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Garlic and ginger extracts modulated broiler chicks innate immune responses and enhanced multidrug resistant *Escherichia coli* O78 clearance

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ABSTRACT

Administration of antibiotics as feed additives in broilers resulted in prompting of some undesirable effects such as the rising emergence of multi-drug resistant (MDR) bacteria, so scrutinizing for new alternatives like herbs is the up to date task for global health. This study was designed to determine the *in-vitro* antibacterial and *ex-vivo* immunomodulatory efficacy of garlic (*Allium sativum*) and ginger (*Zingiber officinale*) extracts post dietary supplementation for 900-one-day-old Sasso broiler chicks. The *in-vivo* protective actions of these extracts against avian pathogenic MDR *Escherichia coli* (*E. coli*) O78 challenge was evaluated after 21 days of extracts supplementation. Garlic extract exhibited broader antimicrobial spectra against MDR *E. coli* O78 and *S. aureus* isolates. Through the 21 days of garlic or ginger dietary supplementation, the chicks' innate immune response was modulated *via* various mechanisms including phagocytosis augmentation, bactericidal activity enhancement and nitric oxide (NO) production reduction, together with triggering the IL-1β, IL-6 and IFN-γ cytokines expression levels in comparison with the non-supplemented chicks. It is tempting to speculate that protection against pathogenic *E. coli* O78 challenge was high in chicks supplemented with each individual extract with severe reduction in the bacterial colony forming units in chicks' vital organs that confirm the extracts immunomodulatory activity and provide a mechanism(s) of their protective actions. Our data suggest promising useful insights to garlic and ginger dietary supplementation in broilers that may be safe for consumers from antibiotic toxic metabolites' residues and protective against the risk of infection with bacterial pathogens.

1. Introduction

Feed additives such as antibiotics are extensively used to improve growth performance in poultry. The extensive and continuous use of antibiotics may cause unfavorable side effects those cause risks to the environment in addition to the immunosuppression of the host. The damaging consequences of this intensification are the emergence of multi-drug resistant (MDR) bacteria, the presence of antibiotic residues in addition to the toxic metabolites remaining in meat byproducts [1]. Amongst the MDR bacteria, avian pathogenic *E. coli* (APEC) strains are common causes of large number of disease conditions in poultry. Colibacillosis is an important avian disease caused by *E. coli* strains, particularly of serogroup O78. It is one of the primary causes of morbidity, mortality and condemnation of broiler carcasses with extensive economic losses in the poultry industry worldwide and a consequent tremendous burden on human healthcare [2].

The continuous increasing of MDR bacteria coupled with the slow pace of newer antibiotics development are attracting the attention for providing effective new, safe and inexpensive control strategies. Recently, extensive researches have been initiated to determine the feasibility of using herbs as alternative feed additives to alleviate these problems. They involved in the modulation of host immune responses in addition the prevention of a number of infections caused by the pathogenic MDR bacteria [3].

Thousands of plant species have also been used *in-vitro* as new therapeutic drugs against a wide range of Gram positive and Gram negative bacteria [4]. Plants of the genus *Allium* are known for their production of many compounds those possess pharmacological properties. Garlic (*Allium sativum*) contains over than 100 phytotherapeutic compounds with a broad spectrum of promising antimicrobial efficacy against MDR microbes [4]. Some studies have been shown that garlic compounds are able to perform immunomodulatory effects such as modulation of cytokines and immune cells proliferation [5]. Another commonly used herbal product in traditional medicine all over the

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world is ginger (*Zingiber officinale*). It contains many natural organic materials and its main compounds have shown various pharmacological effects those augment the host resistance to infectious diseases by increasing its non-specific and specific immune mechanisms [6]. Furthermore, ginger contains several compounds such as gingerol, shogaols, gingerdiol, gingerdione, and some relating phenolic ketone derivatives those possess antioxidant activities.

Over years, studies evaluated the effects of supplementing feed with herbal products such as garlic and ginger compounds in poultry industry. Most of these studies had been carried out *in-vitro* and still need further confirmation *in-vivo* either in experimental models or in the targeted hosts. Therefore, the present study was undertaken to examine whether garlic or ginger extracts could exhibit *in-vitro* antibacterial activities and *ex-vivo* immunomodulatory actions on the innate immunity parameters such as phagocytosis, bactericidal activity, and nitric oxide (NO) production together with pro-inflammatory cytokines productions alteration, and whether these altered parameters could improve the bacterial clearance post *in-vivo* MDR *Escherichia coli* (*E. coli*) O78 challenge.

2. Materials and methods

2.1. Ethical approval

All animal experiments were conducted in accordance with the guidelines approved by the Institutional Animal Ethics Committee (IAEC) in Faculty of Veterinary Medicine, Zagazig University, Egypt.

2.2. Preparation of aqueous extracts

2.2.1. Garlic bulbs

Fresh garlic (*Allium Sativum L.*) bulbs were purchased from a local retail food store (Sharkia, Egypt). Garlic extract was prepared as described elsewhere [7].

