Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Salmonella in Nile Tilapia, Mullet, and Catfish, Molecular and Antimicrobial Profiles, Along with A Reduction Trial Utilizing Acetic and Ascorbic Acids

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Abstract

The purpose of this study is to identify prevalence, serotypes, virulence-associated genes, and the antimicrobial profile of *Salmonella* spp. in catfish, tilapia, and mullet fish sold in El-Beheira governorate, Egypt. An additional innovation in *Salmonella* growth control was the use of acetic acid and ascorbic acid. The present investigation's findings showed that 20% of the mullet, 35% of the catfish, and 20% of the Nile tilapia tested samples were positive for *Salmonella* spp. Additionally, the *inva* gene was present in 100% of the isolates, *hila* in 85.71%, and *stn* in 57.14% of the isolates, according to the PCR results. Multidrug resistance profiling was clearly seen in the recovered *Salmonella* isolates. Reductions of 30.4%, 54.3%, 21.7%, and 39.1% in *Salmonella* spp. were seen in *Oreochromis niloticus* samples after using acetic acid 1% and 2% and ascorbic acid 1% and 2%, respectively, due to their substantial inhibitory effects. The results show that Egyptian fishers do not take enough precautions to ensure their hands are clean when working with fish. Thus, it is advised to adhere to stringent hygiene protocols when fishing, processing, and distributing seafood in Egypt.

Keywords: *Salmonella* spp., catfish, tilapia, mullet, antimicrobial resistance, acetic acid, ascorbic acid.

Introduction

It is widely acknowledged that fish is an indispensable source of several nutrients including essential amino acids. The lack of red meat in Egypt has a substantial impact on the country's current level of food security. Based on the findings of Morshdy et al. [1], fish can serve as an alternate source of animal protein. This is especially true when considering the fact that fish is considerably more affordable than chicken and other types of meat.

Through strict adherence to cleanliness standards throughout the entire process of catching, storing, and processing fish, it is possible to reduce the risk of microbial contamination. According to Aberle [2], the primary causes of microbial contamination in fish are the operator (including their hands, hair, and clothing), the exploitation of infected raw materials, storage vessels, and equipment. Consequently, it is vital to maintain a continual monitoring system for the microbiological quality of fish sold at retail outlets in Egypt.

Salmonellosis is recognized as the second most prevalent zoonotic disease in humans, resulting in gastrointestinal illness. *Salmonella typhimurium* and *Salmonella enteritidis* are the principal etiological agents of these illnesses [3]. Ingesting food contaminated with *Salmonella* spp. can lead to *non-typhoidal salmonellosis* (nts), a significant foodborne infection that has recently attracted global attention. Symptoms of *Salmonella* appear 12 to 36 hours following the consumption of contaminated food (the incubation period). The symptoms encompass fever, intense diarrhea, nausea, vomiting, and abdominal cramping [4]. The bacterium requires both virulence

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(Received 03 May 2025, accepted 06 July 2025)
DOI: 10.21608/ejvs.2025.381529.2823

factors and antibiotic resistance to endure host defenses [5].

Broad-spectrum ß-lactams and fluoroquinolones employed to eradicate *Salmonella spp*, including *S. typhimurium and S. kentucky*, have become ineffective against these strains of the pathogen [6]. Bacterial resistance tactics against antibiotics include reduced cell permeability, target alteration or substitution, and enzyme inactivation [7].

Antimicrobial-resistant bacteria, such as *Salmonella*, have arisen due to the widespread application of antibiotics in Egypt's farmed catfish and tilapia [8]. Using natural antimicrobial alternatives is an important way to avoid the development of antibiotic resistance.

Natural preservatives are increasingly used to maintain the quality and extend the shelf life of fish, offering a safer and more eco-friendly alternative to synthetic chemicals. These natural solutions not only help in retaining the fish's texture, flavor, and color but also respond to consumer demand for cleaner, more sustainable food preservation methods [9]. Przekwas et al. [10] asserted that vitamin C, known as ascorbic acid, possesses antibacterial effects in food systems. Research indicates that ascorbic acid can diminish biofilms of foodborne pathogens, such as *staphylococcus aureus, Escherichia coli*, and *listeria monocytogenes*.

This study assessed the incidence rates of *Salmonella* in Nile tilapia, mullet, and catfish sold in El-Beheira governorate, Egypt. The disc diffusion assay was employed to assess the antibiotic resistance of the recovered *Salmonella*. Furthermore, PCR was employed to detect virulence genes including *hila, stn*, and *inva*. Furthermore, a reduction attempt utilizing organic acids, specifically acetic acid and ascorbic acid, was conducted.

