



THE PATHOGEN INHIBITION EFFECTS OF PROBIOTICS AND PREBIOTICS AGAINST *SALMONELLA* SPP. IN CHICKEN

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Abstract

This current study investigated the effect of probiotics and prebiotics on the control of *Salmonella* spp. isolated from chicken. One hundred and eleven bacterial isolates were recovered from different chicken farms, and nine *Salmonella* isolates were detected with 8%. The serogroup analysis of nine *Salmonella* isolates showed three different groups identified as (4) *S. enteritidis*, (3) *S. typhimurium*, and (2) un-typed group. The positive-identified *Salmonella* was using PCR and genus-specific primer OMPCF (outer membrane protein reverse) with a target size of 204 bp. The results of *Salmonella enteritidis* with target size are also 304 bp and target 401 bp for *Salmonella typhimurium*. In addition, one hundred chicks were grouped into five groups (1, 2, 3, 4 and 5) containing 20 broiler chicks. The broilers of groups 1, 2, 3, 4 and 5 were orally inoculated with a dose of 1×10^8 CFU nalidixic acid-resistant *Salmonella enteritidis*. Experimental groups were as follows: Group 1: negative control, Group 2: positive control (infected chickens), Group 3: infected chickens and treated with *B. subtilis* probiotic, Group 4: infected chickens and treated with levoxyl prebiotic, Group 5: infected chickens and treated with panflor antibiotic. Results showed significantly increased survival percentage against those challenged with a virulent strain of nalidixic acid probiotics and prebiotics. The oral application significantly improved the survival percentage against challenges with a virulent strain of nalidixic acid-resistant *Salmonella* spp. The best result of *B. subtilis* was 1×10^8 which reduced the growth of the microorganism under study (*S. typhimurium* – *S. enteritidis*).

Key words: *Salmonella* spp., control, chicken, probiotics, prebiotics

Salmonella enterica represents the most pathogenic species. It can be transmitted to humans and the farm-to-fork continuum, through contaminated foods of animal origin (Jajere, 2019). Additionally, the incredibly greater part of human infections due to non-*enterica* subspecies is identified with a past discouragement in a vulnerable framework. Accordingly, it is suggested that these subspecies are only opportunistic infections. This bacterium has demonstrated persistence for long-term life in dry circumstances while not producing spores. *Salmonella* remained alive for several years in low-moisture products (Keller et al., 2013; Abdel-Shafi et al., 2014; Lamas et al., 2018).

Salmonellosis is a zoonotic disease of national and international importance. *Salmonella* infection in poultry usually causes a variety of acute and chronic diseases. The diseases have been associated with significant economic losses to poultry producers (Gast, 2003; Enan et al., 2013, 2014 a, b, c, 2018). According to the preliminary report from FoodNet on the incidence of infection with food-borne pathogens, the ongoing efforts to reduce cases of salmonellosis associated with consuming contaminated meat, poultry product and other foods are showing success. This report indicates that fewer cul-

tures of raw broiler chicken samples yielded *Salmonella* in 2009 (7.2%) than in 2006 (11.4%) (CDC FoodNet Report, 2009; Sanders, 2017).

Lilly and Stillwell (1965) described the growth-promoting factors produced by microorganisms and were the first to introduce the term “probiotic”. Crawford (1979) defined probiotics as a culture of specific living microorganisms, primarily *Lactobacillus* spp., which implants in the organisms and ensures the rapid and practical establishment of beneficial intestinal populations. In addition, Zhang et al. (2021) investigated the effects of commercial probiotic supplementation to water on broiler chicks’ performance, carcass traits, immune function, and antioxidant capacity. Authors found that probiotic additives positively impact body weight, average daily feed intake (ADFI), and average daily weight gain for female chicks. Similar results were reported by Abd El-Hack et al. (2017, 2018, 2019), Mahrose et al. (2019) and Othman et al. (2019). In contrast, these probiotics significantly reduced ADFI and the feed conversion ratio of male chicks. The present study aimed to investigate the efficacy of probiotics and prebiotics in controlling *Salmonella* spp. isolated from chicken.

