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Ability of clove oil and lactoferrin to eliminate biofilm formation of vibrio species in shrimp

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ABSTRACT

Seafood is consumed globally and holds a major position in the economic market. However, *Vibrio spp.* can contaminate these products, posing a potential health risk to humans. So, the study's goal was to look into the spread of vibrio spp. in shrimp collected from Kafr-elsheikh fish markets. The obtained results revealed 19 vibrio spp isolates were recovered from 100 shrimp samples (19%). The total occurrence in the samples was 6 (6%), 4 (4%), 4 (4%), 3 (3%) and 2 (2%) for *V. paraheamolyticus , V. fluvialis, V. mimicus, V. furnissi, and V. alginolyticus,* respectively. Molecular detection of the identified *Vibrio spp.* isolates demonstrated that 10 isolates carried vibrio-specific 16S rRNA. Concerning the virulence genes profile for isolates of *V. parahaemolyticus*, it proves that the frequencies of the toxR, trh and tdh genes were 6%, 4% and 2% respectively. Moreover, the capability of the detected vibrio spp. to produce biofilm were studied. The six isolates of *V. parahaemolyticus*, 2 isolates of *V. mimicus* and 1 isolate of *V. furnissii* showed the capacity for biofilm production on Congo red media. PCR analysis also indicated that six isolates of *V. parahaemolyticus* harbor VP950 gene associated with biofilm formation. In addition, trials were conducted using clove oil (0.5% and 1%) and lactoferrin (1% and 2%) treatments to reduce *Vibrio parahaemolyticus* counts in artificially inoculated shrimp, stored at 4ºC for 6 days. Furthermore, quality factors like TBARS, pH rate, and sensory traits were evaluated. Both lactoferrin and clove oil treatments notably reduced *V. parahaemolyticus* amounts over time, according to the experiments, although lactoferrin was more effective than clove oil. Furthermore, the TBARS, pH values and sensory traits of the chilled shrimp samples all showed advancements. Overall, it can be concluded that retail shrimp in markets can naturally harbor various *Vibrio* species

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which exhibit potential virulence and biofilm formation, may pose a concern to public health. Moreover, the clove oil and lactoferrin concentrations applied in the research were unable to eliminate contamination by *Vibrio parahaemolyticus* from shrimp, which is one of the basic requirements included in the Egyptian standards (5021/2005), which requires that the product should be completely free from this organism in order to be fit for human consumption, and if it is present, the product will consider unsafe for the health of the consumer and therefore is considered of poor quality.

INTRODUCTION:

Shrimp is considered one of the fishery products that is most frequently traded globally, owing to its attractive flavour and good nutritional qualities **(FAO, 2020).** The presence of elevated levels of polyunsaturated fatty acids, beta-carotene, fat-soluble vitamins, phospholipids, and cholesterol is the primary cause of shrimp's health benefits **(Gulzar et al. 2020).** However, consuming raw seafood has been linked to human infections with Vibrio spp. Aeromonas spp., Salmonella spp., and Listeria spp. **(De Silva et al. 2018).**

Vibrio species are gram-negative, anaerobic facultative, and curved motile rod-shaped bacteria commonly found in marine and estuarine waters, along with in aquaculture environments globally **(Thompson et al. 2004).** Within this genus, 12 species were identified as harmful to humans, 8 out of which are potentially linked to foodborne diseases that affect the digestive system **(Oliver and Japer, 1997).** It was lately found that *V. cholerae, V. parahaemolyticus, V. vulnificus,* and *V. mimicus* are the main pathogens which affect humans **(Vora et al. 2005).** Additionally, in rare cases, foodborne or waterborne sicknesses have been linked to *V. alginolyticus* and *V. fluvialis* **(Ramamurthy et al. 2014.)**

Vibrio parahaemolyticus is a Gram-negative, rod-shaped, flagellated, halophilic, and motile bacterium. It is a major contaminant of seafood. This bacterium has the ability to cause acute gastroenteritis, leading to symptoms such as vomiting and diarrhea, which often result from eating inadequately prepared or undercooked seafoods **(Letchumanan et al. 2014)**

V. parahaemolyticus is a facultative pathogen for humans and accounts for roughly 25% of food-borne illness **(Mok et al. 2019).** Additionally, *V. parahaemolyticus* has several virulence genes contributing to its pathogenicity, notably tdh and trh genes **(Hiyoshi et al. 2010).** Molecular studies have shown a direct link between the hemolysin genes and the illness-causing capability of *V. parahaemolyticus* **(Hasrimi et al. 2018).** The toxR gene serves as a pandemic indicator of *V. parahaemolyticus*. Also, toxR gene sequence is useful in molecular detection of *V. parahaemolyticus* **(Hubbard et al. 2016).** Biofilms formed by *Vibrio parahaemolyticus* have been recorded on several kinds of seafood, like clams, oysters, fish, and shrimp **(Wang et al. 2022).** *Vibrio parahaemolyticus* in its biofilm state exhibits increased virulence **(Zhang et al. 2023).**

The formation of biofilms by foodborne pathogens of public health significance is increasingly concern for the public health and food safety sectors because it enhances microbial effectiveness and antimicrobials resistance **(Sharma, et al. 2016).**

With the rise in illnesses caused by foodborne pathogens, new methods for preserving meat have been explored, although chemical preservatives have long been employed in the food sector. But, there are significant concerns about their potential health risks **(Thompson and Darwish, 2019).** As a result, the food industry is seeking safe alternatives to antimicrobials for use in producing various food items. These alternatives aim to reduce microbial loads, extend shelf life, and create products with unique aromas and flavors.

Lactoferrin (LF) is the primary glycoprotein that binds iron in the milk of different

mammals **(Wang et al. 2017).** Lactoferrin is assumed to serve a preventive function against infections as it plays critical biological roles in the immune system, like delivering iron, antibacterial, antifungal and immunomodulatory actions **(Xiong et al. 2020).** Additionally, LF possesses anti-inflammatory, antiviral, antioxidant, and anticancer properties. Numerous investigations have shown that LF has bacteriostatic and bactericidal properties in opposition to a broad range of microbes **(Jenssen and Hancock, 2009).** Lactoferrin has been discovered to be useful in combating the creation of biofilms **(Niaz et al. 2019).** Another reason for the appeal of lactoferrin is its designation as 'generally recognized as safe (GRAS)', making it a popular choice in the food sector **(Rybarczyk et al. 2017).** Lately, lactoferrin has been utilized in food as a preservative to protect foods from contamination by pathogenic and spoilage bacteria, in addition to fungi. This approach could potentially reduce the reliance on chemical preservatives **(Bruni et al., 2016).**

