Original

Ocurrence of *Vibrio* spp., positive coagulase staphylococci and enteric bacteria in oysters (*Crassostrea gigas)* harvested in the south bay of Santa Catarina island, Brazil

Ocorrência de Vibrio spp., estafilococos coagulase positivo e bactérias entéricas em ostras (Crassostrea gigas) cultivadas na baía sul da ilha de Santa Catarina, Brasil

Roberta Juliano RAMOS[1]*, Murilo Anderson PEREIRA[1], Letícia Adélia MIOTTO[1], Renata D'Aquino FARIA[1], Nelson SILVEIRA JUNIOR[2], Cleide Rosana Werneck VIEIRA[1]

Abstract

The aim of this study was to assess the contamination of oysters (*Crassostrea gigas*), harvested in six different regions of the South Bay of Santa Catarina Island, with Coliforms at 45 °C, *Escherichia coli*, *Vibrio* spp., positive coagulase staphylococci, and *Salmonella* sp. over a period of one year. One hundred eighty oyster samples were collected directly from their culture sites and analyzed. Each sample consisted of a pool of 12 oysters. All of the samples analyzed showed absence of Salmonella, 18 (10%) samples showed presence of *Escherichia coli*, 15 (8.3%) samples were positive for *V. alginolyticus*, and *Vibrio cholerae* was detected in 4 samples (2.2%). The counts of positive-coagulase staphylococci varied from <10 to 1.9×10^2 CFU.g^{-1}, whereas the counts of Coliforms at 45 °C and *E. coli* ranged from <3 to 1.5×10^2 MPN.g^{-1} and <3 and 4.3×10 MPN.g^{-1}, respectively. Counts of *V. parahaemolyticus* and *V. vulnificus* ranged between <3 and 7 MPN.g^{-1}, for both microorganisms. This suggests the need for monitoring these vibrios contamination in oysters. Based on the results of the microbiological assays, the samples analyzed showed acceptable bacteriological quality, i.e., they were within the parameters established by Brazilian Legislation.
Keywords: *microbiological quality; bivalve mollusks; filter-feeders; Escherichia coli; Vibrio parahaemolyticus; Vibrio vulnificus.*

Resumo

O objetivo deste estudo foi avaliar a contaminação de ostras (*Crassostrea gigas*) cultivadas em diferentes regiões da Baía Sul da Ilha de Santa Catarina, por coliformes a 45 °C, *Escherichia coli*, *Vibrio* spp. Estafilococos coagulase positiva e *Salmonella* sp., durante o período de um ano. Foram analisadas 180 amostras, coletadas diretamente no local de cultivo. Todas as amostras analisadas apresentaram ausência de *Salmonella*, 18 (10%) amostras apresentaram presença de *Escherichia coli*, 15 (8,3%) amostras positivas para *Vibrio alginolyticus* e *V. cholerae* foi detectado em 4 amostras (2,2%). As contagens de Estafilococos coagulase positiva variaram de <10 a $1,9 \times 10^2$ UFC.g^{-1}, enquanto que as contagens de coliformes a 45 °C e *E. coli* variaram de <3 a $1,5 \times 10^2$ NMP.g^{-1} e <3 e $4,3 \times 10$ NMP.g^{-1}, respectivamente. As contagens de *V. parahaemolyticus* e *V. vulnificus* variaram de <3 a 7 NMP.g^{-1}, para ambos os microrganismos, sugerindo um monitoramento tanto destas espécies quanto da temperatura das águas marinhas nas regiões de cultivo. Com base nos resultados das análises microbiológicas, as amostras analisadas mostraram qualidade bacteriológica aceitável, ou seja, dentro dos parâmetros estabelecidos na legislação brasileira.
Palavras-chave: *qualidade microbiológica; moluscos bivalves; filtradores; Escherichia coli; Vibrio parahaemolyticus; Vibrio vulnificus.*

1 Introduction

In Brazil, bivalve mollusks production takes place mainly in Santa Catarina State, south Brazil, due to the excellent geographical conditions of this area for marine organism cultures, such as the presence of a large number of bays facilitating the establishment of mollusk farms (COELHO et al., 2003; CORRÊA et al., 2007; OLIVEIRA NETO, 2005). In 2007, about 11.000 t of mollusks were commercialized in Santa Catarina State, the largest producing region of oysters (*Crassostrea gigas*). The major production takes place in sea farms located in the South Bay of Santa Catarina Island (OLIVEIRA NETO, 2007).