Material and Methods

Samples collection

Sixty fish samples were collected randomly from designated markets in the El-Beheira governorate, Egypt. The samples included *Clarias lazera* (catfish), *Oreochromis niloticus* (Nile tilapia), and *Mugil cephalus* (twenty of each species). Prior to shipment to the lab for microbiological analysis, each sample was carefully conserved in a sterile plastic bag, with the correct labels applied, and kept in an icebox. *Salmonella* spp. were checked in all of the samples that collected.

Sample preparation [11].

A pair of sterile shears and a pair of forceps were used to extract the fins from the body. Using sterile forceps, fish were grasped and carefully peeled off all of its scales using a sterile knife. Using a heated spatula, all parts of the body were sterilized. Twenty-five grams of posterior neck muscles were aseptically transferred into a sterile homogenizer flask (MPW-302 homogenizer, Poland) with two hundred twenty-five milliliters of sterile water containing one percent peptone, following the sterile surface's excision with sterile scissors and forceps. To achieve a 1/10 dilution, the liquid was homogenized for 2.5 minutes at 14,000 rpm. After that, it was let stand for around 5 minutes. The buffered peptone water incubated at 18h at 37 °C.

Salmonella species isolation and identification.

According to ISO [11], *Salmonella* spp. were isolated and identified. Summarizing the procedure, 1 mL of each pre-enriched sample homogenate was mixed with 9 mL of Rappaport Vassiliadis (Oxoid, UK), a selective enrichment medium, and left to incubate at 41.5 °C for 24 hours. After transferring a small amount of the enriched culture onto a xylose lysine deoxycholate (XLD) agar plate (Himedia, India) it was left to incubate at 37°C for one day. Following purification, colonies were subcultured onto nutrient agar slopes and incubated at 37 °C for 24 hours if they had black cores or not. The isolated colonies were identified using morphological, biochemical, and serological methods.

Assessing the antibiotic sensitivity of Salmonella strains

The antibiotic resistance of the obtained *Salmonella* strains was evaluated using the disc diffusion technique, as described before [12]. Oxford, United Kingdom-based Oxoid Limited supplied the antimicrobial discs. Nutrient agar plates were used to test the antibiotic susceptibility of *Salmonella* species. Accordingly, the standards established before [13] were followed for the antimicrobial susceptibility tests. In addition, for every *Salmonella* isolate examined, Singh et al. [14] used an algorithm to determine the Multiple Antibiotic Resistance (MAR) index, which is the ratio of the number of antibiotics tested to the total number of resistances found.

The antibiotics that are used are as follows: ampicillin, cephalothin, chloramphenicol, ciprofloxacin, enrofloxacin, erythromycin, gentamicin, kanamycin, nalidixic acid, neomycin, oxacillin, oxytetracycline, penicillin, and trimethoprim/sulfamethoxazole.

Molecular Characterization of Salmonella virulence genes:

Genomic DNA extraction:

The Rappaport Vassiliadis broth that have been incubated overnight was spun at 13000 rpm for 2 minutes at 4^{o}C and the liquid on the top was removed. In bacteria lysis solution that contained 20mM Tris-Hcl, 2mM EDTA, 1.2% Triton X-100 and 20mg/ml lysozyme, and the pellet was

resuspended. The mixture was then incubated at 37°C for 30 minutes [15].

Amplification of genes by Multiplex PCR:

Molecular identification of virulence factors including invasion (*invA)*, enterotoxin (*stn)* and hyper-invasive locus *(hilA*) genes of *Salmonella species* was applied with the primer sequence as illustrated in Table 1. To perform amplification, a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany) was utilized. The ideal combination for a multiplex PCR reaction, which not be more than 25 µL includes 2 µL of DNA template, 5 µL of 5 × PCR buffer, 2.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM deoxynucleotide triphosphate (dNTP), 0.5 µL of 1.2 µM primer mix and 14.2 µL of deionized water[16].