Traditionally, clove oil has long been used both as a seasoning and as an antimicrobial agent in foods and food packaging. Additionally, it treats oral infections by acting as antiseptic. This essential oil is extracted from the clove tree, scientifically known as Syzygium aromaticum **(Gengatharan and Abd Rahim, 2023).** Clove oil's primary active ingredients are eugenol, eugenyl acetate, and caryophyllene. This essential oil is typically yellow or colorless and has a spicy, pungent flavor **(Carson and Hammer, 2011).** It is classified (GRAS) as "Generally Recognized as Safe" by FDA **(Gooderham et al. 2020).** There are numerous uses for this oil in food and dental hygiene sectors. Research has demonstrated its antimicrobial properties against numerous pathogenic and spoilage organisms **(Jimoh et al. 2017).**

So, the goal of this study was to investigate *Vibrio spp* prevalence in retailed shrimps, identify the formation of biofilm and virulence genes in *vibrio parahaemolyticus* isolates, as well as study the effect of lactoferrin and clove oil on reducing *V. parahaemolyticus* growth in artificially contaminated shrimp at refrigeration storage (4∘C)

MATERIALS and METHOD

Sampling method

One hundred shrimp samples were randomly picked from the Kafr-elsheikh Governorate's fish markets. Samples were transported in an ice box to Animal Health Research Institute, Kafr-elsheikh lab, and were processed as soon as they arrived. Shrimp samples were cleaned by using water, followed by disinfection by alcohol and then the flesh were exposed after the carapace was removed.

Isolation and Identification of *Vibrio* spp.:

Ten grams were collected in an aseptic environment and put in a sterile homogenizer containing 90 ml of alkaline peptone water (APW) broth, then incubated for 24hr at 37°C. Then loopful of broth were subcultured on Thiosulfate Citrate Bile Salts Sucrose agar (TCBS) media. Following incubation at 37oC for 24hr. Green and yellow colonies on TCBS media were subjected to further screening procedures, such as gram staining, catalase, oxidase test and citrate test, subculture to peptone water with NaCl of various concentrations (0-3 -6-8-10%), indole test and other biochemical tests as mentioned by **Hosseini et al. (2004)** and **Jayasinghe et al. (2008).** Identification of *Vibrio* isolates were done by biochemical tests in Animal Health Research Institute, Kafr-elsheikh Lab, a bacteriology unit, and by PCR in Reference Laboratory for Veterinary Quality Control on poultry production, Animal Health Research Institute, Dokki, Giza.

Detecting biofilm production of *Vibrio* species:

It was examined by cultivating the *Vibrio* isolates on Congo red agar (CRA) **(Freeman et al., 1989).** *Vibrio* isolates were put into Tryptone Soya Broth and incubated for 24hr at 37ºC, then cultured on CRA plates and incubated for 24hr at 37ºC in an aerobic environment and then left at room temperature overnight. Bacteria that produce biofilms exhibited as black colonies, while non- biofilm producers appeared non-coloured (pink colonies) **(Sechil et al. 2002).**

Molecular identification by PCR:

PCR amplification. 5 µl of DNA template, 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer (Metabionm, Germany), at a concentration of 20 pmol, and 5.5 µl of water were included in a 25 µl reaction. An Applied biosystem 2720 thermal cycler was used to conduct the reaction.

Analysis of the products of PCR. With the use of gradients of 5V/cm, the PCR products were separated using electrophoresis in 1x TBE buffer at room temperature on a 1.5% agarose gel (Applichem, Germany, GmbH). Every gel slot was filled with 15 µl of the PCR products for analysis. The sizes of fragments were measured with a gelpilot 100 bp DNA ladder (Qiagen, gmbh, Germany) and a generuler 100 bp DNA ladder (Fermentas, Thermofisher, Germany). A gel documentation system (Alpha Innotech, Biometra) is used to take the gel pictures and data analysis completed by computer software.

Table 1. Identification of target genes.

Target gene	Primers sequences 5'-3'	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
V.parahaemolyticus toxR	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	368	94˚C 5 min.	94˚C 30 sec.	60˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	Kim *et al.*, 1999
Trh	GGCTCAAAATGGTTAAGCG CATTTCCGCTCTCATATGC	250	94˚C 5 min.	94˚C 30 sec.	54˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Mustapha *et al.*, 2013
Tdh	CCATCTGTCCCTTTTCCTGC CCAAATACATTTTACTTGG	373	94˚C 5 min.	94˚C 30 sec.	54˚C 40 sec.	72˚C 40 sec.	72˚C 10 min.	
Vibrio 16S rRNA	CGGTGAAATGCG-TAGAGAT TTACTAGCGAT-TCCGAGTTC	663	94˚C 5 min.	94˚C 30 sec.	56˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Tarr*et al.*, 2007
Biofilm VP0950	GCCAAACTTCTCAAACAACA ATGAAACGCAATTTAC-CATC	298	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Ashrafudoulla, 2019

Antibacterial impact of clove oil and lactoferrin on the isolated *Vibrio* species:

The antimicrobial action of the clove oil and lactoferrin on some isolated *Vibrio* species were investigated by agar well diffusion method (**Reeves, 1989).** The isolated bacteria were cultured at 37 ºC for 24 hr in tryptic soya broth, then the bacterial suspension was modified to 0.5 McFarland then 100 µL of inoculum of each test bacterium *(V. parahaemolyticus, V. furnissii, V. mimicus, V. fluvialis, and V. alginolyticus*) were spread onto Mueller-Hinton agar by using a swab. By using a sterile cork borer, wells measuring a diameter of 6 mm are made on the agar. Each well is packed with varying concentrations of clove oil; 0.5% and 1%, as well as 1% and 2% of lactoferrin. For one hour, plates were inverted at room temperature to enable the oil and lactoferrin to properly diffuse to the media, and after that incubation for 24 hrs at 37 °C. Inhibition zone surrounding the well was evaluated in millieters and compared to the control well **(Raid et al. 2014).**

Inoculum preparation:

The isolate of *V. parahaemolyticus*, which derived from strains that were previously isolated from shrimp in this study, was kept on trypticase soy agar slants with 3% NaCl at 4º C. A loopful of *V. parahaemolyticus* was aseptically put into 10 ml sterile alkaline peptone water with 3% Nacl, and it was **incubated** independently for 24hrs at 37ºC, Following incubation, counted the pathogen by spread plate method **(FDA, 2001)** then modified using the tube dilution method to 10^7 CFU/ml **(Shirazinejad and Ismail, 2010).**

Shrimp samples preparation:

A total of 5 groups of freshly chilled shrimp (200 grams each = total 1000 grams) were purchased from a local fish market, then all samples were deheading and peeled off manually and washed by distilled water.

