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Prevalence, Antibiogram and Enterotoxin Production of *Staphylococcus aureus* in Nile Tilapia, Mullet and Catfish with a Reduction Trial Using Acetic and Ascorbic Acids

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Abstract:

This study aimed to examine the prevalence of *Staphylococcus aureus (S. aureus)* in Nile tilapia, mullet, and catfish sold in El-Beheira governorate, Egypt. The antibiotic susceptibility of the isolated *S. aureus* strains was assessed. Further, the Reverse Passive Latex Agglutination technique (RPLA) was employed for detection and identification of *S. aureus* enterotoxins. The current results indicated the isolation of *S. aureus* from Nile tilapia, mullet, and catfish, with incidence rates of 60%, 45%, and 70%, respectively. Catfish exhibited a substantially higher overall *S. aureus* count (3.64±2.49 log10 cfu/g) as compared to Nile tilapia (3.44±2.27 log10 cfu/g) and mullet (2.00±1.85 log10 cfu/g), respectively ($p< 0.05$). Accurately, 55%, 35%, and 20% of the analyzed samples of catfish, Nile tilapia, and mullet, respectively, surpassed the Egyptian threshold for *S. aureus* (3.00 log10 cfu/g). The isolated *S. aureus* strains exhibited distinct multidrug resistance patterns. However, RPLA testing of specific *S. aureus* isolates for the presence of Staphylococcal enterotoxin indicated that enterotoxin A was identified in two *S. aureus* isolates obtained from catfish and in one isolate obtained from tilapia. Enterotoxin C was found solely in one strain obtained from mullet. Enterotoxin D was identified in a single sample from mullet and one strain from tilapia. Enterotoxin A+ D was found solely in one isolate obtained from catfish. Experiments aimed at decreasing *S. aureus* counts with 1% and 2% acetic acid and 1% and 2% ascorbic acid yielded reduction percentages of 21.2%, 46.1%, 17.3%, and 28.9%, respectively. Consequently, rigorous hygienic protocols must be applied during handling, processing, and sale of fish, while acetic acid and ascorbic acid can be utilized effectively to mitigate *S. aureus* in fish, thereby reducing their bacterial load.

Keywords: *S. aureus*, Catfish, Tilapia, Mullet, antimicrobial resistance, Acetic acid, Ascorbic acid

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Introduction

Nutritionists agree that fish is an excellent source of protein of high biological value, polyunsaturated fatty acids, vitamins, and minerals including calcium and phosphorus. Red meat scarcity threatens Egyptians' ability to stay fed. Because of its reduced cost compared to other meat sources, fish acts as a substitute source of animal-based protein **(Ghanem et al., 2025**).

The high omega-3 fatty acid content found in fish meat is a significant characteristic. Omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are well-known for their positive effects on cardiovascular health, brain function, and reducing inflammation. Regular consumption of fish has been associated with a reduced risk of heart disease. **(Maulu et al., 2021)**

In order to avoid contaminating fish with microbes, it is crucial to adhere to strict hygiene protocols when capturing, storing, and processing the fish. The most common ways that microbiological contamination can occur are through the operator's own hands, hair, and clothes, as well as through the use of contaminated equipment, storage containers, and raw materials **(Aberle, 2001).** This highlights the critical importance of continuously monitoring the microbiological quality of retail fish in Egypt.

Factors like cleanliness measures, the kind of fish, and how it is handled and stored might affect the prevalence of *Staphylococcus aureus* (*S. aureus*) in fish meat. When *S. aureus* is found in contaminated food products, it can cause food poisoning. According to **Darwish et al. (2022**), it is a major cause of food poisoning and other food-related ailments on a global scale.

Food poisoning and other food-related illnesses are frequently caused by *S. aureus* **(Darwish et al., 2022)**. In the US alone, it's responsible for around 241,000 cases of sickness annually. The symptoms of *S. aureus* enterotoxin poisoning in humans include anorexia, abdominal cramps, vomiting, and diarrhea. These symptoms typically manifest a few hours following consumption of such tainted meal **(Hennekinne et al., 2012).**

Chiang et al., (2008) reported that exoproteins generated in food and consumed by people, known as *staphylococca*l enterotoxins, cause acute gastroenteritis symptoms and are the cause of *Staphylococcal* food poisning.

