

## ***Vibrio parahaemolyticus* and *Salmonella enterica* isolates in fish species captured from the Lagoa dos Patos estuary**

### ***Vibrio parahaemolyticus* e *Salmonella enterica* isolados de pescados capturados no estuário da Lagoa dos Patos**

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#### **Abstract**

Microorganisms that cause human diseases can contaminate fishes in aquatic environments as well as during their capture, handling, and transport. The purpose of this study was to isolate *Vibrio parahaemolyticus* and *Salmonella enterica* from fishes captured in the Lagoa dos Patos estuary that were eviscerated and cleaned for trade. Thirteen fish landing were analyzed and 65 entire fishes and 65 cleaned fishes were studied to determine the presence of *V. parahaemolyticus* and *S. enterica*. Bacterial isolates were compared using rep-PCR. *V. parahaemolyticus* was isolated from one entire *Micropogonias furnieri* and two entire *Mugil platanus*, as well as from three eviscerated *M. platanus*. *S. enterica* was isolated from two eviscerated *Paralichthys orbignyanus*. Identical rep-PCR bands from *V. parahaemolyticus* were observed in entire and eviscerated fishes from the same discharge, suggesting processing failures that neither eliminated the microorganism from the raw material nor prevented cross-contamination. *S. enterica* was not isolated from entire fishes, presumably because contamination occurred due to hygiene and sanitary failures. Our results showed that *M. furnieri* and *M. platanus* captured in the Lagoa dos Patos estuary may host *V. parahaemolyticus* and that this microorganism, as with *S. enterica*, may also persist even after the fish is cleaned. This is the first record of the isolation of *V. parahaemolyticus* from *M. furnieri*.

**Key words:** Fish. Food safety. *Mugil platanus*. Pathogenic microorganisms. *Paralichthys orbignyanus*.

#### **Resumo**

Micro-organismos que causam doenças humanas podem contaminar os peixes no ambiente aquático, durante a captura, manuseio e transporte. O objetivo foi isolar *Vibrio parahaemolyticus* e *Salmonella enterica* de peixes capturados no estuário da Lagoa dos Patos e artesanalmente eviscerados e limpos para o comércio. Treze desembarques foram acompanhados e 65 peixes inteiros e 65 limpos foram analisados quanto à presença de *V. parahaemolyticus* e *S. enterica*. Os isolados foram comparados entre si através de rep-PCR. *V. parahaemolyticus* foi isolado em uma corvina (*Micropogonias furnieri*) e duas tainhas (*Mugil platanus*) inteiras e em três *M. platanus* eviscerados. *S. enterica* foi isolada apenas de dois linguados (*Paralichthys orbignyanus*) eviscerados. O mesmo perfil de bandas do *V. parahaemolyticus*

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pela rep-PCR foram observados nos peixes inteiros e eviscerados do mesmo desembarque, sugerindo falhas de processamento, o qual não eliminou o micro-organismo da matéria-prima ou não preveniu a contaminação cruzada. Como *S. enterica* não foi isolada de peixes inteiros, presume-se que a contaminação aconteceu por causa de falhas higiênicas e sanitárias. Os resultados mostraram que os peixes da espécie *M. furnieri* e *M. platanus* capturados no estuário da Lagoa dos Patos podem hospedar *V. parahaemolyticus* e que este micro-organismo, assim como *S. enterica*, também pode estar presente em peixes eviscerados. Este é o primeiro registro de isolamento de *V. parahaemolyticus* em *M. furnieri*.

**Palavras-chave:** Micro-organismos patogênicos. *Mugil platanus*. *Paralichthys orbignyanus*. Peixe. Segurança alimentar.

## Introduction

Consumption of fish has been increasing in recent years because it provides proteins, contains low saturated fat, is rich in micronutrients, and is also a source of fatty acids that provide benefits to human health (FDA, 2009). Consumption of fish also protects against cardiovascular disease and ischemic stroke (MOZAFFARIAN; RIMM, 2006). The World Health Organization (WHO) recommends that fish be consumed twice per week (WHO, 2003). However, because it is an aliment with intrinsic features favorable to survival of many microorganisms, as pH, activity water and nutrients, fishes are a potential vehicle of pathogens (APHA, 2001). Fish are highly perishable and susceptible to contamination by several microorganisms in aquatic environments, as well as during their capture and transport (VIEIRA, 2004; RESENDE et al., 2009; POPOVIC et al., 2010). Previous studies have implicated food handlers as a major factor in foodborne disease outbreaks due to food contamination, bad hygiene, and other inappropriate practices during meal production (CAVALLI; SALAY, 2007).

