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Major Article

Occurrence of potentially pathogenic *Vibrio* in oysters (*Crassostrea gigas*) and waters from bivalve mollusk cultivations in the South Bay of Santa Catarina

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ABSTRACT

Introduction: This research aimed to identify and quantify potentially pathogenic *Vibrio* from different cultivations of bivalve shellfish in the State of Santa Catarina, Brazil, and water regions in the South Bay, as well as correlate the incidence of these microorganisms with the physicochemical parameters of marine waters. **Methods:** Between October 2008 and March 2009, 60 oyster and seawater samples were collected from six regions of bivalve mollusk cultivation, and these samples were submitted for *Vibrio* counts. **Results:** Twenty-nine (48.3%) oyster samples were revealed to be contaminated with one or more *Vibrio* species. The *Vibrio parahaemolyticus* and *Vibrio vulnificus* counts in the samples ranged from < 0.5 $\log_{10}$ Most Probable Number (MPN) g^{-1} to 2.3 $\log_{10}$ MPN g^{-1} oyster and from < 0.5 $\log_{10}$ MPN g^{-1} to 2.1 $\log_{10}$ MPN g^{-1} oyster, respectively. Of the 60 seawater samples analyzed, 44 (73.3%) showed signs of contamination with one or more *vibrio* species. The counts of *V. parahaemolyticus* and *V. vulnificus* in the samples ranged from < 0.3 $\log_{10}$ MPN·100mL^{-1} to 1.7 $\log_{10}$ MPN·100mL^{-1} seawater and from < 0.3 $\log_{10}$ MPN·100mL^{-1} to 2.0 $\log_{10}$ MPN·100mL^{-1} seawater, respectively. A positive correlation between *V. vulnificus* counts and the seawater temperature as well as a negative correlation between the *V. parahaemolyticus* counts and salinity were observed. **Conclusions:** The results suggest the need to implement strategies to prevent *vibrio* diseases from being transmitted by the consumption of contaminated bivalve shellfish.

Keywords: Oyster. *Crassostrea gigas*. *Vibrio parahaemolyticus*. *Vibrio vulnificus*. Shellfish. Polymerase chain reaction.

INTRODUCTION

In Brazil, the production of bivalve shellfish occurs primarily in the Southern region of the State of Santa Catarina because of the excellent geographical conditions of this area for the cultivation of marine organisms, such as the presence of a large number of bays, which facilitates the establishment of marine farms[1,2]. In 2011, approximately 18.3 tons of shellfish were sold in the State of Santa Catarina, and the largest production of oysters (*Crassostrea gigas*) occurred at marine farms located in the South Bay near the Island of Santa Catarina.

In addition to indicators of fecal contamination, which are widely used to assess the microbiological quality of bivalve mollusks, different species of the *Vibrio* genus occur naturally in marine, coastal and estuary environments. However, some species, such as *Vibrio parahaemolyticus, Vibrio vulnificus* and *Vibrio cholerae*, are potentially pathogenic to humans and may be present in fish and raw or partially cooked shellfish[3]. The possibility of seafood consumers becoming infected with pathogenic *Vibrio* from oyster consumption depends on the microbiological quality of the marine habitat as well as the handling and processing practices of these shellfish[4]. The occurrence of these bacteria is not related to the counts of either *Escherichia coli* or thermotolerant coliforms, which are primarily responsible for gastroenteritis related to seafood consumption[5].

Infections caused by *Vibrio parahaemolyticus* have been reported in Asia[6-9], Europe (Spain[10] and Italy[11]) and American countries (United States[12-16], Chile[17,18], Peru[19] and Brazil[20]).

Pathogenic *V. parahaemolyticus* strains can be differentiated from non-pathogenic strains by their ability to produce thermostable hemolysin (TDH), which is known as the Kanagawa phenomenon. The pathogenicity of *V. parahaemolyticus* is associated with the presence of the *tdh* and *trh* genes[21].

Infections caused by *V. vulnificus* show different clinical presentations, of which the primary septicemia, wound infections, and gastroenteritis are the most prevalent[16]. *Vibrio vulnificus* infections are serious and appear to be rare in Brazil, although there is little information concerning the actual incidence of these infections.

