Molecular Studies on the prophylactic effect of probiotics on *Salmonella typhimurium* infected chicks

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ABSTRACT

The ability of probiotics to protect chicks against *S.typhimurium* was evaluated. Challenge experiment was designed using 120 one day old chicks divided into 4 groups. The challenging *S. typhimurium* was counted in liver and cecum samples and the results shows that counts of the probiotics pre-treated group were significantly reduced when compared to those of positive control group. The phagocytic percent of chicks of positive control group were 38.40%, 26% and 18% while that of pre-treated group were 54.20%, 38.10% and 36.40% in 7, 14 and 21 days old respectively. Phagocytic indexes (PI) of positive control group were 1.0±0.10, 1.0±0.20 and 1.0±0.0 while those of pre-treated group were 1.50±0.20, 1.10±0.08 and 1.10±0.04in 7, 14, and 21 days old respectively. *S. typhimurium* from ceacal digesta were used for extraction of total RNA to assess the expression of 4 virulence genes (*hil*A, *hil*C, *hil*D and *sip*C) using Real Time PCR. The obtained data revealed that CT means of *hil*A, *hil*C, *hil*D and *sip*C of control group and pre-treated group were as follow 20.31 and 17.68, 17.43 and 16.85, 17.03 and 16.72 and 18.78 and 17.98 respectively. It was concluded that probiotics has an impact on *S. typhimurium* and caused an imbalanced virulence gene expression.

Key words: probiotics, *S. typhimurium,* chicks

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1- INTRODUCTION

Intestinal microflora plays a crucial role in host defence as demonstrated by their ability to modulate both innate and acquired immunity at the local as well as systemic levels. This immune response was modulated due to these specific strains of lactic acid bacteria (LAB), which defined as probiotics. The mechanisms underlying the immune modulating properties of probiotics are not fully understood. However, they may be due to the ability of probiotics to balance the intestinal microflora and/or be a consequence of a direct adjuvant effect on the production of immune factors, such as cytokines. Several strains of LAB were shown to enhance nonspecific immunity *in vitro* as well as *in vivo*, including the release of tumor necrosis factor α, interleukin 6, increased phagocytosis and stimulated natural killer cell activity (Isolauri et al., 2001). Probiotics exert direct antibacterial effect on pathogens through production of antibacterial substances, including bacteriocins and acids (Cotter et al., 2005). Analysis of the known genomic sequences of Lactobacillus strains including *L. plantarum*, *L. acidophilus* NCFM, *L. johnsonii* NCC 533 and *L. sakei* predicts abroad group of bacteriocins with highly divergent sequences. These peptides have a relatively narrow spectrum of activity and are mostly toxic to Gram-positive bacteria, including *Lactococcus, Streptococcus, Staphylococcus, Listeria,* and *Mycobacteria* species (Makarova et al., 2006). Probiotics in the gastrointestinal tract decrease adhesion of both pathogens and their toxins to the intestinal epithelium. Several strains of lactobacilli and bifidobacteria are able to compete with pathogenic bacteria, including *Bacteroides vulgates*, *Clostridium histolyticum*, *C. difficile, Enterobacter aerogenes, Listeria monocytogenes*, *Staphylococcus aureus, Salmonella enterica, Yersinia enterocolitica*, enterotoxogenic *E. coli* and enteropathogenic *E. coli* for intestinal cell binding, and they can displace pathogenic bacteria even if the pathogens have attached to intestinal epithelial cells prior to probiotic treatment. However, specific probiotics or probiotic combinations should be selected based on their ability to inhibit or displace a specific pathogen (Collado et al., 2007). In vitro tests demonstrated that molecules secreted by

Bifidobacterium bifidum interfere with both attachment and invasion. The main regulatory genes controlling the virulence factors essential for these pathogenicity steps were efficiently down-regulated when treated with chromatographically separated *B. bifidum* cell free fractions as measured by reporter constructs and confirmed by RT-PCR (Bayoumi and Griffith, 2012).

This study was conducted to evaluate the ability of probiotics to protect chicks against *S. typhimurium* and the alteration in the expression of some virulence genes of *S. typhimurium* isolated from broilers chicks pre-treated with probiotics.

