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Vibriosis and *Aeromonas* infection in shrimp: Isolation, sequencing, and control

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Abstract

Background and Aim: Shrimp is one of the most commonly consumed types of seafood. It is a very nutritious healthy food. Shrimp is low in calories and rich in protein and healthy fats. It also contains a treasure trove of vitamins and minerals. On the negative side, it may be affected by many bacterial diseases which affect its health. Furthermore, it may be incriminated as a vector of foodborne illnesses that range from mild gastrointestinal upset to life-threatening diseases. This study was designed to assess the clinical picture and zoonotic importance of vibriosis and *Aeromonas* infection in live shrimp and to study the antibacterial effect of citric acid (lemon juice) and acetic acid (vinegar) on these pathogens.

Materials and Methods: A total of 170 live shrimp (*Metapenaeus monoceros*) samples were collected from Suez City, Egypt. The samples were examined clinically, and then, they were enriched into alkaline peptone water and cultivated on thiosulfate-citrate-bile salts-sucrose agar and ampicillin MacConkey agar for the isolation of *Vibrio* and *Aeromonas* species, respectively. The recovered isolates were confirmed biochemically and genotypically using duplex polymerase chain reaction (PCR) and sequencing. The germicidal effects of vinegar and lemon on artificially contaminated shrimp samples with *Aeromonas hydrophila* and *Vibrio parahaemolyticus* at different times (0.25, 1, 1.5, and 24 h) and temperatures (5° and 30°C) were studied.

Results: The results revealed that some of the infected shrimp were hypoxic, lethargic with abnormal swimming behavior. In most cases, body appendages, telsons, uropods, and gills took black coloration. In addition, the hepatopancreas appeared soft, swollen, and congested. The prevalence rates of vibriosis in each of the musculature and hepatopancreas were 4.7%, while the prevalence rates of *Aeromonas* infection in the musculature and hepatopancreas were 11.8% and 11.2%, respectively. Duplex PCR showed that *Aeromonas* isolates gave double bands: 237 bp specific for gcat and 500 bp specific for 16S rRNA, while *Vibrio* spp. and *Plesiomonas shigelloides* isolates gave single band at 500 bp. The effect of organic acid treatment showed that acetic acid (vinegar 5%) had increasing reduction rates that reached its maximum level after 24 h; where it caused (100% inhibition) for *A. hydrophil*a at both temperatures and (33.63% and 60% inhibition) for *V. parahaemolyticu*s at refrigerator and room temperatures, respectively. Moreover, acetic acid was more effective at room temperature than at refrigerator temperature. Concerning the effect of lemon juice (citric acid), it was more effective than acetic acid at short marination (0.25 and 1 h) at both temperatures for the two pathogens. Moreover, lemon was more effective at refrigerator temperature than at room temperature at the same aforementioned time. The difference between the reduction effects of the two acids on both pathogens was statistically significant ($p<0.0001$).

Conclusion: Overall, the examined shrimp samples were found to be vectors for *Vibrio* and *Aeromonas* spp. Application of hygienic measures during handling and cooking of shrimp should be esteemed. The organic acid treatment trial showed that vinegar and lemon juice can be used as a safe and economic method to limit the microbial contamination in seafood.

Keywords: Shrimp, vibriosis, *Aeromonas* infection, sequencing, decontamination, citric acid, acetic acid

Introduction

Shrimp is highly valued as a source of animal protein [1]. From another side, seafoods are considered as vectors in human outbreaks [2]. Many bacteria incriminated to cause diseases for shrimp are normal inhabitants for marine environments and are considered as opportunistic pathogens [3] when natural defense mechanisms are suppressed [4]. The most important bacterial pathogens which cause diseases in shrimp are *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. [5].

Vibrio spp. are the most isolated bacteria from diseased shrimps [6]. They are Gram-negative bacteria that inhabit estuarine and coastal waters [7]. Twelve species of the genus *Vibrio* are pathogenic to humans. The most encountered species in seafood-borne infection are *Vibrio Parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus*. The infection is transmitted by ingestion of raw seafood or through wound infection (during swimming in contaminated water or handling infected seafood). The recorded illness includes (1) infection of the eyes, ears, or

open wounds, (2) primary septicemia with symptoms including fever, and blistering skin lesions that lead to extensive necrosis and necessitate amputation of limbs, and (3) gastroenteritis [8].

Vibriosis in shrimp appears as a number of syndromes (oral and enteric vibriosis, appendage and cuticular vibriosis, localized vibriosis of wounds, shell disease, systemic vibriosis, and septic hepatopancreatitis) [9]. *Vibrio harveyi* luminescent strains cause about 80-100% mortality in *Penaeus monodon* hatcheries [10] and *Fenneropenaeus indicus* culture [11]. Furthermore, Li *et al.* [12] recorded that *V. parahaemolyticus* toxic strains which cause acute hepatopancreatic necrosis will severely damage the global shrimp industry, while *V. alginolyticus* was demonstrated to be the most common organism isolated from the diseased shrimps with signs of septicemia [13].

Aeromonas species are Gram-negative bacteria that are ubiquitous in aquatic environments. The genus *Aeromonas* includes the mesophilic aeromonads: *Aeromonas hydrophila*, *Aeromonas caviae, Aeromonas sobria, Aeromonas veronii,* and *Aeromonas schuberti*. They are renowned as emerging enteric pathogens of public health concern. Mesophilic aeromonads are etiologic agents of a broad spectrum of diseases in man such as gastroenteritis, soft tissue, muscle infections, septicemia, and skin disease [8,14].