The overuse of antibiotics in Egypt's catfish and tilapia farms has led to the rise of bacteria that are resistant to these drugs, such as *S. aureus* **(Decimal et al., 2022).**

The antibacterial effects of ascorbic acid, and acetic acid in food systems were reported by **Przekwas et al. (2020).** Scientific research has shown that ascorbic acid can decrease biofilms of food-borne pathogens such as *S. aureus, Escherichia coli,* and *Listeria monocytogenes*.

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. **(El Bialy et al., 2024)**

In the current investigation, the prevalence of *S. aureus* was determined in three different types of fish: Nile tilapia, mullet, and catfish samples in El-Beheira

governorate, Egypt. In order to further confirm the antimicrobial susceptibility of the recovered *S. aureus* isolates, the disc diffusion assay was employed. The enterotoxins produced by *S. aureus* were also identified using RPLA. A reduction trial for *S. aureus* load in tilapia fish was conducted using acetic and ascorbic acids.

Materials and Methods

Sample collection

Sixty random fish samples from different local markets, comprising *Clarias lazera* (Cat fish), *Oreochromis niloticus* (Nile tilapia) and *Mugil cephalus* (twenty of each), were acquired from specific fish markets in El-Beheira governorate of Egypt. Each sample was stored in an individual sterile plastic bag, labeled accordingly, and preserved in an icebox prior to its transport to the laboratory for pathogen identification. All obtained samples were analyzed for the presence of *S. aureus*.

Preparation of samples

The dorsal, pectoral, and ventral fins were excised using sterile scissors and forceps. Fish were grasped with sterile forceps, and scales were excised from the entire body surface using a sterile knife. Body surfaces were sterilized using a heated spatula. The sterilized surface was excised using sterile scissors and forceps under stringent aseptic conditions; 10 grams of the posterior neck muscles were aseptically transferred into a sterile homogenizer flask (homogenizer type MPW–302, Poland) containing 90 ml of sterile 0.1% peptone water. The mixture was homogenized for 2.5 minutes at 14,000 rpm to achieve a 1/10 dilution, then permitted to stand for approximately 5 minutes. The homogenate was placed into a sterile flask, and 1 ml was aliquot into a sterile test tube containing 9 ml of 0.1% peptone water, from which tenfold serial dilutions were generated **(ISO 4833 : 2022).**

Isolation and identification of *Staphylococcus aureus*

One milliliter from each of the previously made serial dilutions was evenly distributed onto Baired Parker agar plates utilizing a sterile bent glass spreader. The plates were maintained in an upright orientation until inoculum was absorbed by the agar for approximately 10 minutes. The inoculated and control plates were inverted and incubated at 37°C for 48 hours. Subsequently, we analyzed them for colony traits. The developed colonies (shiny black colonies) were quantified, and the presumptive *S. aureus* count per gram was determined. Five *S. aureus* colonies were isolated on nutrient agar slopes for further biochemical examination. Colonies of *S. aureus* were detected via morphological, biochemical, and serological methods. *S. aureus* isolates tested positive for catalase, coagulase, and hemolysis, exhibiting yellow colonies with halo zones in the mannitol test during biochemical analysis. **(FDA, 2001)**

Antimicrobial susceptibility of the isolated *S. aureus* isolates

The disc diffusion method described by **Daka *et al*. (2012)** was employed to assess the antibiotic resistance of the isolated *S. aureus* strains. The antimicrobial discs were sourced from Oxoid Limited, located in Hampshire, UK. The antimicrobial susceptibility of *S. aureus* was assessed using nutrient agar plates as the culture medium. Consequently, the antimicrobial susceptibility testing was conducted in accordance with the standards established by **White *et al*. (2001)**.