*Vibrio parahaemolyticus* is a microorganism distributed in marine and estuarine environments worldwide, but is more abundant in warmer regions. It is isolated from fish and its presence represents a danger to consumers (SU; LIU, 2007). This microorganism can cause diarrhea, abdominal pain, vomiting, fever, and headaches. *V. parahaemolyticus* is easily eliminated from fish by heat exposure (HUSS, 1997). The diseases caused by this pathogen are associated with consuming

raw or under-cooked fish and products that are contaminated (CCFH, 2002).

*Salmonella enterica* is found in birds, cattle, swine, and the human gastrointestinal tract. It is the most common microorganism in reports of foodborne diseases (VESTBY et al., 2009). Salmonellosis in humans causes abdominal pain, fever, vomiting, and diarrhea (FOX, 2009). *Salmonella* are also found in fresh fish. Rall et al. (2011) analyzed 33 fresh fish samples that were sold in the town of Botucatu, São Paulo (SP), and *S. enterica* was detected in 3% of these. Introduction of *Salmonella* is common during the handling process and by contact with contaminated water. *Salmonella* occurs naturally in the intestinal tract of humans and other warm-blooded animals; therefore, its presence in food potentially indicates fecal contamination, improper fish handling by employees, contact with poorly sanitized surfaces, or cross-contamination (MARTINS et al., 2002). The Agência Nacional de Vigilância Sanitária (BRASIL, 2001), institution responsible for food control in Brazil, determines the absence of *Salmonella* spp. in 25 grams, for raw and cooled fishes.

The Lagoa dos Patos estuary, located in southern Brazil, occupies an area of 963.8 km<sup>2</sup> (10% of the total area of this lagoon), and receives water from rivers located in its north portion and from Lagoa Mirim, south through the Canal São Gonçalo (CALLIARI, 1998). The estuary represents an importation area for the cultivation of fishes and crustaceans of commercial value, and represents an artisanal fishing area of outstanding importance for fish provision in southern Brazil (REIS, 1999).

To guarantee food quality, proper handling during capture, processing, storage, and commercialization are necessary. According to Baldisserotto (2009), there are no studies regarding the quality of continental fishes sold to consumers in Rio Grande do Sul. In addition, there is a lack of studies documenting the occurrence of foodborne disease agents in fish from the region, particularly in the Lagoa dos Patos estuary, what is indispensable to trace effective plans to the disease control.

In this study, we obtained molecular profiles for *V. parahaemolyticus* and *S. enterica* isolates from entire and eviscerated fish species. Identification of these pathogens at different stages of the epidemiological chain contributes important information in the comprehension of transmissible etiological disease agents and their control measures. This work aimed to establish the similarity between *V. parahaemolyticus* and *S. enterica* isolates from fish before and after cleaning.

## Material and Methods

### *Specimen collection*

Thirteen landing of fishes were captured from the Z-3 fishing community, in the town of Pelotas, Rio Grande do Sul (RS), and at the public market of RS from catches made using artisanal methods in the Lagoa dos Patos estuary. Five entire fishes were randomly collected during each discharge and five after processing (i.e., evisceration and cleaning) to be sold to consumers, totaling 130 samples. The fish were placed in sterile bags and were immediately transferred to the laboratory in isothermal boxes packed with ice to search for the presence of *V. parahaemolyticus* and *S. enterica*. Collections were made from April to October 2014.

### *Vibrio* research

*Vibrio* research was conducted as recommended by the U. S. Food and Drug Administration (FDA)

(KAYSNER; DEPAOLA, 2004) with modifications. The gills and liver from each fish were excised, placed in sterile plastic bags containing 225 mL alkaline peptone water (APW, Himedia, Mumbai, India), massaged for 5 minutes, and incubated at 37°C for 24 hours for enrichment. Cultures were prepared from medium surface material by streaking on thiosulfate citrate bile sucrose agar (TCBS, Himedia, Mumbai, India) and incubated at 37°C for 24 hours to obtain isolated colonies. Up to three typical colonies from each dish were transferred to APW and, following incubation at 37°C for 24 hours, were mixed with 20% glycerol for stock maintenance at -70°C. When necessary, the isolates were recovered in APW at 37°C after 24 hours.

