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Innovative strategies for identifying and controlling *Staphylococcus aureus* in marine fish: Evaluating the impact of organic acids

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ABSTRACT

This study investigated the prevalence of *Staphylococcus aureus* in three types of fish, "Sardine, Chrysophyrus, and Pagrus" and assessed their fitness for consumption based on bacterial counts. The results showed that Sardine samples had the highest contamination rate at 27.5%, followed by Chrysophyrus at 15%, and Pagrus at 10%. Notably, 20%, 12.5%, and 5% of these fish, respectively, exceeded permissible bacterial limits, rendering them unfit for consumption according to EOS. The isolated *S. aureus* strains produced various enterotoxins in Sardine, Chrysophyrus, and Pagrus samples, which were A and A+B, B, and D enterotoxins, respectively. Antimicrobial susceptibility revealed complete resistance to kanamycin and significant resistance to several antibiotics, while showing high susceptibility to Linezolid, Cefepime, and Daptomycin. The Multiple Antibiotic Resistance (MAR) index ranged from 0.071 to 1, averaging 0.412. Molecular analysis confirmed the presence of the *nuc* gene in all strains, while only three strains carried the *mecA* gene, indicating methicillin resistance. The study also demonstrated the efficacy of organic acids in reducing *S. aureus* counts. Treatments with acetic and ascorbic acids (3%) achieved a reduction of 43.7% and 31.4% of the count. Importantly, these treatments did not compromise sensory qualities, with all samples receiving high ratings in sensory evaluations. This suggests that organic acids can be effectively used to enhance food safety without impacting product quality.

1. INTRODUCTION

Societal growth and dietary diversity have prompted individuals to seek out nutritionally valuable food at a lower cost, resulting in a human inclination towards marine products. Fish meat provides the consumers with the required protein source, essential healthy fatty acids, and vitamins (Ali, 2014). So, the safety of this food supply represents great concern. Foodborne illnesses caused by several pathogenic bacteria like *E. coli*, *Salmonella* spp., *Listeria monocytogenes*, and *Staphylococcus aureus* are among the bacteria that commonly infect fish.

Staphylococcus aureus is the third significant foodborne bacteria reported worldwide (Arfatahery et al., 2015). It causes a higher level of mortality and morbidity frequency (Kong et al., 2016). Although the internal organs of fish are sterile, the contamination of fish flesh occurs mainly after harvest through improper handling, preservation, and transportation (Kumar et al., 2016). The foodborne intoxication of *Staphylococcus* occurs within 2-6 hours after consumption of food containing Staphylococcal enterotoxins (Tranter, 1990) even with a tiny amount vary from 20 ng to < 1 ug / g of food (Abu-Ghazaleh, 2013). There were about nine serotypes of enterotoxins that are produced by *Staphylococcus aureus*, among them enterotoxin A, represents the foremost cause for staphylococcal food poisoning (Wallin-Carlquist et al., 2010). It can withstand different inappropriate conditions.

Staphylococcus aureus displays antibiotic tolerance and growing seafood prevalence, which qualifies this bacterium as a resistant strain that affects global antibiotic distribution (Obaidat et al., 2015). Multiple studies have demonstrated that antibiotic-resistant *S. aureus* occurs in fish between 30% through 60% (Kumar et al., 2016; Vaiyapuri et al., 2019). The most significant and widely demonstrated strain of *S. aures* is Methicillin-resistant *Staphylococcus aureus* (MRSA) that infects a wide range of hosts and exhibit resistance against methicillin and other beta-lactam antibiotics through carrying *mecA* genes (Tsubakishita et al., 2010).

The growing concern to use natural preservative enhancing antimicrobial activity directs researchers to examine the effect of organic acids like ascorbic and acetic acid against *Staphylococcus aureus* contamination. As these acids not only reduce the pH of the surrounding area but also there were weakly acids that have ability to penetrate the cell membrane of the bacteria and reduce its cytoplasmic pH so leading to a bactericidal effect (Ji et al., 2023). Those organic acids were safe and have no harmful effects on nature or humans (Ciriminna et al., 2016).

Therefore, the following study aimed to examine the occurrence of *Staphylococcus aureus* isolated from different marine fish with examination of their sensitivity to different antibiotics, their molecular identification, and demonstration to the effect of acetic and citric acid on it.