Furthermore, **Singh *et al*. (2010)** employed an algorithm to compute the Multiple Antibiotic Resistance (MAR) index for each examined *S. aureus* isolate as follows: The MAR index is determined by dividing the quantity of antibiotics tested by the overall number of resistances.

The employed antibiotics include ampicillin (10 g; AM), cephalothin (30 g; CET), chloramphenicol (30 g; C), ciprofloxacin (5 g; CIP), enrofloxacin (5 g; ENR), erythromycin (15 g; E), gentamicin (10 g; GEN), kanamycin (30 g; K), nalidixic acid (30 g; NA), neomycin (30 µg; N), oxacillin (1 µg; OX), oxytetracycline (30 µg; OXY), penicillin (10 IU; P), and trimethoprim/sulfamethoxazole (25 µg; SXT).

Staphylococcal enterotoxins production (Shingaki, 1981).

A reverse passive latex agglutination (RPLA) test was conducted on the clear culture supernatant to detect staphylococcal enterotoxins A, B, C, and D. The kits for this test were produced by Denka Sekeu LTD in Japan.

The microtitre plate (v-type) was configured with each row containing eight wells. Each test sample required the utilization of five rows of wells on the microtitre plate. Precisely, 25 µl of diluent were dispensed into each well of five rows in the microtitre plate utilizing a pipette or dropper. The sample was collected concurrently with five diluents (each 25 µl), and a two-fold dilution of the test sample was performed along each of the five rows. The final well of each row contained merely 25 µl of diluent. In the first, second, third, and fourth rows of the plate, 25 µl of latex suspensions sensitized with anti-enterotoxin A, B, C, and D were introduced into each well.

Precisely, 25 µl aliquots of control latex were dispensed into each well of the fifth row, while the plate was rotated using a micromixer to guarantee thorough mixing of the contents. The plate was sealed with a lid and remained undisturbed on a vibration-free surface at ambient temperature for 20 to 24 hours. Each well in the row was assessed for agglutination against a black background.

The sensitivity of this test kit for detecting enterotoxins is reported to be 0.5 ng/ml in the test extract. Consequently, enterotoxins at concentrations below this threshold produced erroneous results.

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

The methodology implemented was established by **Abbasit et al. (2009),** but with certain adjustments. Thirty fresh *Oreochromis niloticus* samples were meticulously rinsed with tap water and subsequently with distilled water prior to the experiment. Subsequently, all fish specimens were decapitated and disemboweled. The examined fish samples were categorized into six groups, with five samples per group. The initial negative control group was submerged in water with no pathogen; the other 5 groups were submerged in water infused with the pathogen *S. aureus* at 10^6 cfu/g. The second group was left without any treatment. The third and fourth groups were submerged in 1% and 2% acetic acid solutions for 30 minutes, respectively. The 5th and 6th groups were immersed in solutions of 1% and 2% ascorbic acid for 30 minutes, respectively. Subsequent to the designated interval, the examined fish groups were assessed for pathogen contamination. The reduction percentage in the average counts of *S. aureus* were analyzed.

Statistical analysis

The counts of *S. aureus* were converted to base-10 logarithms of cfu/g. The one-way ANOVA method in SPSS v.23 (SPSS Inc., Chicago, Illinois, USA) was employed for data analysis. Tukey's multiple

comparison tests were conducted to identify statistically significant variations in *S. aureus* counts among the samples analyzed. A p-value of 0.05 is considered statistically significant. Data were presented as means ± standard error.

