

## EFFECT OF SOME ORGANIC ACIDS ON *SALMONELLA* TYPHIMURIUM IN CHICKEN MEAT

REHAM, M. ABDEL-WAHHAB<sup>1</sup>; MERVAT, ELBARBARY<sup>2</sup>;  
AMINA, M. EL-AMIN<sup>3</sup> AND SOAD, H.M.EL-SHEIKH<sup>4</sup>

<sup>1</sup> Researcher of Meat Hygiene, Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, Zagazig Provincial Lab.

<sup>2</sup> Researcher of Meat Hygiene, Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, Zagazig Provincial Lab. E-mail address: [Mmustafa99@yahoo.com](mailto:Mmustafa99@yahoo.com).

<sup>3</sup> Researcher of Meat Hygiene, Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, Zagazig Provincial Lab. E-mail address: [vet\\_doceg1982@yahoo.com](mailto:vet_doceg1982@yahoo.com).

<sup>4</sup> Researcher of Meat Hygiene, Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, Zagazig Provincial Lab. E-mail address: [Soad.elshiekh@yahoo.com](mailto:Soad.elshiekh@yahoo.com).  
Mobile: 01281339938

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### ABSTRACT

There are several health problems and economic losses, which are caused by bacterial species on chicken meat. The obligation to reduce the initial load of bacteria should be taken into serious consideration. So, the aim of this work was to detect the incidence of *Salmonella* spp in chicken meat parts with detection of their virulence genes, which were 4%, 8% and 6% in chicken fillet, thigh and drumsticks respectively, with detection of virulence genes % in isolates. This study is an attempt to evaluate the antibacterial effect against *Salmonella* Typhimurium by soaking chicken breast in acetic acid, citric acid and fumaric acid (1% & 2%) solutions for 15 seconds, as well as on sensory properties. The treatments reduced *Salmonella* counts effectively. Logarithmic reduction values of *Salmonella*, for acetic acid 1%, acetic acid 2%, citric acid 1%, citric acid 2%, fumaric acid 1%, and fumaric acid 2% were 0.82, 1.88, 1.07, 2.19, 0.91, and 2.85 log CFU/g, respectively.

**Key Words:** *Salmonella* Typhimurium; organic acids; acetic acid; fumaric acid; chicken meat.

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### INTRODUCTION

Poultry meat and its products are particularly well-liked food all over the world, due to their deliciousness, nutritional

value, wonderful odor, and easy to digest.

Chicken products are becoming more and more popular in Egypt, because they are fast-cooking meat dishes; solve the problem of high-priced fresh meat shortages that are unattainable for many limited-income families. The intact tissues of birds and healthy animals that have been slain are typically sterile, but during processing, the meat may become contaminated by hands, employees, the gut, hide, clothing, knives, or

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*Corresponding author:* Reham, M. Abdel-Wahhab  
*E-mail address:* [dr\\_riham.mohammed@yahoo.com](mailto:dr_riham.mohammed@yahoo.com)  
*Present address:* Researcher of Meat Hygiene, Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, Zagazig Provincial Lab.

surroundings, making it underling or unsuitable quality for people's consumption. A risk to the public's health could come from contaminated poultry and chicken products, Ahmed and Ismail (2010). Salmonellae is the predominant significant bacterium found in poultry meat and is responsible for many food-borne illnesses. (Ahmed, 2014).

Although ingestion of a variety of foods has been linked to Salmonella infection, animal products are a major source of infection (Castillo *et al.*, 2008). Salmonella has historically been spread from animals to people, and meat, especially chicken meat, has a significant impact on the epidemiology of different serovars (Perin *et al.*, 2020).

The two most commonly isolated serovars from food-borne outbreaks worldwide are *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis (Herikstad *et al.*, 2002). There are several virulence genes specific to Salmonella that are significant contributors to pathogenicity (Baumler *et al.*, 2000). At least 80 distinct virulence genes have been found in Typhimurium serovar. It is known that some genes play a role in invasiveness and adhesion, including: Sef (Clouthier *et al.*, 1993), fliC (Jamshidi *et al.*, 2009), another gene associated with production of toxin, as Stn, (Makino *et al.*, 1999).

Due to the health problems and economic losses caused by different species of bacteria in poultry meat, the obligation to reduce the initial load of bacteria needs to be seriously considered. This is an attempt to investigate the antibacterial effects of common food additives. Chemical disinfectants made of organic acids are frequently employed in the food business. Their antimicrobial activity has been studied in a number of food matrices and it is generally regarded as safe for human consumption (Mani-López *et al.*, 2012).

