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Control of *Vibrio* spp. in Commercially Sold Fish Using Bioactive Natural Antimicrobials

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Abstract | *Vibrio* species are significant pathogenic bacteria found in fish that induce gastroenteritis, a major public health issue. The present research investigated the occurrence, virulence gene detection, antibiotic resistance profile, and the antibacterial influence of lemon juice and pomegranate peel extract on *V. cholerae* and *V. parahaemolyticus*. Samples were 150 fish (30 of each Nile tilapia, Nile perch, sardine, Mugil cephalus, and Sea bass) from fish supermarkets. Sardine samples had the highest *Vibrio* count ($4.77 \pm 1.22 \log_{10}$ CFU/g), followed by and Sea bass ($4.62 \pm 1.35 \log_{10}$ CFU/g), *Mugil cephalus* ($3.50 \pm 1.74 \log_{10}$ CFU/g), Nile perch ($3.24 \pm 1.05 \log_{10}$ CFU/g), while Nile tilapia had the lowest count ($2.90 \pm 0.78 \log_{10}$ CFU/g), and 42% of all fish tested positive for *Vibrio* species. The most prevalent isolated *Vibrio* species were *V. cholerae* (38.2%), *V. parahaemolyticus* (31.7%), *V. vulnificus* (19%), and *V. fluvialis* (11.1%). According to a polymerase chain reaction analysis, most *Vibrio* isolates had one or more virulence genes. Antibiotic sensitivity tests showed that the isolates were highly resistant to many commonly used antimicrobial drugs in Egypt. These drugs included ampicillin, tetracycline, and sulfamethoxazole. The average MAR score was 0.530 indicating that the isolates have acquired a hereditary resistance that poses a public health hazard to customers. Moreover, the marinate with 5% lemon juice (LJ) and 3% pomegranate peel (PPE) extract had a positive impact on the diminution of *V. cholerae* and *V. parahaemolyticus* counts during the storage period by 48.47% and 37.3% for the LJ solution and by 41.51 and 40.13% for the PPE. It exhibited the most significant improvement in sensual characteristics. As a result, there is a need to authorize hygienic measures to regulate fish contamination in aquatic environments and markets. Additionally, marinating with natural components in food is a common approach to control *Vibrio* spp. with the primary objective of safe consumer health.

Keywords | *Vibrio*, Fish, PCR, Virulence gene, Natural Antimicrobials

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natural meat preservatives because of their antioxidant and antibacterial properties (Elbarbary and Abdelmotilib, 2023).

Therefore, the current research pointed to examining the occurrence, virulence gene determination, and antimicrobial profile of *Vibrio* in marketed fish samples at Aswan Governorates, Egypt, in light of the risk of bacterial pathogens in fish and their importance to human health. The antibacterial activity of lemon juice and pomegranate peel extracts against *V. cholerae* and *V. parahaemolyticus* was also studied.

MATERIALS AND METHODS

ETHICAL APPROVAL

The Scientific Research Committee and Bioethics Board of Aswan University, Faculty of Veterinary Medicine (5/2024), reviewed and approved the protocols used for this study which follow the Canadian Council on Animal Care guidelines (CCAC, 2005).

SAMPLES COLLECTION AND PREPARATION

Approximately 150 commercial fresh fish samples (30 each of Nile tilapia, Nile perch, Sardine, Mugil cephalus, and Sea bass) weighting of 200-240 g were obtained from several supermarkets at Aswan City, Egypt, during January and February 2024. Under a septic condition, the scales, head, fins, tails, and bones of the fish were removed using a sterile stainless-steel knife after the fish was eviscerated. The two back fillets were stored in the refrigerator at 4°C for bacteriological analysis at the Meat Hygiene Laboratory, Department of Food Hygiene, Faculty of Veterinary Medicine, Aswan University. All samples were transported in an icebox container.

VIBRIO COUNT DETERMINATION

Approximately 25g of fish muscle was standardized in 225mL of sterile alkaline peptone water (APW, Micro Master - India) for 24 h at 37°C incubation. One ml was conveyed to a single tube with 9 ml of peptone water (0.1%), from which a tenfold sequential dilution was set (Elbarbary et al., 2023). Duplicate sterile Petri plates of thiosulfate citrate bile salts sucrose agar media (GM189, HiMedia) at 37°C for 24 h of incubation with one ml of each serial dilution. The number of green or blue-green colonies on plates ranging from 30 to 300 was quantified using a digital colony counter (DC-8 OSK 100086, Kayagaki, Japan), and the outcome was represented as Log₁₀ CFU/g.

VIBRIO ISOLATION AND IDENTIFICATION

Thiosulfate-citrate-bile salt-sucrose agar plates (GM189, HiMedia) were streaked with a loopful of each inoculated APW broth for 24 h at 37°C incubation. Flat yellow, green-blue, yellow-green, and yellow or translucent

Fish is an abundant source of crucial nutrients, such as high-quality protein, fatty acids, phosphates, calcium, and a variety of vitamins (Elbarbary et al., 2024). Despite having a nutrient-dense diet, fish are implicated in the development of numerous foodborne illnesses. *Vibrio* species are among the most prevalent foodborne bacteria (Trinanes and Martinez-Urtaza, 2021). The existence and spreading of *Vibrio* spp. are seasonal and temperature reliant. Presently, vibriosis has a worldwide dispersal, in tropical as well as temperate areas (Tilusha et al., 2022). They are natively found in estuarine, marine and freshwater settings around the world, often alongside aquatic creatures. *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus* are the most significant *Vibrio* species that are related to the ingesting of under cooked fish (Morshdy et al., 2022). *V. cholerae* is the causative of cholera, which is transmitted to humans through the ingestion of improperly cooked fish and is characterized by abdominal cramps, nausea, vomiting, and massive acute diarrhoea (Tang et al., 2018). *V. parahaemolyticus* ranks is one of the most common sources of fish-related diarrhoea in several regions (Fri et al., 2017). Conversely, *V. vulnificus* can result in severe and fatal infections, including gastroenteritis, skin and soft tissue infections, and primary sepsis, which may lead to necrotizing fasciitis and mortality (Dutta et al., 2021). *V. fluvialis* has been classified as an “emerging” disease due to a rise in cholera-like diarrheal outbursts and extra-intestinal infections (Ramamurthy et al., 2014).

