Occurrence of *Vibrio parahaemolyticus* and *Staphylococcus aureus* in seafood

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Abstract

The objective of this study was to establish the occurrence of *Vibrio parahaemolyticus* and *Staphylococcus aureus* in several species of sea fish and mussels (*Mytilus galloprovincialis*). The study included a total of 33 samples of frozen sea fish and 64 samples of fresh wild and farmed mussels purchased from the stores. *V. parahaemolyticus* was isolated and confirmed via PCR in 2 (6%) fish samples (Atlantic cod and Alaska pollock) and 20 (31%) mussel samples. *S. aureus* was also isolated and confirmed via PCR in 2 (6%) fish samples (Argentine hake and Atlantic cod). Significant differences were found in the total bacterial contamination between wild mussels (6.54 log cfu/g) and farmed mussels (6.69 log cfu/g). Total *V. parahaemolyticus* count did not show significant differences either between wild (4.45 log cfu/g) and farmed mussels (4.99 log cfu/g). In wild mussels the *S. aureus* count was found to be 4.50 log cfu/g, while in farmed mussels it was 3.14 log cfu/g. The occurrence of *V. parahaemolyticus* and *S. aureus* in fish and mussels presents a risk to the consumer's health.

Introduction

Fish catch and aquaculture production have been steadily increasing in recent years worldwide. Fish consumption per capita has also increased in recent years, reaching 20.5 kg in 2018 (FAO, 2020). Seafood consumption is associated with a beneficial effect on human health but can still pose a risk if consumed contaminated with infectious agents such as bacteria, viruses, and parasites (Iwamoto *et al.,* 2010). At least ten genera of bacterial pathogens are linked to human diseases following seafood consumption. They can be categorized in three main groups: i) bacteria that are normal inhabitants of seawater (*Vibrio parahaemolyticus*, *V. cholerae*, *V. vulnificus*, *Listeria monocytogenes*, *Clostridium botulinum* and *Aeromonas hydrophila*), ii) intestinal bacteria due to fecal contamination (*Salmonella* spp., pathogenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp. and *Yersinia enterocolitica*) and iii) bacterial contaminants during processing (toxigenic strains of *Bacillus cereus*, *L. monocytogenes*, *Stapylococcus aureus* and *Clostridium perfringens*) (Pereira *et al.,* 2021).

Vibrio spp. are normal part of bacterial flora in water environment. Some species are isolated from healthy hydrobionts and are, therefore, regarded as opportunistic pathogens (Gomez-Gil *et al.,* 2004). Species such as *V. parahaemolyticus* and *V. vulnificus* cause diseases in hydrobionts and humans, whereas *V. cholerae* is a pathogen for humans (Austin and Austin, 2007; Navaneeth *et al.,* 2020; Tan *et al.,* 2020). *Vibrio* spp. is isolated from fish, mussels, oysters, crabs, shrimp. Consumption of raw or insufficiently heat-treated hydrobionts contaminated with vibrios can cause acute gastroenteritis. *V. parahaemolyticus* is a major cause of gastroenteritis associated with the consumption of aquatic organisms worldwide (Li *et al.,* 2019). Toxicoinfections caused by *V. parahaemolyticus* have been reported in Japan, China, Taiwan, Spain, Italy, Chile, Peru, and Brazil. There were 40 food outbreaks caused by *V. parahaemolyticus* between 1973 and 1998 in the United States, involving more than 1000 cases (Daniels *et al.,* 2000). More than 300 food outbreaks caused by *V. parahaemolyticus* between 2003 and 2008 were reported in China with more than 9000 cases and 3940 hospitalized patients (Wu *et al.,* 2014). According to Letchumanan *et al.* (2014), *V. parahaemolyticus* causes 20-30% of food poisoning in Japan and many cases in the Asian countries. Martinez-Urtaza *et al.* (2018) reported sporadic events in Europe apart from Galicia (northwest Spain), which has been declared a "hot spot" for infections caused by *Vibrio* spp. Toxicoinfections caused by pathogenic vibrios are characterized by acute abdominal pain, vomiting, watery or bloody diarrhoea and gastroenteritis (Jahangir Alam *et al.,* 2002; Wagley *et al.,* 2009).

