.5 mg; thiamine 1.5 mg; pyridoxine 3.0 mg; iron, 80 mg; zinc, 40 mg; manganese, 60 mg; iodine, 0.18 mg; copper, 8 mg; selenium, 0.15 mg.

Preparation of *Amphora coffeaeformis* extract

Amphoracoffeaeformis extract was obtained from the Algae Production Unit (APU), National Research Institute (center), Cairo, Egypt. The extract was previously identified for its phytochemical constitute using liquid chromatography-mass spectrometry (Mekkawy et al., 2020).

Preparation of *Star anise*

The dried *Star anise* was purchased from a local market and was added to the diet after grinding into powder.

Animal grouping

From the seventh day of the experiment, the chickens were divided into three groups with three replicates of 30 birds each. The chickens were raised on the floor with given water and diet *ad libitum*. All chickens were vaccinated against Newcastle Disease (ND) and Infectious Bronchitis (IB) strains HIB120 at ninth days of age and against infectious bursal disease (Gumboro) at the 13thday of age. From day 16 to 35, no vaccination was given against any disease. Chickens were randomly divided into three experimental groups, including group 1 (control group), which chickens only received basic ration; group 2, which ration was mixed with *Amphora* at a dose of 1g / kg feed (Mekkawy et al., 2020), and group 3, in which the chickens received a ration mixed with *Star anise* at a dose of 2 g /kg feed (Alhajj et al., 2015; Ding et al., 2017).

Growth performance parameters

The growth performance parameters of broiler chickens have consisted of Body Weight (BW, gram), Body Weight Gain (BWG), Feed Intake (FI), and Feed Conversion Ratio (FCR). All chickens in groups had been weighed individually at the start and the end of the experiment. The body weight gain of chickens (expressed in grams) in each group was calculated by the difference between 2 successive weights (Nwanna, 2003).

Weight gain = (W2- W1)

Where W1 is the mean chicken weight at the beginning of the experiment and W2 is the mean chicken weight at the end of the experiment**.**

The experimental diets were offered regularly to each group. The feeds offered were measured daily, and at the end of the week, the weekly feed consumption was determined by the difference between the weight of feed offered and remained part. The FCR was calculated by dividing the amount of FI (g) during the entire experimental period by the total BWG (g) as outlined by Abd El-Wahed (1998).

FCR = total FI (g) of chicken / total BWG (g) of chicken.

Sample collection

On day 35, blood samples were taken from the wing vein of 10 chickens per group with anticoagulant (EDTA) to obtain plasma. The plasma was separated from the blood cells by centrifugation at 3.000 rounds per minute (rpm) for 30 minutes to determine haematological and biochemical parameters. Thereafter, chickens were slaughtered, and then the liver, and spleen were removed

to estimate the mRNA expression of GH, IGF-1, IFN-γ, IL - 12p35 according to Cinthia et al. (2013).

Determination of haematological parameters

Red Blood Cells (RBCs), White Blood Cells (WBCs), Haemoglobin (Hb) concentration, and Packed Cell Volume (PCV) were estimated using automated technical analyserDirui Bcc-3600. The Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), and Mean Corpuscular Haemoglobin Concentration (MCHC) were determined according to the method describing by Feldman et al. (2000). Differential leukocyte counts (heterophils, eosinophils, basophils, lymphocytes, and monocytes) were counted on blood smears stained with May-Grunwald-Giemsa (Tavares-Dias and Moraes, 2003). Phagocytosis of polymorph nuclear cells using *Candida Albicans* was performed in accordance with the method described by Sornplang et al. (2015).

Phagocytic Activity % = (Number of Heterophils ingesting candida ×100)/ Total number of Heterophils.

Phagocytic index = The total number of ingested candida / Number of active Heterophils.

Determination of biochemical parameters

The assay of total plasma proteins (g/dl) and albumin (g/dl) was carried out by a colorimetric method using commercial Diamond diagnostics kits according to the method described by Cannon et al. (1974) and Doumas et al. (1971), respectively.Serum alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) enzymes were determined colorimetrically using commercial kits of Bio diagnostics according to Reitman and Frankel, (1957). Creatinine (mg/dl) was determined by the colorimetric method using commercial kits of Biomed diagnostics according to Young (2001).Urea (mg/dl) was measured by the colorimetric method using commercial kits of Diamond diagnostics according to the method of Chaney et al. (1962). Protein electrophoresis profile was carried out by a Polyacrylamide Gel Electrophoresis according to Lewis et al. (2006).