Results and Discussion

Foodborne diseases are mostly attributed to *Staphylococcus.aureus*, ranking third worldwide**(CDC 2011).** The variety of foods associated with staphylococcal food poisoning clearly illustrates *S. aureus's* ability to multiply and produce toxins in diverse environmental and nutritional conditions **(Shawish et al., 2016).** *Staphylococcus aureus* was isolated from retail fish, including tilapia, mullet, and catfish, at varying frequencies. Catfish had the highest prevalence rate of *S. aureus* at 70%, followed by tilapia at 60% and mullet at 45% **(Fig. 1).** Simultaneously, catfish exhibited a substantially higher overall *S. aureus* count (3.64 ± 2.49 log10 cfu/g) compared to Nile tilapia (3.44 ± 2.27 log10 cfu/g) and mullet (2.00 ± 1.85 log10 cfu/mL) **(Fig. 2).** In our comparison of the recorded *S. aureus* counts from the current study with the maximum permissible limits established by the Egypt Organization for Standardization **(EOS 3494 : 2005)** at 3 log10 cfu/g, we observed that 55%, 35%, and 20% of the analyzed catfish, tilapia, and mullet, respectively, tested positive **(Fig. 3).**

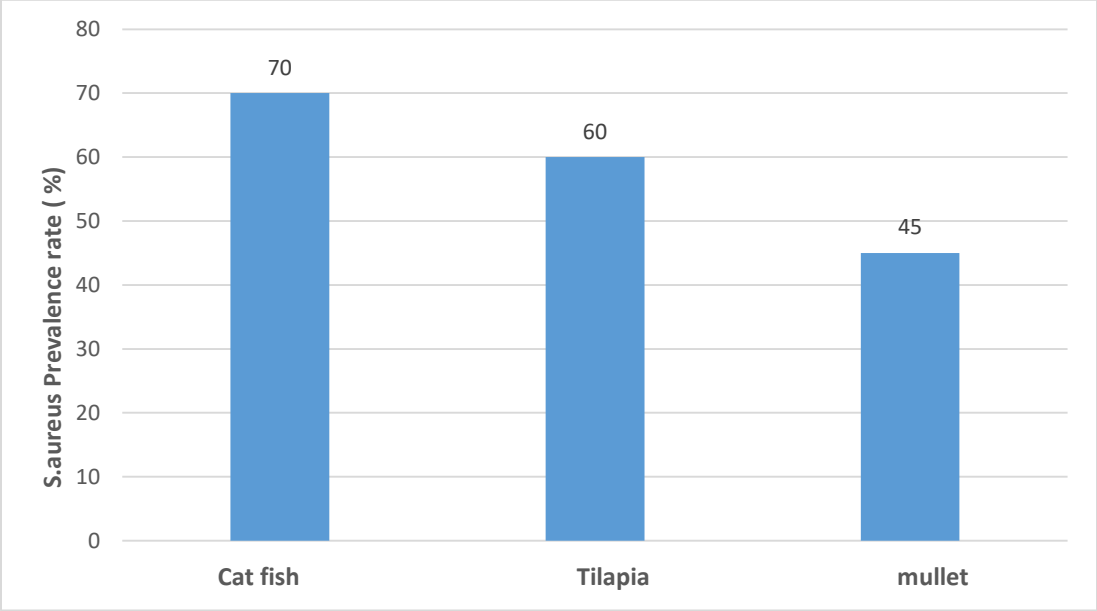

Fig. 1. Prevalence rate (%) of *S. aureus* in the examined catfish, tilapia, and mullet (n = 20/each).