2.2.2. Ginger powder

A commercially available ginger (*Zingiber officinale*) powder was purchased from Mepaco-Medifood Co. (Inshas, Sharkia, Egypt). The ginger aqueous extract was prepared using cold extraction techniques [8]. Both garlic and ginger extracts were further diluted to 32% (w/v) concentration by mixing with appropriate volumes of sterile distilled water.

2.3. Microbial isolates used

Two clinical MDR and pathogenic avian bacterial isolates [*E. coli* O78 and *Staphylococcus aureus* (*S. aureus*)] and one yeast isolate, *Candida albicans* (*C. albicans*) were used in this study. The *E. coli* strain was isolated from the visceral organs of broilers that had died from colibacillosis in a previous publication of one of the co-authors [9]. It was evidenced to be *E. coli* of serotype O78:K80 that was resistant to amoxicillin-clavulanic acid, ciprofloxacin, sulfamethoxazole-trimethoprim, ceftriaxone, streptomycin, tetracycline, doxycycline, chloramphenicol, erythromycin, colistin, rifampicin and gentamycin. Moreover, it was proved to harbor *iroN*, *ompA*, *iss* and *papC* virulence genes and *tetB*, *blaTEM*, *qnrS*, *sulI* and *aadA1* antibiotic resistance genes. *Staphylococcus aureus* strain was kindly supplied from Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University. It was isolated from diseased chickens and it was evidenced to be methicillin resistant *Staphylococcus aureus* (MRSA) being resistant to both oxacillin and cefoxitin. Moreover, it was resistant to ciprofloxacin, gentamycin, sulfamethoxazole-trimethoprim, tetracycline, ceftriaxone and erythromycin. It belonged to SCC*mec* type IVa and *agr* type I and it was proved to have *icaA* and *icaD* virulence genes and *tet*, *erm* and *mecA* antibiotic resistance genes. The serotyping and resistance pattern for *E. coli* strain in addition to its possession of both virulence and resistance genes were all used as re-isolation confirmatory markers. Both *E. coli* O78 and *S. aureus* were used for determining garlic and ginger antimicrobial activities and *E. coli* O78 was used for measuring the immune cells bactericidal activities and in the experimental challenge test. Meanwhile, *C. albicans* was used as a microbial model in phagocytosis assay.

2.4. Antibacterial activities of garlic and ginger extracts

The antibacterial activities of tested extracts were assessed against the bacterial isolates by agar well diffusion [7] and minimal inhibitory concentration (MIC) [10] methods. The inhibition zones were determined as clear areas around the extracts containing wells and they were measured as diameters with subtracted well size (5 mm diameter). The MIC values determined using a broth microdilution technique against *E. coli* and *S. aureus*. The lower the MIC values the most potent the extract [10]. Each experiment was performed in triplicate.

2.5. Experimental chicks and management

The experiments were carried out on a total of 900-one-day-old broiler chicks (Sasso breed). The chicks were acclimatized for a period of one week under standard husbandry conditions prior to the onset of the experiment. Ambient temperature, 29 ± 4 °C; relative humidity, 65 ± 10%; 24 h lighting duration; adequate ventilation and other environmental conditions fully met the requirements laid down for broilers breeding. Feed and water were provided *ad libitum* under hygienic conditions. After acclimatization, the chicks were randomly allotted into 3 groups in a completely randomized design. Chicks in the first group were kept as a control; they were given feed without any extract supplementation. The chicks in the second group were provided with an aqueous garlic extract at concentrations of 10–15% [11], and chicks in the third group were received ginger extract at a concentration of 15 g/kg diet daily for 21 days [12].

2.6. Experimental parameters of the extracts immunomodulatory activities

Heparinized (10 U/mL) blood samples from individual birds were collected from chicks' wing veins and pooled on a group basis directly prior to the extracts feeding (0 time), at 24 h, and then successively at the 7th, 14th and 21st day post garlic or ginger extracts feeding. The pooled blood samples (15 mL) were kept in sterilized tubes and used directly for peripheral blood mononuclear cells (PMNCs) separation [13]. The *ex-vivo* immunomodulatory activities of the extracts were estimated by evaluating the PMNCs phagocytic capacity to engulf 15 *C. albicans* yeast particles or more and *E. coli* O78 bactericidal activities following established methods detailed previously [13]. Moreover, NO production was evaluated in the PMNCs culture supernatants at the same mentioned intervals using Griess reagent (Sigma, St. Louis, MO, USA) following the procedure described earlier [13]. Finally, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was done to determine the *in-vivo* modulation of cytokines (IL-1β, IL-6 and IFN-γ) production. Spleen samples were collected from randomly selected sacrificed chicks of control and extracts fed groups at 12, 24, 48 and 72 h intervals post feeding (n = 6 per each interval). The relative qRT-PCR was performed in triplicate on Applied Biosystems 7500 Fast Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) using the Qiagen QuantiTect Probe RT-PCR kit (Qiagen, Inc., Valencia, CA) according to the supplier`s recommendations. Primers and probes for cytokines and 28S RNA-specific amplification have been described previously [14]. The relative expression of the target genes was calculated by a means of the comparative $2^{-\Delta\Delta Ct}$ method [15].

