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Reduction of Salmonellosis Using Competitive Exclusion Individually and in Combination with Organic Acids in the Experimental Broiler Chicks

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ABSTRACT

Salmonellosis in broiler chickens poses a serious danger to the global poultry sector. Recently, the increasing of antimicrobial resistance in poultry farms requires an alternatives approaches to overcome this problem. Therefore, the influences of competitive exclusion culture (CE) and organic acids as substitutes for antibiotics were investigated at this study. A total of 200 diseased broiler birds were collected from 50 farms located in Egypt's Dakahlia Governorate and examined for Salmonella infection. Salmonella was isolated at rate of 24% (12 isolates) and differentiated serologically into *S*. Typhimurium, *S*. Enteritidis, *S*. Santiago and *S*. Kentucky. The antimicrobial resistance to nalidixic acid, amoxicillin, Ampicillin/sulbactam, streptomycin and tetracycline was recorded at percentages of 100%, 100%, 83.3%, 75% and 75% respectively using disc diffusion method. The effectiveness of organic acids mixture and CE supplementation on *S*. Typhimurium (ST) was studied in vivo using 50 broiler chicks (one day old) (5 groups); Group (1) non treated, non infected group (negative control); group (2) challenged with ST (positive control); group (3) supplemented with CE then challenged with ST; group (4) supplemented with organic acids then challenged with ST; group (5) supplemented with both of and organic acids then challenged with ST. The recorded moratlies in group 2 was 20% and no mortalities were repored in the other experimental groups. No clinical signs were noticed in groups 1 and 5. In group 4, slight diarrhea was recorded at (4dp). The findings in this study showed that the supplementation with CE only/and with organic acids (groups 3 and 5 respectively) prevented ST colonization in chicks cecum. A reduction in ST cecal colonization was reported in group (4) when compared with the positive control (group 2). The supplementation of chicks with organic acids with CE prevented ST colonization in the cecum and improved the growth performance parameters. The findings of this study suggest that supplementating of both organic acids and CE in broiler farms could be a bio-protective and substitute solution for combating multidrug resistant Salmonella and improving growth performance in poultry farms.

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INTRODUCTION

Salmonella is considered as a serious zoonotic foodborne pathogen that occurs on poultry farms producition **(Shang et al. 2018).** Exposure to endemic strains is viewed as a significant danger to the global poultry industry **(El -Sharkawy et al. 2020).** The infected chicks exhibit high mortalities, diarrhea, dehydration and weakness. Postmortem lesions (PM) observed include hepatitis, omphalitis, typhlitis, pneumonia, peritonitis, synovitis and ophthalmitis **(Shivaprasad, 2000).** The increased resistance of Salmonella to antimicrobial agents has become a crucial global public health problem (**Sarker et al. 2021**).

Reduction of Salmonellosis in broiler chickens using effective and safe alternatives to antimicrobial agents is an important issue to minimize the emergence of drug-resistant serotypes **(Hu et al . 2023).** Competitive exclusion (CE) culture considered as a new method to avoid Salmonella infections in poultry **(Schneitz et al. 2016).** It relies on the ability of natural flora to prevent enteric infections from colonizing the intestine **(Jiratitipat et al. 2019).** Furthermore, organic acids have the ability to control Salmonellosis **(Koyuncu et al. 2013)** serveing as an alternative to antibiotics **(Menconi et al. 2013)** and prevent the harmful microbial populations through pH reduction **(Hajati, 2018),** induction of stress response, disruptions of cell membrane, formation of cell membranes pores **(ben Braïek & Smaoui. 2021)** and decreasing in the metabolism of amino acids and carbohydrates in **bacteria cells (Han et al. 2020).**

CE is designed to accelerate the intestinal tract colonization of young commercial birds and also repopulate the gastrointestinal tract after receiving antibiotics. The application of CE culture prepared from adult laying feces has been showed to reduces Salmonella infection **(Sterzo et al. 2005).** The using of CE product in broiler chickens provides a protection from Salmonella infection, improve the broiler performance and improve the intestinal histology **(Jiratitipat et al. 2019).** The treatment of young chicks with CE culture prevents Salmonella infection **(OIE, 2018).**

Bacteria with probiotic properties such as *Pediococcus acidilactic* (**Salehizadeh et al. 2020)** and *Bacillus Spp*. **(Hosseini et al. 2018)** have the ability to attach to intestinal epithelial cells and competitive eliminate of Salmonella colonization .

