

Antibacterial effect of lactic acid on multidrug resistant *Salmonella* isolated from ducks

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Abstract

A total of 100 diseased ducks suffered from mortalities and diarrhea were collected from farms in Dakahlia Governorate (Egypt) then subjected to clinical followed by laboratory diagnosis. Clinical diagnosis was made on the basis of history, signs and postmortem findings of the diseased ducks. Internal organs such as (liver, spleen, heart, intestine) were collected for *Salmonella* isolation, identification, antimicrobial sensitivity testing and detection of some virulence and resistance genes using PCR were done. The effect of lactic acid was studied on the multidrug resistant isolates. *Salmonella* was isolated with an incidence of 7 (7%) and then differentiated serologically and four serotypes were reported (*S. Typhimurium*, *S. Enteritidis*, *S. Kentucky* and *S. Inganda*). Antimicrobial sensitivity testing revealed that *Salmonella* isolates showed moderate sensitivity to ciprofloxacin and norfloxacin and higher resistance to amoxicillin, ampicillin. Among the examined *Salmonella* isolates *invA*, *bcfC* virulence genes were detected with an incidence of (100%) for each gene and *bla*TEM, *tetA*(A) resistance genes with an incidence of (85.7%). the effect of lactic acid on the isolated multidrug resistant *Salmonellae* was studied ; the results revealed that the lowest effective concentration on *Salmonella* isolates was (0.5%).

Keywords: *Salmonella*, lactic acid, Ducks

Introduction

Avian salmonellosis is a problem of economic importance to all phases of poultry industry from production to marketing (Cruchaga *et al.*, 2001). Ducks, duck eggs and duck environmental samples have been recently reported as very important sources of *Salmonella* (Pan *et al.*, 2010) which considered as a Gram negative, non- spore forming bacteria, usually motile and belong to the family of *Enterobacteriaceae* (Bennasar *et al.*, 2000). It's a facultative intracellular pathogen causing localized, systemic infections and chronic asymptomatic carrier state (Su *et al.*, 2011). Paratyphoid infections in all species of young birds are similar and include closed eyes, droopy wings, ruffled feathers, anorexia, profuse watery diarrhea with pasting of the vent (Gast and Beard,

1992). The postmortem findings are enteritis, focal necrotic lesions in mucosal wall of small intestine, enlarged and congested liver with necrotic foci and cheesy cecal cores (Hoop and Pospischill 1993).

Salmonella species have sixty virulence genes and three antigenic (H, O, and Vi) determinants (Groisman and Ochman, 1997). One of the major *Salmonella* virulence genes is *invA* gene which related to a combination of chromosomal and plasmid factors (Sabbagh *et al.*, 2010). The chromosomally located invasion gene *invA* codes for a protein in the inner membrane of bacteria that is necessary for invasion of epithelial cells (Darwin and Miller, 1999). The fimbrial gene (*bcfC*) is located on a fimbrial structure and has a role in cell inva-

sion (Huehn *et al.*, 2010).

Treatment of salmonellosis with antibiotic became difficult due to the emergence of multi-drug resistant strains (Mirza *et al.*, 1996). The extensive use of antibiotics in veterinary medicine resulted in the spreading of multiple antibiotic resistant Salmonellae (Cruchaga *et al.*, 2001).

Organic acids are designated and approved by the Federal Drug Administration as safe substances. Citric, lactic, tartaric, and acetic acids are the most known organic acids used (Mohamed and Abdel-Naeem, 2018). Organic acids offer several advantages as antimicrobials molecule, because of their low cost and the easy manipulation (Lynch *et al.*, 2017). Nowadays, they are used in poultry feed to reduce Salmonella number and other bacteria in digestive tract of animals (El Baaboua *et al.*, 2018).

Lactic Acid is one of the earliest antibacterial substances to be harnessed by humankind (Gladden, 2004) it considered as a weak organic acid and an effective antimicrobial agent (Sapers, 2005), the inhibitory action is mainly due to the compound crossing the plasma membrane in the undissociated state. lactic acid remains undissociated and become in its most active antimicrobial form in low pH (Brul and Coote, 1999).