Preparation of lactoferrin and clove oil concentrations:

Clove oil was supplied by (Harraz, Planta Medical group, Egypt) and Tween 80 was added as a diluent for even dissolving of essential oil concentrations (0.5% and 1%) in distilled water before applying on examined shrimp samples. Bovine Lactoferrin (Pravotin, Hygint pharmaceuticals, Egypt) was employed in this investigation. 1% and 2% LF solution concentrations were prepared using distilled water and sterilized using a 0.45 mm filter and utilized freshly.

Treatments of *peeled* shrimps:

Samples of shrimp were artificially contaminated by *V. parahaemolyticus* occurred by dipping samples in 100 ml of tryptic soya broth with a 24-hour-old culture (107 CFU/ml) and left for 30 min at room temperature to permit attachment. Inoculated shrimp samples were kept in glass beakers covered with glass lids at temperature (30±2°C). *V. parahaemolyticus* was counted to obtain the initial load before application of treatment solutions based on **Terzi and Gucukoglu (2010).** Then shrimp samples were separated into 5 groups (200 grams each). 1st group was dipped in distilled water, the 2nd and 3rd groups were dipped in 0.5% and 1% concentrations of clove oil for 5 min. **(Çoban et al. 2018),** respectively. 4th and 5th groups were dipped in lactoferrin with concentrations 1% and 2% respectively, and held for 2 min. **(Elsayed and Hussei, 2022).** Using sterile forceps to remove the shrimp samples and let them drain for ten minutes, then separately put in sterile polyethylene bags, labeled and kept at 4 ºC. They were analyzed for physicochemical and bacteriological properties promptly at 2 days intervals during store period (at zero, 2nd, 4th and 6th days) and the experiment was conducted in triplicate.

Microbial analysis:

Microbiological procedures for enumerating *V. parahaemolyticus* were carried out based on the standard methods outlined in **(ISO 8914, 1990).** Briefly, 25 grams from shrimp samples were aseptically placed in a stomacher bag contain 225 ml of alkaline peptone water (1% tryptone peptone, 2% NaCl, pH 8.6) and homogenized for 2 minutes using a stomacher. Subsequently, the spread plate method was employed to enumerate *V. parahaemolyticus* by spreading of 0.1 ml aliquots

of 10-fold sterile serial dilutions (1:10, diluent, alkaline peptone water with 2% NaCl) of shrimp homogenates onto solidified thiosulfate citrate bile salt sucrose agar surface (TCBS). The plates were then incubated at 37°C for 24 hours. Colonies that appeared round (2-3 mm in diameter) and bluish-green on TCBS were regarded as positive for *V. parahaemolyticus.* CFU/ml was used to represent the microbial counts and all experiments were carried out in triplicate.

Physico-chemical analysis:

pH measurement:

From each group, 10 gm of shrimp samples were homogenized in 100 mL of distilled water to measure pH by using a pH meter (Hanna Instruments, Milano, Italy) at room temperature **(AOAC, 1990)**.

Determination of thiobarbituric acid (TBARS): was evaluated as outlined in **ES: 63 -10/2006.**

Sensory evaluation:

Conducted by seven trained panelists on a 5-point hedonic scale as stated by **Pelin-Can and Arslan (2011)** (5 = very acceptable, 4= acceptable, 3= middle, 2 = unacceptable, 1= rejected). The evaluation criteria were indicators of color, odor and texture.

Statistical analysis:

The findings are presented as the mean ± standard error (SE). Data significance was assessed using one-way ANOVA, then Duncan's post hoc test for mean comparisons at a significance level of $P < 0.05$, using SPSS software version 22.

RESULTS:

Results inserted in Table (2) showed that a total of 19 isolates of *Vibrio* species were discovered in this investigation, in which *Vibrio parahaemolyticus* (6 isolates) was the most prevalent species, then *V. fluvialis* (4 isolates), *V. mimicus* (4 isolates) followed by *V. furnissii* (3 isolates) and *V. alginolyticus* (2 isolates). All isolates of *V. parahaemolyticus,* and only two strains each of *V. mimicus* and *V. furnissii* could form biofilm and gave black colonies on Congo red media, while other species gave pink colonies.

Table 2. Positive *vibrio* species and biofilm formation in examined shrimp (n= 100) :

Vibrio species	% of positive samples in relation to *vibrio* spp.	% of biofilm forming isolates	
		Positive	Negative
V. parahaemolyticus	6	6	0.0
V. fluvialis	4	0.0	4
V. mimicus	4	2	2
V. furnissii	3	1	2
V. alginolyticus	2	0.0	2
Total	19	9	10

Percentage of *Vibrio.* spp were calculated according to the total no of examined shrimp samples (100)

The presence of virulence or specific genes were examined and shown in Table (3) and (4). The Vibrio species isolates shown positive for specific 16S rRNA genes (figure .1) by a percentage of 10% and *V. parahaemolyticus* positive for toxR gene (figure. 2) by 6% of the isolates. For *V. parahaemolyticus* virulence genes, the results indicated that about 2% of the isolates were positive for tdh gene. However, about 4% showed positive results for trh gene as in figure (3). It was prevalent that isolate number (1) contained both (tdh) and (trh) genes. Furthermore, the current results prove that the 6 isolates of *V. parahaemolyticus* harbored the biofilm-associated gene VP950 (encoding a lipoprotein-related protein) by 6% as shown in figure (4).

N.B Percentage was related to total number of examined samples in which n=100

Table 3. Prevalence of *16S rRNA gene* of randomly selected *Vibrio* isolates*:*

Vibrio isolates	16S rRNA
1	+
2	+
3	+
4	+
5	+
6	+
7	+
8	+
9	+
10	+

N.B Random strains (1-10) from positive isolates were taken to carry out PCR.

Table 4. Distribution of *toxR* gene, virulence genes (*trh* and *tdh*) and biofilm associated gene *VP950* of *Vibrio parahaemolyticus* isolates

V. parahaemolyticus isolates	*toxR*	*trh*	*tdh*	Biofilm *VP0950*
1	+	+	+	+
2	+	-	+	+
3	+	+	-	+
4	+	+	-	+
5	+	+	-	+
6	+	-	-	+