Organic acids have been reported by **Khan et al. (2022)** as antibacterial, immune potentiating, and growth promoters in broilers chickens. A research study conducted by **Hajati, (2018)** found that the using of organic acids improved the bird’s performance and health. Some organic acids like citric, acetic, propionic **(Van Immerseel et al. 2006),** lactic acid **(El Baaboua et al. 2018),** and formic acid **(Ricke et al. 2020)** have to be effective in reducing Salmonella.

MATERIALS and METHODS

Sample collection, preparation and identification

A total of 200 broiler chickens (from 23 to 35 days old) suspected of being infected with Salmonella were obtained from 50 farms located in Dakahlia Governorate, Egypt. Four birds were selected from each farm and underwent a postmortem examination. Samples from the spleen, cecum and liver were obtained under hygienic condition, labeled and immediately transported to Reference Laboratory for Veterinary Quality control on Poultry production (RLQP) (Gamasa Lab.) for the isolation and identification of Salmonella.

The collected internal organs from each bird were pooled together as single sample and Salmonella isolation and identification were conducted according to ISO 6579 (2017). The confirmed Salmonella isolates were then serotyped using somatic (O) and flagellar (H) antigens

Antimicrobial susceptibility testing was performed on the confirmed Salmonella isolates using the disc diffusion method **(Finegold and Martin, 1982)**. The following antimicrobial agents were used: nalidixic acid (30 µg), ciprofloxacin (5 µg), enerofloxacin (5 µg), norfloxacin (10 µg), amoxicillin (10µg), ampicillin/sulbactam (20µg), neomycin (30µg), streptomycin (10µg), oxytetracycline (30µg), and sulfamethoxazole/ trimethoprim (25µg). The diameter of the inhibition zones diameters was interpreted according to **CLSI (2016)** guidelines.

In vivo assay of competitive exclusion and organic acid using in chicks

Ethical approval:

Animal experimental design was reviewed and approved according to the legally protocol of the Animal Health Research Institute (AHRI), Giza, Egypt.

Experimental chicks:

A total of 50 one day old broiler Salmonella free chicks obtained from a commercial hatchery in Dakahlia Governorate were placed in separated cages with a biosecurity level- two (BSL-2) and prored to be free from Salmonella infection. Chicks were divided into 5 groups (ten chicks per group). Cloacal swabs were collected from the chicks and proved to be free from Salmonella infection.

Salmonella challenge:

A multidrug resistant *S*. Typhimurium (ST) was selected from the current study. One colony of ST was inoculated onto 10 ml buffered peptone broth water and then icubated at 37°C for 24 hours. The inoculated ST broth was serially diluted and 0.1 ml containing 10^7 CFU was selected as challenge dose according to Stern et al.(2001).

Preparation of competitive exclusion culture (CE)

1-*Bacillus* spp:

Twenty pooled samples of freshly collected fecal matter were obtained from 10 Salmonella free farms in Egypt's Dakahlia Governorate Fecal aliquots were diluted 1:10 (weight: volume) in buffered peptone water and subjected to vigorously vortexing to insure an even suspension. *Bacillus* spp. Was then prepared according to the method descriped by **Barbosa et al. (2005);** Briefly**,** the suspension was incubated for 20 minutes at 65°C, Subsequent plating of 0.1 ml aliquots of appropriate 10-fold serial dilutions in buffered peptone water (up to 10^5) was done aerobically at 37 ^{0} C for 24 hours on nutrient agar plates that support germination. Colonies were picked and purified by re

-streaking on agar plates with the same media. Growth of pure isolates on Difco sporulation medium (DSM) plates results in the production of spores. The isolates were stored in Difco heart-infusion broth with 30% glycerol. at -80°C.

The antimicrobial activity of *Bacillus* spp. was examined according to **chaiyawan et al. (2010)** with modifications as follow; 5 ml of overnight culture of spore forming isolates were spotted on isolation media (3 spots/ plate) and then incubated for 24 hours at 37^{o}C. ST overnight culture was prepared to a concentration of 10^7 CFU. Then, The mixture of 0.1 ml of ST inoculum and 5 ml of isolation medium containing 0.7% agar was applied on top of the chloroform-treated spots. The spore-forming isolate that displayed an inhibition zone around the spot produced antimicrobial activity.