2- MATERIAL AND METHODS

2.1. Experimental chicks:

One-hundred and twenty, one day old broiler chicks obtained from (commercial poultry company) were used, the chicks reared in separated floor pens under hygienic measures in the experimental poultry unit until the end of experiment (3 weeks) in Faculty of Veterinary Medicine, Zagazig University. Chicks had free access to water and commercial starter diet without supplementation of antibiotics. Then chicks divided into four groups, 30 chicks per each to study the effect of probiotic treatment in reduction of Salmonella infection through down-regulation of virulence genes expression. Two treated groups in addition to control positive and control negative groups were designed. The number of dead chicks were recorded in order to calculate the survival percentage in days 7, 14 and 21.

2.2. Protexin

A commercial probiotic preparation manufactured by Probiotics International Limited, Lopen Head, Somerset TA135JH, United Kingdom, contains 1.0 $x10^9$ CFU/gram of *Lactobacillus acidophilus, Lactobacillus casei, Lactbacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium infantis, Bifidobacterium breve* and *Streptococcus thermophilus.* One group was orally gavaged with 1 ml of probiotic containing 1.0 x 10^7 CFU/ bird starting in the first day and continued to 10^{th} day.

2.3. *Salmonella typhimurium a nalidixic acid-resistant strain*

This strain molecularly typed and kindly supplied by Serology Department, Animal Health Research Institute, Dokki, Giza. Salmonella was cultured in Rappaport-Vassiliadis (RV) enrichment broth at 42°C for 18 h followed by dilution with sterilized phosphate-buffered saline (PBS, pH 7.2) to a final cell count of 1 x 10^4 CFU per ml for chicken infection experiment.

2.4. Preparation of bacterial strains:

Salmonella typhimurium molecularly typed was cultured in buffered peptone water followed by dilution with sterilized phosphate-buffered saline (PBS, pH 7.2) to a final cell count of 1.0 $x10^4$ CFU per ml for experimental infection.

2.5. Experimental design:

One hundred twenty-one day old chicks were divided into four groups, 30 birds each. Group (A): Treated with probiotic from 1^{st} to 10^{th} day with a dose of $1x10^7$ CFU/bird orally. Group (B): Treated with probiotic from 1^{st} to 10^{th} day with a dose of 1.0 $x10^7$ CFU/bird orally then challenged with *Salmonella typhimurium* at 5^{th} day old by 1 ml of 1.0 $x10^4$ CFU/bird. Group (C): Infected with *Salmonella typhimurium* at 5^{th} day old by 1 ml of 1.0 $x10^4$ CFU/bird orally (positive control). Group (D): Non-treated, non-infected (Negative control).

2.6. Collection of samples:

Liver and spleen samples were collected aseptically for Salmonella counts in day 3 and 5 post infection then pure Salmonella culture from ceacal digesta was used for extraction of total RNA, washed, and eluted following the manual of the kit. Livers and spleens were collected from positive control group to examine for possible Salmonella infection (Yang et al., 2014). Blood was collected from the challenged pre-treated and control chicks in days 7, 14 and 21 for conducting of phagocytic activity and indices, as well as the survival percentages of all chicks after challenge was detected at 7, 14, 21 day old.

2.7. Reisolation of Salmonella typhimurium

The procedures for isolation and identification of Salmonella were conducted according to ISO 6579 (2002). The detection of Salmonella needs four successive stages: pre-enrichment in a non-selective broth, enrichment in a selective liquid broth, plating onto selective agar media and detection of suspected Salmonella colonies.

2.8. Identification of Salmonella typhimurium (ISO 6579 (2002)

The identified *Salmonella typhimurium* was further subjected to microscopical examination and colonial appearance. The preliminary identified Salmonella species were further subjected to H_2S production using triple sugar iron agar, Urea hydrolysis test, Lysine decarboxylase test, Indole production test and Citrate utilization test (Qunin et al., 2002).

2.9. Salmonella typhimurium counting on day 3 and 5 post-infection

one gm of cecal digesta and liver homogenate samples were diluted in 1 ml sterile solution of 0.1 % peptone water followed by ten fold serial dilution and then plated on BHI agar medium supplemented with 200µg/ml nalidixic acid to facilitate selection of antibiotic-resistant challenge organism. The mean of the count of the last two dilutions was calculated to measure the final count.