2.7. Challenge experiment

After feeding for 21 days, the birds in the 3 groups were orally

gavaged with 0.2 mL of MDR and pathogenic *E. coli* O78 culture suspension containing 10^9 bacteria/mL and the birds were kept under observation. Clinical symptoms and mortalities in each tested group were reported. Heart, liver and spleen tissues were directly harvested from freshly dead birds and from euthanized birds (n = 25/group) after 7 days post challenge. The specimens were subjected to the re-isolation and identification of the challenging *E. coli* strain using traditional and serological methods in addition to estimation of the number of *E. coli* colony-forming units (CFU)/g tissue. Moreover, re-testing of the antimicrobial susceptibility pattern and the presence of the studied virulence and antibiotic resistance genes for *E. coli* strain was done to ensure that the recovered strain corresponded to the administered one.

2.8. Statistical analysis

The data expressed as mean ± standard deviation was analyzed by one-way analysis of variance (ANOVA), followed by LSD post-hoc tests for pair-wise comparisons and using the GLM procedure of the IBM SPSS statistical software version 16 to compare among different groups.

3. Results

3.1. Antibacterial activities of garlic and ginger extracts

The antibacterial efficacy and bacterial growth inhibition of garlic and ginger extracts against clinical MDR *E. coli* O78 and *S. aureus* strains are depicted in Table (1). Both extracts showed concentration dependent antibacterial activities against both tested bacterial isolates. Garlic extract expressed broad spectra of antibacterial activities against both tested MDR *S. aureus* and *E. coli*. The sensitivity to garlic extract measured by zone diameters was maximum and identical for *S. aureus* (38 to 16 mm), followed by *E. coli* (28 to 13 mm). Further analysis of the garlic antibacterial activity by broth microdilution assay revealed more effective inhibition of *S. aureus* growth than *E. coli* with MIC values of 1.25 and 10 mg/mL, respectively.

Ginger extract demonstrated considerable antibacterial activities against both tested bacterial isolates. Both pathogenic *S. aureus* and *E. coli* isolates were sensitive only to 16 and 32% concentrations of ginger extract with inhibition zone diameters up to 24 and 22 mm, respectively. Moreover, the acceptable antibacterial activity of ginger extract was expressed by MIC values up to 20 mg/mL.

3.2. Ex-vivo assessment of garlic and ginger immunomodulatory activities

3.2.1. Enhancement of phagocytic capacity

Inclusion of garlic or ginger extract as dietary supplements for 21 consecutive days has positively enhanced the *ex-vivo* PMNCs phagocytic activity that was indicated by the significant increase in phagocytic cells capacity to engulf *C. albicans* yeast particles in the supplemented chicks in comparison to the non-supplemented control chicks (Table 2). More than 90% of the phagocytic cells were actively engulfing yeast particle in the tested groups at the determined intervals. A marginal increase in the cells phagocytic capacity to uptake > 15 yeast per phagocyte was observed in the extract receiving birds and this increase was feed time-dependent. A dramatic increase in this phagocytic capacity was observed starting from the 7th day post dietary supplementation, where more than 90 and 70% of garlic and ginger fed birds' phagocytes respectively actively engulfed > 15 yeast particles with respect to more than 30% in the control non-supplemented chicks.

Table 1
Antibacterial activities of various garlic and ginger extracts` concentrations against *E. coli* O78 and *S. aureus* pathogenic isolates by agar well diffusion method.

Plant extract	Concentration (%)	Means of clear inhibition zone diameters (mm)	
		*Escherichia coli*O78	*Staphylococcus aureus*
Garlic	**2**	$13^{h} \pm 2.6$	$16^{gh} \pm 2.6$
	4	$16^{gh} \pm 2.0$	$22^{def} \pm 1.7$
	8	$19^{efg} \pm 7.2$	$27^{bcd} \pm 1.7$
	16	$23^{cdef} \pm 3.6$	$29^{b} \pm 2.6$
	32	$28^{bc} \pm 2.0$	$38^{a} \pm 2.6$
Ginger	**16**	$18^{fgh} \pm 2.6$	$19^{efg} \pm 5.6$
	32	$22^{def} \pm 3.6$	$24^{bcde} \pm 1.0$

Diameter of inhibition zone included well diameter of 5 mm. Means with different superscripts are significant ($p < 0.05$).

3.2.2. Augmentation of bactericidal activity

The PMNCs bactericidal efficacy along the different intervals of the study is depicted in Table 2. The *ex-vivo* cells bactericidal activity against *E. coli* O78 pathogen was prompted in the dietary supplemented birds in a time-dependent manner post supplementation compared to the control birds. The PMNCs bactericidal activity significantly increased starting from the 7th day of extracts supplementation and continued till the 21ststday with an average killing capacity of 97 and 95.8% in garlic and ginger fed birds, respectively in comparison with 61.4% in control birds .