2- ***Pediococcus* spp.:**

The fecal suspension was prepared as previously described, and then *Pediococcus* spp. was isolated and identified according to **Noohi et al. (2016);** the fecal suspension was serially diluted in phosphate buffered saline and plated onto MRS agar, then incubated for 48 hours at 37°C under microaerophillic conditions. The isolates were tested to resist low pH and bile salts, and underwent catalase and oxidase tests.

As mentioned by **Noohi et al. (2016)** with some modification; Pediococcus isolates were tested for their antimicrobial activity using agar spot test; Suspensions of approximately 10^7 CFU/ml of ST were inoculated in Muller Hinton agar plates and then, 100 µl of Pediococcus spp. were separately added to the agar well. The plates were incubated for 24 hours and positive inhibition zone was defined as one milimeter or more (The test replicated twice).

Bacillus and *Pediococcus* isolates were prepared separately with a dose of ($1x10^7$ CFU/ ml) according to **Jiratitipat et al. (2019)** and then 1ml applied to each one liter of drinking water on the fourth day of age for three consecutive days in experimental treated groups .

The organic acid product:

Mixture of organic acids product **(Pro acid, Batch no. 8085 - Amazon Vet. Company, Egypt)** was applied to the experimental chicks. The composition of the product for one liter was: formic acid (150gm), propionic acid (100gm), lactic acid (100gm), citric acid (25gm), phosphoric acid (150gm) and sorbitol (50gm). One mililitre of organic acids product was added to each one liter of the drinking water at the 4^{th} day of age for 3 consecutive days in experimental treated groups.

Experimental design:

Group (1) non treated, non infected group (negative control); group (2) challenged with ST (*Salmonella* Typhimurium) orally with a dose of 0.1 ml containing 10^7 CFU at 7^{th} day of age (positive control); group (3) supplemented with CE wih a dose of one ml from each of *Bacillus* and *Pediococcus* spp. ($1x10^7$ CFU/ ml) that was added to one liter of the drinking water at the 4^{th} day of age for 3 consecutive days then challenged with ST orally with a dose of 0.1 ml containing 10^7 CFU on the 7^{th}

day of age; group (4) supplemented with organic acids (one ml was added to one liter of the drinking water at the 4^{th} day of age for 3 consecutive days) then challenged with ST (0.1 ml containing 10^7 CFU at 7^{th} day of age); group (5) supplemented with both of CE (*Bacillus* and *Pediococcus* spp. ($1x10^7$ CFU/ ml) that was added to one liter of the drinking water on the 4^{th} day of age for 3 consecutive days) and organic acids (one ml was added to one liter of the drinking water on the 4^{th} day of age for 3 consecutive days) then challenged with ST (0.1 ml containing 10^7 CFU at 7^{th} day of age).

The chicks were observed twice daily and the clinical signs, PM lesions and mortalities were recorded allover the experimental period. Dead chick was subjected to PM examinations, Salmonella re-isolation and enumeration. Performance parameters including feed intake, feed conversion ratio, and body weight gain (BWG) were noted. At the end of experiment all chicks were euthanized and samples from cecum were taken aseptically then pH measurement of cecal contents and ST colonization were evaluated according **to Milbradt et al. (2017) and ISO 6579(2017).**

Statistical Analysis

The cecal pH and the growth performance parameters were analysed using SPSS software (version 20). One-Way ANOVA test was used to calculate the mean values, standard errors, and the significance degree between groups. Data were expressed mean with standard error (mean ± SE) as significant at $P < 0.05$.

RESULTS

Salmonella isolation, serotyping and antimicrobial susceptibility

Out of the 50 diseased broiler farms examined in Dakahlia Governorate (Egypt), 12 farms (24%) were positive for Salmonella isolation. Isolates of Salmonella were serologically differentiated resulting in the identification of four serotypes *S*. Typhimurium (6), *S*. Enteritidis (3), *S*. Santiago (2) and *S*. Kentucky (1). The most prevalent serotype was *S*. Typhimurium.