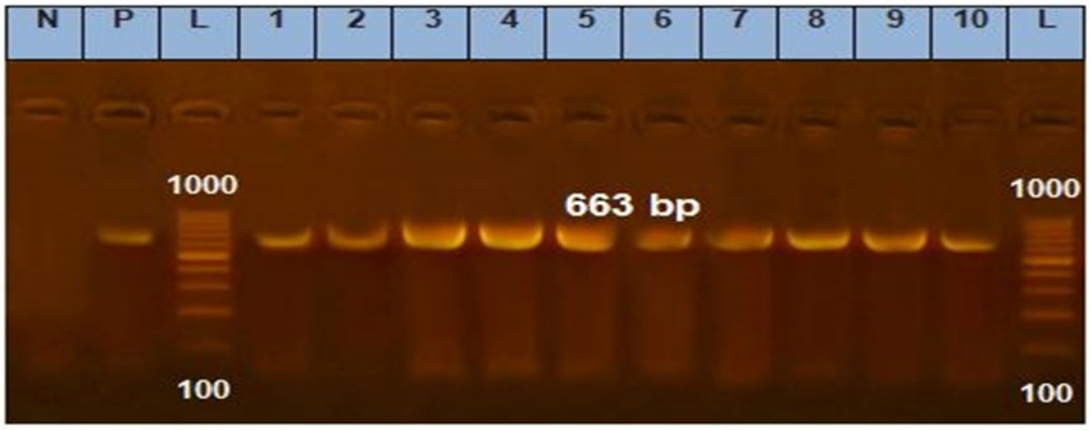

Figure 1. *16S rRNA* gene results at 663 bp for polymerase chain reaction. L (Ladder): DNA ladder (100-1000 bp). lane 1-10 are positive . lane P: Positive control; lane N: Negative control

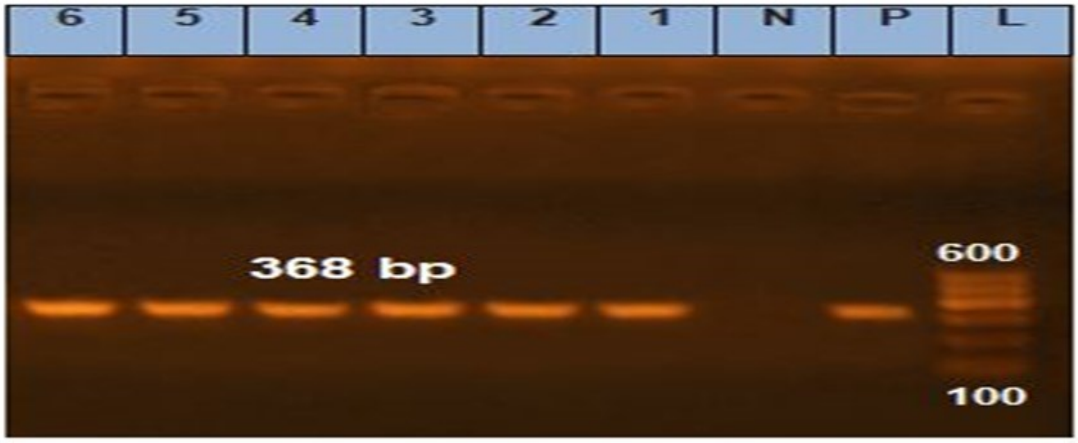

Figure 2. *V. parahaemolyticus toxR* gene results at 368 bp for polymerase chain reaction. L (Ladder): DNA ladder (100-1000 bp). lane 1-6 are positive . laneP: Positive control;lane N: Negative control

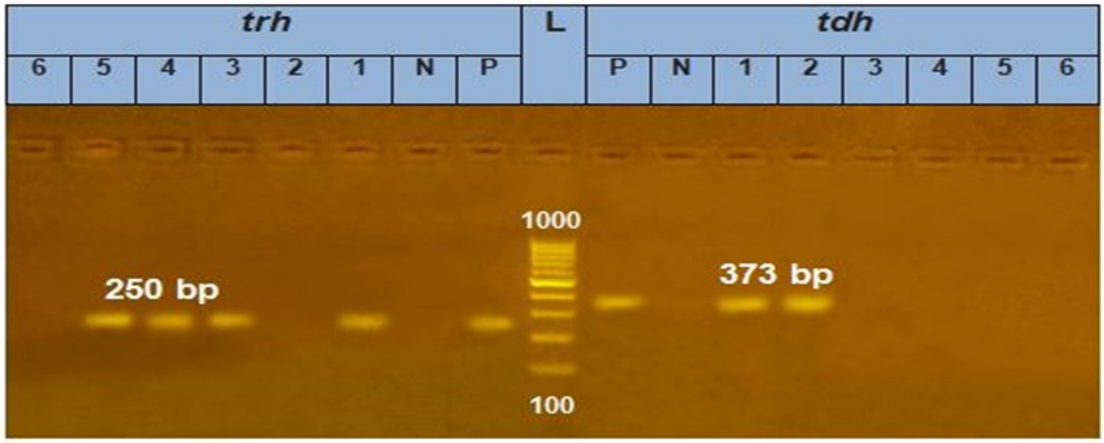

Figure 3. *V. parahaemolyticus* virulence genes (*trh, tdh*) results at 250 bp for polymerase chain reaction. in lane (1, 3, 4, 5) indicating the presence of *(trh)* gene. Amplification of 373 bp.in lane (1, 2) indicating the presence of (*tdh)* gene in samples number (1, 2). L (Ladder): DNA ladder (100-1000 bp). P: Positive control; N: Negative control.

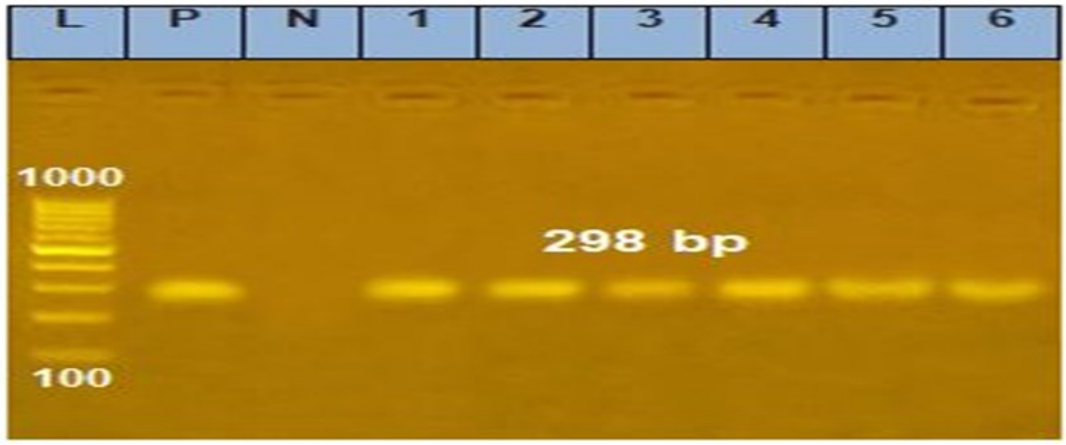

Figure 4. biofilm associated gene *VP950* in *V. parahaemolyticus* results at 298 bp lanes (1-6) are positive. L (Ladder): DNA ladder (100-1000 bp). P: Positive control; N: Negative control.