Aim of study

This study was carried out to evaluate the effectiveness of lactic acid on multidrug resistant Salmonella isolated from diseased ducks in Dakahlia Governorate (Egypt).

Materials and Methods

1. Samples collection and study area.

One hundred ducks (Pekin, Muscovy and Mallard) that suffered from mortalities and diarrhea were collected from farms in Dakahlia Governorate. The age of examined ducks varied from one week to 4 months. Some birds showed clinically high mortality rate and diarrhea but in postmortem examination showed hepatitis, enteritis, pericarditis and prouze liver. Samples from internal organs such as (liver, spleen, heart, intestine) were collected asepti-

cally then labeled and transported directly in the ice box to reference laboratory for Veterinary Quality Control on poultry production Gamasa branch for further examinations.

2. Salmonella isolation, identification and serotyping.

Salmonella was isolated and identified from the collected internal organs according to ISO 6579-1 (2017). All isolates that were biochemically identified as Salmonella were then subjected to serological identifications according to Minor and Popoff, (2000).

3. Antimicrobial susceptibility testing according to Finegold and Martin (1982).

All Salmonella isolates were subjected to antimicrobial susceptibility testing using agar disc diffusion method on Muller Hinton agar plates. The antimicrobial agents were ciprofloxacin (5µg), norfloxacin (10µg), amoxicillin (25 µg), ampicillin (10µg), neomycin (30 µg), streptomycin (10 µg), tetracycline (30 µg) and sulfa methoxazole-trimethoprim (25µg). The antimicrobial agents were categorized into susceptible, intermediate and resistant categories according to (CLSI, 2016).

4. Detection of some virulence and resistance genes in the isolated Salmonellae using conventional PCR

4.1. DNA extraction

DNA was extracted from all Salmonella isolates to detect (*invA* and *bcfC*) virulence genes and (*tetA* (A) (tetracyclin resistance gene) and *bla*TEM (β lactam resistance gene) resistance genes. The extraction was performed using QIAamp DNA mini kit (Qiagen- Germany-GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer.

4.2. Oligonucleotide primers that used were provided from Metabion (Germany) listed in table (1).

Table (1). Oligonucleotide primers sequences, target genes, and cycling conditions for Salmonella virulence and resistance genes.

Target gene	Primers sequences '3-'5	Seg. (bp)	P. dent.	Amplification (35 cycles)			Final ext.	Reference
				Sec. dent.	Annealing	Ext.		
<i>bcfC</i>	ACC AGA GAC ATT GCCTTC C	467	°94C 5min.	°94C 30sec.	°53C 40sec.	°72C 45sec.	°72C 10min.	Huehn <i>et al.</i> , (2010)
	TTC TGC TCG CCG CTA TTC G							
<i>blaTE M</i>	ATCAGCAATAAACCAGC	516	°94C 5min.	°94C 30sec.	°54C 40sec.	°72C 45sec.	°72C 10min.	Colom <i>et al.</i> , (2003)
	CCCCGAAGAACGTTTTC							
<i>TetA (A)</i>	GGTTCACTCGAACGACGTCA	576	°94C 5min.	°94C 30sec.	°50C 40sec.	°72C 45sec.	°72C 10min.	Randall <i>et al.</i> , (2004)
	CTGTCCGACAAGTTGCATGA							
<i>invA</i>	GTGAAATTATCGCCACGTTCCGG- CAA	284	°94C 5min.	°94C 30sec.	°55C 30sec.	°72C 30sec.	°72C 7min.	Oliveira <i>et al.</i> , (2003)
	TCATCGCACCGTCAAAGGAACC							

Seg. (bp)= amplified segment& P. dent.= primary denaturation& Sec. dent.= secondary denaturation& Ext.= extension& Final ext.= final extension

4.3. PCR amplification

PCR amplification. Primers were utilized in a 25- µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

4.4. Analysis of PCR products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Appllichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. A gelpilot 100 bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

5. In vitro sensitivity testing using lactic acid on multidrug resistant Salmonella isolates.

A total of 7 Salmonella isolates that showed multidrug resistance in this study were tested by using an agar disc diffusion method to detect the antibacterial effect of lactic acid according to Rawdkuen *et al.*, (2012).