Staphylococcus aureus is the most important pathogen from genus *Staphylococcus* and is often present in the environment. It is known that the main source of food contamination with staphylococci are people working with food products (Johler *et al.,* 2015). Staphylococci can be present in the nasal cavities, throat, hair, and skin of healthy people and are abundant in wounds, pustules, and abscesses. Approximately 20% of the adult population are permanent carriers of *S. aureus* in their nasal cavity, other 30% are a recurrent carrier, while 50% are not carriers (Wertheim *et al.,* 2005). *S. aureus* is often found in a variety of foods, including fish (Vaiyapuri *et al.,* 2019). Contamination is associated with improper handling and storage, as well as inadequate hygiene practices and secondary contamination. The risk to public health is related to the ability of 50% of *S. aureus* strains to produce thermostable enterotoxins. Symptoms of staphylococcal intoxication usually have rapid onset (1-6 hours) and often include nausea, vomiting, diarrhoea, and abdominal pain (Jablonski and Bohach, 1997). Patients show symptoms when taking enterotoxin at an approximate dose between 20 ng and 1 µg (Bergdoll, 1989). *S. aureus* intoxication ranks third in cases of food poisoning worldwide (Aydin *et al.,* 2011).

Based on the risk of bacterial pathogens in seafood and their significance for human health, the objective of the present study was to establish the occurrence of *Vibrio parahaemolyticus* and *Staphylococcus aureus* in several species of sea fish and mussels (*Mytilus galloprovincialis*).

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Materials and methods

Sampling

The study included a total of 33 samples from frozen sea fish and 64 samples from fresh mussels (*Mytilus galloprovincialis*). The fish were purchased from stores in Stara Zagora and were of the following species: Alaska pollock (*Gadus chalcogrammus*) (n=19), Argentine hake (*Merluccius hubbsi*) (n=5), Atlantic cod (*Gadus morhua*) (n=4), Patagonian grenadier (*Macruronus magellanicus*) (n=3) and Baird's slickhead (*Alepocephalus bairdii*) (n=2). Mussel samples were purchased from regional marketplaces for alive fish and crustaceans in Burgas and Varna, and all mussels were harvested on the day of sampling (Figure 1). Of a total of 64 mussel samples taken from various batches, 34 were wild and 30 were farmed ones. Pooled samples containing 10-15 mussels each were prepared from each batch for further analysis. All samples were transported in refrigerated bags to the microbiological laboratory of the department. The fish were thawed in a refrigerator at 4°C for 24 hours and then a sample was taken from each fish for microbiological examination. The mussels were opened with a sterile scalpel, after which meat was separated for microbiological examination.

Isolation of *Vibrio parahaemolyticus* from fish and mussels

A total of 10 g from the samples were weighed in a Stomacher bag and homogenized with 90 ml Alkaline Peptone Water (HiMedia, India) and incubated at 37°C for 24 hours. The enriched cultures were streaked on selective agar Thiosulfate-citrate-bile salts-sucrose agar (HiMedia, India) and incubated at 37°C for 24 hours. At the end of the incubation period colonies with typical characteristics were counted (size up to 1 mm and dark bluish-green colour).

Identification of *Vibrio parahaemolyticus* via polymerase chain reaction (PCR)

DNA extraction

In an Eppendorf microtube one colony was suspended into 500 µl of sterile distilled and deionized water. The genomic DNA of each isolate was extracted directly from the bacterial suspension by a boiling method, in which the microtubes were placed in a thermoblock (Boeco, Germany) at 98°C for 10 minutes. The suspension was then centrifuged at 14000 rpm^{-1} for 10 minutes in a cooling microcentrifuge at 4°C. Two hundred microlitres of the supernatant containing DNA were transferred in new Eppendorf tubes and used for identification. The concentration and purity of the extracted DNA were measured by means of GeneQuant 1300 spectrophotometer (Biochrom Ltd., Cambridge, UK) and a control electrophoresis was performed to check the suitability of the obtained genomic DNA.