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The concentration of both *V. parahaemolyticus* and *V. vulnificus* in oysters is directly related to water temperature, with a higher concentration of this *Vibrio* present when the oysters are in warm water. Because of this, these microorganisms are rarely isolated when the water temperature is below 15°C[22]. In Brazil, the temperature of the seawater is greater than 20°C for the majority of the year, favoring the occurrence of these microorganisms in different stations.

This study aimed to identify and quantify the potentially pathogenic marine *Vibrio* in fresh oysters (*Crassostrea gigas*) and seawaters from different regions of bivalve shellfish cultivations in the South Bay of the Island of Santa Catarina, Brazil, as well as correlate the incidence of these microorganisms with the physicochemical parameters of the marine waters in these regions.

METHODS

Collection and preparation of the samples

Between October 2008 and March 2009, 60 oyster samples (*Crassostrea gigas*) and 60 seawater samples were collected from six regions of bivalve mollusk farms in the South Bay near the Island Santa Catarina (27° S, 48° W), Brazil. These regions are identified as A, B, C, D, E and F in **Figure 1**. A total of 10 samplings were collected from each region.

Each oyster sample comprised 12 oysters, totaling 720 specimens. Water samples were collected following the methods published by the American Public Health Association[23], which consisted of using sterile 1-liter polypropylene screw-cap containers 50cm below the surface. The seawater temperature and dissolved oxygen levels were measured *in situ* using a YSI-550A dissolved oxygen meter (YSI Incorporated, Ohio, USA), salinity was measured using a refractometer (Alfakit, 211, Florianópolis, Brazil), and the Secchi depth was measured using a Secchi disc; all of these measurements were obtained in the field. However, the water pH was measured in a laboratory with a digital pH meter (Quimis® Q-400, São Paulo, Brazil). All measurements were obtained in triplicate. The oysters and water samples were transported to the laboratory in an isothermal box with packaged potable ice and analyzed within 3h of sampling.

The oysters were scrubbed under tap water to remove debris, allowed to dry, disinfected with 70% ethanol, and opened aseptically using a sterilized knife. The flesh and intervalve liquid were aseptically transferred to sterile bags and homogenized for 1 min, forming a pool of 12 oysters. The oysters' pH was evaluated from the pool of 12 oysters from each sample with a Quimis ® Q-400 digital pH meter. This process was conducted in triplicate.

Isolation and enumeration of *Vibrio* spp

To detect the *Vibrio* spp. in oysters, 50g of each sample was weighed, 450mL of phosphate buffered saline (PBS, Oxoid, Ltd, England) was added to each sample and the mixtures were liquefied in a Bagmixer blender. Serial dilutions up to 10^{-4} were prepared from this homogenate, and 1mL of each dilution was