## MATERIALS AND METHODS

### *Salmonella* spp identification and isolation:

**Preparation of samples:** A number of 150 raw poultry meat samples are divided into 50 pieces each of the drumstick, thigh, and fillet. These samples were collected from various supermarkets and retail stores in the El Sharkia Governorate. Under strict aseptic circumstances, the collected samples were taken and quickly transported in an insulated ice box to the lab for microbiological analysis.

**Bacteriological identification of *Salmonella* spp.:** Salmonella identification was carried out according to ISO (6579:2017) as follows:

**Pre-enrichment non-selective media:** Test portions (25gm) were inoculated into 225 ml of buffered peptone water and incubated at 34-38 C°, for 18 hours±2 hours.

**Enrichment on selective media:** (MKTTn) Muller-Kauffmann Tetrathionate/novobiocin broth and (RVS broth) Rappaport-Vassiliadis medium with soy were inoculated with cultures 1ml got from pre-enrichment non-selective media add to 10ml (MKTTn). Incubate MKTTn broth at 34-38 C° for 24 hours ± 3 hours and the RVS broth at 41.5 C° ± 1 C° for 24 hours ± 3 hours.

**Plating on selective media:** From the previous enrichment, broth streaks a loopful onto the surface of Xylose Lysine Desoxycholate agar medium (X.L.D) was previously prepared, and incubated it at 34-38°C for 24 hours. Suspected colonies were red with or without black centers. Sub cultured to the obtained suspected colonies onto nutrient agar plates and incubated at 37 C° ± 1 C° for 24 ± 3 hours. The obtained colonies (red with or without black centers on XLD media) were plated on nutrient agar media for purification.

**Confirmation tests (biochemical tests):** Voges-Proskauer reaction (VP) (Clark medium), Christensen medium containing urea (Urease production), (TSI) Triple-sugar iron agar media, Lysine decarboxylation medium (Medium containing Lysine) and  $\beta$ -galactosidase detection reagents (ONPG medium)

#### Identification of *Salmonella* Serologically:

The isolates that are biochemically proven to be *Salmonella* organisms were confirmed by serological identification using a rapid diagnostic *Salmonella* antiserum kit, according to the Kauffman-White scheme (Kauffman, 1974).

**Detection of virulence pathogenic factors (stn, hilA, fliC, and sef) of *Salmonella enteritidis* and *Salmonella typhimurium* by using Polymerase Chain Reaction (PCR):**

The QIAamp DNA Mini Kit provides silica-membrane-based nucleic acid purification from different types of samples. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes. The multiplex PCR reactions for (*Stn*, *hilA*, *fliC* & *sef*) were performed with initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 1 minute, primer extension at 72°C for 1 minute, and the final extension at 72°C for 10 minutes.

#### Oligonucleotide primers used in cPCR (Metabion, Germany)

They have a specific sequence and amplify a specific product, as shown in Table (1).

**Table 1:** Primers used for PCR reactions.

Bacterial strain	Target gene	Primer Sequence	Amplified product	Reference
<i>Salmonella</i>	<i>Stn</i>	TTG TGT CGC TAT CAC TGG CAA CC ATT CGT AAC CCG CTC TCG TCC	617 bp	Murugkar <i>et al.</i> , 2003
<i>Salmonella</i>	<i>hilA</i>	CGG AAG CTT ATT TGC GCC ATG CTG AGG TAG GCA TGG ATC CCC GCC GGC GAG ATT GTG	854 bp	Cardona-Castro <i>et al.</i> , 2002
<i>Salmonella Typhimurium</i>	<i>fliC</i>	CGG TGT TGC CCA GGT TGG TAA T ACT GGT AAA GAT GGC T	613 bp	Halim <i>et al.</i> , 2014
<i>Salmonella Enteritidis</i>	<i>sef</i>	GCG AAA ACC AAT GCG ACT GTA CCC ACC AGA AAC ATT CAT CCC	1104 bp	Murugkar <i>et al.</i> , 2003

#### Evaluation of antimicrobial efficiency of organic acids:

**Preparation of inoculum:** the *Salmonella* strains which were obtained from our study were known as *Salmonella enterica* subspecies *enterica* serotype *Typhimurium*. The strains used in this experiment were refreshed on XLD agar incubated at 37 C °

for 24 hours. Colonies were taken, put into brain-heart infusion broth, and then incubated for 24 hours at 37°C. The bacterial growth were centrifuged at 3000 rpm for 15 minutes, twice washing in 10 ml of 0.01 phosphate buffered saline (PBS) and then diluted to obtain 6 log<sub>10</sub> CFU/ml for inoculation of the chicken fillet samples. *S.*

Typhimurium count was detected by tenfold serial dilution and subsequent enumeration on XLD agar (Govaris *et al.*, 2010).