Material and methods

Experimental chicks

Two hundred and thirty chicks, either freshly dead or moribund, aged 1–40 days and of different breeds (Balady, broilers and Saso) were collected from different localities in Sharkia Governorate, Egypt and subjected to clinical and postmortem examination. Specimens from the liver, lung, kidney, heart and yolk sac were aseptically collected and subjected to bacterial isolation and identification.

The commercial probiotic and prebiotic

Lypholac: The commercial probiotic produced by Microbiotech, USA containing *Lactobacillus acidophilus*, 1×10^8 CFU/g.

Levoxyl: The commercial prebiotic produced by New Feed Team (NFT), Italy, contains mannoooligosaccharide and betaglucan.

Bacteriological media

The media contained nutrient agar medium (Oxoid, CMS), buffered peptone water (Difco), Rappaport-Vassiliadis Soy Peptone (RVS) Broth (Merck), MacConkey's agar (Oxoid, CM7), Christensen's urea agar base medium (Difco), Muller-Hinton broth (Oxoid), and Muller-Hinton agar (Oxoid code: CM0337).

Reagents of API 20 E: API NaCl 0.85% medium, 5 ml (Ref. 20 230) or API suspension medium, 5 ml (Ref. 20 150).

Materials used for PCR

Mini Kit – Catalog no. 51304

The contents of the kit: Buffer AL – Buffer ATL – Buffer AW1 (concentrate).

Buffer AW2 (concentrate) – Buffer AE – QIAGEN® protease.

Protease solvent – Proteinase K – Silica membrane (QIAamp Mini Spin Columns), PBS (phosphate buffer saline), Ethanol (96–100%), DNA molecular weight marker, enzyme (2500 bp), primers used in PCR are OMPCF (outer membrane protein c) forward, OMPCF (outer membrane protein c) reverse, ENTf, ENTR, TYPHF, TYPHR.

Isolation and cultivation of *Salmonella* were carried out after (ISO 6579:2002), biotyping using API 20E (BioMerieux, 1992) for the identification of the isolates.

Isolation and identification of bacteria from commercial probiotic preparations were performed according to Collins et al. (1995). Probiotic bacterial strains were re-isolated from lypholac. The identification of growing colonies was carried out using colonial morphology and microscopic examination.

The evaluation of phagocytic activity was done according to Wilkinson (1977). The number of ingested cells represents the phagocytic percentage (p%).

In vitro growth inhibition of *Salmonella* was performed according to Piatek et al. (2020), Serological identification of *Salmonella* was carried out according to Kauffman–White scheme (Kauffman, 1972).

The extraction of DNA was done according to Oliveira et al. (2003). The yield of DNA was stored at -20°C till being used. The agarose gel electrophoresis was performed following Sambrook et al. (1989) method.

Preparation of *Salmonella typhimurium* and *Salmonella enteritidis* resistant strains was performed. The strains were sub-cultured in eight successive broth cultures containing increasing quantities of nalidixic acid, starting with 0.01 gm and ending by 1 g (nalidixic acid/litre).

Experimental design

This work aimed to study the efficacy of using some probiotics and prebiotics against *Salmonella* spp. isolates on experimental seven day-old broiler chicks. One hundred broiler chicks (one-day-old Avian-48) were grouped into five equal groups (1, 2, 3, 4 and 5) containing 20 broiler chicks. Chicks in groups 2, 3, 4 and 5 were orally inoculated with a dose of 1×10^8 CFU of nalidixic acid-resistant *S. enteritidis*. Chicks in group one remained a negative control. Chicks in group 2 served as a positive control. Chicks in group 3 were orally treated with *B. subtilis natto* with a dose of 1×10^8 CFU/bird for five days post-infection. Group 4 was orally treated with Levoxyl (prebiotic) 1 ml/liter in drinking water for five successive days post-infection. Group 5 was treated with panflor 1 ml/liter for five days post-infection. Panflor is a broad-spectrum antibiotic composed of fluorimicol. Broilers were reared separately on floor during the experimental period (6 weeks). Clinical observation of the infected chicks was carried out to record morbidity and gross lesion. Re-isolation trails of inoculated pathogens were performed. The total erythrocytes and total leukocytes were counted according to Schalm et al. (1975).