Gene expression

Analysis of the mRNA expression of genes of Hepatic Growth Hormone and Insulin-like Growth Factor-1

The analysis of the mRNA of the expression of Growth Hormone (GH) and Insulin Growth Factor-1 (IGF-1) was performed at the Central Laboratory of the Faculty of Veterinary Medicine, Benha University, in Egypt. Liver samples were dissected from each group and then immediately frozen at -80°C. Total RNA was extracted from the frozen liver using the RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of RNA were determined using SPECTROstar Nanodrop. Single-stranded cDNA was synthesized from 1000 ng of total RNA according to the manufacturer's protocol for High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cycling conditions were 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. Total RNA and cDNA samples were then stored at -80° C until used. Expression of hepatic GH and IGF-1 genes was analyzed by real time-PCR using sense and antisense primers as previously described (Gasparino et al., 2014) by using the primers sets including GH sense (5 -′AAGGGATCCAAGCTCCTGAT-3′), and antisense (5 -′ATAACCACGTCCCTCAGTGC-3′); IGF-1 sense (5 -′ CACCTAAATCTGCACGCT-3′), and antisense (5 -′ CTTGTGGATGGCATGATCT-3′); and βactin as a housekeeping gene, sense (5ACCCCAAAGCCAACAGA-3′) and anti-sense (5 -′ CCAGAGTCCATCACAATACC-3′). PCR reactions for each gene were performed for each sample analyzed. Each PCR reaction consisted of 1.5 μ l of 1μg/μ l cDNA, 10 μ l SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen), and one μMof each forward and reverse primer for GH and IGF-1 genes while one μM forward and 1.5 μM reverse primer for βactingene and nuclease-free water to a final volume of 20 μl. The reactions were then analyzed on an Applied Biosystems 7500 Fast Real-time PCR Detection system under the conditions of 95°C for ten minutes (holding stage) and 40 cycles of 95°C for 15 seconds (denaturation stage) followed by 60°C for one minute (annealing and extension stage). Changes in gene expression were calculated from the obtained Cycle threshold (Ct) values, which were provided by real-time PCR instrumentation using the comparative Threshold cycle (TC) method for a reference (housekeeping) gene (βactin) (Gasparino et al., 2014).

Analysis of mRNA expression of splenic interferon-gamma and Interleukin 12 genes

Spleen samples were dissected from all groups and then immediately frozen at -80°C. Total RNA was extracted from the frozen spleen using the RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol. The quantity and quality of the RNA were determined by using Spectrostar nanodrop Single-stranded cDNA was synthesized from 1000 ng of total RNA according to the manufacturer's protocol for High Capacity cDNA Reverse

Transcription Kits (Applied Biosystems). Cycling conditions were 25°C for 10 minutes, 37°C for 120 minutes, and 85° C for 5 minutes. Afterward, total RNA and cDNA samples were stored at -80° C until use. Each PCR reaction consisted of 1.5 µl of one µg/µl cDNA, tenµl SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, Qiagen), one µM of each forward and reverse primer for INF-γ and IL-12p35 genes while one µM of forward and 1.5 µM reverse primer for βactin gene and nuclease-free water to a final volume of 20 µl. The Reactions were then analyzed on the Applied Biosystem 7500 Fast Real-time PCR Detection system under the conditions of 95°C for ten minutes (holding stage) and 40 cycles of 95°C for 15 seconds (denaturation stage) followed by 60°C for one minute (annealing and extension stage). Changes in gene expression were calculated from the obtained Cycle threshold (Ct) values provided by real-time PCR instrumentation using the comparative CT method for a reference (housekeeping) gene (βactin) (Gasparino et al., 2014).

Statistical analysis

The results were expressed as Mean ± Standard Error (SE). The relations between means in different groups were tested using a one-way analysis of variance (ANOVA). Duncan test was used for finding the significant differences in SPSS, 16 version. The P-values at 0.05 or lesser were considered significant.