2. MATERIAL AND METHODS

This work follows the ethics of the Scientific Research Ethics Committee, Faculty of Medicine, Benha University, with Ethical Approval Number (Rc 4_4_2025).

2.1. Sampling

A total number of 120 fresh marine fish samples represented by Sardine "*Sardinella pilchardus"*, Chrysophyrus "*Chrysophyrus* auratus", and Pagrus "*Pagrus Pagrus"* (40

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of each) were collected from different fishers at Menoufia government, Egypt, and transferred to the laboratory in separate bags in the ice box.

2.2. Enumeration and identification of S. aureus count (ISO 6888-1, 2021).

In a stomacher bag, 25 g of each sample was homogenized with 90 mL peptone water for 2 minutes and underwent a ten-fold serial dilution. One ml from each dilution was spread over a Baird-Parker agar plate and incubated at 37°C for 48 hours. The produced colonies (shiny black colonies surrounded by a halo zone) were enumerated as presumptive *S. aureus* count/g. The suspected colonies were identified morphologically, biochemically, and serologically by using a reliable latex slide agglutination test, " Staphylase test" (Oxoid Dry Spot Staphytect Plus Kit).

2.3. Detection and typing of enterotoxin (Shingaki et al., 1981).

The clear culture supernatant was tested for staphylococcal enterotoxins A, B, C, and D using the Reverse Passive Latex Agglutination (RPLA) method with SET-RPLA kits from Denka Seiken Ltd., Japan. A microtiter plate was set up with 5 rows of 8 wells each. For each test sample, 25 µl of diluent was added to all wells in the 5 rows. A two-fold dilution of the test sample was prepared across these rows, with the last well in each row containing only diluent. Latex suspensions sensitized to anti-enterotoxins A, B, C, and D were added to the first four rows, while control latex was added to the fifth row. The plate was mixed using a micro mixer and incubated undisturbed at room temperature for 20-24 hours. Agglutination was then assessed against a black background. The sensitivity of the test kit is reported to be 0.5 ng/ml; concentrations below this threshold may yield false-negative results.

2.4. Molecular identification for isolated Staphylococcus aureus.

Molecular identification was performed for the thermonuclease (*nuc*) and Methicillin Resistant S. *aureus* "MRSA" (*mecA*) genes, using the primers demonstrated in Table (1).

Table 1. Primers for the thermonuclease (*nuc*) and Methicillin Resistant S. *aureus* "MRSA" (*mecA*) genes.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
nuc (F)	5′ GCGATTGATGGTGATACGGTT ′3	270	Brakstad et al. (1992)
nuc (R)	5′ AGCCAAGCCTTGACGAACTAAAGC ′3		
mecA (F)	5′ TAGAAATGACTGAAC GTCCG ′3	533	Jukes et al. (2010)
mecA (R)	5′ TTGCGATCA ATGTTACCGTAG ′3		

The extraction steps of DNA follow the instructions labeled in the QIA-amp kit based on the method described by Shah et al. **(**2009). Then the extracted DNA was amplified by using a Thermal Cycler (Master cycler, Hamburg, Germany) through the following steps: an initial denaturation step at 94°C for 5 min followed by 20 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min and final extension at 72°C for 5 min. The DNA fragments were resolved by gel electrophoresis 1.5% agarose gel stained with ethidium bromide solution (0.5µg/ml), visualized under U/V transilluminator, and photographed. A 100 bp plus DNA Ladder was used to determine the fragment sizes.

2.5. Experimental study

2.5.1. Effect of organic acids on control of S. aureus in fish.

The technique adopted by Perviaz et al. (2009) was followed with some modifications. Twenty fresh Sardine samples were washed thoroughly with tap water, followed by distilled water, before they were subjected to the experiment. The tested fish samples were divided into 4 groups (5 samples for each group). The 1st control +ve group was immersed in water containing the pathogen. The 2nd group acts as a control -ve group for sensory evaluation. The 3rd and 4th groups were immersed in a 3% acetic acid and ascorbic acid solution for 30 minutes, respectively. After the appropriate period, all groups were examined for *S. aureus* counts, and the reduction percentage was calculated.

2.5.2. Sensory evaluation (Kilinc and Caki, 2004).

Accurately, 5 panelists evaluated the sensory attributes of Sardine samples. The samples were blind-coded with special codes; the panelists were not informed about the experimental approach. They were asked to give a score for each of appearance, odor, and texture (5 for each trait and total score=15). The panelists were asked to wash their mouths with warm water between samples.