Blood sampling

The first sample was collected without adding an anticoagulant for serum separation. The second sample was collected with EDTA to estimate the total erythrocytic, leukocytic count and different leukocytic counts (DLC).

Statistical analysis

According to Tamhane and Dunlop (2000), the obtained data were statistically analyzed.

Results

Incidence of *Salmonella* spp. among chicken farms

Suspected *Salmonella* was detected with an incidence of 8.1% of farms. This was an incidence of farm infection of 20.5% (Table 1).

Table 1. Incidence of infection in tested farms and samples

Examined farm	Total samples	+ ve sample	% of <i>Salmonella</i> spp.	Total farms	% of farm infection
Abo Hamad	11	2	18	4	25
Abo Kaber	15	1	6.6	3	33.3
Zagazig	15	1	6.6	5	20
Kanayat	13	1	7.6	3	33.3
DearbNegm	15	2	13.3	5	20
El-Salhia	14	1	7.14	6	16.6
Fakos	13	1	7.60	5	20
Menea El-Kamh	15	—	0	3	0
Total	111	9	8.1	34	20.5

Figure 1. Biochemical identification of *Salmonella* isolates using API 20 E kits showing excellent *Salmonella* identification (seven-digit code number: 6704752 id%: 99.9 T index: 0.97)

Identification of *Salmonella* isolates from chicken

API 20 E kits

API 20 E protocol was carried out on nine randomly selected isolates. API 20 E results were parallel with the conventional biochemical identification results for these nine *Salmonella* isolates as both identified the isolates as *Salmonella*. API 20 E revealed different 7-digit profile numbers interpreted through API 20 E analytical profile index (Ref. 20190). Profile 6704752 was the most prevalent one, referring to 4 isolates (Figure 1).

Serotyping of tested *Salmonella* isolates from examined chickens

The serogroup analysis of the nine tested *Salmonella* showed that three groups were identified: *S. enteritidis*, *S. typhimurium* and un-typed. *S. enteritidis* and *S. typhimurium* were the most prevalent serogroups, with 44.4% and 33.3%, respectively. Another serogroup was un-typed with 22.2% (Table 2).

Table 2. Frequency occurrence of different *Salmonella* serogroups isolated from infected chickens

Identified strains	Antigen structure		No strain	Frequency (%)
	O	H		
<i>S. enteritidis</i>	3, 4, 5, 9	c/h: 1.4	4	44.4
<i>S. typhimurium</i>	1, 6, 7	i:1.2	3	33.3
<i>S. spp.</i>	2, 8	—	2	22.2

Molecular identification of tested *Salmonella*

Multiplex PCR was used for genotyping of nine biochemically and serologically identified *Salmonella* isolates. Primers were specific to *Salmonella*, OMPCF (outer membrane protein C forward) and OMPCR (outer membrane protein C reverse), with a target PCR amplicon size of 240 Pb.

The other pairs of primers were specific for the most common *Salmonella* serovars including *S. typhimurium* (target size 401 Pb) and *S. enteritidis* (target size 304 Pb). The positive identified *Salmonella* using PCR and genus-specific primers OMPCF and OMPCR with target PCR amplicon size of 204 bp are presented in Table 3 and Figure 2.

Table 3. Antibiotic sensitivity pattern of various *Salmonella* isolates

Name of antibiotic	Sensitivity	Resistance
Floropenicol	100%	0%
Ciprofloxacin	88.8%	11.2%
Gentamycin	55.5%	43.5%
Amoxycelline	44.4%	55.6%
Kanamycin	33.3%	66.7%
Trimethoprime	33.3%	66.7%
Neomycin	0%	100%
Spectinomycin	0%	100%