RESULTS

The data in table 2 presented that the *Amphora* and *Star anise* supplementation resulted in a significant increase ($p < 0.05$) in the final BW, BWG, and also a significant decrease in the FCR compared to the control group ($p < 0.05$). The data in table 3 indicated that the Amphora and Star anise supplement did not significantly affect the erythrogram. The data in table 4 revealed that the *Amphora* and *Star anise* supplementation resulted in a significant increase ($p <0.05$) in total WBCs count, percentage of heterophils, H/L ratio, globulins (alpha 1 globulin, alpha 2 globulin, beta globulin, and gamma globulin), IgG, IgM, phagocytic activity, phagocytic index, while the total protein and albumin, lymphocyte percentage, and platelets count were not significantly affected. The data in table 5 revealed that the *Amphora* and *Star anise* supplementation did not significantly affect urea, creatinine, AST, and ALT ($p > 0.05$). The data in table 6 revealed that the *Amphora* and *Star anise* supplementation significantly increasedthe expression of hepatic GH and IGF1, and increased splenic INF-Gamma and interleukin12p35 compared to the control group ($p>0.05$).

Table 2. Effect of *Amphora* and *Star anise* supplementation on growth performances of broiler

Groups	Control group	*Amphora* group	*Star anise* group
Initial body weight (g)	192.83 ± 1.37a	195.08 ± 1.23a	194.01 ± 1.30a
Final body weight (g)	2054.9 ± 6.74c	2529.3 ± 10.20a	2297.8 ± 10.59b
Weight gain (g)	1824.60 ± 11.79^{c}	2349.90 ± 12.67^{a}	2195.90 ± 17.55^{b}
FCR	2.43 ± 0.03^{a}	1.62 ± 0.01^{c}	1.82 ± 0.02^{b}

In the same row, Mean ± Standard Error with different letters superscripts are significantly different at $P < 0.05$. FCR: Feed Conversion Rate.

Table 3. Effect of *Amphora* and *Star anise* supplementation on erythrogram of broiler

Groups	Control group	*Amphora* group	*Star anise* group
RBCs count (10^6/mm^3)	2.45 ± 0.08^{a}	2.60 ± 0.06^{a}	2.59 ± 0.07^{a}
PCV %	29.06 ± 0.61^{b}	30.94 ± 0.49^{a}	29.59 ± 0.62^{ab}
Hb (g/dl)	11.29 ± 0.20^{a}	11.68 ± 0.21^{a}	11.61 ± 0.17^{a}
MCV (μ^3)	111.50 ± 1.94^{a}	116.29 ± 1.62^{a}	116.40 ± 1.82^{a}
MCH (Pg)	38.94 ± 0.62^{a}	38.04 ± 0.85^{a}	37.86 ± 0.70^{a}
MCHC %	30.73 ± 0.27^{a}	31.45 ± 0.30^{a}	30.64 ± 0.39^{a}

In the same row, Mean±Standard Error with different letters superscripts are significantly different at $p<0.05$. RBCs: Red Blood Cells; PCV: Packed Cell Volume; MCV: Mean Corpuscular Volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration.

Table 4. Effect of *Amphora* and *Star anise* supplementation on immunity profile of broiler chicken

Groups	**Control group**	***Amphora* group**	***Star anise* group**
WBCs count ($10^3/mm^3$)	20.55 ± 0.17^c	32.99 ± 0.41^a	29.03 ± 0.42^b
Lymphocytes (%)	68.65 ± 0.74^a	67.80 ± 0.31^a	67.63 ± 0.32^a
Heterophils (%)	17.62 ± 0.34^c	27.01 ± 0.19^a	23.73 ± 0.32^b
H/L ratio	0.40 ± 0.003^a	0.35 ± 0.006^b	0.30 ± 0.007^c
Platelets count ($10^3/mm^3$)	310.25 ± 11.51^a	298.25 ± 9.20^a	310.85 ± 8.01^a
Total plasma protein (g/dl)	3.32 ± 0.03^a	3.47 ± 0.07^a	3.41 ± 0.06^a
Albumin (g/dl)	1.83 ± 0.03^a	1.67 ± 0.06^a	1.72 ± 0.06^a
Globulin (g/dl)	1.50 ± 0.02^c	1.80 ± 0.02^a	1.68 ± 0.02^b
Alpha 1 globulin (g/dl)	0.24 ± 0.003^c	0.34 ± 0.006^a	0.32 ± 0.008^b
Alpha 2 globulin (g/dl)	0.32 ± 0.004^a	0.32 ± 0.004^a	0.33 ± 0.005^a
Beta globulin (g/dl)	0.42 ± 0.004^c	0.48 ± 0.010^a	0.45 ± 0.004^b
Gamma globulin (g/dl)	0.51 ± 0.016^c	0.66 ± 0.009^a	0.60 ± 0.018^b
IgM (mg/dl)	50.02 ± 0.8^c	90.02 ± 0.5^a	80.47 ± 1.4^b
IgG(mg/dl)	20.95 ± 0.7^c	40.37 ± 0.5^a	40.14 ± 0.4^b
A/G ratio	1.22 ± 0.03^a	0.93 ± 0.04^b	1.03 ± 0.04^b
Phagocytic activity (%)	49.79 ± 0.72^c	67.73 ± 0.73^a	63.69 ± 0.46^b
Phagocytic index	1.93 ± 0.050^c	2.83 ± 0.040^a	2.67 ± 0.026^b