The outcomes illustrated in table (5) revealed differentiation of the inhibition zone of agar well diffusion method of clove oil in concentrations of 0.5% and 1% and lactoferrin in concentrations 1% and 2% against the isolated *Vibrio* species. The inhibition zone of clove oil against Vibrio species in concentrations 0.5% was at the range of (10-15 mm) and at concentrations 1% (12-17 mm), while the inhibition zone of lactoferrin against *Vibrio* species in concentrations 1% at the range of (14-19 mm) and at concentrations 2% was (15-22 mm).

Table 5. Antibacterial effect of clove oil and lactoferrin against the isolated *Vibrio* species

Species	Isolate	Clove oil		Lactoferrin	
		0.5%	1%	1%	2%
V. parahaemolyticus	VP1	10 mm	12 mm	14 mm	16 mm
	VP2	13 mm	17 mm	18 mm	19 mm
	VP3	11 mm	12 mm	14 mm	15 mm
V. fluvialis	VF1	10 mm	13 mm	15 mm	17 mm
	VF2	12 mm	14 mm	16 mm	17 mm
V. mimicus	VM1	11 mm	15 mm	17 mm	18 mm
	VM2	14 mm	16 mm	17 mm	19 mm
V. furnissii	VFU1	12 mm	15 mm	18 mm	20 mm
	VFU2	15 mm	16 mm	17 mm	18 mm
V. alginolyticus	VA1	11 mm	14 mm	15 mm	17 mm
	VA2	13 mm	17 mm	19 mm	22 mm
Inhibition zone diameter		10-15	12-17	14-19	15-22

N.B Random samples of the positive isolates were taken to examine the antibacterial zone (3 *V. parahaemolyticus* out of 6, 2 *V. fluvialis* out of 4, 2 *V. mimicus* out of 4, 2 *V. furnissii* out of 3 and 2 *V. alginolyticus*).

Table (6) revealed the means counts of *Vibrio parahaemolyticus* (log10cfu/g) experimentally contaminating shrimp samples during storage at 4 ºC for 6 days and treated with different concentrations of clove oil (CO) and lactoferrin (LF). The obtained findings confirmed that varying concentrations of clove oil and lactoferrin exhibited significant reduction effects against *V. parahaemolyticus* producedbiofilm, where at zero day, counts of *V. parahaemolyticus* after inoculation were 7.17 ± 5.91, 7.04 ± 5.46, 6.93 ± 5.04, 6.89 ± 4.60 and 6.69 ± 4.54 log10cfu/g in control, 0.5% CO, 1% CO, 1% LF and 2% LF groups, respectively. By 2nd day of the storage period, such counts decreased to 6.99 ±4.59, 6.82 ±5.07, 6.57 ±5.04 and 6.27 ±4.55 log10cfu/g after treatment with 0.5% CO, 1% CO, 1% LF and 2% LF, respectively, in contrast to the control group which recorded 7.46 ±5.89 log10cfu/g. By the 4th day of storage, control group as well as the treated groups recorded 7.83 ±5.98, 6.51 ±5.04, 6.17 ±4.89, 5.92 ±3.69 and 5.70 ±3.84 log10cfu/g, respectively. At 6th day of the experiment, counts reduced to 6.04 ±4.36, 5.86 ±3.72, 5.50 ±3.76 and 5.04 ±3.57 log10cfu/g, respectively, in treated groups, while in control group increased to 7.98 ±6.04 log10cfu/g.

Table 6. Changes in *V. parahaemolyticus* count (log10 cfu/gm) in control and treated shrimp groups during the storage time at 4º C

Groups	Zero day	2nd day	4th day	6th day
CTR	7.17 ± 5.91^{a}	7.46 ±5.89^{a}	7.83 ±5.98^{a}	7.98 ±6.04^{a}
0.5% CO	7.04 ± 5.46^{b}	6.99 ±4.59^{b}	6.51 ±5.04^{b}	6.04 ±4.36^{b}
1% CO	6.93 ± 5.04^{c}	6.82 ±5.07^{c}	6.17 ±4.89^{bc}	5.86 ±3.72^{bc}
1% LF	6.89 ± 4.60^{c}	6.57 ±5.04^{d}	5.92 ±3.69^{c}	5.50 ±3.76^{c}
2% LF	6.69 ± 4.54^{d}	6.27 ±4.55^{e}	5.70 ±3.84^{c}	5.04 ±3.57^{c}

Values are means ± SE. Different letters in the same column are significantly different at $P< 0.05$. **CO**: Clove oil, **LF**: lactoferrin

Changes of pH values in shrimp groups during the storage time at 4º C showed in table (7). Levels of pH at 0 day in different groups were 6.64, 6.63, 6.62, 6.62 and 6.61, respectively, and rose rapidly and consistently during the storage period to reach 8.12, 7.44, 7.37, 7.28 and 7.14 in control, 0.5% CO, 1% CO, 1% LF and 2% LF groups on 6th day of storage period, respectively

Table 7. Changes of pH values in shrimp groups during the storage time at 4º C

Groups	Zero day	2nd day	4th day	6th day
CTR	6.64±0.006^{a}	7.08±0.006^{a}	7.78±0.006^{a}	8.12±0.006^{a}
0.5% CO	6.63±0.006^{ab}	6.89±0.006^{b}	7.12±0.006^{b}	7.44±0.006^{b}
1% CO	6.62±0.007^{ab}	6.86±0.006^{c}	7.10±0.006^{b}	7.37±0.006^{c}
1% LF	6.62±0.006^{ab}	6.82±0.006^{d}	7.04±0.006^{c}	7.28±0.006^{d}
2% LF	6.61±0.006^{b}	6.79±0.006^{e}	6.98±0.006^{d}	7.14±0.006^{e}

Values are means ± SE. Different letters in the same column are significantly different at $P< 0.05$

The initial TBARS values for the control, 0.5% CO, 1% CO, 1% LF, and 2% LF groups, were 0.91, 0.85, 0.78, 0.81, and 0.72 mg MDA/kg, respectively, as recorded in table (8). There was a notable decline ($p < 0.05$) in TBARS values across all groups that were treated as opposed to the control group at all the storage period. However, TBARS values increased in all groups over storage time

Table 8. Changes in TBARS (mg/kg) values in shrimp groups during the storage time at 4º C