Oysters are filter-feeders that efficiently concentrate microorganisms from polluted habitats, and because they are often consumed raw, they pose a health risk to consumers (CORRÊA et al., 2007; LALOO et al., 2000; PEREIRA et al., 2006; SILVA et al., 2004). The enteric bacteria, originating from the contamination of water with human residues, can readily contaminate the fauna in marine environments, especially molluscan shellfish (CORRÊA et al., 2007, PEREIRA et al., 2006). To guarantee sanitary quality, mollusk cultures should be monitored for contamination by pathogenic microorganisms (CORRÊA et al., 2007).

Vibrios are very common in marine and estuarine water environments and some may cause infections in humans that were exposed to seafood or sea water. Several Vibrio species are pathogenic to humans and may be present in raw or partially cooked shellfish (LHAFI; KÜHNE, 2007; PEREIRA et al., 2007a, b). The concentration of *Vibrio* spp. in oysters is directly

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[1] *Departamento de Ciência e Tecnologia de Alimentos – CAL, Centro de Ciências Agrárias – CCA,Universidade Federal de Santa Catarina – UFSC, Rod. Admar Gonzaga, 1346, CEP 88034-001, Itacorubi, Florianópolis, SC, Brasil, e-mail: robertajulianoramos@yahoo.com.br*
[2] *Associação de Maricultores do Sul da Ilha (AMASI), Florianópolis, SC/Brasil*
**Corresponding author*

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related to water temperature, with a higher concentration being present in oysters from warm water and outbreaks occurring usually during summer months (CABRERA-GARCIA; VASQUEZ-SALINAS; QUINONES-RAMIREZ, 2004; PEREIRA; VIANA; RODRIGUES, 2004; PEREIRA et al., 2007a; YOON et al., 2008). However, no close correlation has been noted between microorganisms of fecal origin and vibrios that are potentially pathogenic for humans (NORMANNO et al., 2006; OLIVER; WARNER; CLELAND, 1983).

In this context, the aim of this study was to assess the contamination of oysters (*Crassostrea gigas*), harvested in six different regions of the South Bay of Santa Catarina Island, with Coliforms at 45 °C, *Escherichia coli*, *Vibrio* spp., positive coagulase staphylococci, and *Salmonella* sp., over a period of one year.

2 Materials and methods

2.1 Sample collection and preparation

The collection sites studied are located in six different geographical regions in the district of Ribeirão da Ilha, in the South Bay of Santa Catarina Island: Caieira da Barra do Sul (27° 48' 849" S and 48° 33' 981" W); Tapera do Ribeirão (27° 46' 989" S and 48° 34 318" W); Costeira do Ribeirão (27° 44' 350" S and 48° 33' 890" W); Freguesia do Ribeirão (27° 43' 17.4" S and 48° 33' 57.8" W), Barro Vermelho (27° 42' 11.1" S and 48° 33' 33.7" W), and Tapera da Base Aérea (27° 41' 397" S and 48° 34' 230" W), which were identified, respectively, as regions: A, B, C, D, E, and F (Figure 1).

The collections were carried out from March 2006 to February 2007, totalizing 15 collections. At each sampling time, the water temperature was recorded. Each pool was composed of 12 oysters. Two samples were collected from each of the six culture areas, totalizing 180 samples over a twelve-month period. The oysters were transported to the Laboratory of Food Microbiology, Department of food Science and Technology at the Federal University of Santa Catarina, in isothermal boxes with packaged potable ice. All specimens were examined within 2 hours of collection.

The oysters were scrubbed under tap water to remove debris, allowed to dry, disinfected with 70% ethanol, and aseptically opened using a sterilized knife. The flesh and intervalve liquid were aseptically placed in sterile bags, making up the pool of

Figure 1. Collection sites in the six different regions at South Bay in Florianopolis, Santa Catarina, Brazil.

each sample, from which two samples of 25 g and one sample of 50 g each were later weighed.