In addition, virulence is recognized as the capability of microbes to attack a host cell to induce illness, and most bacteria require a variety of virulence factors to prompt infection (Mavhungu et al., 2023). The pathogenicity of *Vibrio* strains is assisted by a broad variety of virulence issues encoded by virulence genes. In overall, virulence agents permit pathogens to infect and impairment the host, by allowing pathogenic adherence and entrance, creation and growth, prevention of host defenses, destruction to the host, and lastly exodus from the infected host. Five major virulence factors are occur in vibrio: capsular polysaccharides, adhesive factors, cytotoxins, lipopolysaccharides, and flagella (Deng et al., 2020).

Furthermore, Antibacterials are mostly utilized in fish farms to prevent infections, stop them from spreading, and encourage development. This increases the resistance of *Vibrio* species to various antibacterial and may have an impact on public health because consumers may consume resistant genes or bacteria through food (Binh et al., 2018). Furthermore, the inhibiting effect of certain organic acids on *Vibrio* species is the reason they are employed to control bacterial infection and deploy fish-borne microorganisms throughout food manufacture and handling. Lemon juice (LJ) and pomegranate peel extract (PPE) are regarded as

Table 1: Oligonucleotide primers of genes target for *Vibrio* spp.

Target Species	Target gene	Primers sequences	Cycling conditions	bp
<i>Vibrio</i> spp.	16S rRNA	CGGTGAAATGCGTAGAGAT TTACTAGCGATTCCGAGTTC	Initial at 94 °C/5 min, 35 cycles at 94 °C/30 s, 52 °C/ 30 s and 72 °C/ 60 s. Final extension at 72 °C/10 min	663
<i>V. cholerae</i>	<i>OmpW</i>	CACCAAGAAGGTGACTTTATTGTG GGTTTGTCTGAATTAGCTTCACC	Initial at 94 °C/10 min, 30 cycles at 94 °C/60 s, 59 °C/60 s and 72 °C/2 min. Final at 72 °C/10 min	304
<i>V. parahaemolyticus</i>	<i>toxR</i>	TGTACTGTTGAACGCCTAA CACGT- TCTCATACGAGTG	Initial at 94 °C/5 min, 35 cycles of 94 °C/30 s, 55 °C/30 s and 72 °C/30 s. Final extension at 72 °C/10 min	503
<i>V. vulnificus</i>	<i>vvhA</i>	ACTCAACTATCGTGCACG ACACTGTTCCGACTGTGAG		366
<i>V. fluvialis</i>	<i>toxR</i>	GGATACGGCACTTGAGTAAGACTC GACCAGGGCTTTGAGGTGGACGAC	Initial at 94 °C/5 min, 30 cycles at 94 °C/30 s, 57 °C/60 s and 72 °C/90 s. Final extension at 72 °C/7 min	217

Table 2: Primer sets and thermal profile used in the detection of *Vibrio* virulence genes.

Target gene	Primers sequences	Cycling conditions	bp	Reference
<i>tdh</i>	GGTCTAAATGGCTGACATC CCACTACCACTCTCATATGC	Initial at 96 °C/5 min, 35 cycles of 94 °C/60 s, 55 °C/60 s and 72 °C/60 s. Final 72 °C/7 min	199	Fri <i>et al.</i> , 2017
<i>trh</i>	CATTTCCGCTCTCATATGC GGCTCAAAATGGTTAAGCG		250	
<i>vcgC</i>	AGCTGCCGATAGCGATCT CGCTTAGGATGATCGGTG	Initial at 94 °C/5 min, 35 cycles of 94 °C/40 s, 56 °C/40 s and 72 °C/60 s. Final at 72 °C/7 min	278	
<i>vvh</i>	CCGGCGGTACAGCTTGGCGC CGCCACCCACTTTTCGGG	Initial 96 °C/5 min, 30 cycles of 94 °C/60 s, 63 °C/90 s and 72 °C/90 s. Final at 72 °C/7 min	519	Castello <i>et al.</i> , 2023
<i>prVC</i>	TTAAGCSTTTTCRCTGAGAATG AGTCACTTAACCATAACAACCCG	Initial at 94 °C/2 min, 30 cycles of 94 °C/60 s, 50 °C for 60 s and 72 °C/90 s. Final at 72 °C/10 min	310	
<i>ctxA</i>	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	Initial denaturation at 94 °C/2 min, 30 cycles of 94 °C/60 s, 62 °C/60 s and 72 °C for 60 s. Final at 72 °C/10 min.	564	Tulatorm <i>et al.</i> , 2018
<i>vfh</i>	GCGCGTCAGTGGTGGTGAAG TCGGTCTGAACCGCTCTCGCTT	Initial at 93 °C/15 min, 35 cycles at 92 °C/40 s, 50–62 °C/60 s and 72 °C/90 s. Final at 72 °C/7 min	800	Fri <i>et al.</i> , 2017
<i>stn</i>	GGTGCAACATAATAAACAGTCAA- CAA TAGTGGTATGCGTTGCCAGC		375	

colonies suggesting *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. fluvialis*, respectively, were selected, purified, and subsequently biochemically identified by ISO/ TS 21872-1 (2007) and ISO/ TS 21872-2 (2007).

MOLECULAR CONFIRMATION OF *VIBRIO* SPP. AND THEIR VIRULENCE GENE DETECTION

Probable colonies were cultivated in Tryptone Soy Broth (Oxoid, USA) overnight. Following the manufacturer's instructions, DNA was extracted using a QIAamp DNA Gene^{JET} Genomic DNA Purification Kit (Cat. no.#K0721, Thermo Scientific, USA). 25 µl of the subsequent PCR master mix, 12.5 µl of COSMO PCR RED Master Mix (2x premix), 4.5 µl of PCR grade water, 1 µl each of the forward and reverse primers (20 pmol), and 6 µl of tem-

plate DNA were included in the reaction. To verify that the isolates were members of the genus *Vibrio*, a primer that targeted the 16S rRNA gene was employed. The pathogenic species *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. fluvialis* were further identified from the confirmed isolates using primers specific to each species as well as their virulence genes by multiplex PCR. Tables 1 and 2 display the primer sets and the thermal conditions utilized following the methods of Fri *et al.* (2017). The Bacteriology Unit of the Animal Health Institute in Egypt provided the control positive used in this investigation. The amplified products were permitted to move on a 1.5% agarose gel, and a UV transilluminator was employed to observe the bands (Khairy and Abdelmotilib, 2023).