**Sample preparation for bacterial inoculation:** chicken breast fillets that were proved to be negative in part I of the study were aseptically divided into 7 sets for different additives including the control group, each group subdivided into 3 trials in which the treated samples weighing 25 g. The control, as well as the treated groups, were placed in separate sterile polyethylene bags, inoculated with *S. Typhimurium* (6 log<sub>10</sub> CFU/ml in PBS), then mixed with organic acids of different concentrations after 15 minutes of inoculation.

**Solutions of organic acids:** the concentration of (1 and 2% w/v) of the tested organic acids (acetic acid, citric acid and fumaric acid) was prepared according to manufacturer's instructions. Fumaric acid was reserved at a 50°C from preparation and during the experiment time to evade its precipitation. Treatment trials were done under strict aseptic precautions. Samples were dipped for 15 seconds in acetic, citric and fumaric acid (1&2%) separately. After treatment, samples were aseptically homogenized with 225 ml of 0.1 % sterile peptone water to about 2 minutes at 3000 rpm using sterile homogenizer. Tenfold serial dilution was prepared. *Salmonella*

counts were performed on XLD, as selective media. The values of log reduction were estimated by subtracting counts of samples treated with the different organic acids from counts of control samples, according to (Fernández *et al.*, 2020).

**Statistical analysis and experimental design:** Experiments were done in seven groups separately with three trials in each one. The antimicrobial effectiveness of the three organic acids with different concentrations on *Salmonella typhimurium* count was compared using a one-way ANOVA analysis (Adhikari *et al.*, 2020).

## RESULTS

**Table 2:** Incidence of Salmonellae in chicken parts (chicken fillet, thigh and drumsticks N= 50 for each)

Sample	Positive samples	
	No.	%
Chicken fillet(N=50 )	2	4%
Thigh (N=50 )	4	8%
Drumsticks (N=50 )	3	6%
Total (N=150 )	9	6%

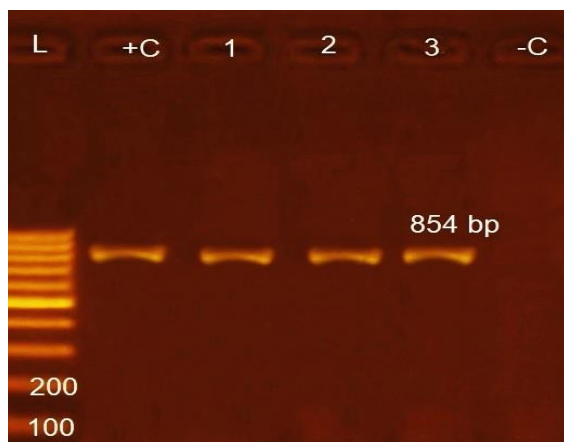
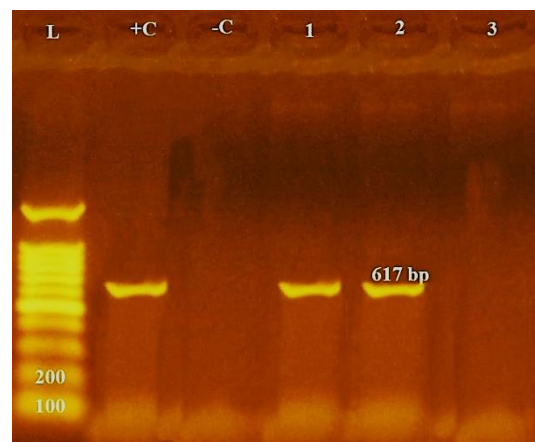
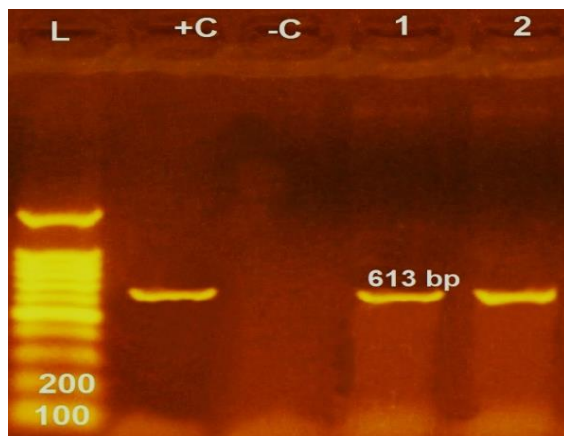
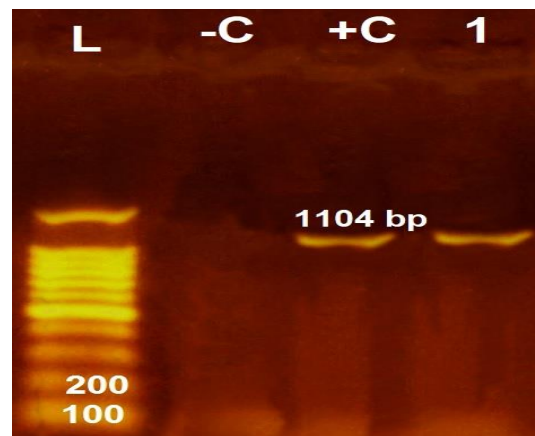
**Table 3:** Identification of Salmonellae serologically.

