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Immune Response Against Salmonella Infection in Chicken Using Cynarin as Immunostimulant

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Abstract

A total of 900 samples of internal organs (liver, spleen, intestine from each bird) was aseptically collected from 300 diseased and apparently healthy Hubbard and Ross broiler chickens of different ages (3-5weeks) from different farms in El-Fayoum Governorate. The rate of isolation of *Salmonella* species from broilers of different ages was (5%), where 15 strains were isolated from 300 broilers. Salmonellae were isolated from diseased and apparently healthy broilers with an incidence of 9.7% and 0.6% respectively. The highest rate of salmonella isolation (6.9%) was recorded in liver of diseased broilers followed by 3.5% in intestine of diseased broilers and only (0.6%) in intestine of apparently healthy broilers, while salmonella species were not isolated from spleen of both apparently healthy and diseased broilers. *S.Enteritidis* was the most predominant isolate (53.3%), followed by *S.Typhimurium* (33.3%) and S.*Infantis* (13.3%). *S.Enteritidis* isolates showed complete resistance to cefotaxime, lincomycin and sulfo-methoxazole trimethoprim. *S.Enteritidis* showed sensitivity to ciprofloxacin with a rate of 50%.

A total of 70 chicks was used to evaluate the protective value of cynarin against *S.Enteritidis*. The mean of optical density (MOD) of serum antibodies IgG in groups treated with cynarin using indirect ELISA were observed.

It was concluded that none of the used antibiotics was 100 % effective; on the other hand, multidrug resistance patterns have been recorded among all isolates examined. Cynarin exhibits high antimicrobial activities against *S.Enteritidis*, and could be used to reduce the usage of antibiotics and its related side effect and highlights the importance of them as complementary tools but not substitutes of integral biosecurity programs against the infection in poultry flocks. Herbs effectiveness on humeral response needs more application on large scales.

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Introduction

Salmonellosis is considered to be one of the most wide- spread foodborne zoonosis in industrial as well as developing countries even though the incidence seems to vary between countries. Food of animal origin, especially from poultry, is an important source of human Salmonella infections. Poultry products, especially undercooked and raw eggs, have been a major risk factor for human infection with salmonella **(Palmer *et al.* 2000; De Buck *et al*. 2004)**.

Antibiotics are extensively used as productivity enhancers in poultry production or to control infectious diseases. Antimicrobial exercise and/or especially abuse are considered to be the most vital selecting force to antimicrobial resistance of bacteria (**Neu, 1992** and **Moreno, *et al.* 2000)**. Antibiotic resistant pathogenic bacteria in animals can pose a risk not only to animal health, but also to humans as food-borne pathogens (**Piddock, 1996** and **Smith, *et al.* 1999)**.

Therefore for salmonella and *E.coli* control, there were a wide range of antimicrobials but antimicrobial resistance has emerged as a global public health problem in recent years (**Harrison and Lederberg, 1998).** For that, there is a worldwide attempt to reduce antibiotic usage, because of its residues in meat, development of resistant bacteria, and imbalance of normal microflora **(Sorum and Sunde, 2001)**. So that it was important to find alternatives to antibiotics.

Herbs and herbal products are incorporated in poultry diets to replace synthetic products in order to stimulate or promote the effective use of feed nutrients which may subsequently result in more rapid body weight gain, higher production rates and improved feed efficiency (**Al-Khdri, 2013**). Moreover, active components of herbs may improve digestion and stimulate the immune function in broilers (**Ghazalah and Ali, 2008**).

Supplementation of spices and herbs could have many benefits to broilers health and performance such as having antioxidative potential (**Hoffman and Wu, 2010)**, antimicrobial activity (**Dorman and Deans, 2000; Lee et al., 2004; Steiner, 2009)**, enhancing digestion by stimulating endogenous enzymes (**Al-Khdri, 2013).**

The aim of the present work was to study the immune-stimulant activity of natural source such as Herbs (Cynarin) against Salmonella isolated from broiler chickens.

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Material and methods

A total of 900 samples of internal organs (liver, spleen, intestine from each bird) were aseptically collected from 300 diseased and apparently healthy Hubbard and Ross broiler chickens of different ages (3-5weeks) from different farms in El-Fayoum Governorate. All samples **Table (1).** Samples were collected in ice box directly to the laboratory for bacteriological examination.