They were isolated from tissues and hemolymph of cultured penaeid shrimp. They cause shell disease with melanization of cuticle, gills, body, and appendages. Moreover, septicemia of juvenile and adult cultured penaeid shrimp and brown spot disease were recorded [3].

By increasing of consumers' needs for safe, natural, and non-toxic preservatives that can be used at household level, organic acids have GRAS (Generally Recognized as Safe) status, and hence, they are used extensively as food preservatives and additives for perishable food items [15]. These compounds are composed of saturated straight-chained monocarboxylic acids and their derivatives [16]. The antimicrobial properties of organic acids are attributed to their low pH. It is established that undissociated forms (uncharged or protonated forms) of organic acids can penetrate the lipid membrane of the bacterial cell, while the dissociated (non-protonated or charged forms) forms cannot. Once the uncharged acid enters the cytoplasm, it dissociates into anions and protons. The bacterial cell has to consume more energy in the form of adenosine triphosphate (ATP) to keep its neutrality and hence depletion of cellular energy and hindering of cell growth occur [17].

The objectives of this study were to record the clinical picture of vibriosis and *Aeromonas* infection in naturally infected shrimp, isolation and identification of the causative organisms by conventional culture method, confirmation of the isolates using duplex polymerase chain reaction (PCR) reaction, sequencing, and finally control of shrimp contamination using acetic acid (vinegar) and citric acid (lemon juice).

Materials and Methods

Ethical approval

The definition of animal (for experimental use) by any of the international consortia or Animal Research Act (ARA) does not include invertebrates (excluding *Cephalopods*) as test animals and hence does not require ethical committee approval [18]. However, we humanely handled the shrimps at the lab, removed them from the transporting seawater and they were left to die before processing of samples.

Sampling and clinical picture [19]

One hundred and seventy live shrimp samples (*Metapenaeus monoceros*) were purchased from fishermen in Suez city, Egypt, during the period from June to September 2016. The samples were placed in sterile seawater in an ice box immediately after capturing and were transported to the Laboratory of Zoonoses, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. Their body weights ranged from 16 to 18 g. The clinical picture of naturally infected shrimps was designated as live, moribund, and freshly dead ones.

Preparation of samples [20]

Hepatopancreas

The carapace was lifted using sterile forceps, and from inside the hepatopancreas, two inoculums were taken using sterile cotton swabs and inoculated into two tubes containing 9 ml of sterile alkaline peptone water (APW, pH: 8, CM1028 Oxoid, UK) and APW (pH: 8) supplemented with 3% NaCl for the isolation of *Aeromonas* and *Vibrio* spp., respectively.

Musculature

The surface was hot seared using hot scissors, and each shrimp sample was aseptically divided into two equal parts (5 g each); one part was stomached with 45 ml of APW in sterile polyethylene sacs, and the other part was stomached with 45 ml of APW supplemented with 3% NaCl for the isolation of *Aeromonas* and *Vibrio* spp., respectively.

Isolation and identification of *Aeromonas* spp. [21,22]

The APW tubes were incubated at 35°C for 24 h. A loopful of the incubated broth was streaked onto well-dried MacConkey-ampicillin agar (20 µg/ml, Lab M, UK) plates to be incubated at 35°C for 48 h. Colorless colonies were selected, purified, and identified.

Isolation and identification of *Vibrio* spp. [23]

The APW tubes supplemented with 3% NaCl were incubated at 35°C for 24 h. A loopful of the incubated broth was streaked onto well-dried thiosulfate-citrate-bile salts-sucrose agar (TCBS, Hardy Diagnostics, USA) plates to be incubated at 35°C for 72-96 h. Yellow, blue, or green colonies were picked up, purified, and identified.

Maintenance of isolates

Isolates were incubated in tryptic soy broth (TSB, Becton Dickinson, GmbH, Germany). 500 µl of the overnight culture was added to 500 µl of 50% glycerol (100% glycerol diluted in sterile distilled H2O) in a 2 ml screw-top tube and was gently mixed and kept frozen at −20°C.

PCR confirmation of isolates

DNA was extracted from isolates by boiling centrifugation method [24]. Duplex PCR technique that allowed detection of *Vibrio* and *Aeromonas* spp. was performed following the technique of Mendes-Marques *et al.* [25]. Primers were designed by Bio Basic Inc., Canada. Briefly, the reaction was carried out in 25 µl reaction that consisted of 5 µl of DNA, 1.5 µl of gcat-f:5'-ctcctggaatcccaagtatcag-3' and 1.5 µl of gcat-r: 5'-ggcaggttgaacagcagtatct-3', 1.5 µl of 16S-f: 5'-acgcaggcggttggataagt-3' and1.5 µl of 16S-r: 5'-ggcaacaaaggacaggggt-3', 12.5 µl of PCR Master Mix (GeneDirex, USA), and 1.5 µl of sterile milliQ water. The PCR reaction was run in a thermal cycler (Techne, England) and programmed for 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, and a final 5 min extension at 72°C. Five µl of PCR product was electrophoresed in 1.5% agarose containing ethidium bromide (0.1-0.5 µg/ml) along with 100 bp DNA ladder for 1 h. The PCR product was viewed and recorded using the SynGene Gel Documentation System. *Aeromonas* spp. give double bands: ~237 bp specific for gcat and ~500 bp specific for 16S rRNA, while *Vibrio* spp. give single band at ~500 bp.