3.2.3. Reduction of nitric oxide (NO) production

The impact of garlic and ginger on broiler chicks NO production was analyzed in the clarified supernatants of PMNCs culture directly prior and post garlic and ginger supplementation. The quantitative changes of NO concentrations in the PMNCs culture supernatants revealed that both extracts were able to significantly reduce NO production when compared to the control group. This reduction in NO levels started from the 7th day and continued till the 21st day of the extracts feeding. The lowest detectable levels in NO concentrations were observed at the 7th day post feeding with a concentration of 12.5 and 14.2 µmol/mL in garlic and ginger fed birds, respectively (Table 2).

3.2.4. Modulation of cytokines expression

The impact of garlic or ginger on broilers` IL-1β, IL-6 and IFN-γ cytokines mRNA expression levels post feeding was measured by qRT-PCR with 28S gene expression serving as a reference gene. Data analysis of qRT-PCR using the $2^{-\Delta\Delta Ct}$ method revealed significant changes in the cytokines expression levels in the extracts supplemented groups. As shown in Fig. 1, garlic extract induced higher levels of all cytokine genes` expressions than those induced by ginger extract at majority of the investigated time points. The cytokine genes` expressions were fluctuated during all time points, with notable peaks at 24 h post feeding the garlic (3.6–11.7-fold) and ginger (2.5 - 6-fold) extracts comparing with the control group. Over expressions of only IL-1β and IFN-γ genes (2- fold) were noticed at 12 h post feeding the garlic and ginger extracts, respectively. At 48 h, garlic extract decreased IL-6 gene expression (0.5- fold), but ginger extract decreased all cytokine genes` expressions (0.3 to 0.6-fold). For both extracts, the pattern of IL-1β gene expression was nearly similar to that of IFN-γ at the time interval of 72 h (1.3 to 1.5-fold).

3.3. Interpretation of challenge test

To carefully test whether the altered parameters of the innate immunity post extracts feeding could augment the *in-vivo* protection, the protection percentage following the induced infection using MDR APEC O78 at the end of the 21st day of consecutive dietary supplementation was reported. Inspection of the internal organs in the extract fed chickens showed no gross pathological changes at least one week post *E. coli* gavaging. The protection percentages against *E. coli* O78 infection were high in garlic and ginger-supplemented birds. No *E. coli* growth or colonization was observed in the garlic received birds' vital organs, but less than 3 log CFU of *E. coli* population was observed in the heart and spleen of birds receiving ginger extract. In contrary, high

Table 2
Effects of dietary supplementation of garlic and ginger extracts on the immunological parameters of broiler chicks at different intervals.

Time course	Phagocytic capacity % (> 15 yeast per cell)			Bactericidal activity (%)			Nitric oxide production (μmol/ mL)		
	G1	G2	G3	G1	G2	G3	G1	G2	G3
0 time	$31.2^{h} \pm 1.9$	$31.3^{h} \pm 2.4$	$31.0^{h} \pm 1.8$	$45.2^{f} \pm 5.8$	$45.4^{f} \pm 5.7$	$45.5^{f} \pm 4.0$	$30.9^{cde} \pm 2.2$	$30.3^{cde} \pm 2.6$	$30.1^{cd} \pm 3.1$
24 hours	$33.3^{gh} \pm 3.4$	$63.3^{d} \pm 3.4$	$55.6^{e} \pm 4.4$	$50.1^{f} \pm 4.9$	$52.8^{ef} \pm 5.7$	$51.1^{f} \pm 6.3$	$32.2^{cd} \pm 2.9$	$30.8^{cde} \pm 2.7$	$30.4^{cde} \pm 3.1$
Day 7	$34.4^{fgh} \pm 4.2$	$95.9^{a} \pm 1.7$	$75.7^{c} \pm 4.4$	$50.3^{f} \pm 6.4$	$86.2^{b} \pm 5.3$	$77.7^{c} \pm 5.4$	$34.8^{c} \pm 3.3$	$12.5^{f} \pm 2.7$	$14.2^{f} \pm 3.2$
Day 14	$36.7^{fg} \pm 3.5$	$95.8^{a} \pm 3.0$	$82.9^{b} \pm 3.4$	$59.8^{de} \pm 3.6$	$95.5^{a} \pm 3.2$	$91.6^{ab} \pm 2.9$	$73.6^{a} \pm 5.9$	$34.6^{c} \pm 2.9$	$35.3^{c} \pm 3.4$
Day 21	$38.8^{f} \pm 3.0$	$97.6^{a} \pm 0.6$	$84.8^{b} \pm 4.5$	$61.4^{d} \pm 4.6$	$97.0^{a} \pm 1.6$	$95.8^{a} \pm 1.9$	$60.5^{b} \pm 2.9$	$25.6^{e} \pm 2.9$	$28.7^{de} \pm 3.2$

G1: control non treated group, G2: garlic treated group, G3: ginger treated group. Means with different superscripts are significant ($p < 0.05$).

mortality rates and severe congestions accompanied with high CFU counts of *E. coli* were detected in the control non-supplemented birds' vital organs; 3.95, 3.41 and 3.39 log CFU/g of heart, liver and spleen samples, respectively (Table 3). The *E. coli* strain isolated from the examined vital organs of chickens in the three tested groups had the same antimicrobial susceptibility pattern and virulence and antibiotic resistance genes of the administered *E. coli* isolate used in the challenge experiment.