2.10. Detection of phagocytic activity: (Wilkinson et al*., 1977)*

2.10.1. Blood samples collection:

Blood samples were collected individually from all experimental chicks at 7, 14 and 21 days old. Through heart puncture, 2.5 ml blood was collected in a sterile plastic centrifuge tube containing heparin (50 IU/ml) for separation of leucocytes

2.10.2. Preparation of Hank's solution (HBSS): (Cruickshank et al, 1975):

HBSS was made by adding one volume of stock solution A and one volume of stock solution B to 18 volume of distilled water. It was sterilized by steaming for 90 minutes. Immediately before use 0.5 ml of sterile 1.4% $NaHCO_3$ solution was added to each 20 ml of Hank's solution.

2.10.3. Preparation of C.albicans:

C.albicans was grown on Saboaraud's 2% dextrose broth for 48 hr at 37°C to obtain the organism in the yeast phase only. The cultures were spinned at 1500 rpm. for ten minutes and the deposit, washed twice with phosphate buffered saline, and filtered through sterile gauze. The yeasts were resuspended in HBSS so as to give a concentration of $5x10^6$ CFU/ml. The yeasts were killed by heating at 100°C in water bath for 30 minutes. A large batch was prepared and divided into small aliquots sufficient for each test. These were stored at -20°C until needed.

2.10.4. Preparation of leucocytes suspension (Wilkinson 1977) and Lucy and Larry 1982):

An amount of 2.5 ml of heparinized blood were carefully layered on surface of equal volumes of Ficoll hypaque sol. 1.077 gradient density in sterile plastic centrifuge tube then centrifuged at 2400 rpm for 30 m. at 18-20 ˚C. The mononuclear cells form white opaque band at Ficoll plasma interface. This layer was aspirated by sterile Pasteur pipette and placed in sterile plastic tube containing HBSS. The separated cells washed three times in HBSS at 2500, 1500 and 1000 rpm respectively each for 10 minutes. Sedimented cells were suspended in 1ml of the RPMI media containing 1% foetal calf serum. The leukocyte cell count was adjusted to $2x10^6$ cell /ml in HBSS after detection of cells viability using trypan blue.

2.10.5. Preparation of pooled normal serum:

Five ml of blood were taken without anticoagulant in a sterile Wasserman tube for separation of serum, which was stored at -20°Cin a small sterile vials until used.

2.10.6. Measurement of phagocytic activity:

An amount of 0.25 ml leucocyte suspension, 0.25 ml heat killed *Candida albicans*, 0.25 ml pooled serum and 0.25 ml HBSS were placed in sterile plastic tube then incubated at 37°C for 30 minutes. They were centrifuged at 2500 rpm for 5 minutes and the supernatant was removed with Pasteur pipette leaving a drop into which the sediment was resuspended. Smears were prepared from the deposit, dried in air and stained with Leishman's stain. Under a light microscope using oil immersion lens, 10 fields, each containing about 20 phagocytes, were examined. The total number of phagocytic cells, the number of phagocytes that ingested yeast cells and the number of ingested yeast cells were determined to calculate the phagocytic percent and phagocytic index (Goddeeris etal.,1986).

2.11. Extraction of bacterial total RNA

RNA was extracted from 1 ml overnight cultures of *S. typhimurium* of different groups according to RNeasy Mini Kit instructions (Qiagen)

2.12. RT-PCR assay:

RT-PCR assay was performed on RNA extracts of *S. typhimurium* isolates from different groups according to Quantitect SYBR Green PCR kit. The volumes and inputs are mentioned in Table (1). The cycling conditions of RT-PCR are mentioned in table (2). The primers used in RT-PCR are listed in table (3). The expression levels of virulence genes were normalized using 16S *rRNA* as an internal housekeeping control, and fold change of target genes was determined by the Stratagene MX3005P software. To estimate the variation of gene expression of different samples, the Ct of each sample was compared with that of the control group according to the "ΔΔ Ct" method stated by (Yuan et al., 2006).

3- RESULTS

3.1. Effect of probiotics on the count of S. typhimurium in the liver and the cecal digesta

The average count of *S. typhimurium* recovered from each group is summarized in Table (4)

3.2. Survival percentage of chicks treated with probiotic and challenged with virulent strain of Salmonella typhimurium

The survival percentage in different groups were calculated and tabulated as shown in Table (5)