Sequencing of selected isolates [26]

Selected isolates were sequenced; the bacterial 16S rRNA gene was amplified using the bacteria-specific primers 27F-P (5'GAGTTTGATCCTGGCTCAG-3') together with the universal primer 1492R-P (5'- GGTTACCTTGTTACGACTT-3'). PCR was performed on a GeneAmp PCR system 9600 (Applied Biosystems, Thermo Fisher Scientific, USA), and the total reaction volume was 50 µl: 25µl AmpliTaq Gold (Applied Biosystems), 2 µl each (10 pM) of forward and reverse primers, 19 µl sterile Milli-Q water, and 2 µl of DNA template. The PCR programming included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1.5 min, final extension at 72°C for 5 min, and holding at 4°C. PCR products were visualized through electrophoresis on a 1% agarose gel with ethidium bromide. Clones (ca. 1500 bp) were sequenced bidirectionally using the forward and reverse primers (Solgent Company Ltd., Korea). Sequence files were scored and compared to the GenBank nucleotide database using the Basic Local Alignment Search Tool.

Decontamination of shrimp samples [27,28]

The antibacterial effects of two organic acids (acetic and citric) against *A. hydrophila* and *V. parahaemolyticus* were studied.

Preparation of inoculating bacteria

TSB was inoculated with *A. hydrophila* colonies and incubated at 35°C for 24 h. Ten-fold serial dilutions were prepared. 100 µl of each dilution was spread evenly onto the surface of MacConkey agar plates and was incubated at 35°C for 24 h. Plates showing 30-300 colonies were counted. An initial count of 7.5 $\log_{10}$ cfu/ml was selected.

V. parahaemolyticus strain was grown into TSB supplemented with 3% NaCl at 35°C for 24 h. Ten-fold serial dilutions were prepared. One hundred µl of each dilution was spread evenly onto surface of TCBS agar plates and was incubated at 35°C for 24 h. An initial count of 8 $\log_{10}$ cfu/ml was selected.

Preparation of organic acid solutions

About 1 L of each of commercial vinegar (5%) and freshly squeezed lemon fruit juice (*Citrus aurantiifolia*) was used to test the antibacterial activity of acetic and citric acids against *Aeromonas* and *Vibrio*. One lemon yields about 45 ml of juice. The juice of the lemon contains about 5-6% citric acid, with a pH of around 2.2.

Inoculating shrimp samples with the bacterial strains

The inoculation was done separately for each pathogen.

Freshly caught shrimp samples were rinsed with sterile saline solution and allowed to drip on sterile metal mesh. Then, they were disinfected by dipping in ethyl alcohol (70%) for 5 min. After that, they were allowed to drip on sterile metal mesh in laminar flow hood. After complete dryness, shrimp samples were dipped for 45 min in 1 L of the bacterial suspension (with 10 times clockwise and counterclockwise shaking every 5 min for the whole period) to allow adhesion of *Aeromonas* to the samples.

The initial bacterial load was determined by ten-fold serial dilutions in sterile saline and 100 µl from each dilution was spread over MacConkey agar plates and incubated at 35°C for 24 h. The experiment was run in triplicates at refrigerator temperature (5±2°C) and at room temperature (30±2°C) according to Diagram-1a and b.

The control consisted of inoculated shrimp samples dipped in sterile saline solution.

After each specified time, treated shrimp samples were picked up using sterile forceps and allowed to dry on a sterile tissue, 10 g of the musculature of treated shrimp samples were aseptically weighed into 90 ml of sterile saline, homogenized in a stomacher, and ten-fold serial dilutions in sterile saline were carried out as aforementioned. The plates showing 30-300 colonies were counted. The $\log_{10}$ cfu/g was calculated.

For *Vibrio*, the same procedures were used except for using 3% NaCl for serial dilution and as a control and using TCBS as spreading medium. The plates showing 30-300 colonies were counted. The $\log_{10}$ cfu/g was calculated.

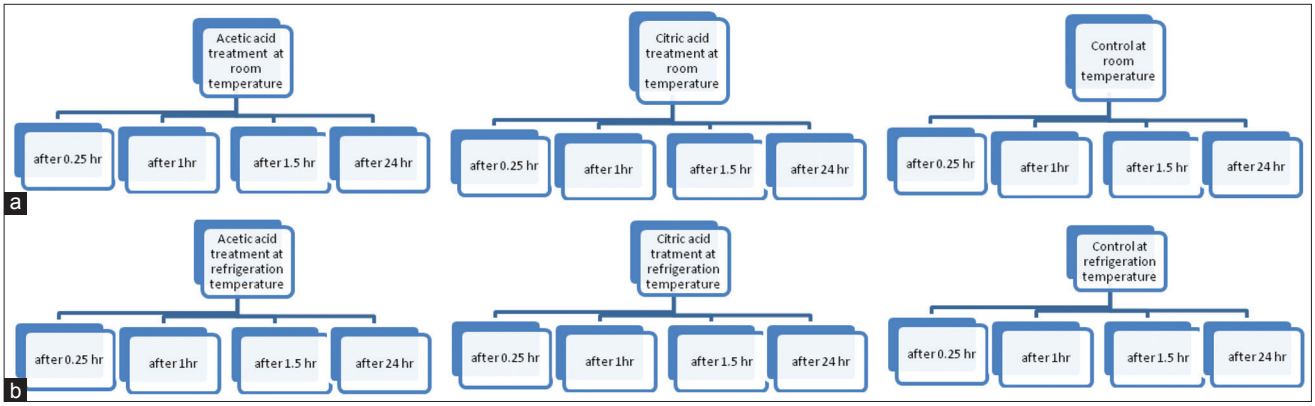

Diagram-1: Experiment (a): At room temperature and (b): At refrigerator temperature.