The effects of organic acids on S. typhimurium load in Nile tilapia

The procedure as out by Abbasit et al. [17] was followed with a few tweaks. Before the experiment, thirty fresh *Oreochromis niloticus* samples were carefully rinsed with tap water and then with distilled water. Afterwards, the heads of all the fish were severed and their organs removed. Five samples from each of the six groups of fish were used for analysis. The first group served as a control and was immersed in water that did not contain any pathogen. The other five groups were then immersed in water that contained *S. typhimurium* at a concentration of 10^6 cfu/g. No therapy was administered to the second group. The third group spent half an hour in a solution of 1% acetic acid and the fourth group half an hour in a solution of 2% acetic acid. Separately, the fifth and sixth groups spent half an hour submerged in ascorbic acid solutions containing 1% and 2%, respectively. The evaluated fish groups were tested for *Salmonella* growth after the specified interval. Reductions in average *Salmonella* counts were examined as a percentage.

Statistical analysis

The obtained results of the examined fish samples were statistically evaluated [18].

Results and Discussion

Salmonella is the predominant cause of bacterial foodborne illness in the United States, resulting in 400 deaths, 15,000 hospitalizations, and 1.4 million non-typhoidal infections annually [19]. It can be deduced from the fish production chain, due to inappropriate handling, inadequate cleanliness, or exposure to contaminated water, that *Salmonella* is a bacterium responsible for severe foodborne infections, despite not being initially recognized as a biological contaminant in fish [20]. The spread of the disease via water has been thoroughly documented [21]. Although the likelihood of acquiring *Salmonella* infection from aquatic food is lower than from other sources, it remains crucial to identify *Salmonella* spp. in aquatic food, as they are a significant contributor to foodborne gastroenteritis. The present study monitored the occurrence of *Salmonella* pathogen in freshwater fish species such as *Clarias lazera*, *Tilapia niloticus* and *Mugil cephalus,* and revealing prevalence of 35%, 20%, and 10% respectively for various serotypes, as illustrated in Figure 1 and Table 2. All identified serovars possessed the *invA* gene, with 85.71% and 57.14% exhibiting the *hilA* and *Stn* genes, respectively. It is stated that *Salmonella* organism should not be found in fish [22]. As a result, all the examined fish samples were found to be above the acceptable limit set by Egyptian standards by percentage 35%, 20% and 10% for catfish, tilapia and mullet respectively as illustrated in Figure 2.

Comparable findings were reported by Elsisy et al. [23], indicating that the prevalence of *Salmonellae* in *Tilapia niloticus* and *Mugil cephalus* was 15% and 10%, respectively. Saad et al. [24] reported higher results, indicating that *Salmonella* species were detected in 24% and 20% of the tested samples of *O. niloticus* and *Mugil cephalus,* respectively. Morshdy et al. [25] documented diminished findings. *Salmonellae* spp. were isolated from tilapia samples with prevalence rates of 13.3% and 7.5%.

The increasing prevalence of microorganisms in fish can be attributed to various factors, including bacterial and fungal infections, poor water quality, and anthropogenic activities leading to aquatic pollution. Throughout the handling and processing phases, the bacterial flora on fish can undergo significant changes, with various bacterial species prevailing at distinct times. Moreover, it has been observed that the prevalence of bacterial populations in fish samples is significantly elevated in certain markets, indicating a potential source of contamination and an increased risk of foodborne diseases, as determined before [26].

Due to the deficiency of red meat, fish farming and aquaculture are seeing global expansion. Antimicrobials are employed alongside these initiatives to avert and control bacterial diseases in fish. However, the improper use of antibiotics in aquaculture may lead to the development of antibiotic resistance [27].

Consequently, *Salmonellae* isolates underwent antimicrobial susceptibility testing. All tested isolates (100%) shown resistance to a minimum of two of the evaluated antimicrobials. *Salmonella* isolates exhibited resistance to the evaluated antimicrobials at the following rates: penicillin G (100%), oxacillin (100%), vancomycin (90%), tetracycline (70%), amikacin (55%), linezolid (50%), cefotaxime (45%), daptomycin (40%), ciprofloxacin (38%), neomycin (23%), colistin (23%), gentamicin (23%),

azithromycin (15%), meropenem (7%), and ceftriaxone (7%).

The computed MAR index for the isolated *Salmonella* spp. varied from 0.125 to 1 with a mean of 0.466 (Table3). These results corroborate the findings of Bayomi et al. [28]. The extensive application of antibiotics for growth promotion and disease management has led to the development of drug-resistant *Salmonella* strains against numerous antibiotics, including penicillin and oxacillin.