2.6. Statistical analysis

The obtained results were statistically evaluated with Duncan by SPSS® version 16.0 according to Feldman et al. (2003).

3. RESULTS

The date tabulated in Table (2) describes the frequency of *S. aureus* in three types of fish samples, "Sardine, Chrysophyrus, and Pagrus", where the highest frequency was seen in Sardine samples, "27.5%", then Chrysophyrus "15%", and finally Pagrus "10%". Based on the *S. aureus* counts, Sardine samples showed a remarkably elevated mean count (cfu/g) exceeding those in Chrysophyrus and Pagrus that were 3.09×10^3, 1.76×10^3, and 7.15×10^2, respectively. The fitness of the tested fish "Sardine, Chrysophyrus, and Pagrus" was determined as 20%, 12.5%, and 5% were unfit for consumption according to EOS (2005) as illustrated in Table (3).

Table 2. Incidence and count (cfu/g) of *Staphylococcus aureus* in examined marine fish samples(n=40)

Fish samples	Number	Positive sample		*S. aureus* counts (cfu/g)		
		No.	%	Min	Max	Mean ± S.E*
Sardine	40	11	27.5	8×10^2	7×10^3	$3.09\times10^3\pm 0.24\times10^{3\ A}$
Chrysophyrus	40	6	15	7×10^2	3×10^3	$1.76\times10^3\pm 0.13\times10^{3\ B}$
Pagrus	40	4	10	4×10^2	1.5×10^3	$7.15\times10^2\pm 0.56\times10^{2\ C}$

*Values with different superscripts in the same column differed significantly at P<0.05.

Table 3. Fitness of the examined fish based on *Staphylococcus* count.

Fish samples	Fit samples		Unfit samples	
	No.	%	No.	%
Sardine	32	80	8	20
Chrysophyrus	35	87.5	5	12.5
Pagrus	38	95	2	5

Fit samples represent fish with no *Staphylococcus* infection or that with a count not exceed10^2/g according to EOS (2005).

The data summarized in Table (4) showed that the typing of enterotoxins in the examined fish was 2 out of 11 Sardine samples were contaminated by enterotoxin A and A+B, while 2 out of 6 Chrysophyrus samples were enterotoxin B, and one of the Pagrus samples was enterotoxin D.

Table 4. The isolated *Staphylococcus aureus* enterotoxins in the fish sample.

Enterotoxin	Sardine		Chrysophyrus		Pagrus	
	No	%*	No	%*	No	%*
A	1	9.09	-	-	-	-
B	-	-	2	33.3		
D	-	-	-	-	1	25
A+B	1	9.09	-	-	-	-
Total	2	18.1	2	33.3	1	25

* Percentage based on the positive samples of each species.

The results of antimicrobial susceptibility for 21 isolated strains of *Staphylococcus aureus* were illustrated in Fig. (1) where all the isolated strains exhibit complete resistant to kanamycin "100%", and slightly resistant to rifampcin, cefotaxime, ampicillin, and tetracycline with percentage 85.7%,76.2%, 71.5%, and 52.4%, respectively. However, it showed a high susceptibility to Linezolid, Cefepime, Daptomycin, Oxacillin, Nitrofurantoin, Levofloxacin, Gentamicin, and Sulphamethoxazol with the following percentage: 95.3%, 90.5%, 81%, 81%, 76.2%, 61.9%, 57.2%, and 57.2%, respectively. The calculated MAR index for the recovered *S. aureus* isolates ranged between 0.071 to 1, with an average of 0.412 (Table 5).

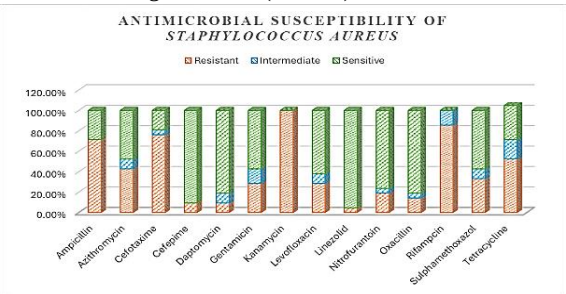

Figure (1). Antimicrobial susceptibility patterns for 21 isolated strains of *Staphylococcus aureus.*

Table 5. Antimicrobial resistant profile of the isolated *Staphylococcus aureus.*