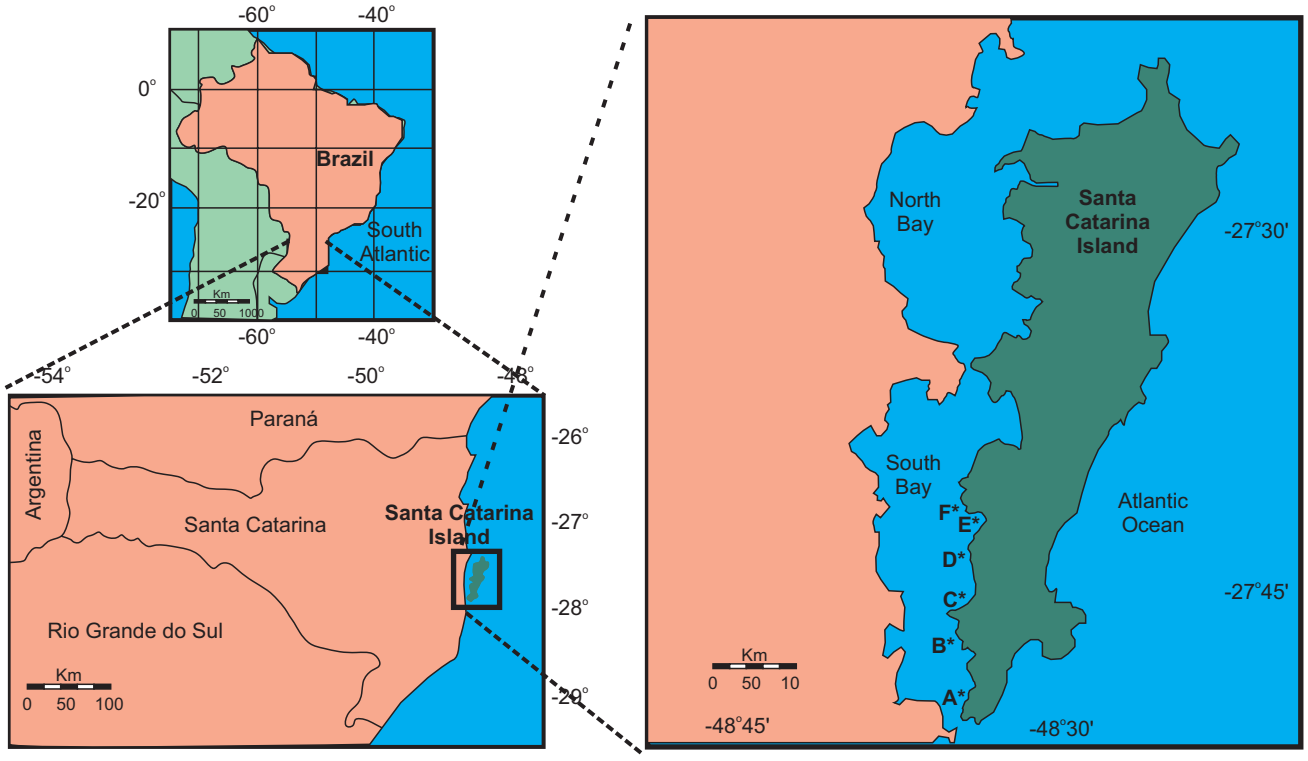

FIGURE 1 - Detailed map of the South Bay, with letters A through F indicating the sampling sites.

inoculated into a tube containing alkaline peptone water (APW) and 3% NaCl to enumerate the most probable number (MPN) of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the sample using a method described in the US Food and Drug Administration Bacterial Analytical Manual[24]. Each dilution was measured in triplicate. Further biochemical differentiation for the identification and confirmation of isolated particles were performed using the Analytical Profile Index (API) 20E system (bioMérieux, France).

For the water analysis, 5 tubes were prepared with alkaline peptone water (APW, Oxoid), concentrated twice with 3% of NaCl, and two sets of 5 tubes were prepared with APW in normal concentration with 3% NaCl. Approximately 10mL of collected water was added to the first set of 5 tubes, whereas 1 and 0.1mL of cultivated water was added to the other two sets. The tubes were incubated at 35°C overnight, and those showing turbidity were sowed onto plates of Thiosulfate Citrate bile Sucrose agar (TCBS, Oxoid). These plates were incubated at 35°C (± 1°C) for 24 hours. The colonies that were suspected to be *Vibrio* spp. were inoculated into triple sugar iron agar (TSI, Oxoid) containing 3% NaCl and subjected to indole motility sulfide agar (SIM, Oxoid) to determine motility, as well as an oxidase test (Newprov oxidase strips), gram coloration and inspection of bacterial morphology. Strains suspected in these tests were further subjected to taxonomic identification of the species using the API 20E kit from bioMérieux with a bacterial suspension containing 0.85% NaCl. The count result was obtained using the MPN table for the series of five tubes for each dilution (10mL, 1mL and 0.1mL) according to section 9221C (APHA, 2005). All of the strains of *V. parahaemolyticus* were genotypically confirmed by detecting the *tlh* gene using multiplex polymerase chain reaction (multiplex-PCR).

Determining pathogenicity

Strains identified as *V. parahaemolyticus* were shipped in Luria Bertani agar containing 3% NaCl to the Microbiology laboratory at the Institute Oswaldo Cruz (IOC) to determine pathogenicity. These strains were also subjected to the following phenotypical virulence tests: urease detection using Urea Agar Base (UAB, Oxoid) and hemolysis of human erythrocytes in Wagatsuma agar to detect the Kanagawa phenomenon[24].