In the same row, Mean ±Standard Error with different letters superscripts are significantly different at $p < 0.05$.WBCs: White Blood Cells; IgM: Immunoglobuline M; IgG: Immunoglobuline G; A/G ratio: albumin / globulin ratio; H/L ratio: Heterophils/ Lymphocytes ratio.

Table 5. Effect of *Amphora* and *Star anise* supplementation on liver function tests and kidney function tests of broiler

Groups	**Control group**	***Amphora* group**	***Star anise* group**
ALT (U/L)	191.60 ± 4.35^a	183.30 ± 3.67^a	187.60 ± 2.97^a
AST(U/L)	36.60 ± 1.21^a	33.90 ± 1.10^a	33.30 ± 1.15^a
Urea (mg/dl)	9.29 ± 0.10^a	8.91 ± 0.13^a	9.35 ± 0.13^a
Creatinine(mg/dl)	0.45 ± 0.010^a	0.42 ± 0.005^a	0.44 ± 0.006^a

In the same row, Mean ± Standard Error with different letters superscripts are significantly different at $p < 0.05$.ALT: Serum alanine aminotransferase; AST: serum aspartate aminotransferase.

Table 6. Effect of *Amphora* and *Star anise*supplementation on mRNA expression of growth hormone, Insulin-like growth factor1, Interferon gamma, Interleukin12p35 of broiler chicken, data represented as fold change among different experimental groups

Groups	**Control group**	***Amphora* group**	***Star anise* group**
GH	1.27 ± 0.11^b	9.11 ± 0.13^a	8.25 ± 0.21^a
IGF1	1.05 ± 0.03^b	3.78 ± 0.08^a	2.73 ± 0.08^a
INF gamma	1.25 ± 0.03^b	7.60 ± 0.15^a	6.36 ± 0.06^a
IL - 12p35	0.90 ± 0.04^b	2.14 ± 0.06^a	1.81 ± 0.05^a

In the same row, mean ± Standard Error with different letters superscripts are significantly different at $P < 0.05$. GH: hepatic growth hormone; IGF-1: insulin-like growth factor-1; INF-γ: splenic interferon-gamma; IL-12p35: Interleukin12p35.

DISCUSSION

Poultry meat is the second largest food product in the world (Manning et al., 2007). In order to endure poultry production the meet global demand, antibiotic replacements were required (Mcdevitt et al., 2006). In the present study, chickens that were fed a diet supplemented with *Amphora coffeaeformis* and *Star Anise* significantly enhanced their growth performance compared to the control group. This result is attributed to the fact that *Amphora coffeaeformis* significantly improved appetite, which led to higher FI and improved growth (Ayoub et al.,

2019). These results were compatible with those reported by Zhao et al. (2004), who concluded that *Amphora*-treated chickens indicated an increased average daily gain and an improvement in the FCR due to the improvement in the digestibility of nutrients.

The improvement in the FCR could also be due to the fact that *Amphora coffeaeformis* contained several nutrients, especially vitamins and minerals that could aid in promoting growth (Belay et al., 1996). The positive effects of *Amphora* could be due to its bioactive compounds, which have antioxidant, anti-inflammatory, antiviral, and antibacterial effects (Rajput and Mishra, 2012; Salahuddin et al., 2017).