2.2 Microbiological analysis

Coliforms at 45 °C and *Escherichia coli* were evaluated according to APHA (AMERICAN..., 2001). Twenty-five grams of each sample was weighed, added to 225 mL of peptone water 0.1%, and homogenized in a Bagmixer blender (Interscience, France). Further dilutions (10^{-2}, 10^{-3}) were then prepared from this dilution, and 1 mL of each dilution was inoculated into each tube of a series of three tubes containing Lauryl Sulphate Triptose broth (LST). They were incubated at 35 °C (±1 °C) for 48 hours. From each tube of LST with turbidity and production of gas, 100 µL from the positive tubes were transferred to tubes containing Escherichia coli broth (EC). The tubes of EC were incubated in water bath at 45.0 °C ± 0.5 °C for 48 hours. The EC tubes showing turbidity and gas production were streaked on Eosin-Methylene Blue Agar (EMB) and incubated at 35 °C (±1 °C) for 24 hours. The typical colonies of *Escherichia coli* were submitted to the following biochemical tests: Indole, Methyl Red, Voges Proskauer, and Citrate (IMViC). The final result of coliforms at 45 °C was obtained from the table of the Most Probable Number (MPN) by the combination of the number of positive tubes, i.e., those showing turbidity and gas production; while the counts of *E. coli* were performed from the biochemical confirmation of the typical colonies isolated in the test IMViC, also obtained by the positive isolated combination from the MPN table.

Positive coagulase staphylococci was evaluated according to APHA (AMERICAN..., 2001). Twenty-five grams of the sample was weighed, added to 225 mL of peptone water 0.1%, and homogenized in a Bagmixer blender (Interscience, France). Further dilutions (10^{-2}, 10^{-3}) were then prepared from this dilution. From each one of these first three dilutions, 1 mL was distributed into 3 Baird Parker agar plates (BP), which were incubated at 35 °C (±1 °C) for 48 hours. Three typical colonies were subcultured for Brain-Heart Infusion broth (BHI) and incubated at 35 °C (±1° C) for 24 hours for catalase and coagulase tests. The results were expressed as coagulase-positive staphylococci/g. When the coagulase reaction was negative, the result was expressed as <10 Colony Forming Units per gram of sample ($CFU.g^{-1}$).

For detection of *Salmonella* sp. (AMERICAN..., 2001), a pre-enrichment of 25 g of sample was performed in 225 mL of buffered peptone water (BPW) which was incubated at 35 °C (±1 °C) for 24 hours. After 24 hours of pre-enrichment, aliquots were transferred simultaneously to tetrathionate broth (TTB) and Rappaport-Vassiliadis broth (RV) and were incubated at 42 °C (±1° C) for 24 hours. The selective enrichment cultures were streaked onto the surface of Brilliant-green Phenol-red Lactose Sucrose (BPLS) and Xylose Lysine Deoxycholate (XLD) agars, and they were incubated at 35 °C (±1 °C) for 24 hours. The typical colonies of *Salmonella* sp. were submitted to biochemical screening in triple sugar iron agar (TSI), lysine iron agar (LIA), and urea agar (UA). Colonies suspected of being *Salmonella* sp. were analyzed by complementary biochemical tests: Dulcitol, Indole, Malonate, MRVP - Methyl Red Voges Proskauer, and Citrate.

Vibrio spp. was evaluated according to Kaysner and De Paola Junior(2001). 50 g of the sample was weighed, 450 mL phosphate buffered saline (PBS) were added, and the mixture was homogenized using a Bagmixer blender. Decimal dilutions up to 10^{-4} were prepared from this dilution, and 1 mL of each dilution was inoculated into one tube of a series of three tubes containing alkaline peptone water (APW) for the enumeration of the *Vibrio parahaemolyticus* and *Vibrio vulnificus* using the most probable number (MPN) method, as described in the US Food and Drug Administration Bacterial Analytical Manual (KAYSNER; DE PAOLA JUNIOR, 2001). After 18 hours of incubation at 35 °C (±1 °C), a loopful of the tubes showing growth were streaked onto thiosulfate-citrate-bile-sals-sucrose agar (TCBS) plates and incubated at 35 °C (±1° C) for 18-24 hours. Both sucrose-positive (yellow on TCBS agar) and sucrose-negative (blue-green on TCBS agar) colonies were submitted for confirmation as gram-negative and oxidase-positive. Further biochemical differentiation for identification and confirmation of isolated items were performed using the API 20E system (bioMérieux).

2.3 Statistical analysis

Since the detection of *Salmonella* sp., *V. cholerae* and *V. alginolyticus* was not quantitative, the results were expressed as isolation percentage using the presence or absence of these bacteria in each sample. Coliforms at 45 °C, *Escherichia coli*, *V. parahaemolyticus*, and *V. vulnificus* were expressed as isolation percentage and as MPN enumeration. The results for the *V. parahaemolyticus* and *V. vulnificus* isolation were then statistically analyzed to observe any correlation between the presence of vibrios and water temperature. Differences in the incidence of the *Vibrio* spp. in oysters in different seasons of the year were evaluated by ANOVA ($p < 0.05$), and mean differences were evaluated using Tukey`s test. Statistical analyses of the data were performed with the *Statistica® 6.0* software.