Genotypic confirmation of pathogenicity was performed using multiplex PCR detection of the *tdh* (thermostable direct hemolysin) and *trh* (thermostable direct hemolysin-related hemolysin) genes using the following primers: VPTDH-L: 5'-gta aag gtc tct gac ttt tgg ac-3' and VPTDH-R: 5'-tgg aat aga acc ttc atc ttc acc-3'; VPTRH-L: 5'-ttg gct tcg ata ttt tca gta tct-3' and VPTRH-R: 5'-cat aac aaa cat atg ccc att tcc g-3', respectively. The *tlh* (thermolabile hemolysin) gene, which is a species-specific marker for *V. parahaemolyticus,* was amplified using the primers L-TL: 5'-aaa gcg gat tat gca gaa gca ctg-3' and R-TL: 5'-gct act ttc tag cat cat ttt ctc tgc-3', as described by Bacteriological Analytical Manual/Food and Drug Administration (BAM/FDA)[24].

Statistical analysis

Results of the microbiological tests were transformed into log values and assumed to be normally distributed; statistical analyses were performed using Statistica 7.0® software (Stat-Soft, Inc., USA). To facilitate the statistical analyses of quantitative data obtained by the most probable number, counts for *V. parahaemolyticus* and *V. vulnificus* when the levels were below the limit of detection were substituted for 2 MPN g^{-1} oyster and 1.7 MPN·100mL^{-1} seawater. A test of significance of the observed differences in *V. parahaemolyticus* and *V. vulnificus* levels regarding the environmental parameters in oysters and seawater across the six sites sampled was conducted using one-way analysis of variance (ANOVA). An alpha level of 0.05 was considered to be the minimum level for statistical significance.

The influence of the physicochemical microbiological counts in seawater was evaluated by analyzing the nonparametric Spearman rank correlation, whereas the correlation between the physical and chemical parameters and incidence of *Vibrio* spp. was assessed by the Pearson correlation.

RESULTS

Collections were performed from October 2008 to March 2009, which included the spring and summer seasons, and the temperature of the seawater ranged from 20ºC and 29°C. The average temperature of the six geographically studied regions was 24.3°C ± 2.2.

Of the 60 examined oyster samples, 29 (48.3%) were found to contain potentially pathogenic *Vibrio* spp. (**Table 1**). The most frequently isolated species from the oysters were *V. parahaemolyticus* (21 isolates, 35%), *V. vulnificus* (6 isolates, 10%) and *V. alginolyticus* (4 isolates, 6.7%). *V. cholerae* was not isolated from any of the 60 analyzed samples, and *V. fluvialis* was isolated from only one sample. The *V. parahaemolyticus* counts ranged from < 0.5 $\log_{10}$ MPN g^{-1} oyster (non-detectable) to 2.3 $\log_{10}$ MPN g^{-1} oyster, with the mean level of *V. parahaemolyticus* in the oyster samples at 1.2 $\log_{10}$ MPN·g^{-1} oyster. The *V. vulnificus* counts ranged from < 0.5 $\log_{10}$ MPN·g^{-1} oyster (non-detectable) to 2.1 $\log_{10}$ MPN·g^{-1} oyster, and the mean level of *V. vulnificus* was 0.8 $\log_{10}$ MPN g^{-1} oyster. In December and January, which is summertime in Brazil, the highest counts of *V. vulnificus* and *V. parahaemolyticus* were observed in the oyster samples. Interestingly, two *Vibrio* species coexisted in three oyster samples (**Table 1**).

During the entire monitoring period, 32 *Vibrio* strains were isolated from the oyster samples. In the C region, the incidence of *Vibrio* was higher compared to the other regions.

Based on ANOVA (**Table 2**), there were no significant differences in the mean $\log_{10}$ density of total counts of *Vibrio parahaemolyticus* and *V. vulnificus* in the oyster samples from the six sites tested ($p > 0.05$).