It is common and well-known technique to apply organic acids to fish surfaces, primarily by dipping or spraying. Numerous studies have demonstrated that organic acids and the salts they contain may prevent bacteria from growing in certain fish species. Because of its antibacterial properties, a unique icing system including 800 mg/kg of citric, ascorbic, and lactic acid was found to be a beneficial mixture for fish preservation [29]. Experiments aimed at decreasing *Salmonella* contamination using 1% and 2% acetic acid and 1% and 2% ascorbic acid yielded reduction percentages of 30.4%, 54.3%, 21.7%, and 39.1%, respectively. These results are remarkably identical to those recorded before [30].

Conclusion

This investigation isolated *Salmonella* spp. from catfish, tilapia, and mullet, showing inadequate sanitary standards in fish production, management, and handling. Acetic acid and ascorbic acid may serve as a novel approach to diminish *Salmonella* contamination levels. Consequently, meticulous attention to sanitation is essential when processing and preparing fish, necessitating the implementation of stringent hygienic measures.

Acknowledgments

We would like to thank all stuff members at the Meat Hygiene and Control Department, Faculty of Veterinary Medicine, Damanhour University, Egypt,

Funding statement

This study didn't receive any funding support

Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study was conducted according to the ethical guidelines of Damanhour University, Egypt.

TABLE 1. Primer sequences of virulence-associated genes used in this study

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
invA (F)	**5′** TATCGCCACGTTCGGCAA **′3**		**[31]**
invA (R)	**5′** TCGCACCGTCAAAGGAACC **′3**	275	
stn (F)	**5′**TTGTGTCGCTATCACTGGCAACC **′3**		**[32]**
stn (R)	**5′** ATTCGTAACCCGCTCTCGTCC **′3**	617	
hilA (F)	**5′**CGGAAGCTTATTTGCGCCATGCTGAGGTAG**′3**		**[33]**
hilA (R)	**5′** GCATGGATCCCCGCCGGCGAGATTGTG **′3**	854	

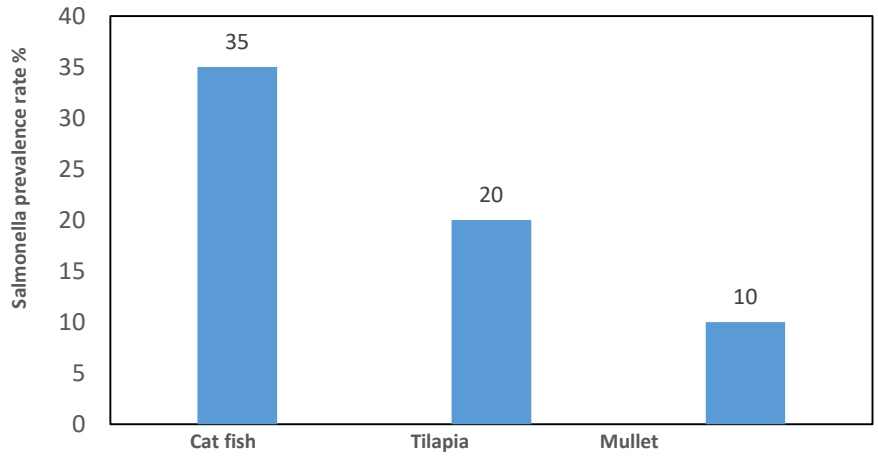

Fig. 1. Prevalence rate (%) of *Salmonella* spp in the examined catfish, tilapia, and mullet (n = 20/each).

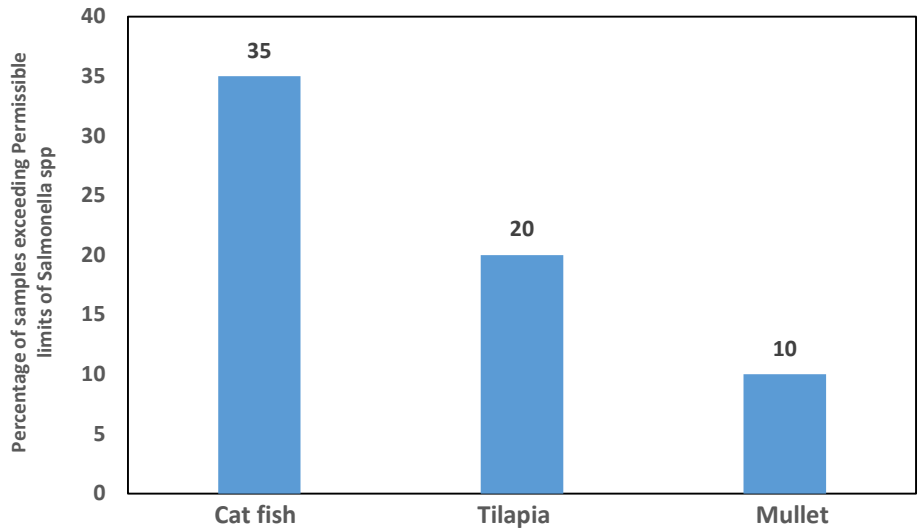

Fig. 2. Percentages of samples exceeding permissible limits of *Salmonellae* in the examined catfish, tilapia, and mullet (n= 20/each).