The disc diffusion technique was employed to test the identified *Vibrio* spp., *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. fluvialis*, following the rules established by the Clinical and Laboratory Standards Institute (CLSI, 2020). The data were evaluated using CLSI Clinical and Laboratory Standards (CLSI, 2018). The antimicrobials that were tested included florfenicol (FFC, 30µg), doxycycline (DO, 30µg), amoxicillin (AX, 25µg), ciprofloxacin (CIP, 5µg), erythromycin (E, 15µg), ampicillin (AM, 10µg), tetracycline (T, 30 µg), penicillin (P, 10µg), nalidixic acid (NAL, 30 µg), sulfamethoxazole (Sul, 25µg), cefoxitin (CEF, 10 µg), norfloxacin (NOR, 10 µg), and gentamicin (G, 10 µg).

Pure colonies of the recognized isolates were cultured overnight in Tryptone Soya Broth (HiMedia) at 28 °C. Subsequently, 100 µL of culture broth was applied to Mueller-Hinton agar (Hi-Media) by sterile glass rods, and antibiotic discs were meticulously smeared and incubated at 28°C for 24 h. Multiple antibiotic resistance (MAR) was calculated following the formula: $MAR = A/B$, where A is the quantity of the antibiotic to which the isolate is resistant, and B is the quantity of the total antibiotic employed in the investigation. A MAR index < 0.2 suggested that the strain originated from a low-risk supply of contamination. On the other hand, isolates from high-risk sources of pollution have been recognized by a MAR >0.2 (Haifa-Harvani et al., 2022).

IN VITRO *VIBRIO* SPP. REDUCTION BY LEMON AND POMEGRANATE PEEL EXTRACT IN FISH FILLETS

The technique described by Elbarbary and Abdelmotilib (2023) confirmed that *V. cholerae* and *V. parahaemolyticus* were positive for the occurrence of more than one virulence gene, with a few minor modifications. Fish fillets were tested for the presence of *Vibrio* spp., and the negative fillets were utilized in the current experiments. Five groups of five raw fish fillets (each weighing 100 g) were employed for each experiment. Each fillet in each group was pipetted with one ml of each *V. cholerae* and *V. parahaemolyticus* broth, which had been adjusted to 0.5 McFarland concentrations. The fish fillet that had been infected with *V. cholerae* and *V. parahaemolyticus* was dipped in solutions of LJ (3% and 5%) and PPE (3% and 5%) that were furnished by the National Research Center. The infected fillets were subsequently maintained at 25°C for 60 minutes. Each inoculation involved five groups: the untreated control group received a bacteria inoculation (C) only; groups were immersed in solutions containing 3% LJ (G1), 5% LJ (G2), 3% PPE (G3), and 5% PPE (G4). The antibacterial impact of LJ and PPE against *V. cholerae* and *V. parahaemolyticus* was determined by measuring the total bacterial count in triplicate after exposure time (zero, 12, 24, 48, and 72 h), and the mean values with standard errors were recorded.

ORGANOLEPTIC PROPERTIES OF LJ AND PPE MARINATION

A scoring test was employed by 27 panelists who are staff members of the Faculty of Veterinary Medicine, Aswan University, to evaluate the sensory attributes of fish fillet (100 g) samples that were marinated for 60 min at 4°C using different doses of natural LJ and PPE. The fillet samples were randomly assigned codes, and the judges were not informed how the study was done. They were requested to evaluate the overall satisfaction, texture, flavour, odour, and appearance (Nady et al., 2024).

DATA ANALYSIS

One-way analysis of difference was utilized to evaluate all statistics (ANOVA). Every value was shown as means ± S.E. $p < 0.05$ is considered a significant variance.

Table 3: Prevalence total *Vibrio* count ($\log_{10}$ CFU/g) of examined samples (n = 30 each)

Species	Count $\log_{10}$ CFU/g		
	Minimum	Maximum	Mean ± SE
Nile tilapia	2.80	3.81	2.90±0.78 ^c
Nile perch	2.71	4.92	3.24±1.05 ^b
Sardine	3.88	6.72	4.77±1.22 ^a
Mugil cephalus	2.91	4.91	3.50±1.74 ^b
Sea bass	3.81	5.68	4.62±1.35 ^a

$p < 0.0001$ is considered extremely significantly different. Mean values with the same letters in each column have no significant difference.

RESULTS AND DISCUSSION

The results available in Table 3 demonstrate the differences in the total *Vibrio* count ($\log_{10}$ CFU/g) found in the examined samples. There were significant differences between the samples ($p < 0.05$). Total *Vibrio* count was the greatest in Sardine (4.77±1.22 $\log_{10}$ CFU/g) and Sea bass (4.62±1.35 $\log_{10}$ CFU/g), followed by *Mugil cephalus* (3.50±1.74 $\log_{10}$ CFU/g), Nile perch (3.24±1.05 $\log_{10}$ CFU/g), while Nile tilapia had the lowest count (2.90±0.78 $\log_{10}$ CFU/g). Bacterial investigation of the fish confirmed that *Vibrio* species exist with an overall ratio of 42% (Table 4). The maximum detected rate was in Nile perch (80%); however, the lowest detected rate was in Sardine and Sea bass samples (60% of each). The main detected *Vibrio* species were *V. cholerae* (38.2%), followed by *V. parahaemolyticus* (31.7%), *V. vulnificus* (19%), and the lowest percentage was *V. fluvialis* (11.1%).

It was found that out of 63 positive *Vibrio* strains depending on conventional identification, 42 isolates belonged to the genus *Vibrio* depending on the 16S rRNA gene (Figure 1).

Table 4: Prevalence and species of *Vibrio* isolates in the examined samples (n=30 of each).

Species	Positive No. (%)		Vibrio species							
	No.	%	<i>V. parahaemolyticus</i>		<i>V. cholerae</i>		<i>V. vulnificus</i>		<i>V. fluvialis</i>	
			No.	%	No.	%	No.	%	No.	%
Nile tilapia	6	20%	2	3.2	3	4.8	1	1.6	0	0
Nile perch	9	30%	3	4.8	4	6.3	1	1.6	1	1.6
Sardine	18	60%	5	8.0	8	12.7	3	4.8	2	3.2
Mugil cephalus	12	40%	4	6.3	4	6.3	3	4.8	1	1.6
Sea bass	18	60%	6	9.5	5	8.0	4	6.3	3	4.8
Total	63	42%	20	31.7	24	38.1	12	19	7	11.1

Positive samples % was calculated from the total examined while *Vibrio* spp. % was calculated from total positive isolates.