Statistical analysis

Comparison of the isolation percentages of *Aeromonas* and *Vibrio* was done using Fisher's exact test (SPSS version 20). Comparison of means of treatments and control was done using Kruskal–Wallis test (non-parametric ANOVA). p-value was set at ≤0.05 (SPSS version 20).

Log_{10} reduction and reduction percentages were calculated using Excel software [29].

$$\log_{10} \text{reduction} = (A) - (B).$$

Where A= $\log_{10}$ number of viable microorganisms before treatment.

B= $\log_{10}$ number of viable microorganisms after treatment.

$$\text{Log reduction } \% = \frac{(A - B)}{A} \times 100$$

If the number is negative, this indicates a $\log_{10}$ increase in number and percentage.

Results

Clinical picture

Some of the naturally infected shrimp (*M. monoceros*) appeared hypoxic and lethargic with abnormal swimming behavior near tank edges and at the water surface. Gill covers were somewhat raised up. Some cases showed white spots on the carapaces, and others had black patches on the cuticle (Figure-1a). In most cases, body appendages, telsons, uropods, and gills took black coloration with deformity of pleopods (Figure-1a and b). Reddish coloration of pereopods and pleopods was recorded in a few cases. Hepatopancreas appeared soft, swollen, and congested (Figure-1c).

Prevalence of vibriosis and *Aeromonas* infections

Among the 170 examined samples, the total prevalence of vibriosis was 7/170, 4.12%. On the other hand, 16 (9.41%) of 170 tested shrimp samples were infected with *Aeromonas* (data were not shown in tables). Hepatopancreas and musculature showed the same prevalence of vibriosis (4.7%). For *Aeromonas*, nearly similar prevalences in hepatopancreas (11.2%) and musculature (11.8%) were recorded. The difference between the total prevalence

Figure-1: Infected *Metapenaeus monoceros* showing, (a): Black patches on cuticle (arrows) and black coloration of body appendages, telsons, and uropods. (b): Black coloration of gills and pleopods (swimmers) deformity. (c): Congested hepatopancreas underneath the carapace (arrow).

rates of *Aeromonas* infection and vibriosis was not considered significant. Odds ratio = 2.419 (95% confidence interval: 0.9687-6.042), p=0.0821 (Table-1).

Biochemically, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *A. hydrophila,* and *A. sobria* were identified.

On sequencing of some biochemically confirmed isolates and others having doubtful results, *A. hydrophila*, *A. sobria*, *A. veronii*, *Plesiomonas shigelloides*, *Pseudomonas putida,* and *Pseudomonas fluorescens* were confirmed. Concerning *Vibrio* isolates, they gave conflicting sequence result and could not be identified.

Duplex PCR showed that *Aeromonas* spp. gave double bands: 237 bp specific for gcat and 500 bp specific for 16S rRNA, while *Vibrio* and *P. shigelloides* gave single band at 500 bp (Figure-2).

Effect of decontamination using organic acids on *A. hydrophila* and *V. parahaemolyticus* counts

Table-2 and Figure-3 depict the effect of acetic acid treatment on *A. hydrophila* count at room temperature for 0.25, 1, 1.5, and 24 h. The log reduction values were 1.28, 1.39, 2.03, and 7.5 and log reduction percentages were 17.07, 18.5, 27.07, and 100, while, for *V. parahaemolyticus*, the log reductions were 0.43, 0.52, 1.6, and 4.8 and the log reduction percentages

Table-1: Prevalence of vibriosis and *Aeromonas* infection in the examined shrimp (*Metapenaeus monoceros*) samples.

Number of examined samples	Vibriosis		*Aeromonas* infection	
	Hepatopancreas	Musculature	Hepatopancreas	Musculature
	n (%)	n (%)	n (%)	n (%)
170	8 (4.7)	8 (4.7)	19 (11.2)	20 (11.8)

The odds ratio=2.419 (95% CI: 0.9687-6.042), p=0.0821. CI=Confidence interval

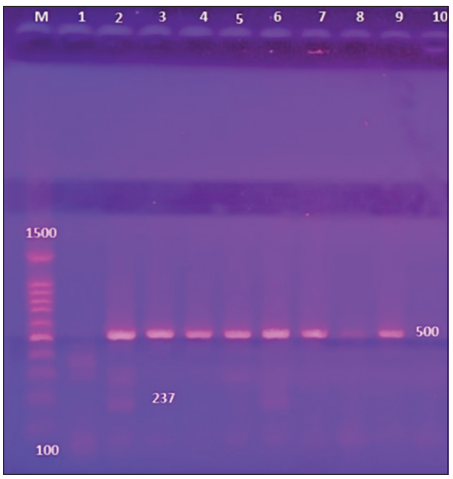

Figure-2: Amplification of gcat and 16 S rRNA genes. Lane M: 100 bp DNA ladder. Lane 1: negative control. Lanes 2 and 6: *Aeromonas hydrophila* and *Aeromonas veronii* gave double bands: 237 bp specific for gcat and 500 bp specific for 16S rRNA. Lanes 3, 4, 8, and 9: *Vibrio* gave 500 bp specific for 16S rRNA. Lanes 5 and 7: *Plesiomonas shigelloides* gave 500 bp specific for 16S rRNA.