### *Salmonella enterica* research

*S. enterica* isolates were obtained according to FDA recommendations (ANDREWS; HAMMACK, 2011) with modifications. Briefly, 25 g of intestine from each fish was placed in a sterile bag with 225 mL of buffered peptone water (BPW, Acumedia, Lansing, Michigan, USA), massaged for 5 minutes, and incubated at 35°C for 24 hours. After pre-enrichment, 1 mL of this solution was transferred into 10 mL of tetrathionate (TT, MicroMed, Rio de Janeiro, Brazil) broth. Another broth was prepared by transferring 0.1 mL of this solution into 10 mL of Rappaport-Vassiliadis (RV, MicroMed, Rio de Janeiro, Brazil). Both broths were incubated in a water bath at 42°C for 24 hours. An aliquot of each broth was cultured by streaking on dishes with brilliant green phenol red lactose sucrose agar (BPLS, MicroMed, Rio de Janeiro, Brazil) and xylose lysine deoxycholate agar (XLD, Acumedia, Lansing, Michigan, USA), followed by a 24-hour incubation at 35°C. Up to three colonies with *S. enterica*- compatible characteristic were selected and grown on triple sugar iron medium (TSI, Acumedia, Lansing, Michigan, USA), lysine iron agar (LIA, Acumedia, Lansing, Michigan, USA),

and urea broth (Synth, Diadema, SP, Brazil). Colonies consistent with the characteristics of *S. enterica* underwent serological tests with sera against antigen O (Probac do Brazil, SP, Brazil) to verify serum agglutination.

#### *Isolates obtained from marketable fishes*

Using the procedures describe above, transverse cuts of approximately 25 g were taken from processed fish and placed in BPW and APW to detect *S. enterica* and *Vibrio*, respectively.

#### *DNA extraction*

DNA of bacterial isolates was extracted following the methods of Sambrook and Russel (2001). An APW culture was incubated at 37°C for 24 hours, and the pellet obtained by centrifugation of 1 mL of culture was resuspended in 100 µL of STES buffer [Tris-HCl 0.2 M, NaCl 0.5 M, SDS 0.1% (m/v), EDTA 0.01 M, pH 7.6]. Next, 50 µL of glass beads and 100 µL of phenolchloroform were added to the suspension. The suspension was then homogenized for 1 minute, followed by centrifugation at 13.000 × g for 5 minutes. The supernatant was collected and precipitated in 2 volumes of absolute ethanol and 0.1 volume of 5M NaCl at -70° C for 30 minutes.

This mixture was then centrifuged at 13.000 × g for 20 minutes, and the pellet was washed with 70% ethanol. Following elution in 40 µL of buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 7.4), 1 µL of RNase was added (10 µg/µL). The extracted DNA was stored at -70° C.

#### *V. parahaemolyticus identification*

*Vibrio* isolates were analyzed by polymerase chain reaction (PCR) to sequence the toxR gene (Table 1), and to identify *V. parahaemolyticus* following the protocol of Kim et al. (1999) with modifications. Each reaction had a final volume of 20 µL. Ten microliters of Master Mix (Qiagen, SP, Brazil), 1 µL (10 pmol) of each primer, 1.2 µL DNA, and 6.8 µL water were used to complete the reaction volume. Amplification was performed in a TC-3000 thermocycler (Techne, Staffordshire, UK) under the following conditions: initial denaturation of 96°C for 5 minutes, followed by 20 cycles of denaturation at 94°C for 1 minute, annealing of the primers at 63°C for 1.5 minutes, extension at 72°C for 1.5 minutes, and a final extension at 72°C for 7 minutes. The PCR products were stained with GelRed (Uniscience, SP, Brazil), and electrophoresis was performed on a 1.8% agarose gel. *V. parahaemolyticus* ATCC 17802 DNA was used as a positive control and pure water as a negative control.

**Table 1.** Primers used in *Vibrio parahaemolyticus* identification from fishes captured in the Lagoa dos Patos estuary during the months of April through October 2014.