3 Results and discussion

The counts of coliforms at 45 °C in the oysters ranged from <3 to 1.5×10^2 $MPN.g^{-1}$, and 147 (81.6 %) samples showed counts equal to <3 $MPN.g^{-1}$. The highest count was seen in the month of December, in region B; however, region E showed higher number of positive samples for Coliforms at 45 °C in relation to the other areas (Table 1).

Only one (0.6%) sample showed high values of MPN for Coliforms at 45 °C exceeding the limit allowed by the Resolution 12/2001. The Department of Sanitary Vigilance has determined the maximum acceptable level of 1×10^2 MPN per gram of Coliforms at 45 °C in raw seafood-based dishes (BRASIL, 2001). Although oysters are not mentioned explicitly in this paragraph, they may be considered raw seafood since they are traditionally consumed in natura.

Pereira et al., 2006 found levels of contamination for Coliforms at 45 °C in oysters *Crassostrea gigas* from the culture area in Ribeirão da Ilha, in South Bay, with counts

Table 1. Counting and isolation percentage Coliforms at 45 °C, *Escherichia coli*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* in the 15 collection oysters in six different regions of the South Bay of Santa Catarina Island.

Microorganisms region (n)	Minimum counting	Maximum counting	Positive samples
Coliforms 45 °C	(MPN.g^{-1})[a]	(MPN.g^{-1})[a]	n (%)
A (30)	<3.0	4.0	2 (6.7)
B (30)	<3.0	1.5×10^2	4 (13.3)
C (30)	<3.0	4.0	2 (6.7)
D (30)	<3.0	9.0	9 (30.0)
E (30)	<3.0	9.3×10	12 (40.0)
F (30)	<3.0	9.3×10	4 (13.3)
Escherichia coli	(MPN.g^{-1})[a]	(MPN.g^{-1})[a]	n (%)
A (30)	<3.0	<3.0	Zero (0.0)
B (30)	<3.0	9.0	3 (10.0)
C (30)	<3.0	4.0	1 (3.3)
D (30)	<3.0	9.0	4 (13.3)
E (30)	<3.0	9.0	8 (26.7)
F (30)	<3.0	4.3×10	2 (6.7)
Vibrio parahaemolyticus	(MPN.g^{-1})[a]	(MPN.g^{-1})[a]	n (%)
A (30)	<3.0	4.0	1 (3.3)
B (30)	<3.0	<3.0	Zero (0.0)
C (30)	<3.0	<3.0	Zero (0.0)
D (30)	<3.0	4.0	2 (6.7)
E (30)	<3.0	3.0	3 (10.0)
F (30)	<3.0	7.0	2 (6.7)
Vibrio vulnificus	(MPN.g^{-1})[a]	(MPN.g^{-1})[a]	n (%)
A (30)	<3.0	4.0	2 (6.7)
B (30)	<3.0	<3.0	Zero (0.0)
C (30)	<3.0	6.0	2 (6.7)
D (30)	<3.0	<3.0	Zero (0.0)
E (30)	<3.0	4.0	3 (10.0)
F (30)	<3.0	7.0	2 (6.7)
Positive coagulase staphylococcus	(CFU.g^{-1})[b]	(CFU.g^{-1})[b]	n (%)
A (30)	<10.0	1.0×10^2	4 (13.3)
B (30)	<10.0	9.3×10	1 (3.3)
C (30)	<10.0	4.0×10	1 (3.3)
D (30)	<10.0	1.9×10^2	4 (13.3)
E (30)	<10.0	3.0×10	2 (6.7)
F (30)	<10.0	3.0×10	2 (6.7)

Note: [a]MPN.g^{-1} = most probable number per gram; [b] CFU.g^{-1} = Colony forming unit/gram of sample.

that ranged between <3 and 9.3×10 MPN.g^{-1}, and 73% of the samples showed counts equal to <3 MPN.g^{-1}. Such levels are similar to those obtained in this study. Silva et al. (2004), evaluating the microbiological quality of mangrove oysters (*Crassostrea rhizophorae*), collected at a natural oyster bed in the estuary of Cocó river in Fortaleza, Ceará, Brazil, found higher levels for Coliforms a 45 °C than those observed in the present study, ranged from <1.8 to 9.2×10^2 MPN.g^{-1}. In a study carried out by Téllez et al. (1999), monitoring the quality of oysters (*Crassostrea virginica*) cultivated in the state of Tamaulipas, Mexico, low counts of Coliforms at 45 °C with highest counts of 1.4×10 MPN.100g^{-1} were found.