Bacteriological examination:

According to **OIE (2004),** one gram of each sample was inoculated in 9ml buffered peptone water and incubated over night at 37ºC for 18h as pre-enrichment for salmonellae. 0.1ml of pre-enrichment culture was transferred to RV and selinite F broth and incubated for 24hrs at 41.5ºC (as selective enrichment). A loopful from both enrichment incubated broth (RV and selinite F broth) was streaked on both S.S agar and XLD medium and incubated for 24hrs at 37ºC. Glistening colonies with black center were picked up on trypticase soya agar for purification for further microscopically examination, biochemical and serological identification. The purified isolates of *E.coli* and salmonellae were examined by different biochemical reactions according to **Quinn *et al.* (2002).** The organisms were serotyped by **Kauffmann and Das-Kauffmann, (2001)** using Salmonella O and H poly and monovalent antisera (Mast Assure).

Sensitivity tests:

Sensitivity tests for bacterial isolates were conducted using disc diffusion technique according to **CLSI (2012)**. Different types of antimicrobial discs with variable concentrations were used to detect the susceptibility of isolates. These discs were obtained from (Oxoid): Amoxycellin (AML 10µg), Ciprofloxacin (CIP 5µg), amoxycellin clavulinic acid (AMC 30µg), ampicillin (AMP 10µg), chloramphinicol (C 30µg), sulfo-methoxazole trimethoprim (SXT 25µg), gentamicine (CN 10µg), spiramycin (SP 100µg), cefotaxime (CtX 30µg), doxycyclin (DO 30µg), lincomycin (MY10µg) and clindamycin (DA 2µg).

Experimental design:

A total of 70 chicks was used. Before the beginning of the experiment, 10 chicks were chosen randomly, sacrificed then postmortem and bacteriological examination were performed to make sure they are free of *S.Enteritidis.*

The remained chicks were reared on cage system and fed on unmedicated commercial ration. Feed and water were consumed ad-libitum, continuous source of light was supplied. These chicks were used to evaluate the protective value of cynarin against *S.Enteritidis*.

The chickens were vaccinated against Newcastle disease at 7 and 18 days of age using Hitchner B1 and Lasota vaccine and against Gumboro disease at 12 day. Vaccines were administrated via eye drop method. Chicks were divided into 3 groups (20 chicks per group) as follow: **Group (1) was** fed without addition of cynarin (control negative), **Group (2)** was fed with addition of cynarin in drinking water at the first day of rearing and challenged at 18 days old with LD_{50} of *S.Enteritidis.* The cynarin used in the experiment was used as competitive exclusion product in drinking water starting. It is a commercial preparation composed of Cynara Scolymus; Star Anise oil extract; Caraway extract; Menthol oil extract; Liquorice root extract; Cinnamon oil extract; Organic Zinc; Organic Selenium; Biotin and Excipient Dill leaves guava (Multikraft Productions Handlesgmbh,Austria)**.Group (3)** was fed without addition Cynarin in drinking water and challenged at 18 days old with *S.Enteritidis (contrl positive).* Reisolation of *S.Enteritidis* and collection of blood samples for indirect ELISA. All birds were kept under observation for 2 weeks post inoculation and observed daily for mortality, clinical signs **(Marilda *et al*., 1990)**.

Immunological response:

ELISA was applied to determine the effect of the tested cynarin on the production of antibody against *Salmonella Eneritidis* after experimental infections.
ELISA plates were coated using 100µl of 1/10 diluted prepared antigen (10^{11}) of *S.Enteritidis* (prepared according to **Nalbantsoy, (2012**) in coating buffer. The plates were then incubated at 4^{o}C overnight. The plates were washed 3 times used 200µl of washing buffer, then blocked using 100µl/well of the blocking buffer (1g bovine serum albumen and 15ml Tween 20in 100ml phosphate buffer saline BPS) for 2 hours on horizontal shaker at 37ºC. The blocking buffer was decanted by inverting and flipping the plate and washed 3 times as before. Each sample was duplicated; where100µl of diluted serum sample (1:100) was added to each well. The plates were incubated in a humid chamber at 37^{O}C for 1 hour. The plates were inverted and flipped as before and washed three times using 300µl/well of the washing buffer. 100µl of the anti-chicken conjugated peroxidase diluted as recommended in dilution buffer were then added to each well of the ELISA plates. The plates were incubated in a humid chamber at 37^{O}C for 1 hour and then washed as before. 100µl of TMB peroxidase substrate indicator

mixture (Synbiotics) were added to each well of the ELISA plates. The plates were then incubated in a dark place at room temperature for 15 minutes to allow color development. Further color development was stopped by adding 100µl stopping solution to each well. Read at optical density 450nm. Interpretation of results was conducted according to the prepared hyperimmune serum based on a standard procedure **(Le Minor and Rohde, 1989)**