Whatman no. 1 filter paper was cut into a disc form 5 mm in diameter with a sterilized hole-punch. They were then sterilized with UV light for 30 minutes (Cooksey, 2000). Lactic acid was diluted at different concentrations (0.5%, 1.0%, 1.5%, 3% and 5%) then the discs were soaked without over or under wetting and stored at 4°C in the refrigerator till use.

The diameters of the inhibitory zone surrounding the paper discs of lactic acid were recorded as an indication of inhibition of the microbial growth and The evaluation of the inhibitory activity was carried out in quadruplicate by measuring the inhibition zones in millimeters. according to Finegold and Martin (1982);

Results

1. Salmonella isolation, identification and serotyping

The cultural and biochemical characteristics of the isolated Salmonellae were similar to that recorded in OIE, (2004).

Seven Salmonellae were isolated from internal organs of 100 diseased ducks with an incidence of (7%). Salmonella isolates were differentiated serologically and the results revealed presence of four *S. Typhimurium*, one *S. Enteritidis*, one *S. Kentucky* and one *S. Inganda*. The most common serotype isolated was *S. Typhimurium*.

2. Antimicrobial Susceptibility pattern of the isolated Salmonellae

Results of antimicrobial susceptibility testing revealed that Salmonella isolates showed moderate sensitivity to ciprofloxacin and norfloxacin which have the highest activity in between the used antimicrobial agents. All isolates

showed totally resistance to amoxicillin, ampicillin. Meanwhile 6 isolates showed resistance to streptomycin and tetracycline (**Tables 2 & 3**).

Table (2). Antimicrobial Susceptibility pattern of isolated Salmonellae.

Antimicrobial Agent	1			2			3			4			5			6			7		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Amoxicillin	-	-	R	-	-	R	-	-	R	-	-	R	-	-	R	-	-	R	-	-	R
Ampicillin	-	-	R	-	-	R	-	-	R	-	-	R	-	-	R	-	-	R	-	-	R
Ciprofloxacin	S	-	-	S	-	-	S	-	-	-	-	R	S	-	-	S	-	-	-	-	R
Sulfamethoxazole-trimethoprim	-	I	-	-	I	-	-	-	R	-	-	R	-	-	R	S	-	-	S	-	-
Neomycin	S	-	-	-	-	R	-	I	-	-	I	-	-	-	R	-	-	R	S	-	-
Norfloxacin	S	-	-	S	-	-	R	-	-	-	-	R	S	-	-	S	-	-	-	-	R
Streptomycin	-	-	R	-	-	R	-	-	R	S	-	-	-	-	R	-	-	R	-	-	R
Tetracycline	-	-	R	-	-	R	-	-	R	S	-	-	-	-	R	-	-	R	-	-	R

The antimicrobial agents were categorized into susceptible, intermediate and resistant categories according to (CLSI, 2016). (1, 2, 3 and 4) = *S. Typhimurium*, 5 = *S. Kentucky*, 6 = *S. Enteritidis* and 7 = *S. Inganda*.

Table (3) Number of sensitive, intermediate and resistant Salmonella isolates against different antimicrobial agents.

Antimicrobial agent	Salmonella (7 isolates)		
	Sensitive	Intermediate	Resistant
Amoxicillin	0	0	7
Ampicillin	0	0	7
Ciprofloxacin	5	0	2
Sulfamethoxazole-trimethoprim	2	2	3
Neomycin	2	2	3
Norfloxacin	4	0	3
Streptomycin	1	0	6
Tetracycline	1	0	6

3. Molecular detection of *bla*TEM, *tetA* (A) (resistance gene) and *invA*, *bcfC* virulence genes using PCR.

PCR was a good tool for accurate detection of *tetA* (A) and *bla*TEM resistance genes

among Salmonella isolates that showed resistance to tetracycline and β lactams antibiotics. From **figure (1) and (2)**; the two genes were reported with an incidence of (85.7%) for each of them.