3.3. The effect of using the probiotic on phagocytic activity

The highest phagocytic percent in group A was 48.1% at age of 7 day old whereas the lowest one 36.2% at age of 21 day old. But the highest phagocytic index was 1.2±0.2 at age of 7 day old whereas the lowest one was 1.2±0.12 at age of 21 day old. The highest phagocytic percent in group B was 54.2% at age of 7 day old whereas the lowest one 36.4% at age of 21 day old. But the highest phagocytic index was 1.5±0.20 at age of 7 day old whereas the lowest one was 1.1±0.04 at age of 21 day old. The highest phagocytic percent in group C was 38.4% at age of 7 day old whereas the lowest one 18.0% at age of 21 day old. But the highest phagocytic index was 1.0±0.10 at age of 7 day old whereas the lowest one was 1.0±0.00 at age of 21 day old. The highest phagocytic percent in group D was 42.5% at age of 7 day old whereas the lowest one 32.4% at age of 21 day old. But the highest phagocytic index was 1.2±1.16 at age of 14 day old whereas the lowest one was 1.0±0.00 at age of 21 day old. As mentioned in Table (6).

3.4. The effect of probiotics on the expression of virulence genes

The expression of SPI-1 virulence genes *hil*A, *hil*C, *hil*D and *sip*C of *S. typhimurium* in the cecal digesta of chickens was investigated by RT- PCR assays in order to compare gene expression in both groups (positive control group) (C) and pre-treated group (B), There is a significant (*P value* <0.05) down regulation and fold differences in group (B) as mentioned in Tables (7), (8), (9) and (10) and Figures (1), (2), (3) and (4).

4- DISCUSSION

Salmonella is one of the most important pathogens that is responsible for food poisoning in developing countries (Cerro et al., 2002). Poultry are one of the important reservoirs of Salmonellae that can be transmitted to human through the food-chain. Pathogenesis of Salmonellosis depends up on a large number of factors controlled by an array of genes responsible for the actual virulence of Salmonella (Murugkar et al., 2003).

In this study, Salmonella count in the liver homogenate from chicks in the positive control group in the 3rd post-infection were $1.4x10^5$ CFU/g while in the group treated with probiotic Salmonella cell count was $1.1x10^3$ CFU/g. But in the 5th day post-infection Salmonella cell count was

Table (1) Mater Mix of Quantitect SYBR Green PCR.

Component	Volume / reaction
2x Quantitect SYBR Green PCR Master Mix	12.5 µl
Reverse transcriptase	0.25 µl
Forward primer (50 pmol)	0.5 µl
Reverse primer (50 pmol)	0.5 µl
RNase Free Water	4.25 µl
Template DNA	7 µl
Total	25 µl

Table (2) Cycling conditions of SYBR Green real time PCR

		Amplification (40 cycles)					Dissociation curve (1 cycle)		
Target gene	Reverse transcription	Primary denaturation	Secondary denaturation	Annealing (optics on)	Extension	Secondary denaturation	Annealing	Final denaturation
Target genes	50ºC 30 min.	94ºC 5 min.	94ºC 15 sec	60ºC 30 sec	72ºC 30 sec	94ºC 1 min.	60ºC 1 min.	94ºC 1 min.

Table (3): Primers used for real-time quantitative PCR identification of the *Salmonella typhimurium*

Gene	Primer sequence (5`-3`)	Reference
1- 16S rRNA	F:CAGAAGAAGCACCGGCTAACTC R:GCGCTTTACGCCCAGTAATT	Yang et al., (2014)
2- *hilA*	F:CATGGCTGGTCAGTTGGAG R:CGTAATTCATCGCCTAAACG	
3-*hil*C	F:GGACTTGTTGCCAGGGATG R:TGACCATTTGCGGGTGAG	
4-*hil*D	F:ACTCGAGATACCGACGCAAC R:CTTCTGGCAGGAAAGTCAGG	
5-*sip*C	F:CTGTGGCTTTCAGTGGTCAG R:TGCGTTGTCCGGTAGTATTTC	

Table (4): Salmonella count in liver and cecal digesta on day 3 and day 5 post infections (PI)

Group	Average Salmonella count on day 3 PI		Average Salmonella count on day 5 PI.	
	Cecal digesta	Liver	Cecal digesta	Liver
A	-		-	
B	$1.9x10^3$ CFU/g	$1.1x10^3$ CFU/g	$1.5x10^3$ CFU/g	$1.2x10^3$ CFU/g
C (+ve control)	$1.5x10^6$ CFU/g	$1.4x10^5$ CFU/g	$1.3x10^5$ CFU/g	$1.2x10^5$ CFU/g
D (–ve control)	-		-	

Table (5) Survival percentages of all group after challenge at 7, 14 and 21 day old