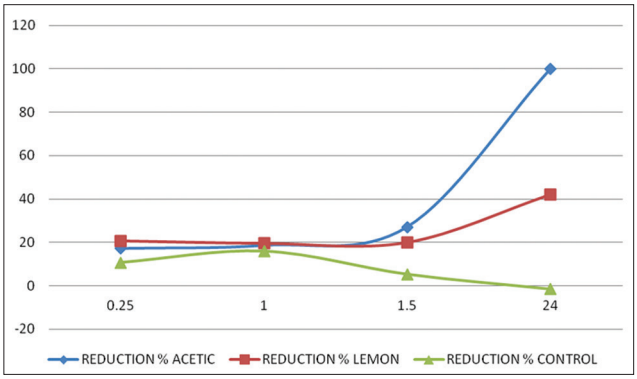

Figure-3: Reduction percentages of *Aeromonas hydrophila* count at room temperature using different treatments.

were 5.38, 6.5, 20, and 60, respectively (Table-3 and Figure-4).

In the acetic acid treatment experiment at refrigerator temperature with *A. hydrophila* for 0.25, 1, 1.5, and 24 h, the log reductions were 1.13, 1.39, 1.9, and 7.5 and the log reduction percentages were 15.07, 18.53, 25.33, and 100, respectively (Table-2 and Figure-5), while the log reductions for *V. parahaemolyticus* were 0.35, 0.42, 1.46, and 2.69 and the reduction percentages were 4.38, 5.25, 18.25, and 33.63, respectively (Table-3 and Figure-6).

With respect to the effect of citric acid treatment on the count of *A. hydrophila* at room temperature for 0.25, 1, 1.5, and 24 h, the log reductions reached 1.55,

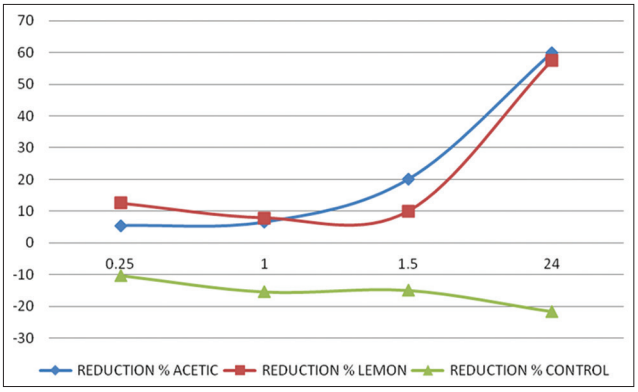

Figure-4: Reduction percentages of *Vibrio parahaemolyticus* count at room temperature using different treatments.

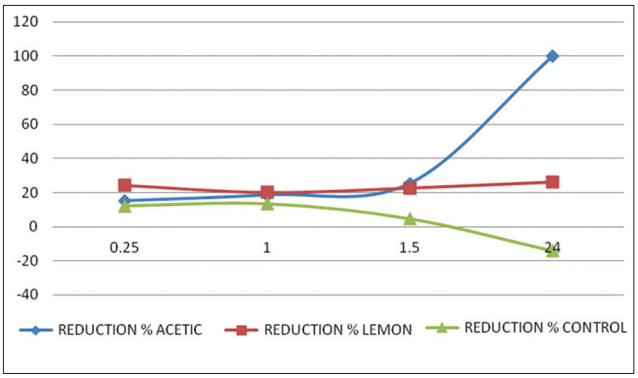

Figure-5: Reduction percentages of *Aeromonas hydrophila* count at refrigerator temperature using different treatments.

1.47, 1.53, and 3.15 and the reduction percentages were 20.67, 19.61, 20.4, and 42, respectively (Table-2 and Figure-3), while, for *Vibrio*, the log reductions were 1, 0.44, 0.8, and 4.61 and the reduction percentages were 12.5, 5.5, 10, and 57.63 (Table-3 and Figure-4).

In the citric acid treatment experiment at refrigerator temperature with *Aeromonas* for 0.25, 1, 1.5, and 24 h, the log reductions were 1.82, 1.5, 1.7, and 1.97 and the reduction percentages were 24.27, 20, 22.67, and 26.27 (Table-2 and Figure-5), while, for *Vibrio*, the log reductions were 1.06, 0.63, 0.53, and 1.99 and the reduction percentages were 13.25, 7.9, 6.63, and 24.88, respectively (Table-3 and Figure-6).

Overall, citric acid was more effective at refrigerator temperature than citric acid at room temperature at 0.25 and 1 h. Furthermore, citric acid was more effective than acetic acid at short marination (0.25 and 1 h) at both temperatures for the two pathogens. After that time, acetic acid showed superiority.

Table-2: Effect of organic acid treatment on *Aeromonas hydrophila* count at different temperatures.