Table (1) displays the result of the disc diffusion tests. It was found that all of the examined isolates were completely resistant to nalidixic acid and amoxicillin (100%). Ampicillin/ sulbactam, streptomycin and tetracycline displayed high resistance rates with percentages of (100%), (100%) , (83.3%), (75%) and (75%) respectively.

Table 1. Antimicrobial susceptibility of the isolated Salmonellae

Antimicrobial agent	**Salmonella (12 isolates)**	
	Sensitive NO. (%)*	**Resistant NO. (%)***
nalidixic acid	0 (0%)	12 (100%)
Ciprofloxacin	4 (33.3%)	8 (66.7%)
Enerofloxacin	6 (50%)	6 (50%)
Norfloxacin	4 (33.3%)	8 (66.7%)
Amoxicillin	0 (0%)	12 (100%)
ampicillin/sulbactam	2 (16.7%)	10 (83.3%)
streptomycin	3 (25%)	9 (75%)
Neomycin	6 (50%)	6 (50%)
Tetracycline	3 (25%)	9 (75%)
Sulfamrthoxazole-Trimethoprim	7 (58.3%)	5 (41.7%)

*Percentages calculated by dividing the number of isolates that revealed resistance or sensitivity on total number of isolates.

In vivo evaluation of competitive exclusion and organic acids application:

Clinical symptoms and PM lesions were evaluated in experimental chicks

The chicks were monitored twice daily throughout the duration of the experiment in order to track any changes. The Clinical symptoms, mortalities, and PM lesions were recorded as follow:

With exception of the positive control (group 2), which experienced a mortality rate of 20%, no deathes were observed in the experimental groups. During the experimental period the chicks exhibit clinical signs such as weakness, dehydration, decrease feed intake, pasted vent and diarrhea.These symptoms first appeared on 3^{rd} day post challenge (3dpc) in group 2. Few chicks in group 4 showed slight signs of diarrhea that started at 4 days post-inoculation (4dp) and stopped at 5 days post-inoculation (5dp). No clinical signs were observed in groups 1 and 5.

The recorded post- mortum lesions showed congested liver, enlarged gall bladder, omphalitis, peritonitis, and enlarged cecum group 2. Chicks in groups 4 showed no post- mortum lesions except for 2 chicks that showed enlarged cecum. Chicks in group 1, 3 and 5 showed no post- mortum lesions.

Salmonella recovery from cecum of the experimental chicks

The cecum of the chicks under experiment was subjected to Salmonella Typhimurum (ST) isolation detection. As previously mentioned; no colonization was recorded in groups 1, 3 and 5. Chicks in group 4 which supplemented with organic acid alone showed reduction in the number of viable Salmonella cells rather than eliminating the organism (2.5 x10^1 CFU/gm). However in group 2, the ST enumeration in cecum of chicks ranged from (4.4x10^6 CFU/gm) (**table, 2**).

Table 2. the mean count of ST (colony forming units/g) in cecum of chicks under experiment

Group no. / Item	Group 1	Group 2	Group 3	Group 4	Group 5
Mean of ST (CFU/g)	0	$4.4x10^6$	0	$2.5x10^1$	0

Measurement of Cecal Contents pH

The pH of cecal contents was measured at the ending of experiment (12th day of age). The mean values with standard error (mean± standard error) of pH was 6.65 ±0.016 in group (1) (control negative), 6.78±0. 013 in group 2 (control positive), 6.68±0.013 in group 3 (supplemented with CE), 6.5±0.00 in group 4 (supplemented with organic acids mixture) and 6.5 in group 5 (supplemented with organic acids mixture and CE) (**table, 3**). The cecal pH in groups 3, 4 and 5 was significant lower when compared with group 2.

Table 3. The Cecal Contents pH Measurement (mean± standard error)

Group no. / Item	Group 1	Group 2	Group 3	Group 4	Group 5
Cecal pH	$6.65±0.017^b$	$6.78±0.013^a$	$6.68±0.013^b$	$6.5±0.00^c$	$6.5±0.00^c$

Mean values expressed as mean ± SEM (mean± standard error). Means with the different letters (a-c) show significant differences between groups at $P< 0.05$.