No.	Identified strains	Group	Antigenic structure		
			O	H	
Chicken fillet	1	<i>Salmonella wingrove</i>	C2	6,8	c : 1,2
	2	<i>Salmonella kentucky</i>	C3	8,20	i : Z6
Thigh	3	<i>Salmonella kentucky</i>	C3	8,20	i : Z6
	4	<i>Salmonella enteritidis</i>	D1	1,9,12	g,m : -
	5	<i>Salmonella Typhimurium</i>	B	1,4,5,12	i : 1,2
Drum sticks	6	<i>Salmonella apeyeme</i>	C3	8,20	Z38 : -
	7	<i>Salmonella Typhimurium</i>	B	1,4,5,12	i : 1,2
	8	<i>Salmonella tsevie</i>	B	1,4,12	i : e,n,z15
	9	<i>Salmonella kentucky</i>	C3	8,20	i : Z6

**Table 4:** Virulence genes incidence of different strains of *Salmonella* isolated from the examined samples (n= 3 strains).

Virulence genes	<i>S.Typhimurium</i> (n=2)		<i>S. enteritidis</i> (n=1)	
	No.	%	No.	%
<b>stn</b>	2	100	0	0
<b>hilA</b>	2	100	1	100
fliC	2	100	--	--
sef	--	--	1	100

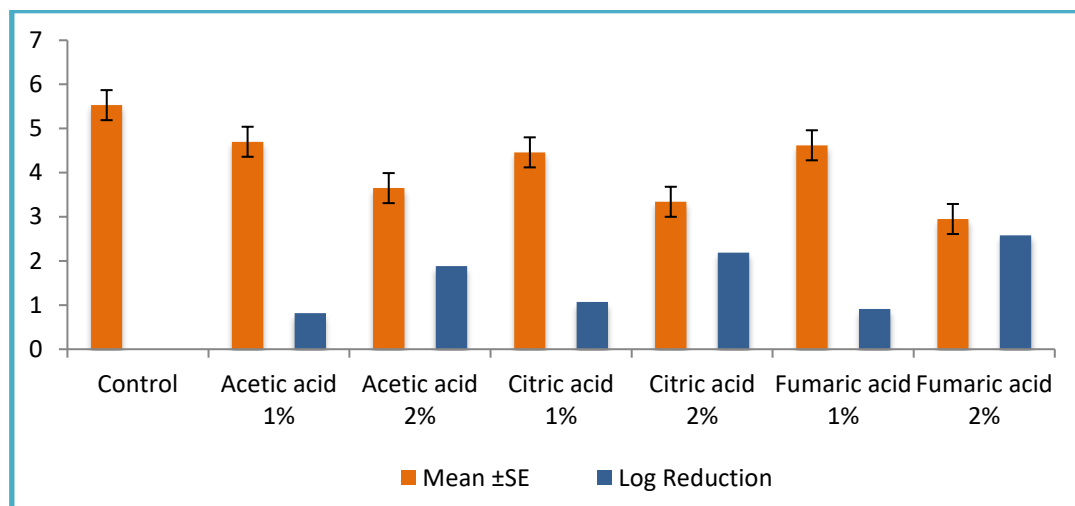
*stn*: Enterotoxin gene *hilA*: hyper-invasive locus gene  
*fliC*: flagellin gene *sef*: fimbrial gene