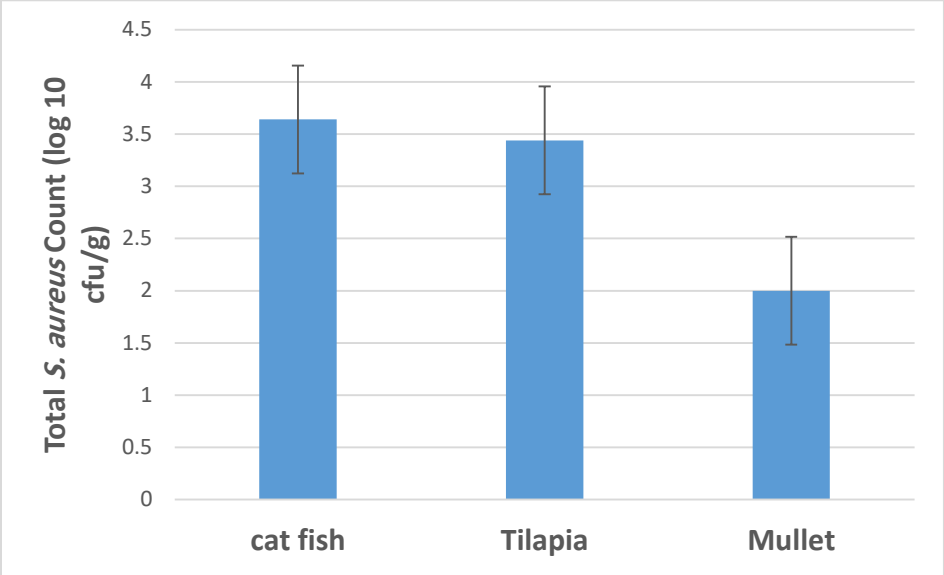

Fig. 2. Total *S. aureus* count (log10 cfu/g) in the examined catfish, Tilapia, and Mullet (n= 20/each).

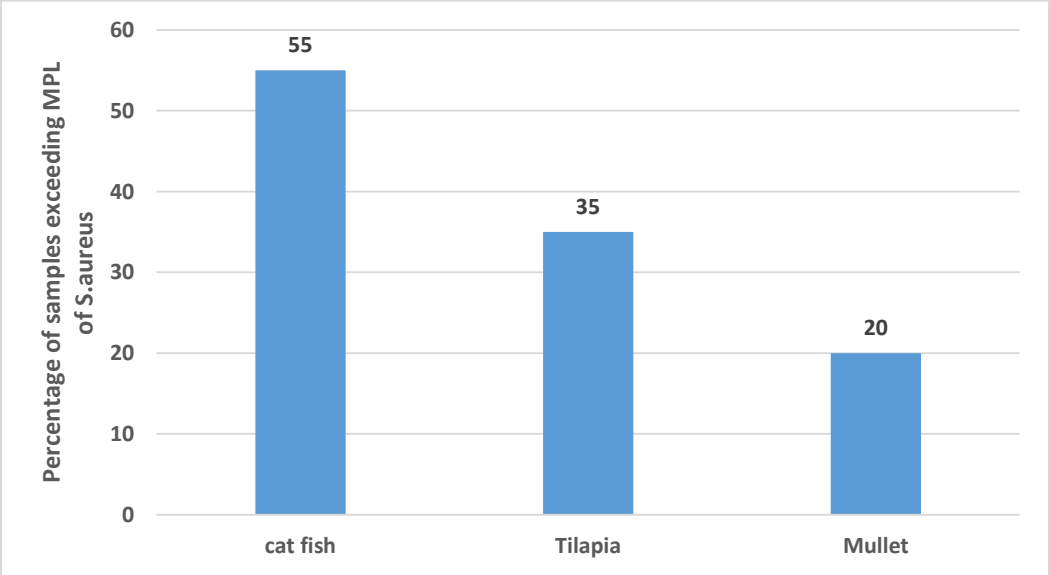

Fig. 3. Percentages of samples exceeding maximum permissible limits of *S. aureus* (3 log10 cfu/g) in the examined catfish, tilapia, and mullet (n= 20/each) compared to EOS No 3494 : 2005.

Likely, **El-Shaer et al. (2012)** reported that the *S. aureus* counts in the examined fish samples varied from 2.39 to 3.52, 3.58 to 4.23, and 3.59 to 4.91 log 10 cfu/g for mullet, tilapia, and catfish, respectively. Similar findings were also reported by **Morshdy et al. (2018).**

The increasing prevalence of *S. aureus* in fish could be attributed to various factors, including bacterial infections, poor water quality, and anthropogenic activities leading to aquatic pollution. Throughout the handling and processing phases, the bacterial flora in fish can undergo

significant changes, with various bacterial species prevailing at distinct stages. Moreover, the bacterial count in fish samples is significantly elevated in certain markets. This indicates a possible source of contamination and an elevated risk of foodborne diseases, a result similarly drawn by **Dar et al. (2020).**