Primer	Sequence (5'-3')	Amplification size (bp)	Reference
ToxR-a	GTCTTCTGACGCAATCGTTG	368	Kim et al. (1999)
ToxR-b	ATACGAGTGGTTGCTGTCATG		

#### *Rep-PCR*

To compare species strains, *S. enterica* and *V. parahaemolyticus* DNAs were analyzed by rep-PCR using PCR-(GTG)<sub>5</sub> (VERSALOVIC et al., 1994), in accordance with Rasschaert et al. (2005). The rep-PCR reaction mixture used was as follows:

2.5 µL DNA, 2µL 5'-GTGGTGGTGGTGGTG-3' oligonucleotide, 12.5 µL Master Mix, and 8 µL water to complete the reaction volume. The amplification conditions used were as follows: 1 cycle at 94°C for 5 minutes, 30 subsequent cycles at 95°C for 30 seconds, followed by 1 minute at 45°C

and 5 minutes at 60°C, and finally 1 cycle at 60°C for 16 minutes. For band visualization at different amplified genome regions, the PCR products were stained with GelRed, and electrophoresis was performed on a 2% agarose gel. DNAs from the ATCC 17802 strain of *V. parahaemolyticus* and from strain ATCC 2017 of *S. enterica* were used as positive controls. The negative control consisted of a mixture of all reaction components except DNA.

## Results

Thirteen landing of fishes captured from the Lagoa dos Patos estuary were analyzed, and 130 fishes (65 entire and 65 eviscerated) were sampled and analyzed for the presence of *V. parahaemolyticus*

and *S. enterica*. The fish species captured and studied were corvina (*Micropogonias furnieri*) and mullet (*Mugil platanus*), both from landing made at Z-3, and sole (*Paralichthys orbignyanus*), obtained at the public market in RS. The three species harbored some of the microorganisms studied (Table 2). This is the first record of *V. parahaemolyticus* isolation from *M. furnieri*.

*V. parahaemolyticus* was isolated from three (4.61%) of 65 entire fish samples and from three (4.61%) of 65 eviscerated fish samples (Table 2). *S. enterica* was identified in two (3.07%) eviscerated fish samples coming from the same discharge (Table 2). This bacterium was not isolated from entire fish samples.

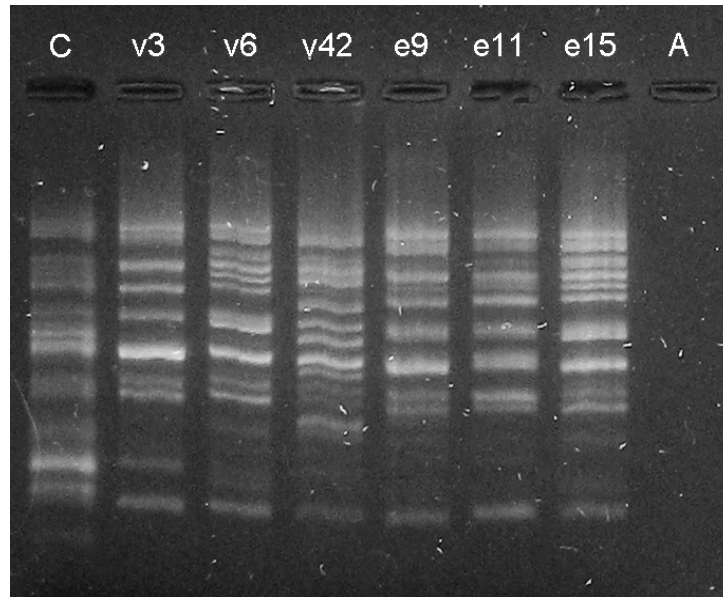
**Table 2.** Incidence and quantity of bacteria isolated from different fish species collected in the Lagoa dos Patos estuary during 2014.

Landing	Months	Fishes	<i>V. parahaemolyticus</i>		<i>S. enterica</i>	
			Entire fishes	Eviscerated Fishes	Entire fishes	Eviscerated fishes
1	April	<i>M. platanus</i>	1	0	0	0
2	April	<i>M. platanus</i>	1	1	0	0
3	April	<i>M. platanus</i>	0	2	0	0
4	June	<i>P. orbignyanus</i>	0	0	0	0
5	July	<i>P. orbignyanus</i>	0	0	0	2
6	September	<i>P. orbignyanus</i>	0	0	0	0
7	October	<i>P. orbignyanus</i>	0	0	0	0
8	October	<i>M. furnieri</i>	1	0	0	0
9	October	<i>M. furnieri</i>	0	0	0	0
10	October	<i>M. furnieri</i>	0	0	0	0
11	October	<i>M. furnieri</i>	0	0	0	0
12	October	<i>M. furnieri</i>	0	0	0	0
13	October	<i>M. furnieri</i>	0	0	0	0