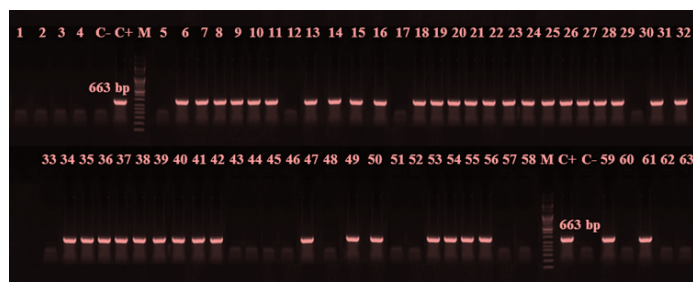


Figure 1: Electrophoretic profile of amplification products of 16S rRNA gene *Vibrio* spp. at 663 bp. M: Marker (50bp), C+: positive control, C-: negative control.

Molecular confirmation of *Vibrio* spp. revealed that the most detected species were *V. cholerae* (17/42), *V. parahaemolyticus* (12/42), *V. vulnificus* (9/42), and *V. fluvialis* (4/42), as shown in Figure 2. It was found that 9/17 and 7/17 *V. cholerae* isolates had *prVC* and *ctxA* virulence genes (Figure 3A), 5/12 and 4/12 *V. parahaemolyticus* isolates had *tdh* and *trh* genes (Figure 3B), 5/9 *V. vulnificus* isolates had one or more of *vcg* and *vvh* genes (Figure 3C), and 2/4 *V. fluvialis* isolates had one or both *stn* or *vfb* genes (Figure 3D).

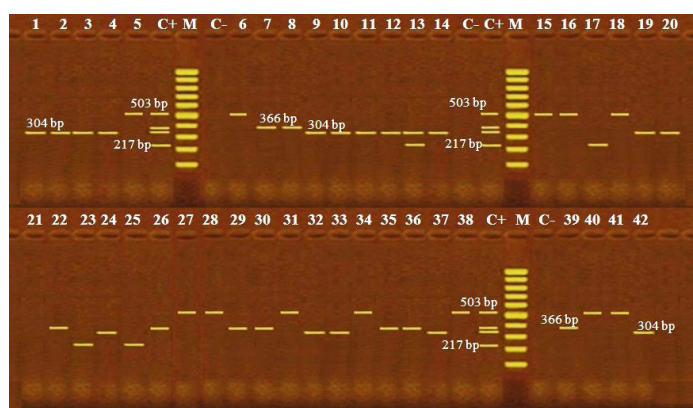


Figure 2: Electrophoretic profile of amplification products for identification of *Vibrio* spp. *OmpW* gene of *V. cholerae* at 304 bp, *toxR* gene of *V. parahaemolyticus* at 503 bp, *vvhA* gene of *V. vulnificus* at 366 bp, and *toxR* gene of *V. fluvialis* at 217 bp. M: Marker (50bp), C+: positive control, C-: negative control.

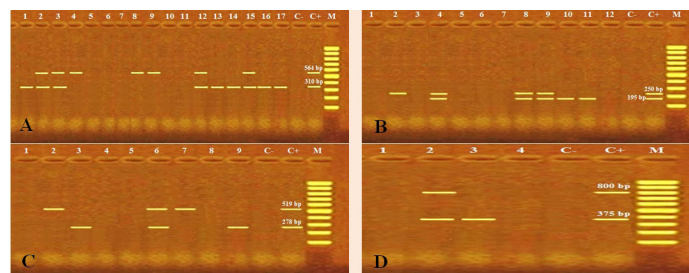


Figure 3: Electrophoretic profile of amplification products for identified *Vibrio* spp. virulence genes. A: PCR amplified *V. cholerae* *prVC* (310 bp) and *ctxA* (564 bp) genes. B: PCR amplified *V. parahaemolyticus* *tdh* (195 bp) and *trh* (250 bp) genes. C: PCR amplified *V. vulnificus* *tdh* (195 bp) and *vvh* (519 bp) genes. D: PCR amplified *V. fluvialis* *stn* (375 bp) and *vfb* (800 bp) genes. M: Marker (50bp), C+: positive control, C-: negative control.

The antimicrobial susceptibility shown in Table 5 shows that *V. cholerae* strains (n = 17) were completely resistant to ampicillin (100%), while the susceptibility to florfenicol was shown in 64.7% of the isolates, followed by cefoxitin (53%). All verified *V. parahaemolyticus* strains (n = 12) were resistant to ampicillin and tetracycline (100%), and a large proportion of strains were resistant to all the verified antibiotics. Additionally, all investigated *V. vulnificus* isolates (n = 9) were susceptible to florfenicol, nalidixic acid, and cefoxitin (100%) while those resistant to tetracycline and sulfamethoxazole reached 100%. Furthermore, all *V. fluvialis* isolates (n = 4) were resistant to tetracycline (100%), but susceptible to florfenicol and cefoxitin (100%). The MRI of *Vibrio* strains alternated from 0.214 to 0.846, with an average of 0.530 (Table 6).

On the other hand, Table 7 evaluates the decontamination of *V. cholerae* and *V. parahaemolyticus* after altered exposure time following dipping in 3% and 5% LJ solution and PPE. *V. cholerae* and *V. parahaemolyticus* in the evaluated samples over 72 h diminished by 4.59 and 3.53 log₁₀ CFU/g (48.47% and 37.3%) in the LJ solution and by 3.93 and 3.80 log₁₀ CFU/g (41.51 and 40.13%) in the PPE. There were not significant differences between the samples at the

Table 5: The interpretation of antimicrobial susceptibility and resistance of *V. species* (n=42).