Table 3
E. coli O78 count levels (CFU/g tissue) and protection percentages following a challenge infection at the end of the 21st day in control and experimental groups fed on garlic or ginger extracts.

Group	Mean ± SD of *E. coli* O78 **log** count levels (CFU/g tissue) after a challenge infection			Protection percentage
	Heart	Liver	Spleen	
G1	$3.95^{a} \pm 0.04$	$3.41^{b} \pm 0.29$	$3.39^{b} \pm 0.33$	$40.0^{A} \pm 8.2$
G2	–	–	–	$95.8^{B} \pm 4.6$
G3	Less than 3	–	$2.89^{c} \pm 0.09$	$90.0^{B} \pm 6.4$

G1: control non treated group, G2: garlic treated group, G3: ginger treated group, CFU: colony forming unit. Means with different small superscripts are significant ($p < 0.05$).

4. Discussion

Herbal plants clearly exhibited *in-vitro* potent immunomodulatory activities with a rare use of animal subjects in the research as reported previously [5,16]. However, the precise *in-vivo* and *ex-vivo* mode of actions of herbal plants is not fully elucidated yet. Hence, our attempt here was to look more closely at the herbal plants' mechanisms of action from the *ex*-and *in-vivo* immunomodulation points of view in a medium-scale experimental poultry model. Previous researches suggested that a pharmacological strategy directed towards multiple targets could result in more efficient therapeutic outcomes [17]. Moreover, the use of whole plants instead of isolated chemicals may offer a safer clinical strategy in the treatment of many diseases [3].

To find safe alternative antimicrobials to fight the continuing spread of MDR bacterial strains in poultry, garlic and/or ginger antibacterial efficacy against MDR *E. coli* and *S. aureus* as common poultry pathogens was investigated. Both extracts showed promising *in-vitro* antibacterial activities against these bacteria in a dose-dependent manner and this was in a link with several studies conducted previously [5]. Another study carried out in Malaysia confirmed that garlic has definite broad-spectrum antibacterial properties and is effective against a wide spectrum of microbes as compared with antibiotics [18]. The garlic *in-vitro* potent antibacterial activity documented here may be linked to the presence of bioactive phenolic and non-phenolic compounds such as alliin, allicin and allyl isothiocyanate [18]. Previous evidence [19] supported our observed antibacterial dose-dependent findings of garlic extract. Like the results of the present study, the antimicrobial activity of the ginger extract has been reported against *S. aureus* [20]. Ginger extract as reported previously [21] significantly reduced the counts of *S. aureus* and *E. coli* when it was applied to chicken meat as microbial decontaminants. The antimicrobial activity of ginger may be attributed to its main active fractions; gingerol, gingerdiol, and gingerdione [20,21]. Garlic and ginger antimicrobial activities were suggested to act through alteration of cell wall permeability, thereby permitting the loss of macromolecules from the cell interior. The extracts bioactive compounds may also act through interference with membrane functions and interaction with membrane proteins [19].

The defensive functions of phagocytic cells are required for immediate control of pathogens and prevention of progressive diseases` consequences resulted from overcoming phagocytosis defense line. As reported in Table (2), phagocytic cells capacity to uptake yeast particles significantly enhanced in the extracts supplemented birds, and it started earlier post feeding and continued along the experiments indicating the immunostimulatory activities of the extracts. Earlier studies had reported that garlic [22] and ginger [23] administration as feed additives in poultry enhanced phagocytic cells activity to *C. ablicans*. The exact mechanism by which garlic and ginger interacts with the phagocytic cells is not yet known. We believe that these extracts may act through up-regulation of a diverse array of identifying molecular patterns required for *C. albicans* recognition. Other scenarios including enhancement of cytokines regulating phagocytic cells cooperation, cytoskeleton reorganization, stimulating the secretion of opsonizing immunoglobulin, or activation of lymphocytes that direct phagocytes'

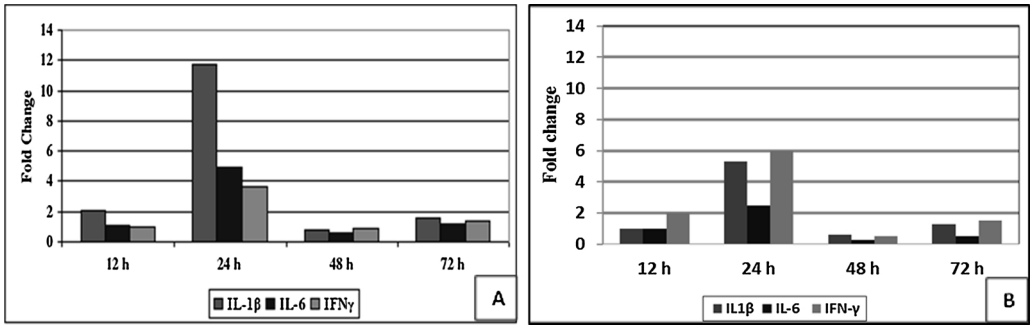

Fig. 1. Cytokine genes` expressions in the spleen of chickens at 12, 24, 48 and 72 h post feeding of broiler chickens with garlic (A) and ginger (B) extracts.

actions could not excluded.