Age/day Group		7 days	14 days	21 days
A	*	3/30	2/27	2/25
	**	90%	92.5%	92%
B	*	2/30	2/28	1/26
	**	93.3%	92.8%	96%
C	*	12/30	8/18	5/10
	**	60%	55.5%	50%
D	*	4/30	3/26	3/23
	**	86%	88%	87%

Table (6): Phagocytic percentages (P %) and phagocytic index PI in different groups at 7, 14 and 21 day old

Groups	Sample	*hilA* CT (Individual)	CT (Mean)	*16S rRNA* CT (Individual)	CT (Mean)	Fold change to control	Fold change to control (Collective)
Control (C)	1	17.96	20.31	20.65	20.31	-	-
	2	18.01		20.73		-	
	3	17.88		19.83		-	
	4	18.74		20.02		-	
Treated (B)	5	17.96	17.68	19.76	19.50	0.7631	0.7738
	6	18.01		20.43		1.1728	
	7	17.95		19.56		0.6690	
	8	16.78		18.25		0.6071	

Table (7) Cycle threshold (CT) and Fold change of *hil*A virulence gene

Age/day	7 day		14 day		21 day	
Groups	P %	PI	P %	PI	P %	PI
A	48.1	1.2±0.20	42.4	1.2±0.12	36.2	1.2±0.12
B	54.2	1.5±0.20	38.1	1.1±0.08	36.4	1.1±0.04
C	38.4	1.0±0.10	26.0	1.0±0.2	18.0	1.0±0.0
D	42.5	1.12±0.26	36.4	1.2±1.16	32.4	1.0±0.0

Table (8) Cycle threshold (CT) and Fold change of *hil*C virulence geneold change to control (Collective)

Groups	Sample	*hilC*		*16S rRNA*		Fold change to control	
		CT (Individual)	CT (Mean)	CT (Individual)	CT (Mean)		
Control (C)	1	18.72	17.03	20.65	20.31	-	-
	2	16.65		20.73		-	
	3	15.65		19.83		-	
	4	17.10		20.02		-	
Treated (B)	5	16.85	16.72	19.76	19.50	0.7738	0.7423
	6	17.23		20.43		0.9461	
	7	17.43		19.56		0.4506	
	8	15.37		18.25		0.7579	

Table (9) Cycle threshold (CT) and Fold change of *hil*D virulence gene

Groups	Sample	*hilD*		*16S rRNA*		Fold change to control	Fold change to control (Collective)
		CT (Individual)	CT (Mean)	CT (Individual)	CT (Mean)		
Control (C)	1	16.54	17.43	20.65	20.31	-	-
	2	17.20		20.73		-	
	3	18.55		19.83		-	
	4	17.45		20.02		-	
Treated (B)	5	17.00	16.85	19.76	19.50	0.9202	0.8526
	6	16.92		20.43		1.5476	
	7	17.23		19.56		0.6830	
	8	16.23		18.25		0.5510	

Table (10) Cycle threshold (CT) and Fold change of *sipC* virulence gene

Groups	Sample	*sipC*		*16S rRNA*		Fold change to control	Fold change to control (Collective)
		CT (Individual)	CT (Mean)	CT (Individual)	CT (Mean)		
Control (C)	1	19.14	18.78	20.65	20.31	-	-
	2	19.04		20.73		-	
	3	18.99		19.83		-	
	4	17.96		20.02		-	
Treated (B)	5	18.99	17.98	19.76	19.50	0.5905	0.7738
	6	17.92		20.43		2.1735	
	7	18.79		19.56		0.50	
	8	16.23		18.25		0.5586	

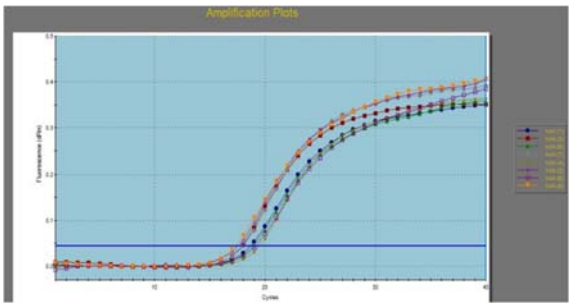

Figure (1) *hil*A virulence gene expression of *S. typhimurium* by SYBR green RT-PCR