Antimicrobial agents	<i>V. cholerae</i>			<i>V. parabaemolyticus</i>			<i>V. vulnificus</i>			<i>V. fluvialis</i>		
	n= 17			n= 12			n= 9			n= 4		
	R %	I %	S %	R %	I %	S %	R %	I %	S %	R %	I %	S %
Florfenicol (30µg)	29.4	17.6	64.7	50	16.7	33.3	0	0	100	0	0	100
Doxycycline (30µg)	53	5.9	41.2	66.7	0	33.3	44.4	22.2	33.3	75	0	25
Amoxicillin (25µg)	64.7	17.6	17.6	58.3	8.3	33.3	33.3	44.4	22.2	50	25	25
Ciprofloxacin (5µg)	47	29.4	23.5	33.3	25	41.7	44.4	22.2	33.3	75	25	0
Erythromycin (15µg)	35.3	23.5	41.2	41.7	25	33.3	66.7	0	33.3	50	0	50
Ampicillin (10µg)	100	0	0	100	0	0	77.8	11.1	11.1	75	25	0
Tetracycline (30µg)	82.3	5.9	11.8	100	0	0	100	0	0	100	0	0
Penicillin (10µg)	47	23.5	29.4	58.3	16.7	25	77.8	22.2	0	50	25	25
Nalidixic acid (30µg)	35.3	35.3	29.4	50	33.3	16.7	0	0	100	25	50	25
Sulfamethoxazole (25µg)	70.6	17.6	11.8	91.7	8.3	0	100	0	0	75	25	0
Cefoxitin (10µg)	29.4	17.6	53	41.7	33.3	25	0	0	100	0	0	100
Norfloxacin (10µg)	47	23.5	29.4	58.3	16.7	25	55.6	0	44.4	25	25	50
Gentamicin (10µg)	53	41.2	5.9	66.7	16.7	16.7	66.7	22.2	11.1	75	25	0

R: Resistant, I: Intermediate, S: Sensitive

Table 6: Antimicrobial resistance profile of *Vibrio species* (n=36).

Species	Isolates NO.	Antimicrobial resistance profile	No. of antibiotic	MAR index
<i>V. cholerae</i>	7	FF, DO, AX, CIP, E, AM, T, SUL, CEF, NR, G	11	0.846
	5	FF, DO, AX, E, P, N, SUL, AM, NR	9	0.692
	3	DO, CIP, AM, T, N, SUL, CEF, NR	8	0.615
	2	AX, CIP, E, AM, T, CEF, G	7	0.538
<i>V. parabaemolyticus</i>	6	FF, DO, AX, E, T, P, AM, SUL, NR	9	0.692
	3	FF, DO, E, AM, T, P, N, CEF, G	9	0.692
	2	DO, AX, AM, T, P, G	6	0.462
	1	AM, T, SUL, NR	4	0.307
<i>V. vulnificus</i>	5	FF, CIP, T, P, N, CEF, NR, G	8	0.615
	3	DO, AX, AM, T, SUL, NR	6	0.462
	1	DO, AXE, CIP, SUL	4	0.286
<i>V. fluvialis</i>	2	AX, CIP, AM, T, N, SUL, CEF, G	8	0.615
	1	DO, AX, CIP, T, SUL	5	0.384
	1	AX, CIP, T	3	0.214
Average				0.530

MAR: Multiple Antibiotic Resistant indexes. FF: florfenicol, DO: doxycycline, AX: amoxicillin, CIP: ciprofloxacin, E: erythromycin AM: ampicillin, T: tetracycline, P: penicillin, N: nalidixic acid, SUL: sulfamethoxazole, CEF: cefoxitin, NR: norfloxacin, and G: gentamicin.

initial of the treatments but over the time it increased significantly ($p<0.05$).

A sensory assessment of a fish fillet prepared with an altered concentration of LJ and PPE is shown in Table 8. The study confirmed the group was immersed in a solution of 5% LJ as well as the group was dipped in a solution of 3% PPE possessed superior sensory criteria, with no significant variance in between, but significantly varied in contrast with the control and other treated groups.

VIBRIO COUNT, ISOLATION AND IDENTIFICATION

Fish is critical for supplying a consistent supply of protein for the human diet in quickly expanding coastal countries such as Egypt. This source is susceptible to uncontrollable bacterial diseases, mainly those caused by virulent and drug-resistant pathogens like *Vibrio* spp. (Saad et al., 2024). *Vibrio* species are fish-borne bacteria of the water supply that are primarily found in a variety of fish. These pathogens raise humans' vul

Table 7: Effect of lemon juice and pomegranate peel extract on artificially inoculated fish fillet with *V. cholera* and *V. parahaemolyticus* after different exposure times.

T (hrs)	V. cholerae count (log ₁₀ CFU/g)					V. parahaemolyticus count (log ₁₀ CFU/g)			
	C	G1	G2	G3	G4	G1	G2	G3	G4
Zero	7.58±0.13 ^a	7.55±0.17 ^a	7.52±0.73 ^a	7.56±0.52 ^a	7.53±0.84 ^a	7.53±0.48 ^a	7.48±0.13 ^b	7.55±0.24 ^a	7.52±0.18 ^a
	R. count	0.03	0.06	0.02	0.05	0.03	0.10	0.03	0.06
	R. %	0.41	0.81	0.31	0.66	0.41	1.32	0.41	0.81
12	7.83±0.44 ^a	7.49±0.42 ^a	6.94±0.38 ^b	7.52±0.73 ^a	6.95±0.42 ^b	7.46±0.73 ^a	6.83±0.13 ^b	7.49±0.84 ^a	6.87±0.64 ^b
	R. count	0.34	0.89	0.31	0.88	0.37	1.00	0.34	0.96
	R. %	4.34	11.41	3.96	11.24	4.73	12.81	4.34	12.31
24	8.64±0.75 ^a	6.45±0.64 ^c	6.27±0.28 ^c	6.81±0.22 ^b	6.76±0.16 ^b	6.40±0.77 ^c	5.89±0.13 ^d	6.83±0.39 ^b	5.95±0.47 ^d
	R. count	2.19	2.37	1.83	1.88	2.24	2.75	1.81	2.69
	R. %	25.35	27.34	21.18	21.81	26	31.83	21	31.13
48	9.47±0.32 ^a	6.37±0.27 ^c	5.43±0.55 ^d	6.66±0.72 ^b	6.29±0.83 ^c	5.83±0.36 ^d	5.63±0.13 ^d	5.86±0.59 ^d	5.75±0.83 ^d
	R. count	3.1	4.04	2.81	3.18	3.64	3.84	3.61	3.80
	R. %	35.88	46.76	32.52	36.81	38.44	40.55	38.12	40.13
72	Spoiled	5.87±0.84 ^a	4.88±0.27 ^b	5.97±0.78 ^a	5.94±0.47 ^a	5.69±0.58 ^b	5.54±0.85 ^b	5.76±0.77 ^b	5.67±0.59 ^b
	R. count	3.60	4.59	3.50	3.53	3.78	3.93	3.71	3.80
	R. %	38.01	48.47	37	37.3	40	41.51	39.2	40.13

C: The control group only got a microorganism inoculation; G1: group submerged in a solution of 3% LJ; G2: group was submerged in a solution of 5% LJ; G3: group was submerged in a solution of 3% PPE; G4: group was submerged in a solution of 5% PPE. ^{a-d} Values within the same raw have different superscript letters that are significantly different at $p<0.05$. Values are expressed as mean± standard error (SE) of three determinations.