Effect of organic acids mixture and CE on chick's growth performance

Parameters of growth performance including body weight ,feed intake and feed conversation ratio (BWG), (FI), and (FCR) were recorded at 6th day of age before ST challenge and shown **in table (4);** The mean values (mean± standard error) of BWG recorded in chicks of groups 3, 4 and 5 supplemented with CE, organic acids and CE with organic acids respectively, were slightly higher than groups 1 and 2 (not supplemented with CE or organic acids). The body weight gain and FI in groups 3, 4 and 5 was non significantly different. No differences were recorded in the FCR between group 3 and 4 meanwhile the FCR was slightly improved in group 5 when compared with other groups.

Parameters of growth performance including BWG, FI, and FCR were also recorded at the 12th day of age challenge and shown **in table 4.** The mean values of BWG recorded in chicks of groups3, 4 and 5 were significant higher than the control groups 1 and 2. The BWG in group 5 was significant higher than groups 3 and 4. Group 1 showed a lower (BWG and FCR) but higher (FI) when compared to groups 3, 4 and 5. Group 2 exhibited very poore growth performance when compared with the other groups. However, the application of organic acid with CE in group (5) improved the FCR when compared with other groups.

In conclusion, both BWG and FCR in group 5 at the end of the experiment were significantly improved when compared with other groups.These findings suggest, that the application of CE with organic acids not only prevents ST colonization, but also had the ability to enhance growth performance parameters.

Table 4. chicks' average body weight, weight gain, and feed conversion rate

Parameter Group No.	at 6th days of age body weight gain (BWG) (g)	feed intake (FI) (g)	feed conversion ratio (FCR)	at 12th days of age body weight gain (BWG) (g)	feed intake (FI) (g)	feed conversion ratio (FCR)
Group 1	127±1.13^{b}*	106.1±0.74^{ab}	0.64±0.006^{a}	344.8±3.23^{c}	394.5±1.76^{a}	1.02±0.006^{b}
Group 2	128.5±0.62^{b}	107.1±0.64^{a}	0.64±0.004^{a}	156.7±3.81^{d}	286.4±6.82^{c}	1.46±0.02^{a}
Group 3	133±0.79^{a}	104.3±0.68^{c}	0.60±0.003^{b}	356.8±1.78^{b}	374.4±0.722^{b}	0.95±0.01^{c}
Group 4	133±0.33^{a}	104±0.6^{c}	0.60±0.002^{b}	354.3±2.01^{b}	376.9±1.18^{b}	0.96±0.005^{c}
Group 5	134.6±0.62^{a}	102±0.81^{c}	0.59±0.003^{c}	372.8±0.81^{a}	369.4±0.56^{b}	0.9±0.001^{d}

* Mean values expressed as mean ± SEM (mean± standard error). Means with the different letters (a-d) show significant differences between groups at $P< 0.05$.

DISCUSSION

In this study 12 broiler farms out of 50 farms in Dakahlia Governorate, Egypt were found to be positive for Salmonella isolation (24%). These results were remarkably similar to those of **Soliman et al. (2018),** who isolated Salmonella from broiler flocks with a percentage of (20.24%) in Egypt. However, higher percentages were recoreded **by ElSharkawy et al. (2017) with 38% and Islam et al. (2016)** with a percentage of 66.67% in Bangladesh.

However, **ElSharkawy et al. (2017)** recordrd higher percentage (38%) and **Islam et al. (2016)** in Bangladesh. isolated Salmonella with a percentage (66.67%) . Meanwhile, lower percentages were reported by **Hassan et al. (2018) and Abd El-Ghany et al. (2012)** who isolated Salmonellae from chicken farms in Assiut and Kalubia in Egypt, with a percentages of 5.2% and 7.03% respectively. The geographic location, the seasonal variation and the breeds of the examined broiler may have contributed to the variety of Salmonella isolates used in this study compared to earlier studies.

Four Salmonella serotypes were recorded in this study; *S*. Typhimurium, *S*. Enteritidis, *S*. Santiago and *S*. Kentucky. These findings are consistent with the finding of **Hassan et al. (2018)** who isolated S. Enteritidis S. Typhimurium, and S. Kentuky from broiler farms in Assiut, Egypt. However our finding differs from a study performed by **Soliman et al. (2018)** who recorded that S. Enteritidis is the most common serotype isolated from broiler chickens.