Of the 60 seawater samples collected, 44 (73.3%) had one or more *Vibrio* species. The most frequently isolated species from the seawater were *V. parahaemolyticus* (27 isolates, 45%), *V. alginolyticus* (17 isolates, 28.3%) and *V. vulnificus* (8 isolates, 13.3%). *V. cholerae*, the only *Vibrio* species surveyed that originates from waters contaminated by sewage, was not isolated in any of the 60 samples analyzed, and *V. fluvialis* was found in only one sample.

TABLE 1 - Prevalence of *Vibrio* in oysters and seawater.

		Positive samples	
Samples (n)	Microorganisms	n	%
Oysters (60)	*Vibrio* spp.	29	48.3
	Vibrio parahaemolyticus	18	30.0
	Vibrio vulnificus	6	10.0
	Vibrio parahaemolyticus and *Vibrio alginolyticus*	3	5.0
Seawaters (60)	*Vibrio alginolyticus*	1	1.7
	Vibrio fluvialis	1	1.7
	Vibrio spp.	44	73.3
	Vibrio parahaemolyticus	20	33.3
	Vibrio alginolyticus	10	16.7
	Vibrio parahaemolyticus and *Vibrio alginolyticus*	5	8.3
	Vibrio vulnificus	4	6.7
	Vibrio vulnificus and *Vibrio alginolyticus*	2	3.3
	Vibrio vulnificus and *Vibrio parahaemolyticus*	2	3.3
	Vibrio fluvialis	1	1.7

TABLE 2 - Results of microbiological analysis and seawater parameters of samples collected from the South Bay of Santa Catarina, Brazil.

	Seawater parameters		*VP* in oysters log MPN·g^{-1}	*VP* in seawater log MPN·100mL^{-1}	*VV* in oyster log MPN·g^{-1}	*VP* in seawater log MPN·100mL^{-1}
Site	T (°C)	S (ppm)	mean ± SD*	mean ± SD*	mean ± SD*	mean ± SD*
A	22.9 ± 2.0^{a}	33.9 ± 3.6^{a}	0.4 ± 0.2^{a}	0.5 ± 0.4^{a}	0.6 ± 0.6^{a}	0.3 ± 0.2^{a}
B	23.3 ± 1.9^{ab}	33.3 ± 3.3^{a}	0.8 ± 0.7^{a}	0.4 ± 0.3^{a}	0.4 ± 0.2^{a}	0.3 ± 0.2^{a}
C	24.6 ± 2.0^{ab}	32.8 ± 3.5^{a}	0.9 ± 0.6^{a}	0.6 ± 0.5^{a}	0.3 ± 0.0^{a}	0.3 ± 0.2^{a}
D	24.9 ± 1.9^{ab}	32.5 ± 3.5^{a}	0.4 ± 0.3^{a}	0.5 ± 0.4^{a}	0.4 ± 0.4^{a}	0.40 ± 0.6^{a}
E	24.9 ± 2.0^{ab}	30.7 ± 6.5^{a}	0.7 ± 0.7^{a}	0.8 ± 0.4^{a}	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}
F	25.2 ± 2.3^{b}	31.2 ± 4.0^{a}	0.8 ± 0.0^{a}	0.5 ± 0.5^{a}	0.7 ± 0.6^{a}	0.4 ± 0.4^{a}

T: temperature; **S (ppm):** salinity parts-per-million; ***VP*: *Vibrio parahaemolyticus***; ***VV*: *Vibrio vulnificus;*** **MPN**: most probable number; **SD:** standard deviation**.** * means (n = 3) in the column with different superscripts (a, b) are significantly different ($p < 0.05$).

The *Vibrio parahaemolyticus* counts ranged from < 0.3 $\log_{10}$ MPN·100mL^{-1} seawater (non-detectable) to 1.7 $\log_{10}$ MPN·100mL^{-1} seawater, and the mean level of *V. parahaemolyticus* in the seawater samples was 0.8 $\log_{10}$ MPN·100mL^{-1}, whereas the *V. vulnificus* counts ranged from < 0.3 $\log_{10}$ MPN 100mL^{-1} seawater (non-detectable) to 2.0 $\log_{10}$ MPN·100mL^{-1} seawater, and the mean level of *V. vulnificus* was 0.6 $\log_{10}$ MPN 100mL^{-1} seawater. Two *Vibrio* species coexisted in nine seawater samples (**Table 1**).