Time (h)	At refrigerator temperature									At room temperature								
	Acetic acid			Citric acid			Control			Acetic acid			Citric acid			Control		
	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%
0.25	6.37	1.13	15.07	5.68	1.82	24.27	6.59	0.91	12.13	6.22	1.28	17.07	5.95	1.55	20.67	6.70	0.8	10.67
1	6.11	1.39	18.53	6	1.5	20	6.5	1	13.33	6.11	1.39	18.5	6.03	1.47	19.61	6.31	1.19	15.87
1.5	5.60	1.9	25.33	5.8	1.7	22.67	7.16	0.34	4.53	5.47	2.03	27.07	5.97	1.53	20.4	7.1	0.4	5.33
24	0	7.5	100	5.53	1.97	26.27	8.57	−1.07	−14.27	0	7.5	100	4.35	3.15	42	7.61	−0.11	−1.47

R means reduction. The negative sign indicates a log_{10} increase and log_{10} increase %. Kruskal–Wallis value=105.02, $P<0.0001$. Control treatment comprised inoculated shrimp samples dipped in sterile saline solution

Table 3: Effect of organic acid treatment on *Vibrio parahaemolyticus* count at different temperatures.

Time (h)	At refrigerator temperature									At room temperature								
	Acetic acid			Citric acid			Control			Acetic acid			Citric acid			Control		
	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%	Log_{10} means	Log_{10} R	R%
0.25	7.65	0.35	4.38	6.94	1.06	13.25	8.63	−0.63	−7.88	7.57	0.43	5.38	7	1	12.5	8.82	−0.82	−10.25
1	7.58	0.42	5.25	7.37	0.63	7.9	8.67	−0.67	−8.38	7.48	0.52	6.5	7.56	0.44	5.5	9.24	−1.24	−15.50
1.5	6.54	1.46	18.25	7.47	0.53	6.63	9.18	−1.18	−14.75	6.40	1.6	20	7.2	0.8	10	9.19	−1.19	−14.88
24	5.31	2.69	33.63	6.01	1.99	24.88	7.09	0.91	11.38	3.20	4.8	60	3.39	4.61	57.63	9.73	−1.73	−21.63

R means reduction. The negative sign indicates a log_{10} increase and log_{10} increase %. Kruskal–Wallis=85.884, $p<0.0001$. Control treatment comprised inoculated shrimp samples dipped in sterile 3% NaCl solution

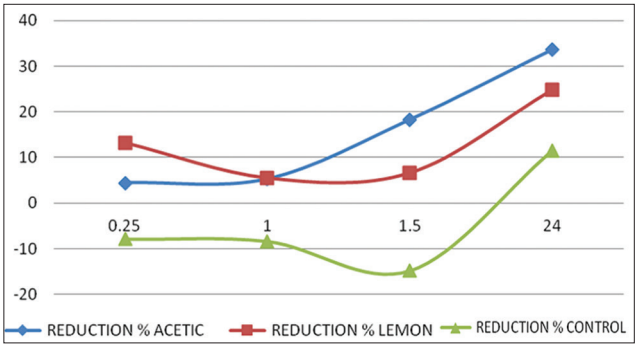

Figure-6: Reduction percentages of *Vibrio parahaemolyticus* count at refrigerator temperature using different treatments.

While for acetic acid, it showed an ascending increase in the reduction percentages to reach 100 % reduction for *Aeromonas* at 24 h at both temperatures and (33.63% and 60%) reduction for *V. parahaemolyticus* at refrigerator and room temperatures, respectively. Overall, the reduction effect using acetic acid was more effective at room temperature than that at refrigerator temperature for both microorganisms.

The difference between the reduction effects of the two acids on both pathogens was significant. Kruskal–Wallis value = 105.02, 85.88, $p<0.0001$ for *A. hydrophila* and *V. parahaemolyticus*, respectively.

Discussion

Clinical picture of infected shrimp

In the present study, the infected *M. monoceros* appeared hypoxic and lethargic with abnormal swimming behavior near tank edges and at water surface. These results were similar to what was recorded by Alavandi *et al.* [5] and Biju and Gunalan [30] due to vibriosis and Cheng *et al.* [31] due to *A. veronii* infection in the ornamental shrimp (*Caridina cf. babaulti*). This may be due to gills' infection. As gills are covered by thin exoskeleton, so they are easily penetrated by bacterial pathogens [32]. Some cases showed white spots on the carapace as what was recorded by Ali and Abo-Esa [33]. Furthermore, black patches were seen on the cuticle. Body appendages, telsons, uropods, and gills took black coloration. These results were recorded due to vibriosis and *Aeromonas* spp. infection [5], while Zaki *et al.* [11] recorded tissue and appendages necrosis in *F. indicus* culture infected with vibriosis. These may be due to the distribution of melanophores in the affected area and that makes a black ring which separates healthy tissue from the affected one [34]. The most important bacterial pathogens which cause shell disease in shrimp are *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp. [5]. Shrimp with vibriosis has body discoloration. Reddish coloration was recorded on pereiopods and pleopods as a result of expansion of chromatophores [5]. A special group of *Vibrio* has a luminescence character as heavily infected shrimp appears "glow-in-the-dark" [10,35]. Hepatopancreas is the target organ of most bacterial infections of shrimp [35]. In the current study, hepatopancreas of the infected shrimp appeared to be soft, swollen, and congested, while Ali and Abo-Esa [33] recorded paleness and hemorrhagic patches on it in *Penaeus semisulcatus* affected by *V. alginolyticus*. It appeared atrophied and pale due to acute hepatopancreatic necrosis disease caused by *V. parahaemolyticus* strains [12]. Furthermore, Aly and El-Attar [36] recorded the same signs and lesions due to *P. fluorescens* in freshwater shrimp in Egypt.