**Figure (1):** Electrophoresis of agarose gel of multiplex PCR of *hilA* gene (854 base pairs)**Figure (2):** Agarose gel electrophoresis of multiplex PCR of *stn* gene (617 bp)**Figure (3):** Agarose gel electrophoresis of multiplex PCR of *fliC* gene (613 bp)**Figure (4):** Agarose gel electrophoresis of multiplex PCR of *sef* gene (1104 bp)

**Table 5:** The mean counts  $\pm$ S.E. ( $\log_{10}$  CFU/g) of the inoculated *Salmonella* Typhimurium in chicken fillet samples after additives treatments (n = 3 for each group).

Treated groups	Control	Acetic acid 1%	Acetic acid 2%	Citric acid 1%	Citric acid 2%	Fumaric acid 1%	Fumaric acid 2%
Mean $\pm$ SE	5.53 $\pm$ 0.03 <sup>a</sup>	4.70 $\pm$ 0.38 <sup>b</sup>	3.65 $\pm$ 0.05 <sup>c</sup>	4.46 $\pm$ 0.04 <sup>b</sup>	3.34 $\pm$ 0.06 <sup>d</sup>	4.62 $\pm$ 0.20 <sup>b</sup>	2.95 $\pm$ 0.05 <sup>e</sup>
Log Reduction	---	0.82	1.88	1.07	2.19	0.91	2.85

Means  $\pm$ SE within the same row with different superscripts are significantly different at ( $p < 0.05$ ) according to Duncan's multiple comparisons.

**Figure 5**

## DISCUSSION

Food safety threats posed by food-borne infections, such as *Salmonella*, continue to be of a great concern for the industry of food. *Salmonella* disease is a major public health issue and more important worldwide in developing countries (Wang *et al.*, 2008). The frequency of *Salmonella* serotypes found by isolation from chicken flesh samples tested in Table 2 was 4%, 8%, and 6% in fillets, thigh, and drumsticks, respectively. As shown in Table 3., serologically identified salmonellae were *Salmonella* wingrove, *Salmonella* kentucky, *Salmonella* enteritidis, *Salmonella*

Typhimurium, *Salmonella* apyeme and *Salmonella* tsevie.

The incidence of *Salmonella* in poultry meat was resulted from contamination within manual handling, slaughter and by workers (Carraminana *et al.*, 1997). Organic matter dispersed on the bird surface provides a harbor for salmonella, acts as a contamination source to scalding boiling tanks and facilitates cross-infection between chickens. The plucker's rubber fingers have multiple cracks that allow organic matter to migrate and cause cross-contamination between chickens. In addition, cross-contamination can occur during the evisceration stage due to leakage of intestinal contents (Berrang *et al.*, 2011).

PCR findings declared in Table (4) and Figures (1-4) showed a total of 3 *Salmonella* serotypes (2 *S. Typhimurium* & 1 *S. enteritidis*) were tested using multiplex PCR for the presence of virulence genes, including *hilA*, *stn*, *fliC* and *seF*. A complex phenomenon, diarrhea was caused by *Salmonella*, and was resulted by multiple pathogenic mechanisms, like production of enterotoxin. The production of enterotoxin is activated by *stn* and it plays an important role in developing gastroenteritis through the enterotoxin production (Chopra *et al.*, 1987).

In our study, *stn* gene was found in 2 of 3 isolates (66 %). Comparable results obtained by Ammar *et al.* (2016) and Eldesouky *et al.* (2016), who found *stn* gene in 58.82% and 41.17% of isolates, respectively. The *hilA* gene is a key component of *Salmonella* pathogenicity, because it's required for colonization of bacteria of the host intestine extracellular space, (Murray and Lee, 2000). Based on our results, *hilA* was detected in 3 of 3 isolates (100%), closing the results of Craciunas *et al.* (2012) who detected *hilA* gene in all isolates (100%) as it is important for cell invasion. Meanwhile, Ammar *et al.* (2016) and Eldesouky *et al.* (2016) detected *hilA* gene in 88.24% and 64.70% of isolates, respectively.

The flagellin (*fliC*) gene encodes the main flagella component in *Salmonella enterica* serovar *Typhimurium* (Aldridge *et al.*, 2006). As evidenced by PCR results, two *S. Typhimurium* isolates (100%) generated an identical band of 613 bp, the predicted size for the *fliC* gene.