Since *E. coli* is a better indicator of fecal contamination than the other genres and species of the coliforms group, determination of its incidence in a coliform population is desirable (JAY, 2005). The counts of *E. coli* obtained in the oysters from the six different regions were low, ranging between <3 and 4.3×10 MPN.g^{-1}, and 162 (90%) samples showed counts below detectable levels (<3 MPN.g^{-1}). In a study carried out by Oliveira et al. (2006), monitoring the quality of oysters *Crassostrea gigas* cultivated in Alaska, USA, the count of *E. coli* in the oysters ranged between <3 and 2.3×10 MPN.g^{-1}. These results are very similar to those obtained in this present study. In a study performed by Pereira et al. (2006), *E. coli* was found in 9% of the samples of oysters *Crassostrea gigas* collected in three different culture areas in Florianópolis, State of Santa Catarina. Such results are in agreement with the results shown in this study, with 18 (10%) positive samples (Table 1); however, this study showed lower numbers than the maximum allowed by Brazilian legislation for Coliforms at 45 °C.

Region E showed the highest levels of *E. coli* and Coliforms at 45 °C in the mollusks, eight (26.7%) samples were contaminated, probably due the higher population density and geographical characteristics of this region, while region A did not show any sample contaminated by *E. coli* (Table 1). It is interesting to point out that, although the region E showed the highest frequency of contaminated samples for all researched microorganisms, cell counts were very low, i.e., well below the limits established by legislation.

The counts of positive coagulase staphylococci in the samples ranged between <10 and 1.9×10^2 CFU.g^{-1} of sample (Table 1); the highest counts obtained in regions C, D, and E were registered in the month of September. This did not happen in the other regions although no significant statistical differences were observed at a level of significance of 5%, between the averages of counts of positive coagulase staphylococci in the different regions studied (H: 2.1008; $p > 0.05$).

All the samples analyzed are within the limits established by the RDC 12/2001 - ANVISA, which has determined a maximum count of 10^3 CFU.g^{-1} of positive coagulase staphylococcus for in natura bivalve mollusks (BRASIL, 2001). The samples analyzed also showed counts well below these limits. According to Jay (2005), the presence of *Staphylococcus* can be expected, even if in small amounts, in almost all foods of animal origin.

Pereira et al. (2006), evaluating 45 samples of oysters from culture areas located in Florianópolis, found only one sample contaminated by positive coagulase staphylococci, while Ayulo, Machado and Scussel (1994), evaluating samples of bivalve mollusks from coastal regions of Santa Catarina, detected the presence of *Staphylococcus aureus* in 60% of the samples. In this present study, about 8% (14/180) of the samples showed contamination by positive coagulase staphylococci.

All of the 180 samples analyzed showed absence of *Salmonella* sp., complying with the current legislation, RDC 12/2001 - ANVISA, which has established the absence of *Salmonella* sp. in in natura, refrigerated or frozen bivalve mollusks (BRASIL, 2001). Pereira et al. (2006) did not find *Salmonella* sp. in any of the 90 samples analyzed; 30 of which were from the region of Ribeirão da Ilha in the South Bay. Such result is in agreement with those found in this study.

Although *Salmonella* sp. was not isolated in any of the samples of this study, this bacterium is among the most important cause of foodborne diseases.

Of the 180 samples analyzed, 25 (13.9%) contained one or more vibrios (Table 2). The most frequently isolated species were *V. alginolyticus* (15 isolates, 8.3%), *V. vulnificus* (9 isolates, 5.0%), and *Vibrio parahaemolyticus* (8 isolates, 4.4%). *Vibrio cholerae* was detected in 4 samples (2.2%), and *Vibrio fluvialis* was detected only in one sample (0.6%). Part of the samples was contaminated with more than one *Vibrio* species. Two *Vibrio* species were found in 4.4% of the samples, and 1.1% showed a contamination with three *Vibrio* species. The percentage of the samples containing one or more *Vibrio* species varied over the investigation period. In the last four summer samplings, it was observed a stronger presence of *Vibrio* spp. compared with the other seasons of the year ($p < 0.05$). When the water temperature was at its highest, the highest level of *Vibrio*-positive samples was found, and positive correlation ($r = 0.51$; $p < 0.01$) was observed between the incidence of *Vibrio* spp. in oysters and temperature of marine waters (Figure 2). These findings are in agreement with those of several authors (HLADY; KLONTZ, 1996; LHAFI; KÜHNE, 2007; MATTÉ et al., 1994), who reported a considerable rise in the numbers of *Vibrio* spp. isolated from seafood samples during the summer months.