Prevalence and public health significance of vibriosis and *Aeromonas* infection in shrimp

Vibrio spp. has been encountered in seafood-borne infection which is transmitted by ingestion or handling of raw seafood. The disease varied from gastroenteritis to septicemic life-threatening illness [8]. *Aeromonas* species are emerging enteric pathogens of public health concern. They inhabit the aquatic environment. They can cause a broad spectrum of diseases in man such as gastroenteritis, wound infection, septicemia, and skin disease [8,14].

The available literature included various levels of *Vibrio* and *Aeromonas* infection in shrimp. El-Hadi *et al.* [37] detected *Vibrio* spp. in 4.6% of examined shellfish in Malaysia. Eight *Vibrio* spp. were recovered from shrimp that included *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus*, *Vibrio metschinkovii*, *Vibrio mimicus*, *Vibrio damsela,* and *Vibrio fluvialis*.

In a study conducted in India [38], found that *V. alginolyticus* (3-19%), *V. parahaemolyticus* (2-13%), *V. harveyi* (1-7%), and *V. vulnificus* (1-4%) were the predominant *Vibrio* species identified in water, sediment, and shrimp samples by standard biochemical testing. Koralage *et al.* [39] investigated the prevalence of *Vibrio* spp. in farmed shrimp (*P. monodon*) in Sri Lanka. Overall, 98.1% of the farms and 95.1% of the ponds were positive for *Vibrio* spp. In shrimp, at the pond level, *V. parahaemolyticus* (91.2%) was the most common, followed by *V. alginolyticus* (18.8%), *V. cholerae* non-O1/non-O139 (4.1%), and *V. vulnificus* (2.4%). Abd-Elghany and Sallam [40] verified by molecular PCR that 20/120 (16.7%) of shellfish samples were positive for *V. parahaemolyticus*. The prevalence among shrimp collected from different fish shops in Mansoura city, Egypt, was 9 (22.5%) of 40 examined samples [40].

A much higher prevalence (55·8%) was recorded in shrimp samples collected in the city of Agadir, Morocco [41], where *V. parahaemolyticus* was recovered from 25 of 299 samples (8·4%), *V. cholerae* from six samples (2%), and *V. alginolyticus* from 161 samples (53·8%). Biju and Gunalan [30] revealed that the overall occurrence of *Vibrio* species from moribund shrimp samples from three major Indian shrimp farm villages was 29.01 %. Odeyemi [42] reported the incidence of 48.3 % of *V. parahaemolyticus* in shrimps.

In contrast, in Croatia [43], the prevalence of *V. parahaemolyticus* in shellfish was 15%. However, the examined crustaceans were negative.

In India [35], *Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp. were isolated from the hepatopancreas, hemolymph, intestine, gills, and eroded portion of the exoskeleton of cultured *P. monodon*. Luminous *V. harveyi* was the dominant bacterial flora in the affected organs, followed by non-luminous *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, other *Vibrio* spp., *Aeromonas* spp., and *Pseudomonas* spp.

With respect to *A. hydrophila*, Vivekanandhana *et al.* [44] analyzed 278 prawn samples from the major fish market of Coimbatore, South India. They recorded that the prevalence of *A. hydrophila* in *Penaeus indicus*, *P. monodon,* and *P. semisulcatus* was 16.58%, 13.20%, and 25.52%, respectively.

In the same line, Khamesipour *et al.* [45] examined 36 shrimp samples in Iran. Fourteen (38.9%) *A. hydrophila* strains were confirmed by conventional bacteriological techniques, while five strains (13.89%) were identified by PCR targeting 16S rRNA gene.

In our study, we could identify *P. shigelloides* using duplex PCR and by sequencing. However, the reference study [25] did not mention that *Plesiomonas* could be amplified using their specified PCR protocol. *P. shigelloides* is included within the bacterial family *Vibrionaceae* which also contains the genus *Vibrio.* Both *P. shigelloides* and some *Vibrio* spp. are known causes of food poisoning in man [46]. Many cases of *Plesiomonas*-associated eye and wound infections have been documented [46,47]. *P. shigelloides* is present in freshwater ecosystems (rivers, lakes, and surface waters) and marine estuaries in tropical and temperate climates [48]. *P. shigelloides*, *A. hydrophila,* and *Pseudomonas* spp. strains had been isolated from sick guppy fishes (*Poecilia reticulata*) that belonged to aquarium fish rearing farms in Istanbul Province, Turkey [49]. The sick fish suffered from ascites, emaciation, and fin rot. *P. shigelloides* had also been isolated from the gut of wild and cultured banana prawns, *Penaeus merguiensis* [50].

The other spp. recovered on sequencing were *Pseudomonas putida* and *P. fluorescens*. *Pseudomonas* spp. are opportunistic human pathogens. They are prevalent in patients with skin infections who are considered as the main source of water contamination; they can also cause diarrhea and even septicemia [51-53]. They had also been reported in seafood [20,54,55]. Nevertheless, public health hazard arises to food consumers when their count exceeds10^6-10^7 cfu/g of product [56].

Decontamination of shrimp using organic acids

Consumers' protection against foodborne pathogens is a global public health issue. Certain organic acids have been used to limit microbial contamination and dissemination of foodborne pathogens in pre-harvest and post-harvest food production and processing, thus prolonging food shelf life and conserving food appealing to consumers [57]. The antibacterial activity of organic acids may vary depending on the physiological status of the organism and the physicochemical characteristics of their external environment [16].