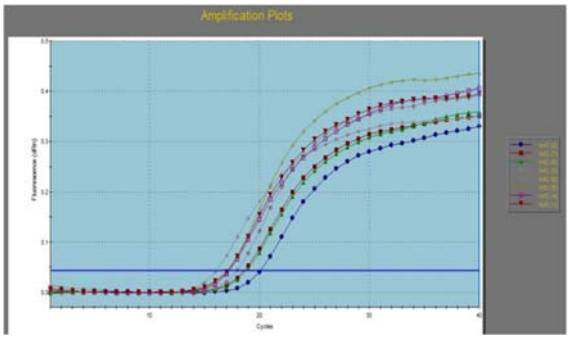

Figure (2) *hil*C virulence gene expression of *S.typhimurium* by SYBR green RT-PCR

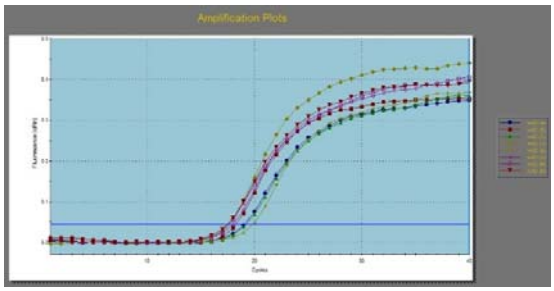

Figure (3) *hil*D virulence gene expression of *S.typhimurium* by SYBR green RT-PCR

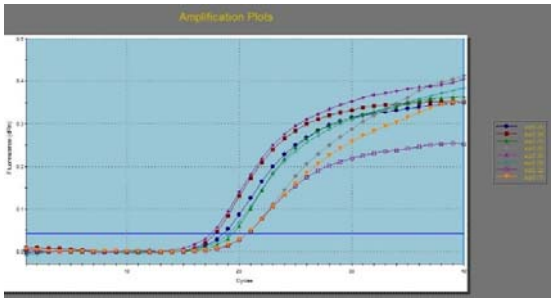

Figure (4) *sipC* virulence gene expression of *S. typhimurium* by SYBR green RT-PCR

$1.2x10^5$ CFU/g in the positive control group while in group treated with probiotic was $1.2x10^3$ CFU/g indicating significant decrease in Salmonella cell count that observed by (Akbari et al., 2008) who reported that there are several strategies currently exist for control of *Salmonella enterica* serovar Typhimurium colonization in the chicken intestine, among which the use of probiotics is of note.

Additionally, mortality rates in chicks of positive control group were 40%, 44.50% and 50% in 7, 14 and 21 day old respectively, while chicks treated with probiotic and challenged by *Salmonella typhimurium* the mortality rates were 6.70%, 7.20%and 4% at 7, 14 and 21 day old. These results agree with (Pairat et al., 2015) who showed that *Salmonella enteritidis* (SE) challenged group without Lactobacillus treatment, the mortality was 100% by 7 day old. SE challenged group pre-treated with Lactobacillus exhibited a significantly higher survival rate at 1, 2, and 7 days post treatment. This may be attributed to the low expression of cytokines stimulating function of neutrophils of 1-day old chicks when compared to older chicks (Chen et al., 2012).

The phagocytic percentages in chicks of the positive control group which challenged with *S. typhimurium* without probiotic treatment were 38.40%, 26% and 18% at 7, 14 and 21 day old while the phagocytic indexes (PI) were 1.0±0.10, 1.0±0.20 and 1.0±0.0 in 7, 14, and 21 day old respectively.

In chicks treated with probiotic and challenged by *S. typhimurium* the phagocytic percentages were 54.20%, 38.10% and 36.40% while the phagocytic indexes (PI) were 1.50±0.20, 1.10±0.08 and 1.10±0.04 in 7, 14, and 21 day old respectively. These results are getting along with (Pairat et al., 2015) who found that chick heterophils were activated after challenged with SE. although, some infected chick may show a heterophil engulfing SE before treatment with Lactobacillus but all heterophils engulfed only one SE per heterophil. SE challenged group and treated by Lactobacillus supplementation can significantly improve (P<0.05) phagocytosis and PI value at 24 and 48 h after treatment. Supplementation of Lactobacillus probiotic at 1 h post SE challenged can eradicate SE infection in broiler chicks by 24 h after treatment. This result is conflicting with the study of (Higgins et al., 2010) who reported Lactobacillus treatment after 24 h of Salmonella infection cannot reduce SE in chicks.