Key No	Antimicrobial resistance profile	MAR index
1	K, R, CF, AM, T, AZ, SXT, G, L, N, X, DA, FEP, LZ	1
2	K, R, CF, AM, T, AZ, SXT, G, L, N, OX, DA, FEP	0.928
3	K, R, CF, AM, T, AZ, SXT, G, L, N, OX	0.785
4	K, R, CF, AM, T, AZ, SXT, G, L, N	0.714
5	K, R, CF, AM, T, AZ, SXT, G, L	0.642
6	K, R, CF, AM, T, AZ, SXT, G, L	0.642
7	K, R, CF, AM, T, AZ, SXT	0.500
8	K, R, CF, AM, T, AZ	0.428
9	K, R, CF, AM, T, AZ	0.428
10	K, R, CF, AM, T	0.357
11	K, R, CF, AM, T	0.357
12	K, R, CF, AM	0.286
13	K, R, CF, AM	0.286
14	K, R, CF, AM	0.286
15	K, R, CF, AM	0.286
16	K, R, CF	0.214
17	K, R	0.143
18	K, R	0.143
19	K	0.071
20	K	0.071
21	K	0.071
	Average	0.412

The molecular examination for the Thermonuclease gene (*nuc*) and the Methicillin Resistant *S. aureus* "MRSA" gene (*mecA*) was illustrated in Fig. (2). All the examined strains carried the *nuc* gene, which amplified at 270 bp. While only three strains, 6, 9, and 18, carried *the mecA* gene, which amplified at 533 bp.

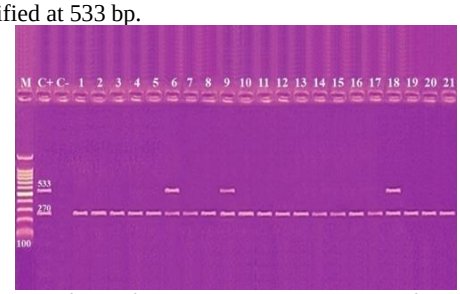

Figure (2). Amplification of *nuc* (270bp) and *mecA* (533bp) genes of *Staphylococcus aureus* by using multiplex PCR. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive for *nuc* and *mecA* genes. Lane C-: Control negative. Lanes from 1 to 18: Positive *S. aureus* strains for *nuc* gene. Lanes 6, 9 & 18: Positive *S. aureus* strains for *nuc* and *mecA* genes.

The effect of addition of organic acid 3% on the viability of *Staphylococcus aureus* was demonstrated in Table (6) Whereas the count of *Staphylococcus aureus* (cfu/g) in the control group was 1.53×10^6 while in the treated samples with acetic acid 3%, it reduces to 8.61×10^5 with marked reduction percentage of 43.7%, although the treated ones with ascorbic acid 3% was showed count 1.05×10^6 with reduction percentage of 31.4%. In addition, the sensory evaluation of the treated samples was examined and recorded in Table (7). Samples of the control and treated ones were of high ratings in sensory evaluation, with scores indicating "Very good" quality across all traits assessed. The treatment with 3% acetic acid showed the highest overall score, suggesting it may enhance sensory attributes slightly more than ascorbic acid while maintaining high quality in appearance, odor, and texture. This indicates that organic acid treatments can be beneficial without compromising sensory qualities in Sardine products.

Table 6. Influence of organic acids (3%) on the viability of *S. aureus* in the examined samples of Sardine (n=5).

Treatment	Mean ± S.E (cfu/g)	Reduction %
Control	$1.53\times10^6\pm 0.12\times10^{6\,A}$	-------
3% Acetic acid	$8.61\times10^5\pm 0.79\times10^{5\,C}$	43.7
3% Ascorbic acid	$1.05\times10^6\pm 0.94\times10^{6\,D}$	31.4

Values with different superscripts within the same column differed significantly at P<0.05.

Table 7. Sensory evaluation between control and organic acid treated samples (n=5).