In the current study, citric acid was found to be more effective in reducing *A. hydrophila* and *V. parahaemolyticus* counts than acetic acid at short marination period (0.25 and 1 h) at both temperatures. After that, time acetic acid outperformed. Furthermore, citric acid was more effective at refrigerator temperature than citric acid at room temperature at the same time. To sum up, acetic acid showed an ascending increase in the reduction percentages to reach 100% reduction for *Aeromonas* after 24 h at both temperatures and 33.63% and 60% reduction for *V. parahaemolyticus* at refrigerator and room temperatures, respectively.The reduction effect using acetic acid was more effective at room temperature than that at refrigerator temperature for both microorganisms.

Our result is supported by Mathur's findings [28] that lime juice is effective in inactivation of both *V. parahaemolyticus* and *Salmonella* Enterica. He also found that refrigeration while marinating the fish had a significant impact on S*almonella* reduction. A reduction ~0.5 log was observed in the first 10 min after application of lime juice on *Salmonella* in ceviche marinated at room temperature. The reductions remained almost constant until time 60 min. An extended incubation for 150 min produced a log reduction of 1.3. Log reductions in *V. parahaemolyticus* in experiments at room temperature varied from >4.5 to >5.2, while, at refrigerator temperature, log reductions varied from >3.5 to >4.3. When he tested the inhibitory effect of lime juice, without the fish matrix, he found more than 5 log reductions in counts on both bacteria.

Our observation that lemon juice was more effective at refrigerator temperature than lemon at room temperature at 0.25 and 1 h is supported by Mathur's postulation [28] that when cells are exposed concurrently to two stresses (acid and refrigeration), this results in a greater reduction.

Furthermore, Mata *et al.* [58] studied the effect of lime juice on *V. cholera*e O1 on commercially prepared ceviche (from mahi-mahi fish), ceviche prepared from the fish contaminated in the laboratory and also by adding the bacteria directly to lime juice without the fish matrix. Their results were as follows: For commercial ceviche, no bacteria were recovered after 15-30 min of exposure, while a 4 log reduction in ceviche prepared in a laboratory was observed after 30 min. For addition of *Vibrio* directly to the lime juice, a 3 log reduction within 5 min and complete elimination after 2 h were observed. The buffering effect of shrimp or fish tissues was thought as a reason for reducing the antimicrobial effect of acidic sauces [28,58].

In the experiment performed by Rodrigues *et al.* [59], limes were shown to limit foodborne transmission of *Vibrio cholera.* In the same line, Tomotake *et al.* [60] found that lime fruit juices were effective against *Vibrio* species.

Lime juice contains essential oils, saponins, and flavonoid compounds which have an antibacterial and bacteriostatic character. These compounds inhibit metabolism and growth of bacteria [61,62]. The citrus peels contain essential oils (rich in phenolic compounds) which are responsible for the antimicrobial activity of squeezed citrus fruits [63].

Our finding that acetic acid outperformed citric acid was supported by Bang and Drake [64] who recorded a reduction of 6 log of *Vibrio vulnificus* counts within 15 min at pH 3.5 with citric acid. At pH 3.5, acetic acid was more effective than citric acid. A concentration of 0.5% acetic acid was recommended for marinating seafood [57]. Furthermore, the concentrations of 0.07% acetic acid and above completely inhibited *V. harveyi* growth [65].

Accordingly, vinegar is esteemed as a natural antimicrobial product which can improve the shelf life and safety of food products, providing acceptable organoleptic quality at a fair price and reducing economic loss due to spoilage of food products.

The emerging challenging problem is that organic acids have been observed to induce an acid tolerance response (ATR) which enhances the survivability of acid-sensitive pathogens exposed to low pH and this is probably linked to increased virulence. The finding that *Vibrio* was more resistant to acid treatment is supported by previous researches [64,66,67] which stated that all *Vibrio* spp. were found to be susceptible to low pH but gradually developed an ATR pattern if the stress was regulated. Adaptation to one stress also seems to provoke other physiological adaptations, resulting in adaptability to multiple stresses. In a study by Wong *et al.* [68] on *V. parahaemolyticus*, the acid adapted cells showed greater survival at low salinity and after thermal inactivation. In like manner, acid adaptation of *V. parahaemolyticus* with an accumulation of the decarboxylation product of lysine was reported [69]. Lysine decarboxylation is the mechanism thought to be related to acid tolerance response.

Furthermore, the fact that *V. parahaemolyticus* has a short generation time, it only takes 9 min in culture medium and 12 min in a host to produce one generation [70] may account for its smaller reduction percentages.

Due to the antibacterial effect of organic acids, they have been applied to animal and human nutrition. Feeds treated with organic acids are believed to limit pathogen colonization by conversion of these acids into their respective antibacterial forms once they have entered the digestive tract of humans, animals, fish, and shrimps consuming the treated feed [17,65,71-74].

Conclusion

Bacterial shrimp diseases as vibriosis and *Aeromonas* infections affect its health. The presence of *Vibrio*, *Aeromonas,* and other potential human pathogens in shrimp samples raises public health concern. Hence, hygienic measures should be practiced during handling and cooking of seafood. Organic acid treatment showed that vinegar and lemon juice could be used as a safe and economic method to limit the microbial contamination in seafood and to decrease the seafood borne-associated illness.

Authors' Contributions

HMF was responsible for the collection of samples, statistical analysis, designing, carrying out of the experiments, writing, and revising of the manuscript. MEMM shared in the collection of samples, statistical analysis, performance of the experiments, writing, and revising of the manuscript. Both authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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