In reaction to the deficiency of red meat, fish farming and aquaculture are proliferating worldwide. Antimicrobials are employed alongside these initiatives to avert and address bacterial diseases in fish. However, the improper use of antibiotics in aquaculture might lead to the development of antibiotic resistance **(Alsayeqh et al., 2021).** Consequently, the isolated *S. aureus* strains underwent antibiotic susceptibility testing. Notably, all examined isolates (100%) shown resistance to a minimum of two of the evaluated antimicrobials. Penicillin G (90%), cefotaxime (75%), sulfamethoxazole (70%), colistin (60%), azithromycin (55%), ceftriaxone (45%), and tetracycline (40%) shown ineffectiveness against the tested *S. aureus* strains. Ciprofloxacin (35%), amikacin (25%), oxacillin (10%), linezolid (8%), and vancomycin (5%) were also not effective. The computed MAR index for the isolated *S. aureus* strains varied from 0.063 to 1 with a mean of 0.427 **(Table 1).**

Likewise, 99.1% of *S. aureus* isolated from gills exhibited resistance to ampicillin, but 53.3% from the gastrointestinal tract demonstrated resistance to levofloxacin. *Staphylococcus aureus* isolated from water samples shown complete resistance to ciprofloxacin, norfloxacin, gentamicin, amoxicillin, rifampicin, erythromycin, ampicillin, and levofloxacin, whereas 50% showed resistance to streptomycin and chloramphenicol **(Matouke et al., 2019).** This study may have identified antibiotic-resistant *S. aureus* due to selection pressure from human activities, stemming from the misuse of antibiotics leading to residual medicines in aquatic environments.

Table (1): Antimicrobial resistance profile of *S. aureus* strains isolated from the examined fish samples (n=35).

Key No	Antimicrobial resistance profile	MAR index
1	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK, M, OX, LZ, V, DA**	1
2	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK, M, OX, LZ, V**	0.937
3	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK, M, OX, LZ**	0.875
4	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK, M**	0.750
5	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK, M**	0.750
6	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK**	0.688
7	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK**	0.688
8	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK**	0.688
9	**N, P, CF, SXT, CO, AZ, CR, T, G, CP, AK**	0.688
10	**N, P, CF, SXT, CO, AZ, CR, T, G, CP**	0.625

11	**N, P, CF, SXT, CO, AZ, CR, T, G, CP**	0.625
12	**N, P, CF, SXT, CO, AZ, CR, T, G, CP**	0.625
13	**N, P, CF, SXT, CO, AZ, CR, T, G, CP**	0.625
14	**N, P, CF, SXT, CO, AZ, CR, T**	0.500
15	**N, P, CF, SXT, CO, AZ, CR**	0.437
16	**N, P, CF, SXT, CO, AZ, CR**	0.437
17	**N, P, CF, SXT, CO, AZ**	0.375
18	**N, P, CF, SXT, CO, AZ**	0.375
19	**N, P, CF, SXT, CO, AZ**	0.375
20	**N, P, CF, SXT, CO**	0.313
21	**N, P, CF, SXT, CO**	0.313
22	**N, P, CF, SXT**	0.250
23	**N, P, CF, SXT**	0.250
24	**N, P, CF, SXT**	0.250
25	**N, P, CF, SXT**	0.250
26	**N, P, CF, SXT**	0.250
27	**N, P, CF**	0.187
28	**N, P**	0.125
29	**N, P**	0.125
30	**N, P**	0.125
31	**N, P**	0.125
32	**N, P**	0.125
33	**N**	0.063
34	**N**	0.063
35	**N**	0.063
Average 0.427		

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

A test employing RPLA to detect staphylococcal enterotoxin in specific *S. aureus* isolates revealed the presence of enterotoxin A in three isolates, two derived from catfish and one from tilapia. Enterotoxin C was found solely in one strain obtained from mullet. Enterotoxin D was found in a single sample from mullet and one strain from tilapia. Enterotoxin A+D was found solely in one isolate obtained from catfish as illustrated in **(Table 2).** The obtained results go in agreement with results investigated by **Morshdy et al. (2018).**

Table (2): Prevalence of entertotoxins producing *S. aureus* strains isolated from the examined samples of fish (n= 20).