Results and Discussion

Salmonellosis is considered to be one of the most wide- spread foodborne zoonosis in industrial as well as developing countries even though the incidence seems to vary between countries. Food of animal origin, especially from poultry, is an important source of human Salmonella infections. (**Palmer, *et al.* 2000 and De Buck et al. 2004**). These variations may be attributed to the diverse pathogenicity of *E.coli* for chicks which had been correlated with numerous extrinsic and intrinsic bird factors and conditions, exposure to other infectious agents, virulence of the microorganisms, level and duration of exposure, while intrinsic factors includes the age, route of exposure, active and passive immune status and breed of chicken **(Deb and Harry, 1976 and Gaven, 1978).**

All isolated Salmonella enteric serovars (15 strains), morphologically characterized by gram negative short rods or bacilli, non lactose fermenter on MacConkey agar, glistening colonies with or without black center on S.S agar or XLD agar, hemolytic on blood agar, motile, negative for oxidase, urease, Lysine decarboxylase and Indole. positive to Cimmon`s citrate test, methyl red, ferment glucose and dulcitol and produce H_2S on TSI (**Cowan and Steel, 1974 and Quinne *et al.*2002).**

In the present study, the rate of isolation of *Salmonella* species from broilers of different ages was (5%), where 15 strains were isolated from 300 broilers. Salmonellae were isolated from diseased and apparently healthy broilers with an incidence of 9.7% and 0.6% respectively. The highest rate of salmonella isolation (6.9%) was recorded in liver of diseased broilers followed by 3.5% in intestine of diseased broilers and only (0.6%) in intestine of apparently healthy broilers, while salmonella species were not isolated from spleen of both apparently healthy and diseased broilers as shown in **(Table 2). Hazem (2010)** isolated from broiler chicken, 6 *S.Enteritidis* serotype out of total 19 salmonella serotypes. It was estimated that the national prevalence of *S.Enteritidis* was 6.3% **(Betancor *et al.* 2010).).**

The result of serotyping of salmonella isolates as explained in **Table (3)** revealed that *S.Enteritidis* was the most predominant isolate (53.3%), followed by *S.Typhimurium* (33.3%) and S.*Infantis* (13.3%).

Salmonella Enteritidis and *S.Typhimurium* are presented separately from other sero-types of Salmonella because, on the one hand, these bacteria are often specifically cited in zoonosis control legislation, and, secondly, because there are differences in the epidemiology as compared to other salmonellae. These are the predominant sero-types associated with human disease in most countries. **(Bangtrakulnonth, *et al.* 2004 and Bishop *et al.* 2007)**

Poppe, *et al.* (1991) isolated *S.infantis* in a rate of 8.8% and **Roy, *et al.* (2002)** isolated *S. Infantis* in a rate of 4.12%.

The sensitivity of *S.Enteritidis* to different antibacterial agents was illustrated in **Table (4). Where** *S.Enteritidis* showed complete resistance to cefotaxime, lincomycin and sulfo-methoxazole trimethoprim. *S.Enteritidis* showed sensitivity to ciprofloxacin with a rate of 50%. These results were nearly agreed with **Osman *et al.* (2014) and Pulido-Landínez *et al.* (2014).**

The results of antibiotic susceptibility of our study are invariance with some studies and in accordance with others, indicating that antibiotic susceptibility pattern varies with different isolates, time and development of multiple drug resistant *E.coli* and *S.Enteritidis* as reported by (**Holmberg *et al.* 1984 and Sharada *et al.* 2010)**. Antibiotic resistant pathogenic bacteria in animals can pose a risk not only to animal health, but also to humans as food-borne pathogens **(Rahimi, 2013).**

Among the many purported alternatives to the use of antibiotics are the incorporation of either prebiotics or synbiotic (probiotics) into feed and/or drinking water. The utilization of plants, herbs nutritional immunomodulators are becoming popular due to their low toxicity, fewer side effects, being cost-effectitive and other beneficial advantages for safeguarding health of humans and their companion animals including poultry **(Mahima *et al.* 2012 and 2013**). In the present study, its aimed to use herbs as alternative to antibiotics treatment and immunomodiolators for *S.Enteritidis.*

The contents of cynarin used in this study are Cynara Scolymus, Star Anise oil extract, menthol oil extract, caraway extract, liquorice root extract, cinnamon oil extract, organic zinc, organic selenium, biotin and Eexcipient dill leaves guava

The mean of optical density (MOD) of serum antibodies IgG in groups treated with cynarin using indirect ELISA were observed. MOD OF IgG in groups (2) and (5 treated with cynarin were showed significant increase at the second week (2.021±0.018 and 1.989±0.234 respectively) than groups (1) and (3) not treated with cynarin (0.431±0.218, 0.431±0.218 and 0.490±0.145 respectively). At 3rd and 4th week of

experiment (post infection), group (2) infected with *S.Enteritidis* showed significance difference (1.221±0.118 and 0.967±0.218 respectively) than group (1) control (–ve) and group (3) infected with *S.Enteritidis* (0.699±0.234 and 0.810±0.207 respectively) **Table (5).**