The antimicrobial susceptibility tests revealed that all of the examined isolates were resistant to nalidixic acid and amoxicillin. Ampicillin/sulbactam, streptomycin and tetracycline showed high resistance with percentages of 83.3%, 75% and 75% respectively. These outcomes aliganed partially with the finding of **Alam et al. (2020)** who mentioned that Salmonella isolated from broiler in Bangladesh showed higher resistance to ampicillin (82.85%), streptomycin (77.14%) and tetracycline (97.14%), and **Sarker et al. (2021)** who showed that non typhoidal Salmonella isolated from chickens in Bangladesh exhibited resistance to nalidixic acid (100%), ampicillin (40%) and amoxicillin (25%) and **Ahmed and Shimamoto, (2012)** who found that Salmonella isolated from diseased broiler in Egypt displayed multidrug resistance against ampicillin, streptomycin, tetracycline and nalidixic acid (94.0%), (94.0%), (88.0%) and (70.0%) respectively.

In the livestock industry, the use of probiotic feed additives and competitive exclusion agents plays an important role as a cost-effective alternative to improving breeding performance and controlling animal disease **(Barbosa et al. 2005).** By studying the effects of CE and organic acids application on chicks after challenge with multidrug-resistant ST, the results revealed no mortalities, PM lesions, clinical signs and ST cecal colonization in all groups except for positive control (group 2) which showed (20%) mortalities and group (4) which showed slight diarrhea, enlarged cecum. The mean of pH values were lower in groups 4 and 5 but with in the normal range in the other groups including group 3.

In this study, using of CE alone (group 3) showed no ST colonization in the cecum. However, some studies performed by **Hume et al. (1996)** recorded a significant reduction of ST cecal colonization when chickens treated orally with CE culture, and **Jiratitipat et al. (2019)** recorded a significant reduction of SE cecal colonization after treatment with CE culture in chickens. Regarding the supplementation of organic acids in the drinking water in group 4, the ST infection showed a reduction of ST colonization compared to the positive control group. Previous studies, such as **Koyuncu et al. (2013), Menconi et al. (2013), Dittoe et al. (2018) and Adhikari et al. (2020),** recorded that application of organic acids reduced Salmonella colonization in cecum. The composition of organic acids administration time and the chicks' health conditions may be responsible for these findings.

In this study, the use of CE alone (group, 3) and with organic acids (group, 5) reduced ST colonization in the cecum of the chicks under experiment. This is in consistent with, sterizo et al. (2007), who mentioned that the using of probiotic either individually or in combination acidifier reduced the colonization of SE in the cecal contents of chickens. These results may be due the activity of CE in preventing enteric pathogens colonization, in addition to the ability of organic acids to change pH which inhibit harmful microbes.

The growth performance parameters recorded at 6th day of age before ST challenge showed that BWG in chicks of groups 3, 4 and 5 were slightly higher than the control groups 1 and 2. The BWG and FI in groups 3, 4 and 5 were non significantly different. No differences were recorded in the FCR between group 3 and 4 meanwhile the FCR was slightly improved in group 5 when compared with other groups.

At 12th day of age, BWG recorded in chicks of groups 3, 4 and 5 were significant higher than the control groups. BWG in group 5 was significant higher than groups 3 and 4. Group 1 showed a lower (BWG and FCR) and higher (FI) when compared with groups 3, 4 and 5.The application of organic acid with CE in group 5 in comparison with other groups improved the FCR. The findings attributed to researchers such as **Dittoe et al. (2018), Adil et al. (2010), Hamid et al. (2018) and Adhikari et al. (2020).**

Although the mean pH measured in group (3) was with in the normal range and lowered in group 4 and 5, ST colonization was compeletly prevented in all chicks of groups 3 and 5. The ability of organic acids alone in prevention of colonization was lower than CE alone or when applied with CE. The growth performance parameters were improved in group 5 (supplemented with CE and organic acids) compared to other groups. These findings suggested the use of CE with organic acids is a helpful solution in controlling ST infection and improve the growth performance of broiler chicks.

CONCLUSION

The effectiveness of organic acids mixture and CE supplementation on multidrug resistant ST was studied in vivo and the results concluded that the supplementation of both organic acids with CE had a beneficial effect to control ST cecal colonization and to improve the chicks' growth performance. These results should be betaken into consideration as one of the possible methods to control the spread of Salmonella in farms of poultry.

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