As a study preliminary outcome, bactericidal efficacy of *ex-vivo* boosting of cells was significantly observed at the 7th days post supplementation and continued progressively indicating the importance of using such extracts as infection limiting natural additives. Bioactive compound(s) of such extracts have been reported to interact with innate lymphocytes and natural killer cells [24], activation of macrophages and B cells as major antigen presenting cells, increasing immunoglobulin production, or through regulation of cytokines secretion as detailed previously [5] and as reported in this study. Previously, ginger extract triggered TNF-α cytokines production and lymphocytes proliferation [25] that enhance bactericidal activity. As reviewed recently [16], activation of lysozyme and nitric oxide modulation as bactericidal enhancing elements cannot be excluded.

Data in the current work reported that NO levels were significantly reduced in birds receiving the extracts at the 7th day to the 21st day post supplementation. The NO down-regulation after administration of garlic and ginger additives may reduce the pathological harms arising due to the excess in its production [26]. The underlined mechanisms behind NO reduction in the supplemented poultry may be due to a decreased or absence of nitric oxide synthase or a limited availability of L-arginine, as well as increased degradation of NO by reaction with oxidized serum low density lipoproteins or superoxide anions [27].

This work represents the first systematic study of the simultaneous changes in pro-inflammatory cytokines` expressions following dietary inclusion of garlic and ginger extracts. Although many studies have focused on the induction of cytokines mRNA expressions by herb components *in-vitro* using conventional techniques such as semi-quantitative RT-PCR and ELISA [28], we think that our study is the first report, albeit limited, which investigated the *ex-vivo* activity of garlic and/or ginger extracts on broilers` mRNA expression of IL-1β, IL-6 and IFN-γ cytokines at the molecular level using qRT-PCR technique. The obtained data indicated that both extracts up-regulated the mRNA expressions of the investigated cytokines. These results suggest that these herbal feed additives confer modulating activities that could stimulate the broilers' immune system. Garlic extract had been proven previously to modulate the metabolism and function of macrophages and consequently stimulated the release of pro-inflammatory cytokines such as IL-1β and IL-6 and IFN-γ [29]. Similarly to garlic, but to a lesser extent, ginger extract exerted an up-regulatory activity on pro-inflammatory cytokine genes` expressions that was consistent with those from another study conducted in Japan reporting enhancement of mRNA expressions of IL-1β and IFN-γ cytokines [28]. This lends a distinct support to the significant immunomodulatory activity of ginger extract. In contrary, other reports had conflicting results on garlic and ginger effects on the same tested pro-inflammatory cytokines. In Australia, IL-1, IL-6 and IFN-γ cytokines decreased significantly in the presence of garlic extract [30]. In Iran, significant reductions in IL-1β and IL-6 concentrations post ginger administration were reported [31]. Dissimilar immunomodulatory cytokines results of both tested extracts with the reported data might be attributed to their nature, diverse compositions, extraction and purification methods, concentrations and/or if the whole extract or a specific derived compound(s) were used.

To overcome the drawback of the the herbal extracts` *in-vitro* studied activities, *in-vivo* extract protective activity testing was performed using APEC O78 that is known as a major cause of colibacillosis and chronic respiratory disease complex in birds resulting in major economic losses due to high mortality and decrease in productivity [32]. Herein, APEC O78 infection was induced experimentally post feeding for 21 consecutive days. Unexpectedly, the extracts supplemented birds resisted the induced infection with limited mortality rates those did not exceed 10% compared to 60% in the control non-supplemented birds. Moreover, severe reduction in *E. coli* counts was also noticed in the chicks' vital organs in ginger receiving, but not in garlic supplemented birds. Therefore, we strongly suggest that the bioactive phenolic and non-phenolic compounds in garlic [19] and active fractions in ginger [20] are major agents supporting the phagocytic capacities, the enhanced cells bactericidal activity, the reduced NO levels and the triggered pleiotropic cytokines quantities; those could all induce this prominent protection against *E. coli* induced infection. The bioactive compounds of garlic and ginger should be purified and tested to determine the exact component(s) responsible for immunomodulation and protection. Understanding of the mechanisms through which phytochemicals interact with the immune system and influence its different components is necessary to appreciate the use of herbal plants as immunostimulators in veterinary medicinal products.