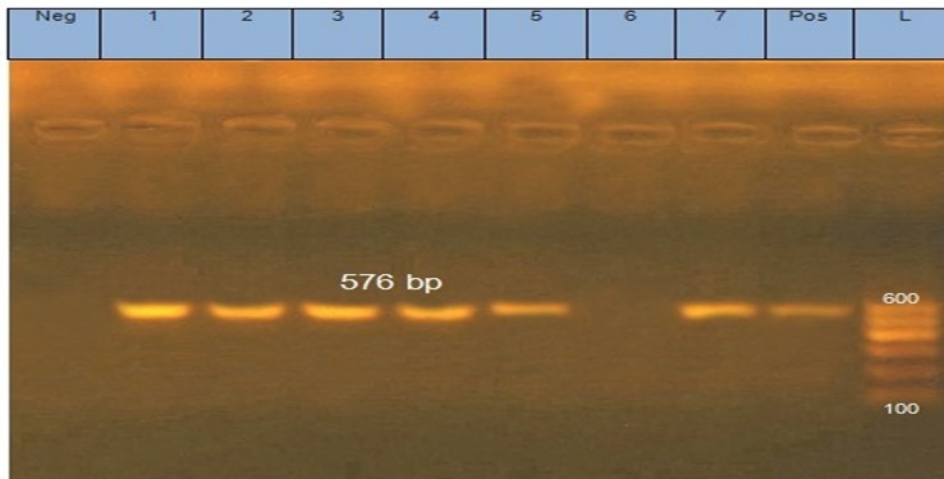


Figure (1). Agarose gel electrophoresis of PCR products for Salmonella isolates to detect *tetA* (A) gene in genomic DNA. Lane L: 100-600 bp DNA ladder. Neg: Negative control, Pos: Positive control. Lane: 1, 2, 3, 4, 5 and 7 were positive but lane 6 was negative.

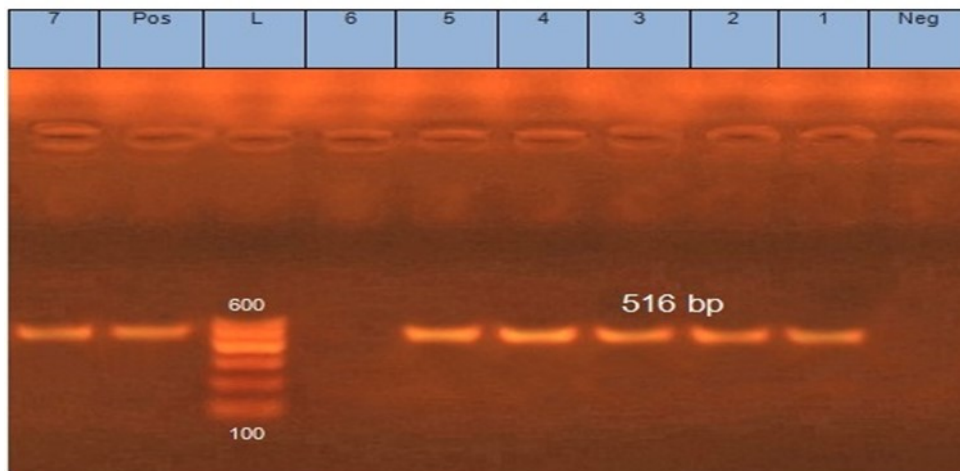


Figure (2). Agarose gel electrophoresis of PCR products for Salmonella isolates to detect *bla*TEM gene in genomic DNA. Lane L: 100-600 bp DNA ladder. Neg: Negative control, Pos: Positive control. Lane: 1, 2, 3, 4, 5 and 7 were positive but lane 6 was negative.

From figure (3) and (4), *invA* and *bcfC* (virulence genes) were reported in all ex-

amined Salmonellae with a percentage of (100%).