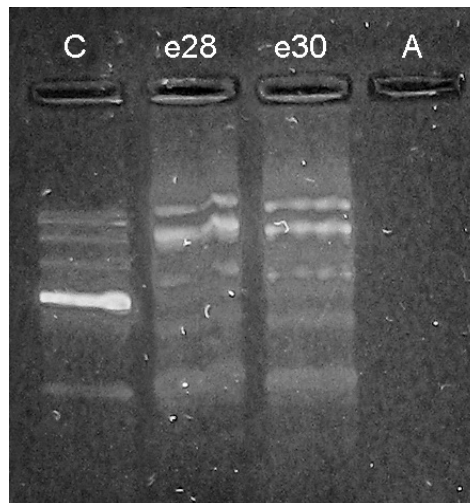
In the rep-PCR analysis of *V. parahaemolyticus*, four strains displayed an identical banding profile (Figure1), one from an entire fish (v6) and one from an eviscerated fish (e9) from the second discharge, and two (e11 and e15) from the third discharge, both

from cleaned fishes. The other two strains (v3 and v42) came from different landing and displayed distinct banding profiles. Regarding *S. enterica*, both isolated strains showed identical bands by rep-PCR (Figure2).

**Figure 1.** Comparative analysis of *Vibrio parahaemolyticus* strains from fishes captured in the Lagoa dos Patos estuary from April through October 2014 by rep-PCR. C: Reference strain (ATCC 17802); v: entire fish; e: eviscerated fish; A: negative control.



**Figure 2.** Comparative analysis of *Salmonella enterica* strains from fishes captured in the Lagoa dos Patos estuary from April through October 2014 by rep-PCR. C: Reference strain (ATCC 2017); e28 and e30: eviscerated fishes; A: negative control.



## Discussion

Microorganisms were isolated only from collections made in April through October, which may be a result of environmental temperature. *V. parahaemolyticus* is more commonly found in tropical and temperate climates (MAGALHÃES et al., 1991) where temperatures are warmer. In cold months, this bacterium is present mostly in marine

mud; however, in hotter months *V. parahaemolyticus* may be free in water or in fishes and mollusks (BUTT et al., 2004). As a result, in collections from the colder months of June to September, no microorganisms were isolated. Chen et al. (2005) isolated *V. parahaemolyticus* from 2.67% (3 of 112) of tuna (*Thunnus* spp.) obtained in SP where the climate is considered subtropical, as well as

in the region where our work was performed, in which the microorganism was isolated from 4.61% (6 of 130) of the analyzed samples. Pereira et al. (2007) isolated *V. parahaemolyticus* from 11.6% of 86 mussel (*Perna perna*) samples from the town of Niterói, Rio de Janeiro (RJ), which has a tropical climate. In Fortaleza, Ceará, where the climate is semiarid, Vieira et al. (2004) analyzed 90 samples of freshwater crabs (*Ucides cordatus*), of which 8.9% tested positive for *V. parahaemolyticus*. In our work, and that of Chen et al. (2005), collections were made during cold months, which might have influenced the number of bacterial isolates obtained. Our results were lower in comparison to the studies of Vieira et al. (2004) and Pereira et al. (2007), which were conducted in climates with warmer temperatures. *V. parahaemolyticus* is most frequently found in estuaries, sediments, mollusks, and crustaceans (ICMSF, 1978). These factors might help explain differences in the percentage of bacteria found in the works cited above, since *Thunnus* is a pelagic fish, *Perna perna* is a strainer shellfish, and *Ucides cordatus* is a crustacean that feeds on detritus. On the other hand, in our study *V. parahaemolyticus* was not isolated from *P. orbignyanus*, a benthic fish that lives in intimate contact with the estuary substrate. One possible explanation for this finding is the small sample size (20 entire fishes from four landing), or that *P. orbignyanus* is not an appropriate host for *V. parahaemolyticus*.