5. Conclusion

From the data obtained in this study, garlic or ginger extract has shown dose-dependent *in-vitro* antibacterial activities. Additionally, using garlic or ginger as dietary supplementation for at least three weeks enhanced the *in-vivo* clearance of APEC O78 that could be discussed through the different *ex-vivo* innate immunomodulatory scenarios reported herein post supplementation such as promotion of phagocytosis, enhancement of immune cells microbicidal activity, triggering the cytokines production levels or through maintaining the birds more healthier *via* reduction of NO secretion. So, we recommend the use of garlic and ginger as natural feed additives not only to enhance immune response, but also to replace the antibiotics in broilers chickens. However, there will be still a need to establish dosages and standards for using these natural products friendly as feed additives in poultry.

Authors' contributions

G.A.E. and M.I.A contributed equally in the conception and design of the study, formulation of overarching research goals and aims, acquisition of data, analysis and interpretation of the results, writing the paper and revising it critically for important intellectual contents. A.M.A. and A.M.A. contributed in the design of the study and the creation of experimental models. M.A. and G.A.E. helped in provision of the study materials, reagents, laboratory samples, animals and instrumentation and the application of the experiment. All the authors have approved the final article version to be submitted.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial, financial relationships, or any scientific conflict that could be construed a potential conflict of interest.

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References

[1] S.R. Shakya, Medicinal uses of ginger (*Zingiber officinale* Roscoe) improves growth and enhances immunity in aquaculture, Int. J. Chem. Stud. 3 (2015) 83–87.
[2] C. Ewers, T. Janssen, L.H. Wieler, Avian pathogenic *Escherichia coli* (APEC), Berl. Munch. Tierarztl. Wochenschr. 116 (2003) 381–395.
[3] G. Alice, Recent contributions to development of herbal-based immunomodulators for farm animals, J. Cell. Immunol. Serum Biol. 3 (2016) 68–77.
[4] R. Arreola, S. Quintero-Fabián, R.I. López-Roa, E.O. Flores-Gutiérrez, J.P. Reyes-Grajeda, L. Carrera-Quintanar, D. Ortuño-Sahagún, Immunomodulation and anti-inflammatory effects of garlic compounds, J. Immunol. Res. 2015 (2015) 1–13, https://doi.org/10.1155/2015/401630.
[5] G. Li, X. Ma, L. Deng, X. Zhao, Y. Wei, Z. Gao, J. Jia, J. Xu, C. Sun, Fresh garlic extract enhances the antimicrobial activities of antibiotics on resistant strains in vitro, Jundishapur J. Microbiol. (2015) 8, https://doi.org/10.5812/JJM.14814.
[6] D. Bauer, E. Mazzio, K.F. Soliman, E. Taka, E. Oriaku, T. Womble, S. Darling-Reed, Diallyl disulfide inhibits TNFá-induced CCL2 release by MDA-MB-231 cells,