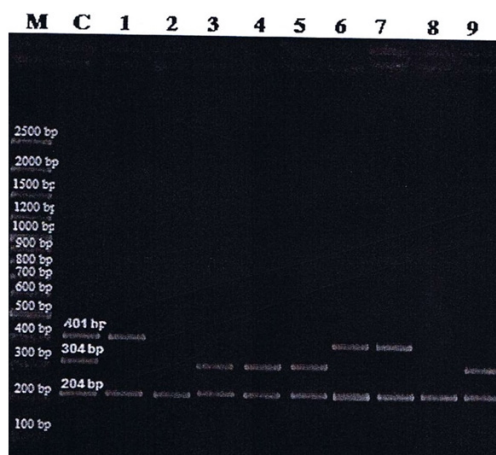


Figure 2. Agarose gel electrophoresis for PCR results of serotyping of all isolates for detecting *Salmonella* genus by a molecular method using PCR techniques

Also, the results of *Salmonella enterica* were positive by using PCR and species-specific primers (ENTF and ENTR) with a target size of 304 bp for detecting *S. enteritidis* and primers (TYPHF and TYPHR) with a target size of 401 bp for detecting *S. typhimurium* are presented in Figure 2.

Lane M.: 2500 base-pair size marker ladder; Lane c: positive referring to *Salmonella* spp. (204 bp), *S. enteritidis* (304 bp), *S. typhimurium* (401 bp). Lane 1, 6, 7 *S. typhimurium* with amplicon size (304 bp). Lane 3, 4, 5, 9: refers to the species *S. enteritidis*. Lane 1, 6, 7: refers to *S. typhimurium*. Lane 2, 8: to the un-typed *Salmonella*, Lane 3, 4, 5, 9 refers to *S. enteritidis* with amplicon size (304 bp). All Lanes 1–9 showed +ve amplicon and molecular size 204 bp for *Salmonella* spp.

PCR for detection of the most common virulence genes in different *Salmonella* isolates

Using five sets of primers, PCR was used to detect virulence genes that proved to play an important role in the virulence of *Salmonella* isolates (AvrA, Stn, Mgtc, SopB, BcfC). It was applied to five different *Salmonella* isolates. The PCR products of the obtained virulence genes gave characteristic bands at 600, 422, 677, 517 and 467 bp for Stn, Avra, Mgtc, SopB and BcfC virulence genes, respectively (Figures 3–7).

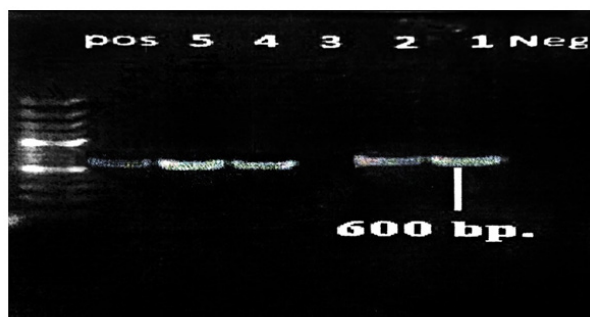


Figure 3. Agarose gel electrophoresis of *Salmonella* DNA products of Stn virulence gene lanes 1, 3, 4 and 5. *Salmonella* field isolates had Stn virulence gene

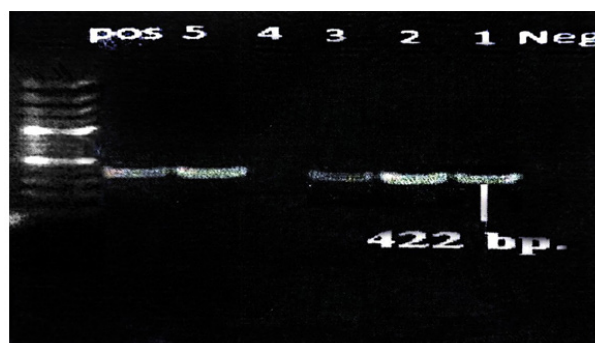


Figure 4. Agarose gel electrophoresis of *Salmonella* DNA products of AvrA virulence gene lanes 1, 2, 3 and 5. *Salmonella* field isolates had AvrA virulence genes



Figure 5. Agarose gel electrophoresis of *Salmonella* DNA products of Mgtc virulence gene lanes 1, 2, 3, 4 and 5. *Salmonella* field had Mgtc virulence gene