Table 8: Effect of lemon juice and pomegranate peel extract marination on organoleptic properties of fish fillet.

Treat- ment	Sensory criteria					
	Appear- ance (5)	odour (5)	Texture (5)	Flavor (5)	Overall (20) *	Grade
C	4.0	3.0	4.0	4.0	17.0 ^c	Good
G1	4.3	5.0	4	5.0	18.3 ^b	Very good
G2	5.0	5.0	5.0	5.0	20.0 ^a	Excellent
G3	5.0	5.0	5.0	5.0	20.0 ^a	Excellent
G4	4.0	4.6	5.0	4.4	18.0 ^c	Good

* >5 spoiled, 5-10 poor, 10-15 middle, 15-18 good, 18-19 very good and 20 excellent. C: The control group only got a microorganism inoculation; G1: group submerged in a solution of 3% LJ; G2: group was submerged in a solution of 5% LJ; G3: group was submerged in a solution of 3% PPE; G4: group was submerged in a solution of 5% PPE. ^{a-d} Values within the same column have different superscript letters that are significantly different at $p<0.05$.

nerability to public health hazards (Semenza and Paz, 2021). In the present research, the total *Vibrio* count varied significantly across the samples ($p<0.05$). Sardine and sea bass had the highest count (4.77 ± 1.22 log₁₀ CFU/g and 4.62 ± 1.35 log₁₀ CFU/g, respectively), followed by Mugil cephalus and Nile perch (3.50 ± 1.74 log₁₀ CFU/g and 3.24 ± 1.05 log₁₀ CFU/g).

Nile tilapia had the lowest count (2.90 ± 0.78 log₁₀ CFU/g). These different outcomes may be imputed to species variances, in addition to low salinity in freshwater, as *Vibrio* is more predominant in brackish and marine water (Noga, 2010). In contrast, Stratev *et al.* (2021) reported a high count of 5.90 ± 5.84 log₁₀ CFU/g, while Lamon *et al.* (2019) demonstrated a decreased count (2.04 log₁₀ CFU/g) of *Vibrio* spp.

Furthermore, 42% of the samples include *Vibrio* spp. In Nile perch, the isolation rate was the highest at 80%, with *V. cholerae* (38.2%) being the most prevalent isolated *Vibrio* spp. *V. parahaemolyticus* (31.7%) and *V. vulnificus* (19%) were the next most prevalent, and *V. fluvialis* (11.1%) was the least prevalent. Although *V. cholerae* and *V. parahaemolyticus* are a halophilic bacterium and linked to marine water (Almejjhim *et al.*, 2021). Parallel findings were observed by Saad *et al.* (2024), who observed that 37% of *Vibrio* spp. were isolated, with *V. parahaemolyticus* being identified at a higher incidence of 18%, followed by *V. vulnificus* at 11%. Furthermore, Castello *et al.* (2023) found that *Vibrio* spp. contaminated 27.4% of the fish they studied, with *V. parahaemolyticus* accounting for 26.1%, *V. cholerae* for 7.3%, and *V. vulnificus* for 3% of the infec-

tions. According to El-Sharaby et al. (2018), 39% of the fish that were evaluated had *Vibrio* spp. contamination, of which 10.25% had *V. fluvialis* and 16.67% had *V. vulnificus*. Additionally, Morshdy et al. (2022) reported a greater isolation rate, finding that 52% of *Vibrio* spp. were detected in fish, 42.3% of which were classified as *V. parahaemolyticus*, and 1.92% of which were identified as *V. cholerae*. Furthermore, Suresh et al. (2018) found that 82.85% of the *Vibrio* spp. found in freshwater fish were *V. parahaemolyticus* (6.9%), *V. vulnificus* (2.3%), and *V. cholerae* (3.45%). However, Ahmed et al. (2018) verified a low *Vibrio* detection rate of 16%, while *V. parahaemolyticus* and *V. cholerae* were found in 15.1% and 0.9% of the samples, respectively. According to Yan et al. (2019), *V. cholerae* (10.33%), *V. parahaemolyticus* (3.89%), and *V. vulnificus* (0.76%) were the most frequently isolated *Vibrio* species. The disparities in the prevalence proportions of identified bacteria may be attributable to a variety of environmental factors, including the total inspected samples, differences in host susceptibility, geographical dissemination, sodium concentration, and sampling duration (Saad et al., 2024).

MOLECULAR CONFIRMATION OF *VIBRIO* SPP. AND THEIR VIRULENCE GENE DETECTION

However, traditional phenotypic and biochemical identification approaches are inadequate for *Vibrio* strains obtained from fish. The identification of *Vibrio* spp. is impeded by the occurrence of both false positive and negative outcomes in all biochemically recognized approaches. As a result, PCR analyses are necessary for their recognition (Haifa-Haryani et al., 2022). Depending on the 16S rRNA gene, 42 of the 63 positive *Vibrio* spp. were identified as belonging to the *Vibrio* genus. The 16S rRNA is a widely used molecular indicator for recognizing and characterizing isolated pure cultures, as well as assessing bacterial density using metagenomic assays (Rajendhran et al., 2011). However, the 16S rRNA gene has limited discriminatory strength, resulting in misidentification of *Vibrio* spp. when related to other investigated genes (Sawabe et al., 2013). Furthermore, the 16S rRNA shared by *Vibrio* spp. is over 97% similar, which complicates the differentiation of closely related species (Zhang et al., 2014). Because of this constraint, alternative housekeeping genes were utilized as markers to assess bacterial species diversity.