Star anise had a positive effect on the live BW, BWG, and FCR of broiler chickens (Al-hajj et al., 2015). The improvement in broiler performance could be due to the active component in *Star anise*, anethole, which could lead to better digestion by inducing the secretion of endogenous enzymes, better absorption, and improved microbial balance in the gut. Amad et al. (2011) stated that the inclusion of essential oils from thyme and *Star anise* resulted in an improvement of the digestibility of the crude protein, crude ash, crude fat, calcium, and phosphorus. This enhancement in the digestibility also led to an increased surface area for absorption in the intestine and improved nutrient absorption. Additionally, it was found that essential oils had a stimulatory effect on pancreatic enzymes (Rao et al., 2003).

The present study revealed no significant difference in blood parameters between the treatment groups the control group. The present results were in agreement with Soltan et al. (2008) and Mekkawy et al. (2020), who reported that there were no significant differences in blood profiles in groups that supplemented with *Star anise* seed and *Amphora*.

In the present study, the addition of *Amphora* and *Star anise* improved the immunity indices in broilers such as phagocytic activity, gamma globulins, IgM, and IgG. *Amphora coffeaeformis*activated the immune system of the chickens and became resistant to pathogenic bacteria, which was consistent with the finding of Mariey et al. (2012) and Jamil et al. (2015). This activation of the immune system could be due to *Amphora,* which was rich in different pigments and polyphenolic compounds, catechin, gallic acid, and p-coumaric acid (El-Sayed et al., 2018). Also, Jaswir et al. (2011) revealed that *Amphora coffeaeformis* was known to be a potent radical scavenger due to the presence of β-carotene and fucoxanthin, which were often used as food additives in addition to the various nutraceutical applications such as pro-vitamin A, antioxidant, anticancer, and anti-obesity.

Feeding chickens with *Star anise* resulted in a significant increase in the immunity indices of all groups. It might be due to the fact that *Star anise* modulates the immune system by stimulating various immune cells such as macrophages, monocytes, Natural Killer Cell (NKC) and effects on cytokines/chemokines in various *in vitro* and *in vivo* trials (Shahrajabian et al., 2019; Sung et al., 2012 a, b).

The results of *Amohora* which activated the immune system agreed with Kang et al. (2013), who found a significant increase in the lymphocyte counts in algae-fed broilers compared to other chickens. The phagocytic activity was also significantly increased in treated animals that were fed with algae and improved the immune response (An et al., 2010). Also, Khan et al. (2012) reported that the immunomodulatory effects of natural feed additives were linked to their ability to increase phagocytosis of potential macrophages, the production of interleukins, interferon-Y, and tumor necrosis factor, increase the secretory metabolism of macrophages, antigen-presenting cells, and antioxidant functions.

The present findings were in accordance with Abdelnour et al. (2019), who noted that IgG and IgM levels were increased in broilers that consumed food containing algae. Immunoglobulins increasing could be because several types of unicellular microalgae are excellent sources of immunoregulatory polysaccharides, such as β-glucan, βcarotene, and vitamin B12, which play a vital role in inflammatory and immune responses in animals and humans, which promote the macrophages activity and immune cells to increase the production of interferon- γ protein. Therefore, *Amphora* could stimulate the ability of the immune system to fight against pathogens and foreign proteins (Mason, 2001), which were in accordance with (Abdo and Zeinb, 2004), who was indicated that the herbal supplements could improve the immune response since globulin levels were used as an indicator of immune responses and a source of antibody production. Soltan et al. (2008) reported that *Star anise* supplementation in the broiler diet significantly increased lymphocyte counts compared to the control group, possibly be due to an aromatic plant like *Star anise* seeds, which contain many essential B-complex nutritional vitamins such as pyridoxine, niacin, riboflavin, thiamine, and magnesium, copper, potassium, manganese, zinc, and iron. In addition, *Star anise* seeds contained the amounts of antioxidant nutritional vitamins, such as vitamin C and vitamin A (Zhou et al., 2016). Herbal Supplements could

increase the immune response as globulin levels have been used as an indicator of immune responses and antibody sources (Abdo and Zeinb, 2004).