There is no published work about the effects of herb infusions on antibody titers. However, **Al-Ankari *et al*. (2004)** reported that mint powder had no effects on antibody titers to NDV vaccine. While **Sadeghi *et al.* (2012)** found that Cinnamon and herbal mix infusions significantly improved the immune response of birds to the NDV vaccine. Also, **Van Furt (1982)** reported that *cinnamomum zeylanicum* essential oil showed stimulatory effects on macrophages. Macrophages play an important role in the initiation and regulation of the immune response (**Albercht 1979; Kende 1982**). **Song *et al.* (2012)** found that Selinium improves the expression of genes that are related to enhanced immunity of pigs.

It was concluded that none of the used antibiotics was 100 % effective; on the other hand, multidrug resistance patterns have been recorded among all isolates examined. Cynarin exhibits high antimicrobial activities against *S.Enteritidis*, and could be used to reduce the usage of antibiotics and its related side effect and highlights the importance of them as complementary tools but not substitutes of integral biosecurity programs against the infection in poultry flocks. Herbs effectiveness on humeral response needs more application on large scales.

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Table (1): Types of samples collected from diseased and apparently healthy broilers:

Status	Total number of chicken	Type of samples	No. of samples
Apparently healthy broiler (156)	**156**	**Liver**	156
		spleen	156
		Intestine	156
Total		468	
Diseased broilers* (144)	**144**	**Liver**	144
		spleen	144
		Intestine	144
Total		432	
Total No. of samples		900	

Table (2): Isolation rate of *Salmonella* spp. recovered from samples collected from diseased and apparently healthy

Broilers	Type of samples	Total No. of samples	Rate of isolation of salmonella species	
			No.	%
Apparently healthy broiler (156)	**Liver**	156	0	0
	Spleen	156	0	0
	Intestine	156	1	0.6
Total (156)			1	0.6
Diseased broilers (144)	**Liver**	144	9	6.9
	Spleen	144	0	0
	Intestine	144	5	3.5
Total (144)			14	9.7
Total (300)			15	5

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Table (3): Serotyping of salmonella isolates

Serotypes	No.	%	Antigenic structure			
			Group	Somatic (O) antigen	Flagellar (H) antigen	
					Phase I	Phase II
S.Enteritidis	8	53.3	D_1	9	g,m	-
S.Typhimurium	5	33.3	B	4,5,12	I	1,2
S. Infantis	2	13.3	C_1	6,7	R	1,5
Total	15	100				

Table (4): Sensitivity of isolated *S.Enteritidis* (8 isolates) to antibacterial agents

Chemotherapeutic Discs	**Conc (µg)**	**Resistant**		**Intermediate**		**Sensitive**	
		No.	**%***	**No.**	**%**	**No.**	**%**
β-lactamas classes							
Amoxycillin	10	7	87.5	1	12.5	0	0
Amoxycillin clavulinic acid	30	0	0	6	75	2	25
Ampicillin	10	7	87.5	0	0	1	12.5
Cifotaxime	30	8	100	0	0	0	0
Fluroquiinolones class							
Ciprofloxacin	5	0	0	4	50	4	50
Phenicol group							
Chloramphinicol	30	4	50	1	12.5	3	37.5
Tetracyclines class							
Doxycyclin	30	5	62.5	1	12.5	2	25
Aminoglycosides class							
Gentamycin	10	5	62.5	0	0	3	37.5
Macrolides class							
Spiramycin	100	4	50	2	25	2	25
Folate pathway inhibitors							
Sulfo-methoxazole trimethoprim	25	8	100	0	0	0	0

Table (5): Mean of Optical Density (MOD) IgG in chickens treated with cynarin

Groups	Old of chicken	OD ± SD Before challenge	OD ± SD after challenge*
Group 1 (control -ve)	1st week	0.230±0.101	
	2nd week	0.431±0.218[c]	
	3rd week	0.699±0.234	
	4th week	0.810±0.207[b]	
Group 2	1st week	0.420±0.145c	
	2nd week	2.021±0.018[a]	
	3rd week		1.221±0.118[b]
	4th week		0.967±0.218[b]
Group 3 (control +ve)	1st week	0.330±0.101	
	2nd week	0.431±0.218[c]	
	3rd week		0.257±0.189[d]
	4th week		0.188±0.111[d]

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