Anticancer Res. 34 (2014) 2763–2770.
[7] S. Yadav, N.A. Trivedi, J.D. Bhatt, Antimicrobial activity of fresh garlic juice: an *in vitro* study, AYU 36 (2015) 203–207, https://doi.org/10.4103/0974-8520.175548.
[8] W.M. Ali, N. Othman, M. Tawfiq, R.T. Sulieman, Evaluation of antibacterial effects of ginger extract when used as one component of the root canal sealers; (An *in vitro* study), Tikrit Journal of Pharmaceutical Sciences. 8 (2012) 303–311.
[9] A.M. Ammar, Marwa I. Abd El-Hamid, Samah E.A. Eid, Amal S. El Oksh, Insights into antimicrobial resistance and virulence genes of emergent multidrug resistant avian pathogenic *Escherichia coli* in Egypt: how closely related are they? Rev. Mã©d Vã©t 166 (2015) 304–314.
[10] Ishaku Leo Elisha, Francien S. Botha, Lyndy Joy McGaw, Jacobus Nicolaas Eloff, The antibacterial activity of extracts of nine plant species with good activity against Escherichia coli against five other bacteria and cytotoxicity of extracts, BMC Complement. Altern. Med. 17 (2017) 133, https://doi.org/10.1186/s12906-017-1645-z.
[11] M. Sakthi Priya, T.R. Gopala Krishna Murthy, T. Vijayanand, Optimization of level of feeding herbal preparation in reducing faecal bacterial load in layer chicken, J. Pharmacogn. Phytochem. 6 (2017) 98–104.
[12] A. Akbarian, A. Golian, A. Sheikh Ahmadi, H. Moravej, Effects of ginger root (*Zingiber officinale*) on egg yolk cholesterol, antioxidant status and performance of laying hens, J. Appl. Anim. Res. 39 (2011) 19–21, https://doi.org/10.1080/09712119.2011.558612.
[13] G.A. Elmowalid, A Simple method for generation of functional sheep peripheral blood monocytes-derived-macrophages in vitro, Glob. Vet. 9 (2012) 590–599, https://doi.org/10.5829/idosi.gv.2012.9.5.6650.
[14] P. Kaiser, P. Wigley, J. Burnside, P.A. Barrow, E.E. Galyov, L. Rothwell, Differential cytokine expression in avian cells in response to invasion by *Salmonella* typhimurium, *Salmonella* enteritidis and *Salmonella* gallinarum, Microbiology 146 (2000) 3217–3226, https://doi.org/10.1099/00221287-146-12-3217.
[15] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2-^{\Delta\Delta CT}$ Method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[16] S.A. Ragland, A.K. Criss, From bacterial killing to immune modulation: recent insights into the functions of lysozyme, PLoS Pathog. 13 (2017) e1006512, , https://doi.org/10.1371/journal.ppat.1006512.
[17] P. Csermely, V. Agoston, S. Pongor, The efficiency of multi-target drugs: the network approach might help drug design, Trends Pharmacol. Sci. 26 (2005) 178–182, https://doi.org/10.1016/j.tips.2005.02.007.
[18] C.F. Low, P.P. Chong, P.V.C. Yong, C.S.Y. Lim, Z. Ahmad, F. Othman, Inhibition of hyphae formation and *SIR2* expression in *Candida albicans* treated with fresh *Allium sativum* (garlic) extract, J. Appl. Microbiol. 105 (2008) 2169–2177, https://doi.org/10.1111/j.1365-2672.2008.03912.x.
[19] R.R. Hafidh, Inhibition of growth of highly resistant bacterial and fungal pathogens by a natural product, Open Microbiol. J. 5 (2011) 96–106, https://doi.org/10.2174/1874285801105010096.
[20] M. Abdel-Raouf, M. Nabil, M. El-Sayed, Antimicrobial activities of some herbs extracts on food borne bacteria, J. Am. Sci. 10 (2014) 76–85.
[21] S. Sudarshan, N. Fairoze, S.W. Ruban, S.R. Badhe, B.V. Raghunath, Effect of aqueous extract and essential oils of ginger and garlic as decontaminat in chicken meat, Res. J. Poult. Sci. 3 (2010) 58–61.
[22] N.S. Saleh, A.S. Abd El-Latif, T.S. Allam, E.W. Ghazy, The Effects of rosemary (*Rosemarinus afficinalis*) and Garlic (*Allium sativum*) essential oils on performance, hematological, biochemical and immunological parameters of broiler chickens, Br. J. Poult. Sci. 2 (2013) 16–24, https://doi.org/10.5829/idosi.bjps.2013.2.2.74145.
[23] N. Saleh, T. Allam, A.A. El-latif, E. Ghazy, The effects of dietary supplementation of different levels of thyme (*Thymus vulgaris*) and ginger (*Zingiber officinale*) essential oils on performance, hematological, biochemical and immunological parameters of broiler chickens, Glob. Vet. 12 (2014) 736–744, https://doi.org/10.5829/idosi.gv.2014.12.06.83189.
[24] S.S. Percival, Aged garlic extract modifies human immunity, J. Nutr. 146 (2016) 433S–436S, https://doi.org/10.3945/jn.115.210427.
[25] M.S. Mojani, A. Rahmat, R. Ramasamy, V.H. Sarmadi, P. Sandrasaigaran, S. Vellasamy, S.M.A. Hejazi, Metabolic and immunologic alterations of ginger rhizome among streptozotocin-nicotinamide induced diabetic rats, Mal. J. Nutr. 22 (2016) 421–432.
[26] Z. Amirghofran, S. Malek-Hosseini, H. Golmoghaddam, F. Kalantar, M. Shabani, Inhibition of nitric oxide production and proinflammatory cytokines by several medicinal plants, Iran. J. Immunol. 8 (2011) 159–169 doi: IJIv8i3A3.
[27] C. Nathan, Qwen Xie, Nitric oxide synthases: roles, tolls, and controls, Cell 78 (1994) 915–918, https://doi.org/10.1016/0092-8674(94)90266-6.
[28] X.X. Huang, M. Yamashiki, K. Nakatani, T. Nobori, A. Mase, Semi-quantitative analysis of cytokine mRNA expression induced by the herbal medicine Sho-saiko-to (TJ-9) using a Gel Doc system, J. Clin. Lab. Anal. 15 (2001) 199–209.
[29] H.P. Chang, S.Y. Huang, Y.H. Chen, Modulation of cytokine secretion by garlic oil derivatives is associated with suppressed nitric oxide production in stimulated macrophages, J. Agric. Food Chem. 53 (2005) 2530–2534.
[30] G. Hodge, S. Hodge, P. Han, Allium sativum (garlic) suppresses leukocyte inflammatory cytokine production *in vitro*: potential therapeutic use in the treatment of inflammatory bowel disease, Cytometry 48 (2002) 209–215, https://doi.org/10.1002/cyto.10133.
[31] F. Zehsaz, N. Farhangi, L. Mirheidari, The effect of *Zingiber officinale* R. Rhizomes (ginger) on plasma pro-inflammatory cytokine levels in well-trained male endurance runners, Cent. J. Immunol. 39 (2014) 174–180, https://doi.org/10.5114/ceji.2014.43719.
[32] Y. Otaki, Poultry disease control programme in Japan, Asian livestock 20 (1995) 65–67.