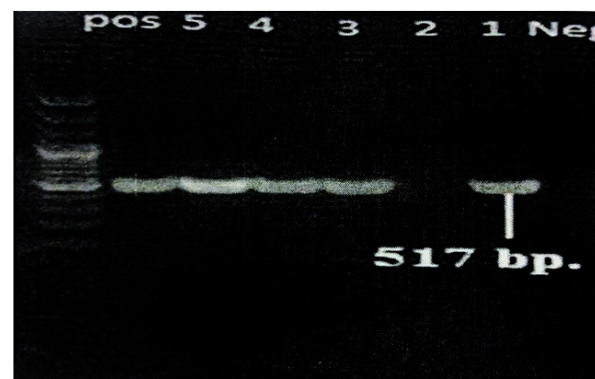


Figure 6. Agarose gel electrophoresis of *Salmonella* DNA products of SopB virulence gene lanes 1, 3, 4 and 5. *Salmonella* field had SopB virulence gene

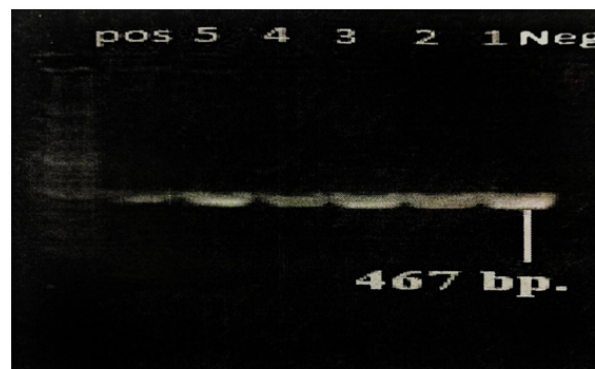


Figure 7. Agarose gel electrophoresis of *Salmonella* DNA products of BcfC virulence gene lanes 1, 2, 3, 4 and 5. *Salmonella* field isolates had the BcfC virulence gene

Antibiotic sensitivity

The nine isolates of *Salmonella* showed that isolates were highly sensitive to Florphenicol (100%), Ciprofloxacin (88.8%), Gentamycin (55.5%), Amoxycelline (44.4%), Kanamycin (33.3%), Trimethoprim (33.3%), Neomycin (0%) and Spectinomycin (0%), (Table 3).

Bio-control of *Salmonella* (*typhimurium* and *enteritidis*) by *B. subtilis*

Results of the *in vitro* growth showed that the best result of *Bacillus subtilis* was 1×10^8 , which reduced the growth of microorganisms under study (*S. typhimurium* and *S. enteritidis*). Probiotics were used to determine the antimicrobial activity and produced microbial inhibition zone in *Salmonella* spp. (Table 4).

Table 4. The antimicrobial activity of probiotic on produced microbial inhibition zone in *Salmonella* spp.

Concentration of <i>B. subtilis</i>	Zone inhibition of <i>S. typhimurium</i>	Zone inhibition of <i>S. enteritidis</i>
1×10^{10}	2.3 cm	2.1 cm
1×10^8	2.3 cm	2.1 cm
1×10^7	2.0 cm	1.95 cm

– Performance parameters (body weight and weight gain) at 28 days post-infection in group 2 broilers showed a significantly lower body weight than the control (Table 5).

– Broilers treated with probiotics and prebiotics showed a significant increase ($P < 0.05$) in total erythrocytic and total leukocytic counts all over the experimental period compared with the infected broilers only (Table 6 and Figure 8).

Table 5. The effect of probiotic and prebiotic on mortality and performance parameters

Group	No. of broilers	Dose/ Broilers	Route of administration	Mortality	Reisolation	Body weight gain		
						7 days PI	14 days PI	28 days PI
Control group (healthy chickens)	20	0	–	0	0	380± 40 a	840± 112 a	1956± 140 a
Infected chicken group	20	1×10^8	Orally	8/20	7/20	142± 33 e	360± 85 d	1540± 160
Infected group and treated with <i>B. subtilis</i> (probiotic)	20	1×10^8	Orally	1/20	0/20	220± 52 b	640± 69 b	1970± 80 a
Infected group and treated with levoxyl (prebiotic)	20	1×10^8	Orally	2/20	1/20	283± 39 c	674± 110 b	1940± 70 a
Infected group and treated with panflor (antibiotic)	20	1×10^8	Orally	2/20	2/20	253± 60 d	549± 120 c	1820± 90 b

PI: post infection.

a, b, c, d, e – means in the same column with different letters are significantly different ($P < 0.05$).