Spearman correlation analysis indicated a significant positive correlation between the $\log_{10}$ total of *V. vulnificus* counts in the seawater and oysters at the same site of the South Bay ($p < 0.05$); however, the same was not observed for *V. parahaemolyticus* ($p > 0.05$).

The influence of temperature on the concentration of *Vibrio* in the marine waters was only observed with *V. vulnificus* ($p < 0.05$). For *V. parahaemolyticus* ($p > 0.05$), no Spearman correlation was established, presumably due to samplings that were conducted during the spring and summer months when water temperatures remained at approximately 24°C on average, resulting in the continuous detection of different species of *Vibrio*.

A negative Spearman correlation was observed between the counts of *V. parahaemolyticus* and salinity ($p < 0.05$); however, this was not observed with *V. vulnificus* ($p > 0.05$). The seawater clarity, dissolved oxygen and pH measurements were not correlated with the level of contamination with either *V. parahaemolyticus* ($p > 0.05$) or *V. vulnificus* ($p > 0.05$). The incidence of different species of marine vibrio in the water did not correlate with any of the physicochemical parameters measured ($p > 0.05$). The pH values of the oysters from the different regions of the South Bay ranged between 5.8 and 6.8, and the average pH observed was 6.2 ± 0.2. Although Brazilian laws have set pH limits for fish, there are none for mollusks.

Only one of the 48 strains isolated from oyster and seawater samples (confirmed to be *V. parahaemolyticus* by *tlh* detection) was urease-positive, but the strain did not produce b-hemolysis halos in Wagatsuma agar. The *tdh* gene *was* detected in four strains, and *trh* was detected in 23 strains (**Figure 2**).

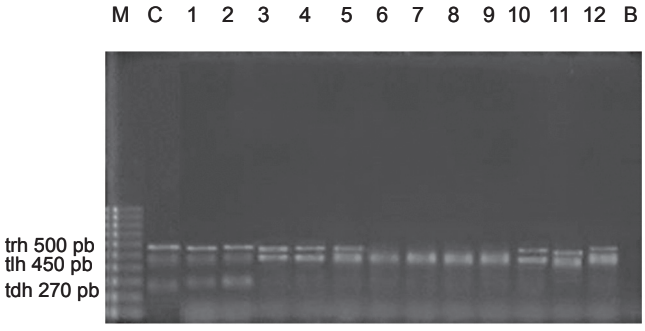

FIGURE 2 - Agarose gel electrophoresis of the multiplex PCR products obtained from the collected *Vibrio parahaemolyticus* strains. Lanes: M: 100bp molecular weight marker; C: positive control (*tlh*-, *trh*- and *tdh*-positive *V. parahaemolyticus*); B: negative control; 1 and 2, positive for *trh* and *tdh*; 3, 4, 5, 10, 11 and 12, positive for *trh*; 1 - 12, positive for *tlh*.

DISCUSSION

Similar mean water temperatures were observed at the six sites investigated except the temperatures of regions A and F, where a significant difference was observed ($p < 0.05$). This most likely occurred because the collections took place early in the morning in the A region and approximately 12h later in the F region, over which time the water temperature experiences a natural increase.

The average temperature of 24.3°C in the water of the six sampling stations in South Bay of Santa Catarina Island was favorable for the development of *Vibrio* because pathogenic *Vibrio* are more frequently isolated in an aquatic environment with temperatures varying between 10 and 30°C[25]. According to Strom and Paranjpye[26], *V. vulnificus* proliferates in areas when the water temperature exceeds 18°C. The lowest recorded temperature of all of the samples was 20°C.

Several studies have suggested a strong influence of temperature on the concentration of vibrio in marine waters[27-30]. However, this study only established a correlation with *V. vulnificus* ($p < 0.05$). For *V. parahaemolyticus* ($p > 0.05$), it was not possible to establish any correlation, presumably due to the average water temperature of 24°C, which enabled the continuous detection of different species of *Vibrio*.

In this study, the prevalence and concentration of *V. parahaemolyticus* were lower compared to studies performed in southeastern regions of Brazil[31-33]. Although no isolated Kanagawa-positive strains have been isolated, 6.7% of the strains isolated in this study contained *tdh* and 38.3% of the strains contained *trh*, both of which are related to the pathogenicity of *V. parahaemolyticus*.