Multi-strains or high dose ($\geq 10^6$CFU/ml) of probiotic supplementation showed a positive effect on eradication of intestinal infection as similar report of (Khodadad et al., 2013)

Infection with *Salmonella enterica* seovar Typhimurium is initiated by entry of Salmonellae into intestinal epithelial cells. The expression of invasion genes is tightly regulated by environmental conditions, as well as by many bacterial factors including the key regulator *hil*A. One mechanism by which probiotics may antagonize intestinal pathogens is by down-regulating the gene expression which is confirmed by this study (Sigrid et al., 2005).

Salmonella species have a repertoire of virulence genes. Some of these are distributed on large genomic regions of 10-200 kbp known as Salmonella pathogenicity islands (SPIs) (Hensel, 2004), while others are not located on SPIs such as the chromosomally-encoded *stn* (Salmonella enterotoxin) gene (Prager et al., 1995 and Bäumler et al., 1996). Moreover, *hil*A encode a transcriptional regulator that activates the expression of invasion gene and it is also required for the regulation of the type III secretion system (Lostroh et al., 2000 and Lesnick et al., 2001).

S. typhimurium harbours 80 virulence genes. Four genes (*hil*A, *hil*C, *hil*D and *sip*C) which are located in SPI-1 and have important roles in the intestinal invasion of Salmonella were tested by RT-PCR after treatment with probiotic. The expression of *hil*A, *hil*C, *hil*D and *sip*C genes was significantly down-regulated, the obtained data revealed that CT mean of *hil*A for control group was 20.31 and for treated group was 17.68 with a fold change 0.77. CT mean of hilD for control group was 17.43 and for treated group was 16.85 with a fold change 0.85. CT mean sipC for control group was 18.78 and for treated group was 17.98 with a fold change 0.77. CT mean hilC for control group was 17.03 and for treated group was 16.72 with a fold change 0.74 which come in harmony with that obtained by (Yang et al., 2014).

Many virulence genes were higher expressed in *Salmonella enterica* subsp. *enterica* serovar typhimurium N-15(N-15) during co-culture with *Bifidobacterium thermophilum* RBL67 and such increased expression may enhance infection rate. However, this would contradict with previous results showing reduced invasion capacity of Salmonella to cells in presence of RBL67.

Invasion of Salmonella follows sequential expression of first flagellar genes, followed by genes encoded on SPI-1, and eventually type 1 fimbrial genes. Flagellar genes were repressed while genes of SPI-1 and type 1 fimbriae genes were activated in co-culture compared to mono-culture. This showed that the balance in virulence gene expression is disturbed by presence of RBL67. (Tanner et al., 2016).

Microarray analysis revealed gene expression differences among all treatment groups. At 12 h, 170 genes were expressed at significantly different levels ($P < 0.05$), with a minimum difference in expression of 1.2-fold. At 24 h, the number of differentially regulated genes with a minimum 1.2-fold change was 201. Pathway analysis revealed that at both time points, genes associated with the nuclear factor kappa B complex, as well as genes involved in apoptosis, were significantly regulated. Based on this analysis, probiotic-induced differential regulation of the genes growth arrest-specific 2 (GAS2) and cysteine-rich, angiogenic inducer, 61 (CYR61) may result in increased apoptosis in the cecae of chicks. Because Salmonella is an intracellular pathogen, we suggest that increased apoptosis may be a mechanism by which the probiotic culture reduces Salmonella infection (Higgins et al., 2011). These PCR results confirmed the result in the present study.

Enteric pathogens such as *Salmonella enterica* serovar Typhimurium and Enterohaemorrhagic Escherichia coli require an initial indispensable step of attachment or invasion of enterocytes before they can produce systemic disease and translocate to their target organs. Prevention of either of these steps will result in an a virulent state and limit their pathogenicity. In vitro tests demonstrated that molecules secreted by *Bifidobacterium bifidum* interfere with both attachment and invasion. The main regulatory genes controlling the virulence factors essential for these pathogenicity steps were efficiently down-regulated when treated with chromatographically separated *B. bifidum* cell free fractions as measured by reporter constructs and confirmed by RT-PCR. Moreover, the ability of both pathogens to colonize eukaryotic cells was significantly reduced, and the capacity of Salmonella to survive and multiply within macrophages was also diminished upon treatment with these bioactive molecules. These results indicate that probiotic Bifidobacteria strains may represent an effective alternative approach to control food-borne enteric pathogens. (Bayoumi et al., 2012) which corresponds closely to the present study.

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