Milan et al. (2015) analyzed 10 entire *M. platanus* samples caught in the Lagoa dos Patos estuary and found 40% (4 of 10) of the samples contaminated by *V. parahaemolyticus*. In a different study, Ramos et al. (2014) isolated *V. parahaemolyticus* from 35% (21 of 60) of oysters samples in Santa Catarina (SC). Both Milan et al. (2015) and Ramos et al. (2014) made these collections during warmer months in contrast to our study.

*V. parahaemolyticus* has also been isolated from different fish species in studies from other locations. Herrera et al. (2006) found *V. parahaemolyticus* in two of 50 samples (4%) from fillets and slices of

different fish species (e.g., conger eel, swordfish, sole, grouper, and whiting) in Spain. These percentages are similar to our results. In contrast, Torres and Fernandez (1993) studied 57 samples of seafood (e.g., fresh oysters, fish, and shrimp) collected almost yearlong in Guadalajara, Mexico, and observed *V. parahaemolyticus* in 45.6% of all samples with the greatest incidence in fishes (71.4%), followed by oysters (44%), and shrimp (27.6%).

All fishes analyzed in the present study were from the Lagoa dos Patos where there is an exchange of water with the Atlantic Ocean through a waterway approximately 20 km long and 0.5 to 3 km wide (BONILHA; ASMUS, 1994). In addition to rain and evaporation, wind also controls water circulation, distribution of salinity, and water levels (GARCIA, 1998). These factors influence microorganism abundance through the entrance of water and other contaminated species of animals. *V. parahaemolyticus* is most frequently found in water that varies widely in salinity, similar to that found in estuaries and coastal areas, and its occurrence declines in areas of low salinity (KELLY; STROH, 1988). In the Lagoa dos Patos during the summer months, there is a greater occurrence of salt water entering the estuary (ANACLETO; GOMES, 2006). Our collections were made during fall, winter, and spring, when the salinity of the water might have contributed to low *V. parahaemolyticus* incidence.

Fishes contaminated with *S. enterica* have been reported in other studies. Onyango et al. (2009) analyzed 120 samples of tilapia from the Nile River that were captured in Kenya and found 9 samples (14.3%) with *S. Typhimurium*, 7 (11.1%) with *S. Typhi* and 4 (6.3%) with *S. Enteritidis*. Dams et al. (1996) found *S. enterica* in 20% (1 of 5) of entire whiting fish samples (*Cynoscion striatus*), in 80% (4 of 5) of the frozen fillets analyzed, and in 40% (2 of 5) of entire frozen fish in Florianópolis, SC, indicating possible contamination sources, such as the use of non-potable water, improper storage of fish, cross-contamination of clean and gutted fish,

contact with insects, and contamination by handlers. Lorenzon et al. (2010) found *S. enterica* in 2% of eviscerated fish and 4% of entire fish (50 samples total) in fishing communities located in northeast SP.

During the second discharge, it is thought that the fish remained contaminated with the same bacterial strain even after evisceration and cleaning, indicating that these processes were inadequate in eliminating the microorganisms. During the third discharge, there was probably cross-contamination from another contaminated fish. These observations reflect the precarious hygiene-sanitary measures that have been adopted during processing, as they are not only incapable of eliminating fish-associated bacterial contamination, but also contribute to disseminating undesirable microorganisms from contaminated entire fishes to processed fishes ready to be sold.

In the instance where bacteria were isolated from two eviscerated fish that originated from a discharge that contained no bacterial isolates from an entire fish, it is presumed that hygiene and sanitary faults during the evisceration process caused the contamination, such as using contaminated utensils. This argument is supported by the fact that *S. enterica* was not isolated from any entire fish sample from any discharge, suggesting that its infection of fishes in the Lagoa dos Patos estuary, if it occurs, is low.

It is important that hygiene and sanitary standards are adopted during all stages of fish processing, in order to minimize natural contamination of food by *V. parahaemolyticus* and/or *S. enterica*, and to prevent cross-contamination, thereby avoiding risks to consumer health.

## Conclusion

Fish of the species *M. furnieri* and *M. platanus* may host *V. parahaemolyticus*. This microorganism, like *S. enterica*, may also be present in cleaned fish available at the market.

These results reinforce the importance of adopting corrective and preventive hygiene and sanitary standards through good manufacturing practices, as well as the necessity of close supervision of the fishing production chain by a competent party in order to reduce the occurrence of foodborne diseases in humans.

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