Vibrio alginolyticus was the most prevalent species confirming published research findings by authors from other countries, both in Europe and in the United States (LHAFI; KÜHNE, 2007; MATTÉ et al., 1994; RIPABELLI et al., 1999). The counts of *V. parahaemolyticus* and *V. vulnificus* in the samples ranged between <3 and 7 MPN.g^{-1} of sample for both bacteria (Table 1). The Department of Sanitary Vigilance has determined the maximum acceptable level of 10^3 per gram of *V. parahaemolyticus* in raw seafood-based dishes

Table 2. Prevalence of *Vibrio* spp. in 180 oysters samples from the South Bay of Santa Catarina Island.

Microorganisms	Positive samples	
	No.	(%)
Vibrio spp.	25	13.9
V. alginolyticus	7	3.9
V. parahaemolyticus	1	0.6
V. vulnificus	4	2.2
V. cholerae	2	1.1
V. alginolyticus and *V. parahaemolyticus*	3	1.7
V. parahaemolyticus and *V. vulnificus*	2	1.1
V. vulnificus and *V. alginolyticus*	2	1.1
V. alginolyticus and *V. cholerae*	1	0.6
V. fluvialis	1	0.6
V. vulnificus and *V. alginolyticus* and *V. parahaemolyticus*	1	0.6
V. cholerae and *V. alginolyticus* and *V. parahaemolyticus*	1	0.6

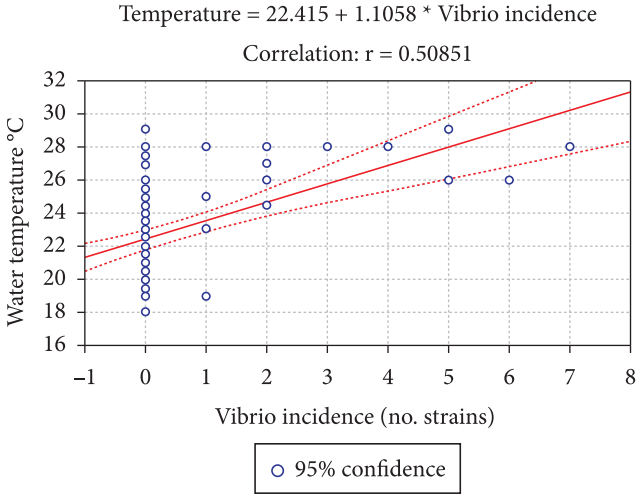

Figure 2. Correlation between incidence of *Vibrio* spp. in oysters and temperature of marine waters in six different regions of the South Bay of Santa Catarina Island.

(BRASIL, 2001). Hence, all samples analyzed are within the limits established by Brazilian Legislation. The presence the *V. vulnificus,* even at low counting, indicate need for continuous monitoring of bacterial contamination in oysters.

4 Conclusion

The high percentage of samples with counts of coliforms at 45 °C equal to <3 MPN.g^{-1} suggests that the oysters produced in the South Bay show acceptable hygienic and sanitary quality. The great majority of the samples showed absence of *E. coli*, and in the samples in which the presence of this microorganism was confirmed, the counts were very low. However, since *E. coli* is the best indicator of recent fecal contamination, and because it is a bacterium which, depending on the species, can be highly pathogenic to humans, the control of this microorganism in oysters, which are frequently consumed raw, is essential.

The counts of positive coagulase staphylococcus in the samples of oysters were well below the limits allowed by the current legislation, RDC 12/2001 - ANVISA, probably due to the fact that the oyster meat is enclosed inside their shells, with no direct contact with the manipulators.

All of the samples analyzed showed absence of *Salmonella* sp. in 25 g, which, therefore, comply with the standards established by Brazilian legislation, RDC 12/2001 - ANVISA

The results obtained in the present study confirm the needs to improve shellfish-borne disease control strategies focusing on pathogenic vibrios. Therefore, classical fecal indicators are not suitable for oyster quality control by itself. The risk to public health from the presence of vibrios depends on the consumer health and the concentration and pathogenicity of the pathogen.

The safest way to eat oysters is by ensuring they are cooked before consumption.

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