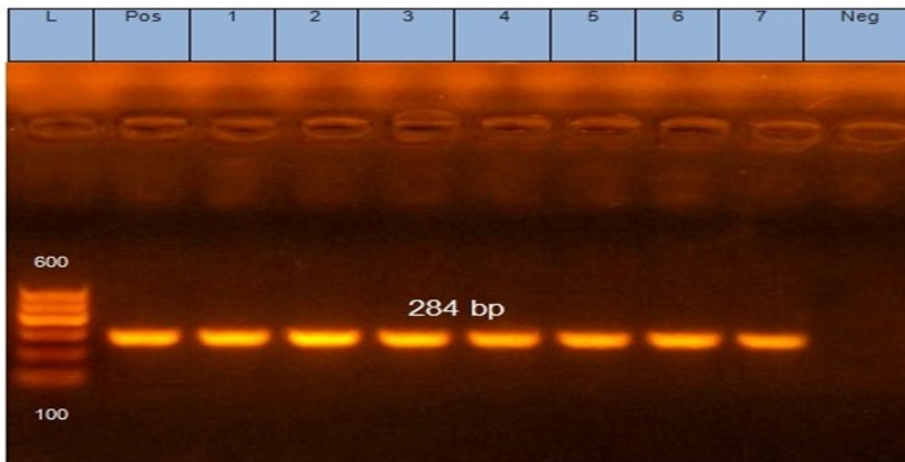


Figure (3). Agarose gel electrophoresis of PCR products for *Salmonella* isolates to detect *invA* gene in genomic DNA. Lane L: 100-600 bp DNA ladder. Neg: Negative control, Pos: Positive control. Lane: 1, 2, 3, 4, 5, 6 and 7 were positive.

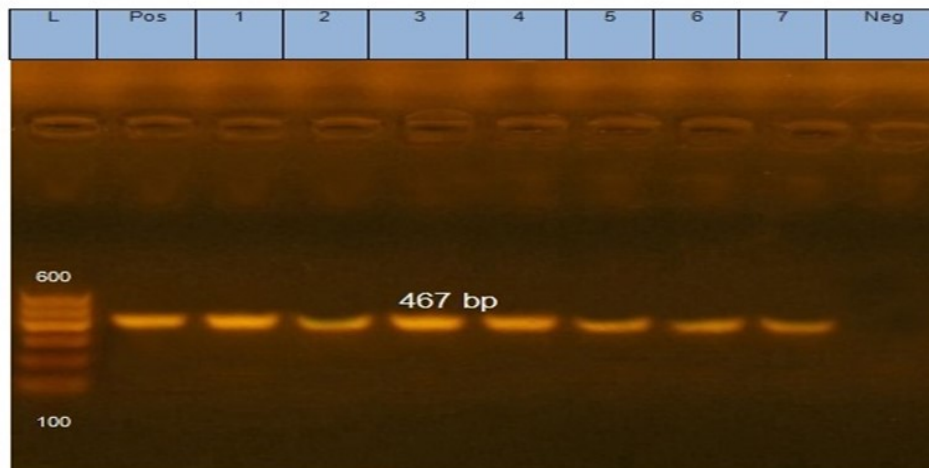


Figure (4). Agarose gel electrophoresis of PCR products for *Salmonella* isolates to detect *bcfC* gene in genomic DNA. Lane L: 100-600 bp DNA ladder. Neg: Negative control, Pos: Positive control. Lane: 1, 2, 3, 4, 5, 6 and 7 were positive.

4. Results of antimicrobial susceptibility testing using lactic acid

The inhibition zones of antimicrobial susceptibility testing using lactic acid on 7 multidrug resistant *Salmonella* isolates revealed that all of the used concentrations of lactic acid (0.5%, 1.0%, 1.5%, 3% and 5%) had inhibitory effect were the same measurements

Discussion

In this study seven *Salmonella* isolates were reported from ducklings in Dakahlia Governorate with an incidence of (7%). These results varied to some extent with **Lam *et al.*, (2002)** who isolated *Salmonella* from ducks in Vietnam with a percentage of (18%) in duck-

lings less than one month old and with (10%) in one day old ducklings; **Hoszowski and Wasyl (2005)** who isolated *Salmonella* from duck broilers in Poland with percentage of (14.3%). Meanwhile a study performed by **Asawy and El-Latif (2010)** in Dakahlia Governorate, Egypt to isolate *Salmonella* from ducks in different ages and the incidence was (3.3%). The results in this study varied greatly from **Rahman *et al.*, (2016)** who isolated *Salmonella* from ducks with a an incidence of (39.58%).