Kanagawa-negative strains carrying the *tdh* gene produce TDH and are therefore potentially toxigenic. The only urease-positive strain, which also contained the *trh* gene, has been previously described by other authors[4,34]. According to these studies, urease positivity is an indication of virulence.

In this study, more than half of the isolates were *V. parahaemolyticus*; this percentage of isolates is different from that previously observed in another study conducted in the same region[35]. The variability in the incidence of different species of *Vibrio* may be related to the fact that this study was conducted in the warmer spring and summer seasons, whereas the other study covered all four seasons. Vieira et al.[4] also observed a higher prevalence of *V. parahaemolyticus* (30.3% of those isolated) in the oyster samples.

In Brazil, the bacteriological quality of the aquaculture water areas is evaluated by either fecal coliforms or the *Escherichia coli* test[36]. Through Resolution 12/2001, the Department of Sanitary Vigilance[37] determined the maximum acceptable level of *V. parahaemolyticus* in raw seafood-based dishes to be 10^3 per gram. Though oysters are not mentioned explicitly in this definition, they should be considered as raw seafood because they are traditionally consumed in nature. Considering this legislation as a parameter put the results observed in our study into context, the *V. parahaemolyticus* counts reported here vary below the maximum-allowed limit.

The United States, through the Guide for the Control of Molluscan Shellfish, which was published by the National Shellfish Sanitation Program[38], has established action levels and levels of concern for *V. parahaemolyticus* levels that are equal to or greater than an MPN count of 10,000 per gram, as well as Kanagawa positive or negative strains. However, the recommended attention to *V. vulnificus* does not establish limits for this microorganism, which must be analyzed on a case-by-case basis.

Many factors are involved in the distribution and survival of microorganisms in estuarine ecosystems, such as biotic and abiotic parameters of the surrounding seawater (i.e., temperature, salinity, pH and turbidity)[30,32,39]. The concentration of *V. parahaemolyticus* in seawater increases with rising water temperatures and corresponds to a seasonal increase, with sporadic cases reported in the warmer months[40]. According to the Centers for Disease Control (CDC)[13], outbreaks of

V. parahaemolyticus infections in the Pacific Northwest and Texas occur primarily during the summer months. However, this study showed no correlation between the *V. parahaemolyticus* counts and the water temperature, most likely due to the minimal temperature variation over the study period, whereas a significant correlation was observed between the *V. vulnificus* counts and water temperature, confirming the previously reported results[41,42].

Audemard et al.[43] suggested that unexplored postharvest processing (PHP) methods to eliminate *V. vulnificus* from oysters, which use relaying to high salinity waters, could be an alternative strategy, considering that high salinities appear to negatively affect the survival of *V. vulnificus*. However, this study observed a negative correlation between salinity and *V. parahaemolyticus*, whereas no correlation was observed between salinity and *V. vulnificus*; this result is consistent with results obtained in other studies conducted in shellfish cultivation areas off the Brazilian coast[32,35] and in other parts of the world[41,44].

Although the *V. parahaemolyticus* and *V. vulnificus* counts observed in the waters can be considered as *low*, it is important to note that in filtering shellfish such as oysters and mussels, these microorganisms are concentrated in their guts and in other tissues, with levels reaching up to 10^6 bacteria per gram of shellfish[30].

Although only eight (13.3%) samples in this study were positive for *V. vulnificus* (even at low concentrations), these data should serve as an indication for the need for constant monitoring of this species in areas of bivalve shellfish cultivation due to the ability of this organism to cause serious infections that are often fatal[30].

The results of this study suggest the need to improve strategies to prevent the occurrence of diseases transmitted by consumption of bivalve shellfish contaminated with pathogenic *vibrio*. This translates to monitoring not only indicators of fecal contamination, such as *Escherichia coli*, but also the potentially pathogenic *Vibrio*. Additionally, establishing time and temperature parameters for the handling, transporting, marketing, distribution and consumption of these shellfish is imperative to prevent illnesses due to these foodborne pathogens.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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