Table 6. Effect of probiotic and prebiotic given in drinking water for five successive days on total erythrocyte and total leukocyte count

Parameter Groups	Total RBC counts		Total WBC count	
	7 day PI	14 day PI	7day PI	14 day PI
Control group (healthy chickens)	4.46±0.09 a	4.5±0.07 a	4.71±0.08 a	4.81±0.01 b
Infected chicken group	3.05±0.02 c	3.22±0.03 d	3.81±0.01 c	4.81±0.12 c
Infected group and treated with <i>B. subtilis</i> (probiotic)	3.05±0.02 b	4.04±0.05 b	6.5±0.14 a	5.92±0.01 a
Infected group and treated with levoxyl (prebiotic)	3.48±0.01 b	3.57±0.08 c	4.8±0.02 b	5.1±0.12 a
Infected group and treated with panflor (antibiotic)	3.12±0.07 c	3.42±0.01 c	3.89±0.02 c	3.91±0.12 c

RBC: Red blood cells, WBC: White blood cells, PI: Post infection.

a, b, c, d – means in the same column with different letters are significantly different ($P < 0.05$).

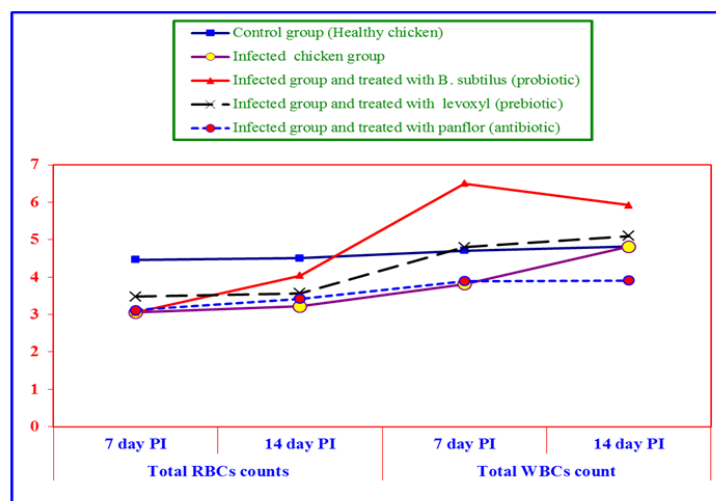


Figure 8. Impacts on total erythrocytic and total leukocytic count are all over the experimental period compared with infected broilers only

– Broilers treated with probiotics and prebiotics showed a significant increase ($P \leq 0.05$) in hemoglobin ratio compared to the untreated broilers and those infected with *Salmonella* spp. (Table 7).

Table 7. Effect of probiotic and prebiotic given in drinking water for five successive days on hemoglobin ratio (Hb) in healthy and experimentally infected broilers with *Salmonella* organism

Groups	Parameter	Hemoglobin ratio (Hb)	
		7 days PI	14 days PI
Control group (healthy chickens)		14.15±0.04 a	14.17±0.02 a
Infected chicken group		9.4±0.07 c	10.39±0.14 c
Infected group and treated with <i>B. subtilis</i> (probiotic)		9.4±0.07 b	13.85±0.14 b
Infected group and treated with levoxyl (prebiotic)		10.8±0.17 b	13.46±0.11 b
Infected group and treated with panflor (antibiotic)		9.55±0.08 c	13.12±0.14 b

PI: Post infection.

a, b, c – means in the same column with different letters are significantly different ($P < 0.05$).

Discussion

In this study, nine pooled samples from poultry aged 1 to 40 days were suspected to be *Salmonella*-positive out of 111 collected pooled samples with an incidence of 8.1% in seven positive farms from a total of 34 farms with a farm infection rate of 20.5%. Microbiological, biochemical and serological examinations confirmed that all suspected isolates belong to the genus *Salmonella*. Also, PCR examination confirmed that all suspected isolates belong to the genus *Salmonella* by using specific primers (Alvarez et al., 2004).