In the present study four isolates were *S. Typhimurium*, one *S. Enteritidis*, one *S. Kentucky* and one *S. Inganda*. The most common serotype was *S. Typhimurium* and this agreed

with **Mituniewicz *et al.*, (2007)** who reported that these serotype was most often isolated from duck flocks. However **Tasi and Hansing (2005)** reported that *S. Potsdam* was the most common serotype isolated from ducks in Taiwan.

Salmonella isolates showed moderate sensitivity to ciprofloxacin and norfloxacin and these results were nearly similar to **Mondai *et al.*, (2008)** who stated that the isolates Salmonellae from ducks were highly sensitive to ciprofloxacin.

All isolates showed totally resistance to amoxicillin, ampicillin. Meanwhile 6 isolates showed resistance to streptomycin and tetracycline. The results in this study agreed with **Pan *et al.*, (2009)** who stated that Salmonella isolates displayed a high level of resistance to ampicillin, streptomycin and tetracycline.

In this study, results of PCR for the detection of *invA* and *bcfC* genes revealed their presence of Salmonella isolates with a percentage of (100%) and these results were agreed with **Lebdah *et al.*, (2017)** who reported this gene in Salmonellae isolated from poultry, **Osman *et al.*, (1992)** who recorded also this gene in all Salmonellae isolated from one day-old ducklings. Meanwhile another study performed by **El-Sayed (2014)** reported the absence of this gene in Salmonella isolated from ducklings.

The *invA* gene was reported in this study with a percentage of (100%) and this agreed with **Elgohary *et al.*, (2017)** who recorded the same results in Salmonella isolated from diarrhoeic ducks.

The *tetA* gene was reported in the present study with a percentage of (85.7%) and these results nearly in coordinated with **Shahada *et al.*, (2006)**; **Lu *et al.*, (2011)** and **Ahmed and Shimamoto (2012)** who reported this gene in Salmonella isolates with a percentage of (89%), (81.2%) and (66.7%) respectively.

The *bla* TEM gene was reported in the present study with a percentage of (85.7%) and these results nearly similar to **Hur *et al.*, (2011)** who reported that *S. Enteritidis* in Korea carried the *bla*TEM gene with a percentage of (90.5%). A study performed by **Lu *et al.*, (2014)** reported also a nearly similar percentage which was (81.2%).

The effect of lactic acid on 7 multidrug resistant Salmonella isolates revealed that all of

the used concentrations (0.5%, 1.0%, 1.5%, 3% and 5%) had inhibitory effect on Salmonella isolates. The results in this study nearly agreed with **Castillo *et al.*, (2001)**, the treatment of (2%) lactic acid reduced levels of *S. Typhimurium* on inoculated carcass surfaces, **Van der Marel *et al.*, (1998)** who reported that using of lactic acid (1%) or (2%) dips and spray washes reduce the bacterial count from 5.2 to 3.7 log CFU/g when prechilled birds immersed for 15 seconds. Another study by **Gyawali and Ibrahim, (2012)** reported that the combination of both 50 ppm Cu and 0.2% lactic acid produced a significant growth inhibition of Salmonella.

Conclusion

Salmonella was isolated from 100 diseased ducks collected from farms located in Dakahlia Governorate (Egypt). The findings of the present study revealed that Salmonella was isolated with a percentage of (7%) and four serotypes were reported (*S. Typhimurium*, *S. Enteritidis*, *S. Kentucky* and *S. Inganda*). Antimicrobial sensitivity test revealed that Salmonella isolates showed moderate sensitivity to ciprofloxacin and norfloxacin and higher resistance to amoxicillin, ampicillin. Two virulence genes such as *invA*, *bcfC* were detected in all isolates and two resistance genes such as *bla*TEM, *tetA(A)* were recorded in 6 isolates. The lowest concentration of lactic acid produced an inhibitory effect on Salmonella isolates was (0.5%) so that it is expected that using of lactic acid as therapeutic agents will be helpful in decreasing incidence of salmonellosis in poultry farms so that it is necessary to perform further investigations to clarify the effect of lactic acid and the method of application in vivo.

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