Groups	Zero day	2nd day	4th day	6th day	MPL
CTR	0.91±0.006[a]	1.88±0.006[a]	3.22±0.005[a]	4.76±0.006[a]	
0.5% CO	0.85±0.005[b]	1.66±0.005[b]	2.77±0.004[b]	3.98±0.006[b]	4.5 mg/kg
1% CO	0.78±0.006[d]	1.48±0.005[c]	2.38±0.006[c]	3.71±0.004[c]	
1% LF	0.81±0.006[c]	1.33±0.004[d]	2.14±0.005[d]	3.51±0.003[d]	
2% LF	0.72±0.005[e]	1.24±0.006[e]	2.03±0.006[e]	3.18±0.002[e]	

Values are means ± SE. Different letters in the same column are significantly different at $P< 0.05$.
MPL: Maximum permissible limits as stated by **Egyptian standards (5021/2005).**

Table (9) revealed the changes in sensory parameters (color, odor, and texture) in all the experimental groups, depending on 5 points as the baseline score for the overall assessment. On zero day, all samples were considered to be of very high quality by the panelists. Sensory scores significantly decreased in both treated and untreated shrimp groups, correlating with storage time, where at 6th day of storage, shrimp treated group with clove oil and lactoferrin received higher ratings for texture, color, and odor as opposed to the control group, which had already become unacceptable by the 4th day. Therefore, it can be observed that the addition of CO and LF may provide a protective effect against chemical and microbial changes because of their antioxidant and antimicrobial effects, thereby preventing negative impacts on sensory properties.

Table 9. Changes in sensory parameters of control and treated shrimp groups during the storage time at 4º C

Groups	CTR	0.5% CO	1% CO	1% LF	2% LF
Zero day	4.99±0.007	4.98±0.006	4.98±0.009	4.99±0.003	5.00±0.003
1st day	3.65±0.009[e]	4.50±0.006[d]	4.55±0.009[c]	4.63±0.006[b]	4.71±0.012[a]
2nd day	3.18±0.006[e]	4.02±0.006[d]	4.10±0.006[c]	4.16±0.006[b]	4.24±0.012[a]
3rd day	2.85±0.015[e]	3.88±0.006[d]	3.97±0.006[c]	4.05±0.006[b]	4.14±0.006[a]
4th day	2.09±0.009[e]	3.18±0.006[d]	3.32±0.006[c]	3.87±0.006[b]	3.98±0.006[a]
5th day	1.87±0.012[e]	2.77±0.005[d]	2.97±0.004[c]	3.34±0.005[b]	3.62±0.003[a]
6th day	1.31±0.009[e]	2.28±0.006[d]	2.56±0.004[c]	3.02±0.003[b]	3.22±0.006[a]

Values are means ± SE. Different letters in the same raw are significantly different at P< 0.05. 5 = very acceptable, 4= acceptable, 3= middle, 2 = unacceptable, 1= rejected

DISCUSSION:

Vibrio species is a harmful bacteria which frequently impacts a variety of marine species. Fish should be analyzed bacteriologically for the presence of *Vibrio* species, since these organisms can cause foodborne illness and are important indicators of fish quality **(Abdel-Latif et al. 2022).** It is necessary to identify *Vibrio* species precisely as a result of increasing Vibrio infections resulting from the consumption and handling of fish that are contaminated with the microbe. Accurate surveillance of *Vibrio* species in fish and seafood products may provide recently intriguing insights for epidemiological research on the primary risk elements connected to this microbe **(Raissy et al. 2015).**

The outcoms demonstrated that 19% (19/100) of samples were infected with *Vibrio* species. These outcomes were almost similar to those acquired by **Asgarpoor et al. (2018),** who revealed incidence of *Vibrio* spp. in shrimp was 17.1%. Additionally, the findings obtained were less than that mentioned by **Ola et al. (2023),** who demonstrated that the percentage of *Vibrio* in shrimp was 40%, and **Amin et al. (2011),** who found out that 57.3% of shrimp contained *Vibrio* species, and **Bakr et al. (2011)** who detected *Vibrio* species in approximately 32 % of all tested shrimp. Our findings exceeded that cited by **Raissy et al. (2015),** who found that 7(12.72%) out of 55 shrimp samples were contaminated with Vibrio species. Moreover**, Fadel and El-Lamie (2019)** revealed that 7/170 (4.12%) of tested shrimp samples infected with *Vibrio.* Additionally, the most frequent species were *Vibrio parahaemolyticus*. This agreed with **Kriem et al. (2015); Xu et al. (2016)** and **Xie et al. (2017)**, who stated that the primary bacteria linked to the shrimp was *V. parahaemolyticus*. And disagreed with **Guardiola et al. (2020),** who found an increased prevalence of *V. cholera* (17.8%) and *V. mimicus* (6.7%) were recovered from shrimp.

The safety of seafood is significantly threatened by *V. parahaemolyticus*, creating a significant obstacle for the fisheries sector **(Aagesen et al .2013).** *V. parahaemolyticus* creates a protective biofilm covering made of self-produced proteins, polysaccharides, and lipids which covers the host's surface **(Berlanga and Guerrero, 2016).** This biofilm plays a key part in its pathogenesis, potentially enhancing its resistance to adverse conditions and treatments **(Roy et al. 2022).** *V. parahaemolyticus*'s capacity for formation of biofilm increases the capability of cells for adhering to floating particles and to shellfish **(Elexson et al. 2014).** Food safety is constanty at risk due to *V. parahaemolyticus's* biofilm, which makes it easier for the bacteria to colonize and persist throughout the food manufacturing chain **(Zhu et al. 2023)**. The obtained outcomes revealed that *V. parahaemolyticus* isolates were biofilm producers and also some isolates of *V. mimicus* and *V. furnissii* can form biofilm. This agreed with **Ashrafudoulla et al. (2019)** who showed that *V. parahaemolyticus* recovered from shrimp and mussels has both biofilm-forming abilities and pathogenic properties. **Han et al. (2016)** cited that 100% of approximately *V. parahaemolyticus* strains isolated from seafoods has the ability to produce biofilms.

PCR-based molecular assays are helpful in identifying the toxR gene in *Vibrio parahaemolyticus* **(Fabbro et al. 2010).** PCR assays targeting toxR gene, along with genes related to biofilm creation and pathogenicity, were performed to molecularly characterize the *Vibrio parahaemolyticus* isolates.

The findings of this investigation revealed that 10% of randomly selected *Vibrio* isolates were positive PCR, whereas **Rojas et al. (2011)** cited that when they targeted the 16S rRNA gene, 82.6% of their isolates of *Vibrio* species were PCR-positive. 6% of the isolates successfully amplified the *Vibrio parahaemolyticus*-specific toxR gene fragment (368 bp); comparable outcomes were mentioned in another investigation concluded by **Mizan et al. (2016).**

Additionally, the findings revealed that the tdh gene was found in 2% of *V. parahaemolyticus* isolates. On the other hand, **Raghunath (2015)** identified the tdh gene in 20.7% of seafood samples from India by using PCR. The

same study used colony hybridization after ST broth enrichment to find tdh-carrying *Vibrio parahaemolyticus* isolates in 19% of samples of seafood. In the meantime, **(Ashrafudoulla et al. 2019)** found that 100% of mussel samples tested positive for tdh using PCR.