The immunity results were consistent with those of Rahmani and Speer (2005), who found a higher percentage of gamma globulins in broilers given herbal ingredients than the control ones. The present results revealed that there was no significant difference between ALT, AST, Urea, and Creatinine in the treated groups compared to the control group, which indicated that *Amphora* and *Star anise* appeared safe and did not have an adverse effect on physiological and nutritional status. Therefore, the utilization of algae in pharmaceutical applications has attracted world wild attention in recent years (Enwereuzoh and Onyeagoro, 2014). *Star anise* will contribute to the development of more phytotherapeutic products that are cheaper, safer, and affordable, and have a lower risk of resistance than conventional therapeutic drugs (Ritter et al., 2014; Sri et al., 2015).

In the current study, the mRNA expression of GH and IGF-1 was significantly increased in the treated chickens compared to the control chickens ($p < 0.05$). Growth hormones and IGF-1 are required to support normal growth (Scanes, 2009). Furthermore, animal growth is closely related to the mRNA expression levels, as intestinal nutrient transporters are required to circulate digestive products from digestion (McCracken and Edinger, 2013).

The results of the current study are compatible with those of Guobin et al. (2011), who reported that IGFs were important positive modulators of body and muscle growth in mammals and chickens.

In the current study, the splenic mRNA expression of IFN-γ was significantly increased in the treated chickens in comparison with the control chickens and the housekeeping gene. Interferon-γ regulated acquired immunity by stimulating lymphocytes and increasing the expression of class II antigens of Major Histocompatibility (MHC). In addition, IFN-γ is a common marker of cellular immunity, and high levels have been correlated with protective immune responses to parasitic infections (Lee et al., 2008). Interferon-gamma (INF) influences the immune system and inhibits tumor growth, and are involved in various immune interactions as inducers, regulators, and effectors of both innate and acquired immunity during the infections (Priyanka and Muralidharan, 2014).

The molecular results of the present study indicated an increase of INF gene transcription levels in the spleen of the treated chickens compared to the control chickens. Blinkova et al. (2001) reported that algae are important positive stimulators for the production of antibodies, cytokines, and also T and B cell activation. Moreover, the available molecular results from INF agreed with Hirahashi et al. (2002), who reported that algae enhanced Natural killer cell functions by INF- γ production and cytolysis. On the other hand, Mohammed AL (2014) stated a significant increase in the INF-γ concentration in the blood serum of both groups of mice treated with two concentrations of herbal additives (1 and 5 mg/kg BW) compared to the control group. Also, Kim et al. (2010) and Lillehoj et al. (2011) reported that supplementing one-day-old chickens diets with medicinal plants indicated higher rates of interleukin interferon (IFN-γ), encoding gene transcripts in comparison with the chickens given a standard diet.

In the current study, the mRNA expression of the IL-12 levels was significantly increased in the treated chickens compared to the control chickens. These results were in agreement with the results of Philbin et al. (2005), who reported that IL-12 differentiates T cells (Trinchieri, 1994), which is known as a factor that activates T cells, that could promote the T cells growth and function. The activation of T cells enhances IFN-γ development (Lesley et al., 2000). The present finding agreed with Ferdous et al. (2008) and Kirshenbaum et al. (2008), who found that adding the herbal additive to broilers' feed increased the expression of IL–12.

CONCLUSION

The supplementation of *Amphora coffeaeformis* and *Star anise* to the diet of broiler chickens for 35 days was sufficient to improve broiler performance by improving BW, BWG, and FCR and to modulate their immunity through increasing total WBC count, the phagocytic activity percentage, the phagocytosis index in plasma, IgM, and IgG, in serum. In addition,*Amphora* and *Star anise* significantly increased the mRNA expression of the hepatic growth hormone gene, the insulin-like growth factor-1 (IGF-1) genes (IGF1), and the mRNA expression of splenic interferon-gamma (INF-γ), and Interleukin12 (IL-12p35) genes.

DECLARATION

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Competing interests

The authors declared that they have no conflicts of interest.

Author's contribution

Sherif Mohamed Shawky, Said Ibrahim Fathalla, and Sahar Hassan Orabi have participated in the creation of the conception, design, and writing of the manuscript. Sherif Mohamed Shawky, Said Ibrahim Fathalla, Sahar Hassan Orabi, Huda Hassan El-Mosalhi, and Ibrahim Said Abu-Alya interpreted the data and reviewed the manuscript, while Huda Hassan El-Mosalhi and Ibrahim Said Abu-Alya managed and analyzed the data. Huda Hassan El-Mosalhi, Said Ibrahim Fathalla, and Ibrahim Said Abu-Alya carried out the sampling and management of the samples.

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