Furthermore, multiplex PCR analysis identified four isolates as *S. enteric* serovar *enteritidis*, three as *S. enterica* serovar *Typhimurium* and two *Salmonellae* un-typed (Alvarez et al., 2004). For the effect of *B. subtilis* on microbial activity, it is noticeable that the use of *B. subtilis* reduced the growth of selected microorganisms (*Salmonella* spp.). This result agrees with those of Khodary and El-Sayed (1997).

In the present study, PCR was used to detect five virulence genes that proved to play a role in the virulence of *Salmonella* genes (Stn, AvrA, Mgtc, SopB, and Bcf). Two isolates had five virulence genes, while three isolates had four virulence genes (Stn, Mgtc, SopB and Bcf). The PCR products of the obtained virulence genes gave characteristic bands at 422, 600, 677, 517, and 467 pb for AvrA, Stn, Mgtc, SopB and Bcf virulence genes, respectively. These results agree with those obtained by Zou et al. (2012), who found that all the 425 isolates tested by PCR were positive for the presence of virulence genes Mg2⁺ transportation system (Mgtc), production enterotoxin (Stn) and *Salmonella* outer protein production (SopB). *Salmonella* on all clinical isolates gave us highlights on the role of this gene in the production of enterotoxin.

Also, MurugKar et al. (2003) confirmed the presence of the Stn gene in all *Salmonella* serotypes and reported that the Stn gene obtained a unique sequence to *Salmonella* strain field samples. The experimental infection of broiler chicks with *Salmonella* spp. showed clinical signs after the incubation period of 36 hours. Similar results were obtained by Reda et al. (2019).

The clinical signs were the information of depression, weakness, off food, ocular discharge, closed eye and greenish diarrhea in the two infected groups. The mortality rates of chicks of groups 2, 3, 4 and 5 were 40%, 5%, 10% and 10%, respectively. The inoculated nalidixic acid-resistant *Salmonella* percentage was high from the caecum, intestine and liver of orally infected chicks (0%, 35%, 0%, 5% and 10%, respectively). Also, Hui and Dus (2001) assured that the highest percentage of *Salmonella* may exist in the intestine and liver. Performance parameters (body weight and weight gain) at 28 days post-infection in group 2 broilers showed the lowest mean body weight, 1540 g, compared to the control group, 1950 g. While in groups 4 and 5, body weights were 1940 g and 1830 g, in group 3. These results are similar to those of Khodary and El-Sayed (1997).

The effect of probiotics and prebiotics after 35 days of treatment showed an increase in feed consumption compared to the control. This result agrees with Andreeva and Dimitrov (2002), who reported increased feed consumption for probiotics and prebiotics-treated groups concerning body weight gain. Also, Jin et al. (1997), Alagawany et al. (2016, 2018) and Soomro et al. (2019) observed good growth elevation with increased feed intake in birds treated with probiotics and prebiotics.

Conclusions

Probiotics and prebiotics are potential tools for reducing intestinal contamination caused by food-borne bacteria. They could be successfully used as nutritional tools in poultry feed to promote growth, modulate intestinal microflora and inhibit pathogens. Lactic acid-producing bacteria and *Bacillus subtilis* species have been recently used as commercial probiotics. So, probiotics and prebiotics could be used as antibiotic alternatives to improve poultry health and production. Based on our findings, the best result of *B. subtilis* was 1×10^8 which reduced the growth of the microorganism under study (*S. typhimurium* – *S. enteritidis*).

Author Contributions

Conceptualization: G.E., S.A., A.A., S.A.; methodology: A.A., S.A.; formal analysis: A.A., M.E.A.E.-H., S.A.; investigation: A.A.; writing – original draft preparation: M.E.A.E.-H., S.A.; writing – review and editing: M.E.A.E.-H., S.A.; visualization.

Ethical approval

Animal maintenance and care adhered to Zagazig University's criteria for the care and use of laboratory animals and those of the Egyptian Research Ethics Committee.

Data Availability Statement

The datasets generated for this study are available on request from the principal or corresponding authors.

Conflict of interest

The author declares no conflicts of interest.

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