It is widely recognized that some strains of *V. parahaemolyticus* induce illnesses in humans and marine fish. However, strains carrying the tdh and trh genes are thought to be virulent markers of pathogenic strains because they produce hemolysin components that cause inflammatory gastroenteritis **(Letchumanan et al. 2015).** Thus, it is essential to identify these genes in isolates in order to reduce any possible hazards to humans. In this study, we discovered that in *V. parahaemolyticus* isolates, the trh gene was significantly more prevalent (4%) than the tdh gene (2%). This trend is in line with **(Di et al. 2017)** found that tdh and trh genes were 6.1% and 15.9% in isolates of Korean seawater, respectively, and in 9.9% and 19.8% of seafood isolates from China **(Li et al. 2020),** respectively. Conversely, in an estuarine system in South Carolina, USA, the tdh gene was found in an elevated rate (48%) than trh gene (8.3%) **(Gutierrez West et al. 2013).**

Likewise, *V. parahaemolyticus* positive for tdh gene was more common than that positive for trh gene in oyster habitats in Taiwan **(Nguyen et al. 2024)** and in coastal waters of Saudi Arabia **(Almejhim et al. 2021).**

Biofilm formation is a major pathogenicity element in pathogenic microbes. Various species of *Vibrio* are capable of producing biofilms which help bacteria to cause illness and strengthen their resilience against challenging conditions, like antibiotics and the host immune system **(De Silva & Heo, 2023).** The outcomes of this study found that all *V. parahaemolyticus* isolates tested positive for the biofilm-associated gene VP950 Figure (4). Similar outcomes were noted by **(Ashrafudoulla et al. 2019),** who stated that 3 biofilm-associated genes were successfully present in each of *V. parahaemolyticus* isolates and **Mizan et al. (2017)** found that all investigated *Vibrio parahaemolyticus* isolates, either pathogenic or non-pathogenic demonstrated abilities of biofilm formation, as verified through detection of biofilm gene. Variations in ability for forming biofilm perhaps attributed to physical conditions such as pH and temperature as well as surfaces to which the cells adhere **(Han et al. 2016).**

Microorganisms related to Foodborne illnesses have turned into a significant global public health issue in all countries because of their elevated rates of illness and death **(WHO, 2015)**. Additionally, over 80% of acute and recurrent bacterial infections in humans and more than 60% of foodborne illness cases are associated with biofilms formation **(Yan et al. 2022).** Natural antimicrobials have been utilized for ensure food safety and preservation. Many studies have demonstrated that lactoferrin and clove oil were considered to be friendly candidates with noteworthy antimicrobial characteristics.

In the food sector, using essential oils as antimicrobial agents is regarded as extra fundamental factor to enhance food's shelf life and safety **(Aminzare et al. 2016).** Several investigations revealed that eugenol, which is the main ingredient of cloves, had antibacterial properties against several pathogens **(Miladi et al. 2017** and **Yadav et al. 2015).** The strong affinity of lactoferrin for iron and the iron-free state in which it exists in body secretions causing lactoferrin to create an environment that is deficient in iron, which prevents bacteria from growing **(Yamauchi et al. 1993).** This study's results demonstrated that clove oil and lactoferrin displayed potent antibacterial effects against Vibrio species, based on the inhibition zones observed around the bacteria. This is parallel to **Elexson et al. (2013),** which stated that cloves significantly reduce growth of *V. parahaemolyticus* and **Ashrafudoulla, et al. (2020)** who indicated that eugenol exhibits a strong antibacterial activity against isolates of *V. parahaemolyticus* that are resistant to antibiotics. In addition to **Acosta-Smith et al. (2018)** who demonstrated that lactoferrin exhibits bactericidal action against *V. cholera, V. alginolyticus, V. vulnificus, V. furnissii,* and *V. fluvialis.* **Mimmi et al. (2022)** found that lactoferrin from milk exhibits antibacterial effects contrary to seven different bacterial strains and

reported that lactoferrin can be applied to decrease microbiological degradation in the food sector. Furthermore, **Jahani et al. (2015)** added that lactoferrin can decrease growth of bacteria and biofilms development, so it ought to be considered as a beneficial antibacterial therapeutic agent.

Regarding the outcomes recorded in table (6), there was a noticeable difference in the initial *V. parahaemolyticus* count (Zero day) between the control and treated groups ($p<0.05$). Throughout the experiment, *V. parahaemolyticus* count in the control group significantly increased gradually ($p<0.05$). In contrast, treated groups on days 2, 4, and 6 of the storage period consistently showed a significantly lower *V. parahaemolyticus* count ($p<0.05$) compared to control group, which had an increased count. The findings indicated that higher concentrations of both clove oil and lactoferrin treatments led to a greater reduction in *V. parahaemolyticus* count. The results also prove that lactoferrin treatment was more effective in suppressing the *V. parahaemolyticus* count in the samples of shrimp as opposed to clove oil treatment, although, both treatments (clove oil and lactoferrin) could not eliminate *V. parahaemolyticus* completely, which considered one of the main requirements of **ES (5021/2005)** as the product should be free from *V. parahaemolyticus* in order for the product to be fit for human consumption. This might be explained by the smaller doses of clove oil used compared to the lactoferrin dose, in order to avoid altering the product's sensory attributes and this is in accordance with **Soyer et al. (2020)** who documented that the incorporation of large amounts of plant extracts into foods may be considered.; nevertheless, that could lead to development of undesirable organoleptic attributes. Unlike lactoferrin, it's odourless **(Gruden and Poklar Ulrih, 2021)** and does not negatively affect odour and taste **(Zakaria et al. 2020).** Our findings were on line with **Kerekes et al. (2019),** who found that most essential oils eradicate bacterial cells from biofilms, and **Mizan et al. (2020)** concluded that the virulence of *V. parahaemolyticus* was diminished by preventing biofilm formation through being exposed to clove oil (CO), thyme oil (TO), and garlic oil treatment. **Acosta-Smith et al. (2018)** reported that bovine LF is peptides of bactericides which eliminate *Vibrio spp.* following direct contact with the organism, and **Leon-Sicairos et al. (2009)** proposed that lactoferrin could be a promising agent in the fight against multidrug-resistant pathogenic *Vibrio* species.