In this investigation, the molecular technique was em-

ployed to identify the isolated *Vibrio* spp. by analyzing the *OmpW* gene of *V. cholerae* at 304 bp, the *toxR* gene of *V. parahaemolyticus* at 503 bp, the *vvhA* of *V. vulnificus* at 366 bp, and the *toxR* of *V. fluvialis* at 217 bp. The molecular approach validated four species in our study: *V. cholerae* (40.5%), *V. parahaemolyticus* (28.6%), *V. fluvialis* (21.4%), and *V. vulnificus* (9.5%), with certain strains retaining one or more virulence factors (Figures 2 and 3). It was discovered that 12 out of 17 *V. cholerae* isolates possessed *prVC* (9/17) and *ctxA* (7/17) virulence genes (Figure 3A), 50% of *V. parahaemolyticus* strains identified in this investigation have either *tdh* or *trh* genes, whereas the remaining strains harboured one or both genes (Figure 3B). Furthermore, 5 of 9 *V. vulnificus* isolates have one or both *vcg* or *vvh* genes (Figure 3C), while 2 of 4 *V. fluvialis* isolates possess one or both *stn* or *vfb* genes (Figure 3D). This is consistent with earlier research that has verified the virulence genes related to *Vibrio* spp. enteropathogenicity (Castello et al., 2023; Haifa-Haryani et al., 2022; Morshdy et al., 2022; Saad et al., 2024). Additionally, despite the absence of the tested virulence genes in certain strains, these strains can harbour other virulence markers and exhibit high antimicrobial resistance rates that may be unsuccessful in treating infections (Silva et al., 2018).

In addition, the *Ctx* gene is a crucial component of *V. cholerae* pathogenicity, generating the cholera toxin that causes diarrhoea in cholera patients (Fri et al., 2017). The pathogenic risk of *V. parahaemolyticus* is suggestively influenced by the thermostable direct hemolysin (*tdh*) and the thermostable direct hemolysin-related (*trh*) gene (Terzi et al., 2016). This is a result of the *tdh* biological actions, which comprise its enterotoxin and cytotoxic properties. The primary gene responsible for the hazardous effects of *V. vulnificus* is the virulence-correlated gene (*vcg*) which has been described as valuable in distinguishing potentially infectious and non-infectious strains (Mavhungu et al., 2023). On the other hand, *Vfb* has a wide range of cytotoxic action and a virulence attribute harboured by *V. fluvialis*, causing inflammatory diarrhoea in vulnerable individual hosts and producing erythrocytes rupture (Mavhungu et al., 2023). Furthermore, the *toxR* gene, which encodes a significant membrane-localized regulatory protein and is frequently implicated in the microbial regulation of a range of exhibition yields, is carried by 28.6% of *V. parahaemolyticus* in the current investigation (Li et al., 2020). Almejhim

et al. (2021) reported the same outcome, indicating that 21.7% of *V. parahaemolyticus* have the *toxR* gene. Nevertheless, Morshdy et al. (2022) and Yen et al. (2021) detected that all *V. parahaemolyticus* isolates have *toxR* genes. Elbarbary et al. (2024) also discovered *toxR* (70%) and *tdh* (50%) genes in *V. parahaemolyticus* using PCR research. Concurrently, Terzi et al. (2016) also reported a low incidence of *tdh* and *trh* (0% and 4.2%, respectively).

Bacterial pathogenicity is dependent on the presence of virulence factors serving as principal orchestrators. Furthermore, according to Hiyoshi et al. (2010), *tdh* and *trh* are the chief virulence genes of *V. parahaemolyticus* in terms of their pathogenicity. Fri et al. (2017) identified *tdh* and *trh* in 3% of the strains tested. However, other research (Al-Othrubí et al., 2014; Letchumanan et al., 2015) found greater percentages of *trh* (40%) and *tdh* (12.3%). All of the *V. parahaemolyticus* confirmed by Li et al. (2020) had the *toxR* gene; the *tdh* and *trh* were present in 9.90 and 19.80%, respectively. In addition, Fri et al. (2017) found that 13.3% and 6.2% of *V. fluvialis* harboured *stn* and *vfb* pathogenic genes. Hence, to avoid food-borne illnesses, it is essential to highlight the requirement for bacteriological risk valuations of food safety and improve active checking efforts, particularly the sanitary supervision of fish and their products.

ANTIMICROBIAL SENSITIVITY

Antimicrobial residues pose a major risk to the public's health when they are found in aquaculture products. This health risk is caused by either the horizontal genetic factor transfer from aquatic surroundings to individuals or the direct dissemination of resistance genes acquired by bacteria (Sun et al., 2015). According to current research, *V. cholerae* is completely susceptible to florfenicol and cefoxitin but has significant proportions of whole or intermediary antibacterial resistance used in therapeutic rules, for instance, ampicillin (100%), tetracycline (82.3%) and sulfamethoxazole (70.6%). Regarding the evaluation of antimicrobial resistance, *V. parahaemolyticus* showed 100% ampicillin and tetracycline resistance and was exceedingly resistant to sulfamethoxazole and gentamicin. Conversely, the present findings verify the resistance of *V. vulnificus* and *V. fluvialis* to antibiotics, such as tetracycline and sulfamethoxazole, which are prescribed by the Centers for Disease Control and Prevention (CDC) for contagion therapy (Serratore et al., 2017).

Additionally, they are highly susceptible to florfenicol, nalidixic acid, and cefoxitin. The results are consistent with those described by other investigators regarding the prevalence of antimicrobial resistance (Castello et al., 2023; Morshdy et al., 2022; Saad et al., 2024; Silva et al., 2018). However, the organism was highly resistant to doxycycline, sulfamethoxazole, and gentamicin, which was also observed in other studies (Ahmed et al., 2018; Sony et al., 2021). Aside from minor differences in proportions achieved, our outcomes established that *Vibrio* spp. isolates were resilient to a wide range of antibiotics, implying that they may have been widely misrepresented, decreasing their efficacy in the handling of *Vibrio* illness (Castello et al., 2023).

The majority of isolates in the present study declare a multiple MAR index within the range of 0.214 to 0.846, with an average of 0.530, which is >0.2. This indicates that the isolates have acquired a hereditary resistance that poses a public health hazard to customers (Elbarbary and Abdelmotilib, 2023). High MAR may be due to the increased chance for resistance genes situated on plasmids to be swapped among environmental isolates through horizontal gene transfer due to the prevalent, uncontrolled use of Antibacterials in the infection handling (Ottaviani et al., 2013). Analogously, earlier reports (Ahmed et al., 2018; Morshdy et al., 2022; Saad et al., 2024; Zaafrane et al., 2022) indicated that *Vibrio* isolates had a high MAR index. The variance in the MAR index could be attributed to variations in geographic dissemination, sample supply, and technique applied. Higher MAR in the current investigation demonstrated that the *Vibrio* isolates are from a high-risk supply; hence, an antimicrobial resistance investigation is required. Public and manufacturing waste water have been drawn as a potential source of resistant isolates in the aquatic ecosystem. A substantial quantity of the antibiotics that people take for medical causes are ousted in their faeces and urine in an active biological form and between 30 to 90% of the antibiotics that animals drink are also rejected in faeces and urine. Antibiotic-resistant bacteria and antibiotics were found to pollute the environment through animal excreta (El-Zamkan et al., 2023). An emerging issue with infectious bacteria present in the aquatic environment, including fish, is their growing resistance to key antibiotics. Consequently, it is imperative to examine the medicine resistance outlines to avoid the dissemination of antibiotic-resistant bacteria and to identify more effective

alternatives (Saad et al., 2024).