TABLE 2: Incidence of *Salmonellae* isolated from the examined samples of fish in El Behira governorate (n=20).

Fish species	*Clarias Lazera*		*Oreochromis niloticus*		*Mugil cephalus*		Group	Antigenic structure	
Salmonellae	No.	%	No.	%	No.	%		O	H
S. enteritidis	1	5	2	10	-	-	**D1**	1,9,12	g,m : -
S. muenster	-	-	1	5	-	-	**E1**	3,10,15,34	e,h : 1,5
S. parathphi A	1	5	-	-	-	-	**A**	1,2,12	i : 1,5
S. rissen	2	10	-	-	-	-	**C1**	6,7,14	f,g : -
S. saintpaul	-	-	-	-	1	5	**B**	4,5,12	e,h : 1,2
S. shangani	-	-	1	5	-	-	**E1**	3,10	d : 1,5
S. typhimurium	3	15	-	-	1	5	**B**	1,4,5,12	i : 1,2
Total	7	35	4	20	2	10			

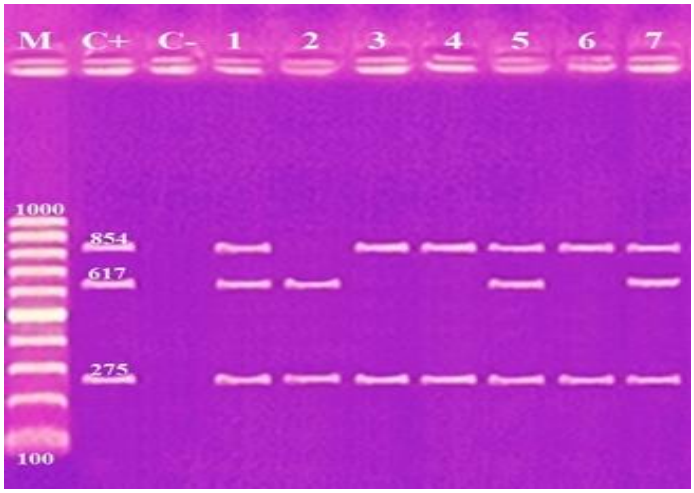

Fig. 3. Agarose gel electrophoresis of multiplex PCR of *invA* (275 bp), *stn* (617 bp) and *hilA* (854 bp) as virulence genes for characterization of *Salmonella* species.

TABLE 3. Antimicrobial resistance profile of *Salmonella* strains isolated from the examined fish samples (n=13).

No	*Salmonella* strains	Antimicrobial resistance profile	MAR index
1	***S. typhimurium***	**P, OX, V, T, AK, LZ, SXT, CF, DA, CP, N, G, CO, AZ, M, CR**	1
2	***S. typhimurium***	**P, OX, V, T, AK, LZ, SXT, CF, DA, CP, N, G, CO**	0.813
3	***S. typhimurium***	**P, OX, V, T, AK, LZ, SXT**	0.437
4	***S. typhimurium***	**P, OX, V**	0.188
5	***S. enteritidis***	**P, OX, V, T, AK, LZ, SXT, CF, DA, CP, N, G, CO, AZ**	0.875
6	***S. enteritidis***	**P, OX, V, T, AK, LZ, SXT, CF**	0.500
7	***S. enteritidis***	**P, OX, V, T**	0.250
8	***S. rissen***	**P, OX, V, T, AK, LZ, SXT, CF, DA, CP**	0.625
9	***S. rissen***	**P, OX**	0.125
10	***S. paratyphi A***	**P, OX, V, T, AK, LZ, SXT, CF, DA, CP**	0.625
11	***S. shangani***	**P, OX, V, T**	0.250
12	***S. muenster***	**P, OX, V**	0.188
13	***S. saintpaul***	**P, OX, V**	0.188
	Average	**0.466**	