Fimbrial antigen of *S. enteritidis* (*sefA*) which is specific for the discovery of *salmonella enteritidis* serovar, was detected, and this agreed with Chagas *et al.* (2013).

These indicate the need to find a suitable approach to prevent salmonella in chicken meat, and show the necessity to increase cleanliness and hygienic principles in poultry slaughter houses.

Concerning of the tested acids effect on the intended pathogen, as shown in Table (5) and Figure (1), it has been cleared that the three additives proved their effectiveness (fumaric 2% > citric 2% > acetic 2% > citric acid 1%). On the other hand, acetic acid 1% and fumaric acid 1% has little significant effect.

Near finding obtained by (Cutter and Betancourt, 2000), they recorded 2 log reduction of *Salmonella Typhimurium* in beef meat after treatment by 2% acetic acid, furthermore, Cosansu and Ayhan (2012) recorded that 1% and 2% acetic acid solutions reduced *Salmonella enteritidis* count in chicken leg meat by 0.85 and 0.95 log, respectively from 4-5 log initial number : while the same concentration of the citric acid reduced the same initial *Salmonella enteritidis* count in breast chicken meat by 0.95 and 1.58 log, respectively, Madushanka *et al.* (2018) concluded that the used acids have an antibacterial effect on *S. Typhimurium* in chicken meat, and Fernandez *et al.* (2021) found that count of *Salmonellae* in fumaric acid treated samples were significantly differ than control samples.

pH range from 6.5 to 7.5 were preferred by *Salmonella* (Huiying, 2011), so their biological function may be lost under extreme pH levels and possible suppression. The three acids used in our study are having quite acidic pH. At room temperature acetic, citric and fumaric acids are shown following pH values 3.51, 3.91 and 2.48 that are antagonistic to the growth of *Salmonella* spp. The used acids not only cause the cytoplasm to become acidified and the difficulties in performing the essential metabolic functions in the bacterial cell but also cause an energy loss because ATP is used to actively export protons, (Davidson *et al.*, 2013)

## CONCLUSION

Approaches to diminish *Salmonellae* count on raw chicken meat should be assumed to increase the safety of chicken meat products and decrease the frequency of human salmonellosis from its consumption, It can be concluded that organic acids (acetic, citric and fumaric) have an antimicrobial effect on *S. Typhimurium* and, so, they can be used for the refinement process of chicken meat.

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### تأثير بعض الاحماض العضوية على ميكروب سالمونيلا تيفيموريوم في لحوم الدجاج

رهام محمد عبد الوهاب ، مرفت مصطفى على البربري ، أمينة محمد الأمين ، سعاد حفنى الشيخ

E-mail: dr\_riham.mohammed@yahoo.com

Assiut University web-site: www.aun.edu.eg

تعد لحوم الدواجن مصدرا مهما من مصادر البروتين الحيوانى نظراً لاحتوائها على العديد من العناصر الغذائية مثل الفيتامينات والأملاح المعدنية وكذلك الأحماض الأمينية الضرورية لبناء جسم الإنسان، ولكن قد يتلوث اللحم أثناء المعالجة من أيدي العمال أو الملابس أو السكاكين أو الجلد أو الأمعاء أو من البيئة مما يؤدي إلى جودة منخفضة أو حتى غير صالحة للاستهلاك البشري. وتعتبر سالمونيلا من أهم مسببات الأمراض البكتيرية الموجودة في لحوم الدجاج والتي تسبب التسمم الغذائى. وقد استهدفت الدراسة الكشف عن تلوث أجزاء الدجاج بميكروب سالمونيلا مع الكشف عن جينات الضراوة وقد كانت النسبة ٤٪ و ٨٪ و ٦٪ في فيليه الدجاج والاوراك والدبابيس على التوالي. وتواجد جينات الضراوة لميكروب سالمونيلا تيفيموريوم وأيضاً سالمونيلا انتريديتس. يقترح أن يتم تدريب العاملين بشكل دورى فضلا عن المراقبة الفعالة والتفتيش على مصانع تجهيز منتجات اللحوم. كما استهدفت تقييم فعالية غمر فيليه الدجاج في محاليل الخليك والستريك وحمض الفورماريك (١٪ و ٢٪) لمدة ١٥ ثانية على سالمونيلا تيفيموريوم. واتضح ان للمعالجات الثلاثة تأثير فعال على تقليل ميكروب سالمونيلا.