IN VITRO *VIBRIO* SPP. REDUCTION BY LEMON AND POMEGRANATE PEEL EXTRACT IN FISH FILLETS

Food shelf life is extended by the use of organic compounds, which also restrict microbial contamination and decrease foodborne pathogens (Lingham et al., 2012). One of the contemporary food handling techniques that use non-thermal procedures to create nutrient-dense, microbe-safe food products is treatment with organic acids such as LJ and PPE because of their antibacterial properties, organic substances are widely employed as food additives and preservers to prolong the storage period of perishable foods (Mathur and Schaffner, 2013; Wang et al., 2015). *V. cholerae* and *V. parahaemolyticus* counts were successfully lowered in the current study after 72 h of marination in LJ and PPE with varying concentrations. Additionally, Table 7 showed an ascending increase in the decline of *V. cholerae* and *V. parahaemolyticus* during the exposure time. Additionally, compared to other treatments, the 5% lemon juice marinade had a greater favourable effect on the decrease of *V. cholerae* and *V. parahaemolyticus* counts during the storage period. The present study indicates that the LJ and PPE may have potent antimicrobial properties against *V. cholerae* and *V. parahaemolyticus* due to their highest concentrations of phenolics and flavonoids ($p < 0.05$).

Moreover, Morshdy et al. (2022), Sushmita (2022), and Tsai et al. (2021) have all demonstrated the antimicrobial action of LJ as a natural reduction of *Vibrio* spp. However, PPE marination has been demonstrated by several researchers (Elbarbary and Abdelmotilib, 2023; Hayrapetyan et al., 2012; Rasuli et al., 2021) that there is a substantial difference ($p < 0.05$) between the PPE concentration and storage length. The antibacterial mechanism of flavonoids, a key constituent in LJ and PPE, involves inhibiting nucleic acid synthesis, disturbing cytoplasmic membrane roles, influence energy metabolism, and impacting biofilm creation and membrane permeability. Flavonols and phenolic acids, in specific, prove important antibacterial act, preventing bacterial virulence factors such as toxins and enzymes, and improving the effectiveness of antibiotics (Sattar et al., 2024). Saponin compounds are antibacterial mixes that destruct bacterial cell membranes. Also, LJ has vitamin C which is convenient as an antioxidant. The main component LJ is organic acid in the form of citric acid which is

contained most in LJ. The citric acid supplies a degree of acidity (pH) which is one of the issues that can prevent bacterial development (Ekawati et al., 2019). In addition, PPE is a naturally occurring substance that has strong antibacterial and antioxidant properties (Rasuli et al., 2021). Many researches have been undertaken on the use of natural antioxidants to improve the oxidative stability of fish products (Akuru et al., 2020). Marination with natural components in food is a typical approach for decreasing bacterial contamination and increasing shelf life (Rasuli et al., 2021).

ORGANOLEPTIC PROPERTIES OF LJ AND PPE MARINATION

The sensory evaluation of fish fillets that were prepared with varying ratios of LJ and PPE during cold storage is illustrated in Table 8. The investigation indicated that LJ 5% and PPE 3% concentrations had the highest sensory criteria, with no significant difference between them, although they changed dramatically with other treatments. It has been proposed that LJ 5% concentrations provide the samples with a more acidic environment, which is not favourable to most bacteria. The findings are also corroborated by prior research (Elbarbary and Abdelmotilib, 2023; Morshdy et al., 2022; Nady et al., 2024). We hypothesize that the variation in average overall scores between tests may also be attributed to personal rating differences, as the panel members were not identical for each test. Furthermore, the scores of the assessors may have been significantly influenced by the significant differences in the samples they evaluated after each storage period. The study's distinctiveness lies in its investigation of the antibacterial properties of lemon juice and PPE. However, the study had limitations. The scope was confined to in vitro conditions, which may not fully replicate in vivo environments. Future research should consider in vivo studies to validate these outcomes. Also, the specific mechanisms through which these extracts exert their antibacterial effects, mainly in combination treatments, warrant further examination.

CONCLUSIONS AND RECOMMENDATIONS

More hygienic measures should be implemented during all processing steps as a result of the current study's findings, which show that a sizable portion of the samples under examination is contaminated with antibiotic-resistant *Vibrio* spp., specifically *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *V. fluvialis*. These bacteria harbour one or more virulence genes, which increases the risk of foodborne illness and may facilitate its transmission. The results indicate that using the marination process with 5% lemon juice and 3%

pomegranate peel extract has a positive effect on reducing *V. cholerae* and *V. parahaemolyticus* counts through the storage period, improving sensual characteristics, extending product shelf life, and being a safe method of preserving fish products. To assist in the elimination of contaminated pathogens, it is recommended that hygienic conditions be consistently enforced in the following areas: food handling, food contact surfaces, personal sanitary practices, and the consumption of fully cooked fish. Further research is required to detect other virulence and antibiotic resistance genes as well as to explore other natural antimicrobials to reach the optimum conditions of marination for the maximum prolongation of the product's shelf life.

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NOVELTY STATEMENT

This study is the first to evaluate the occurrence, virulence gene detection, antibiotic resistance profile, and the antibacterial influence of lemon juice and pomegranate peel extract on *V. cholerae* and *V. parahaemolyticus* in fish samples from supermarkets at Aswan City, Egypt.

AUTHORS' CONTRIBUTIONS

Sohaila El-Hawary, Nady Elbarbary and Reda Gomaa: conducted conceptualization, data curation, and methodology. Nermeen Malak conducted a formal analysis. Hesham Tawfeuk and Suzan Ismail: managed the investigation, validation, and visualisation. Nady Elbarbary and Nermeen Malak: wrote the original draft, revised, and edited the paper. The authors contributed equally and accepted the absolute manuscript.

CONFLICTS OF INTEREST

The authors have declared no conflict of interest.

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