N: Neomycin P: Penicillin-G CF: Cefotaxime SXT: Sulfamethoxazole CO: Colistin AZ:Azithromycin CR: Ceftriaxone T: Tetracycline G: Gentamicin CP: Ciprofloxacin AK: Amikacin M: Meropenem OX: Oxacillin LZ: Linezolid V: Vancomycin DA: Daptomycin

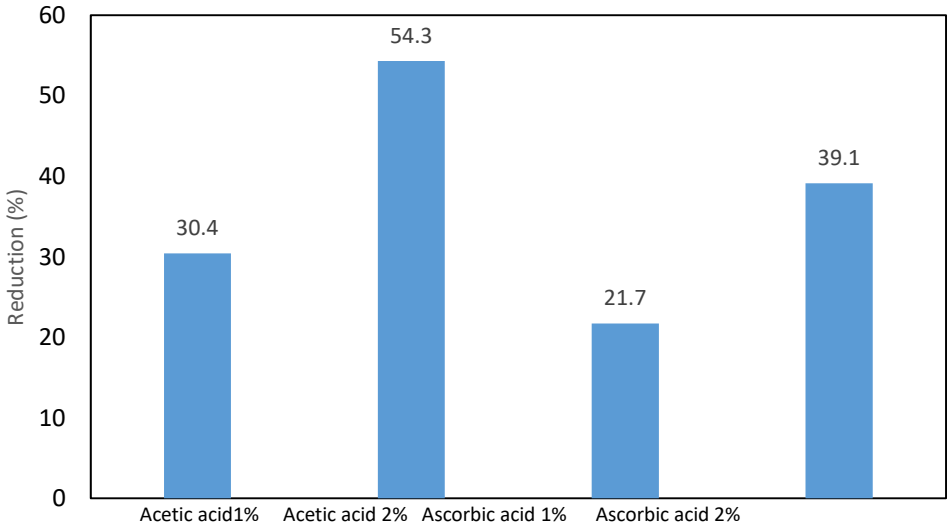

Fig. 4. Effect of organic acid treatments on *S. typhimurium* counts/g in the examined *Oreochromis niloticus* samples.

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إنتشار أنواع السالمونيلا في أسماك السلور والبلطي والبوري: الملفات الجزيئية وأنماط المقاومة للمضادات الحيوية، مع تجربة تقليل التلوث باستخدام حمض الأسيتيك وحمض الأسكوربيك

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الملخص

الغرض من هذه الدراسة هو تحديد نسب تواجد ميكروب السالمونيلا في أسماك السلور (القرموط)، والبلطي، والبوري المعروضة للبيع في محافظة البحيرة، مصروالجينات المرتبطة بالضراوة، والأنماط المصلية وكذلك نمط المقاومة لبعض المضادات الحيوية لبكتيريا السالمونيلا بأنواع الأسماك محل الدراسة. كما تم ابتكار طريقة جديدة للسيطرة علي نمو السالمونيلا باستخدام حمض الأسيتيك وحمض الأسكوربيك. أظهرت نتائج الدراسة الحالية أن: 35% من عينات أسماك السلور و 20% من عينات البوري، و20% من عينات البلطي النيلي كانت موجبة لبكتيريا السالمونيلا. ووفقا لنتائج اختبار تفاعل البلمرة المتسلسل فقد وُجد أن: جين *inva* كان موجودا في 100% من العزلات، جين *hila* في 85,71%، وجين *stn* في 57,14 % من العزلات.كما أظهرت عزلات السالمونيلا نمطًا واضحًا لمقاومة متعددة للمضادات الحيوية. وبالنسبة لتجربة خفض مستويات السالمونيلا باستخدام الأحماض، فقد تم تسجيل نسب انخفاض بلغت: 30,4% و 54,3% عند استخدام حمض الأسيتيك بتركيز 1% و 2% ، و 21,7% و39,1% عند استخدام حمض الأسكوربيك بتركيز 1% و 2% وذلك في عينات البلطي النيلي ، مما يُظهر فعالية كبيرة لهذه المركبات في التثبيط البكتيري. كما تشير النتائج إلى أن الصيادين في مصر لا يتبعون الإجراءات الصحية الكافية لضمان نظافة أيديهم أثناء التعامل مع الأسماك. ولذلك، يُوصى باتباع بروتوكولات صحية صارمة أثناء صيد، وتجهيز، وتوزيع المأكولات البحرية في مصر.

الكلمات الدالة: السالمونيلا ، سمك السلور، البلطي ، البوري ، مقاومة المضادات الحيوية ، حمض الخليك ، حمض الأسكوربيك.