Treatment	Appearance (5)	Odor (5)	Texture (5)	Overall (15)	Grade
Control -ve	4.8	4.6	4.8	14.2	Very good
3% Acetic acid	5	4.8	5	14.8	Very good
3% Ascorbic acid	4.8	5	4.8	14.6	Very good

4. DISCUSSION

Fish become contaminated by microorganisms during their journey from aquatic environments until reaching the table throughout the harvest and processing steps, along with storage until final sales (Trang et al., 2023). The frequency of *Staphylococcus aureus* in examined marine fish in this study is nearly similar to Elkassas et al. (2021), who detected the presence of *Staphylococcus aureus* in 30% of examined sardines. Furthermore, it is higher than those recorded by Morshdy et al. (2022), who isolated *S. aureus* from 15% of examined Sardine fish. While it is lower than that recorded by Hassanien et al. (2016), who found that *Staphylococcus aureus* present in Sardine was 36.6%, in addition to those of Darwish et al. (2023), who isolated *S. aureus* from 64% of Pagrus fish.

Comparing the counts of Staphylococcus aureus with the standard maximum permissible limits recorded by the Egyptian Organization for Standardization (EOS, 2005), where the count should not exceed10^2/g, the present results are higher than those reported by Morshdy et al. (2022). Foodborne illness occurs due to ingestion of food polluted with staphylococcal enterotoxins (SEs) (Darwish et al., 2022), mainly Enterotoxin A is the most prominent enterotoxin, causing foodborne intoxication (Sokari, 1991). In the present study, only 18.1% of examined *Staphylococcus aureus* were isolated from Sardine can produce enterotoxins type A and A+B, whilst 33.3% of examined *Staphylococcus aureus* ones were isolated from Chrysophyrus produced enterotoxin B, and 25% of Pagrus examined samples produced enterotoxin D. This nearly agrees with the findings detected by Darwish et al. (2023) and Hussein et al. (2019).

The increasing international interest in fish farming along with aquaculture activities motivated people to overuse antibiotics to compensate for various bacterial diseases that may infect fish during their growth curve, which leads to the development of antimicrobial resistance (Alsayeqh et al., 2021). Results of the current study in Fig (1) and Table (5) is nearly similar to those of Vázquez-Sánchez et al. (2012)

who determined that all *S. aureus* strains obtained from salted fish displayed resistance to penicillin, chloramphenicol, and ciprofloxacin and showed resistance to tetracycline in 82.4% in cases at Spain. Moreover, Morshdy et al. (2022) detected that the isolated *S. aureus* showed multiple resistance toward several antibiotics with an average MAR of 0.435. Also, Darwish et al. (2023) demonstrate the resistance of the isolated strains to kanamycin, clindamycin, nalidixic acid and Sulphamethoxazole with multiple antibiotic resistance (MAR) index for the recovered *S. aureus* isolates ranged from 0.062 to 1 with an average value of 0.442.

The molecular analysis for the Thermonuclease gene (*nuc*), which was a species-specific gene for *S. aureus* (Studer et al., 2008), was detected in all examined isolates (Fig. 2). Although analysis of the *mecA* gene showed its presence in only three strains out of 21 strains (14.3%), this is nearly similar to findings of Arfatahery et al. (2016) and Zhang et al. (2005).

The present study reveals the efficacy of organic acids (acetic acid and ascorbic acid) in reducing the viability of *Staphylococcus aureus* while maintaining sensory qualities in Sardine fish. Organic acids like acetic and ascorbic acids exert their antimicrobial effects by disrupting cell membrane integrity and creating an acidic environment that inhibits metabolic processes essential for bacterial survival (Raftari et al., 2009; Usanmaz et al., 2024). This action is more pronounced with weak organic acids such as acetic acids compared to strong acids like hydrochloric acid (Zhou and Fey, 2020). Despite the antimicrobial treatment, all samples received high ratings during sensory evaluation, indicating that these treatments do not compromise product quality. Acetic acid treatment showed slightly better performance than ascorbic acid regarding sensory attributes such as appearance, odor, and texture.

5. CONCLUSIONS

The study highlights the significant presence of *Staphylococcus aureus* in marine fish, with contamination rates varying across different species. The findings indicate that Sardine was contaminated than Chrysophyrus, and Pagrus. This contamination poses a risk to consumer health due to the potential production of staphylococcal enterotoxins, which can cause foodborne illness. The isolated strains showed high resistance to several antibiotics like Kanamycin, rifampcin, cefotaxime, ampicillin, and tetracycline, underscoring the issue of antimicrobial resistance in the food chain. However, the use of organic acids like acetic and ascorbic acids offers a promising approach to reducing *S. aureus* counts without compromising product quality. These results emphasize the need for improved handling and processing practices to minimize microbial contamination and ensure the safety of fish products for consumption.

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