Entertotoxin	*Clarias Lazera*		*Oreochromis niloticus*		*Mugil Cephalus*	
	No	**%**	**No**	**%**	**No**	**%**
A	2	10	1	5	-	-
C	-	-	-	-	1	5
D	-	-	1	5	1	5
A+D	1	5	-	-	-	-
Total	3	15	2	10	2	10

Experiments were performed to decrease the *S. aureus* population utilizing acetic acid at concentrations of 1% and 2%, and ascorbic acid at concentrations of 1% and 2%. The findings indicated reduction percentages of 21.2%, 46.1%, 17.3%, and 28.9%, respectively. Consequently, rigorous hygiene protocols must be implemented during the handling, processing, and sale of fish. These obtained results are closely correspond with the findings documented by **Selim et al. (2012)** and **Monirul et al. (2019).**

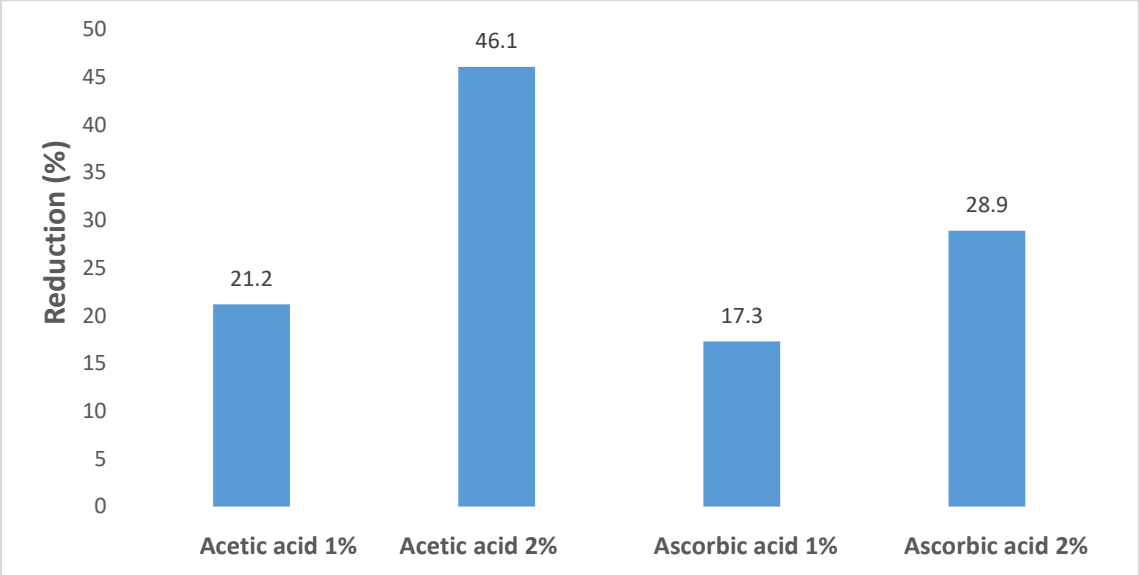

Fig 4: Effect of organic acids at different concentrations on the *S. aureus* counts in the examined samples of *Oreochromis niloticus* (n=5/ each group).

Conclusion

The results of the current investigation demonstrated that *S. aureus* contamination of retailed fish happened at different rates and occasionally went over the established Egyptian limits. The *S. aureus* isolates that were collected also showed a notable resistance to many drugs and a robust tolerance to antibiotics. Additionally, certain *S. aureus* isolates could produce enterotoxins. Acetic acid and ascorbic acid can be used safely for controlling *S. aureus* in fish. Therefore, when processing and preparing fish, great attention to sanitation is required and strict hygienic measures should be adopted.

Conflict of interest

The authors declare they have no conflict of interest.

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