The antimicrobial action of clove oil is primarily because of its content of eugenol, oleic acid, and lipids **(Nzeako et al. 2006).** The noteworthy concentration of eugenol in clove essential oil has accountability for its potent antimicrobial and biological effects. It is well established that eugenol and the phenolic substances in clove essential oil can cause protein to denature and interact with cell membrane phospholipids, altering their permeability and hindering a wide range of pathogens, in addition to various species of yeast **(Gupta and Prakash, 2021).** Regarding lactoferrin, there are two main processes that are responsible for its antimicrobial activity. The first involves iron uptake from infection locations, depriving microbes of this essential component, leading to a bacteriostatic effect, and the second mechanism involves lactoferrin directly interacting with the cytoplasmic membrane, leading to the selective permeability of potassium ions without initially disturbing the cellular pH gradient **(Aguilera et al. 2003).**

The shift in pH levels can indirectly indicate muscle damage and protein alterations. The pH is believed to be directly associated with shrimp degredation and may be influenced by microorganisms and endogenous enzymes within shrimp muscle **(Cen et al. 2021).** This rise in pH value during refrigeration storage is linked to the accumulation of fundamental substances resulting from enzymatic activity **(Lopez-Caballero et al. 2007).** It was noticed that the treated groups had a noticeable reduction ($P< 0.05$) in values of pH than control group, which related to the reduced microbial abundance in the treated samples and the breakdown of proteins, amino acids, and other nitrogenous substances into ammonia, trimethylamine, and other alkaline compounds slowed down. Additionally, the pH value of

samples treated with lactoferrin was relatively less than that of samples treated with clove oil and 2% LF group was more efficient in maintaining the value of pH in the samples than 1% LF group. The pH value of shrimp is regarded as a reliable sign of its quality. Generally, shrimp with a pH of 7.7 or below are considered of the best quality, and pH between 7.7 and less than 7.95. Shrimp are still acceptable but of lower quality, while those with a pH of 7.95 or higher are known as deteriorated products **(Gökoğlu, 2004).** Consequently, samples treated with clove oil and lactoferrin maintained good quality till the 6th day of storage, whereas the quality of control samples was only accepted till 4th day of storage (Table 7). This is if the presence of *V. parahaemolyticus* was not taken into account, but the presence of such a pathogen would make the product unsafe and therefore of poor quality.

The thiobarbituric acid test is frequently employed to measure MDA levels. The decomposition rate of lipid hydroperoxides is indicated by the concentration of malondialdehyde (MDA) (a secondary oxidation compound). The rise in TBARS may be due to partial dehydration of shrimp tissue, which is rich in PUFA, as well as exposure to oxygen **(Bensid et al. 2014).** These also promote lipid oxidation, which leads to damage to the tissues and development of off-flavors and off-odors that shorten the shelf life **(Noordin et al. 2020).**

The analysis showed that clove essential oil and lactoferrin improved the reduction of oxidation in shrimp samples. Clove oil has demonstrated strong antimicrobial and antioxidant properties **(Chaieb et al. 2007),** with its potent antioxidant value attributed to its phenolic compounds **(Das et al. 2021)**. Additionally, LF possesses metal ion-binding abilities that may hinder iron-catalyzed hydroxyl radicals through Fenton reaction, a significant source of reactive oxygen species (ROS). Thus, LF's antioxidant function is probably related to its ability to scavenge iron and decrease the production of ROS **(Esmaeili et al. 2019)**. **Chiu and Kuo (2007)** suggested that LF antioxidant action is most often caused by its capacity to store free iron, a catalyst for lipid oxidation reactions. It is worth noting that the LF-supplemented groups were better than CO groups in delaying lipid oxidative rancidity during preservation of the shrimp. Besides, raising the lactoferrin concentration promoted the decrease of lipid oxidation levels, so that the LF 2% group was the most effective at keeping lower TBARS values in the samples (Table 8). When comparing results to maximum allowable limits per Egyptian standard **(ES: 5021/2005),** which states that TBARS levels in chilled shrimp should not exceed 4.5 mg/kg, the control remained within limits up to the 4th day of storage.

Sensory properties are crucial characteristics of food products that offer an understanding of their acceptance by the public **(Amagwula et al. 2022).** The decline in sensory scores for shrimp treated with clove oil and lactoferrin was significantly less than the control shrimp group ($P < 0.05$). Also, the decline in sensory scores for shrimp dipped in 1% LF and 2% LF was significantly less than for those treated with 0.5% CO and 1% CO over the course of the storage period. Moreover, the 2% LF treatment was the most effective in preserving the color, odor, and texture of the stored shrimp (Table 9). Therefore, it is proposed that lactoferrin might have a greater efficiency than clove oil in maintaining the sensory quality of shrimp. Our results align with those of **Niaz et al. (2019),** who concluded that LF enhances the sensory characteristics of various products. Similarly, **Zakaria et al. (2020)** found that the sensory properties remained acceptable with the addition of LF and noted that LF did not negatively affect odor or taste. Clove oil, as noted by **Hosseini et al. (2019),** contains phytoconstituents with significant antimicrobial and antioxidant potential. These bioactivities are demonstrated here to preserve stored shrimp from spoilage. According to **Chaichi et al. (2021),** applying clove oil to animal food products can reduce unfavorable changes in sensory qualities such as flavor, aroma, color, and texture thanks to its antioxidant property. Moreover, its antimicrobial effects help to lower bacterial counts, inhibit the degradation of non-protein nitrogenous sub-

stances and reduce hydroperoxide formation. **Abdel-Wahab et al. (2020)** demonstrated that clove oil is a potent antimicrobial agent capable of preventing growth of numerous microbial pathogens. Similarly, **Niaz et al. (2019)** reported that lactoferrin exhibits antibacterial activities against a variety of microbes.

CONCLUSION

The findings of this investigation proved that using of either clove oil (0.5% and 1%) or lactoferrin (1% and 2%) could not successfully eliminate *V. parahaemolyticus* properly from contaminated shrimps, as it is considered one of the main requirements of **ES (5021/2005)** which stipulated that chilled shrimps should be free from *V. parahaemolyticus* and available for human consumption. Thus, it could be concluded that treatments used in this experiments did not fulfill the ES requirements. In the event of trying such work again by other researchers, we advised to use other concentrations or use other more effective substances, but at the same time, the concentrations used should not alter the sensory properties of the product. The identification of *Vibrio* spp. in shrimp sold at retail outlets should prompt food safety authorities to implement monitoring programs, conduct food inspections, and develop strategies to reduce foodborne diseases linked to shrimp consumption. To prevent cross-contamination in retail settings, different types of seafood should be stored separately without contact. Although proper cooking can reduce the risk of foodborne illness, there is still a potential danger of *Vibrio* infection from cross-contamination during preparation or from consuming improperly cooked seafood. Thus, both consumers and catering professionals need adequate training to raise awareness and improve food handling and preparation practices.

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