PCR protocol

Primers specific to *toxR* gene were purchased from SGP Biodynamics (Sofia, Bulgaria) (Table 1). The reaction mixture for PCR had volume of 25 µl and contained 2 µl of the extracted DNA, 12.5 µl TopTaq Master Mix (QIAGEN, Germany), 0.2 µl of each primer and 10.1 µl of water free from nucleases. Polymerase chain reaction took place in thermocycler QB-96 (Quanta Biotech, USA) with the following programme: i) initial denaturation at 94°C for 5 min, followed by 30 cycles of ii) denaturation at 94°C for 1 min., iii) annealing at 63°C for 2 min., iv) extension at 72°C for 1.5 min., v) final extension at 72°C for 10 min. The separation of the amplified DNA fragments was done by horizontal electrophoresis in 2% agarose gel TopVision Agarose (Thermo Scientific, USA) at 100 V for 1.30 hours. The gel was stained with safe dye peqGREEN (VWR International, Belgium) and visualized and documented with UV Transilluminator (ImageQuant 150, GE Healthcare). To determine molecular weight marker Gene Ruler 100 bp DNA Ladder (Thermo Scientific, USA) was used.

Isolation of *Staphylococcus aureus* from fish

A total of 10 g of the sample were weighed in a Stomacher bag and homogenized with 90 ml Brain Heart Infusion stock (Merck, Germany) and incubated at 37°C for 24 hours. After those cultures were made on the surface of two petri dishes with pre-spilled Baird-Parker agar (Merck, Germany) containing yolk emulsion and potassium tellurite. The cultured petri dishes were incubated in a thermostat at 37°C for 48 hours. At the end of the incubation period, colonies with typical *S. aureus* characteristics (black colour and lightening of the area around the colony) were counted. In the bacteriological examination, up to 3

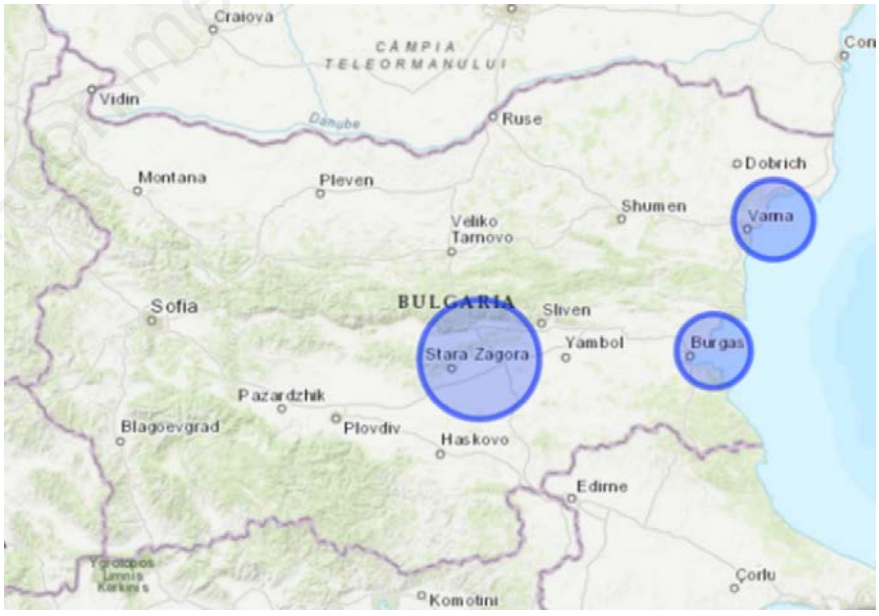

Figure 1. Study area map.

Table 1. Primers used for identification of *Vibrio parahaemolyticus*.

Primer	Gene	Oligonucleotide sequence (5'-3')	Product size (bp)	Source
toxR-F	*toxR*	GTC TTC TGA CGC AAT CGT TG	368	Nelapati and Krishnaiah (2010)
toxR-R		ATA CGA GTG GTT GCT GTC ATG		

colonies with *S. aureus* characteristics were selected, with which cultures were made on Petri dishes with Tryptic Soy agar (Merck, Germany) in order to obtain pure cultures.

Identification of *Staphylococcus aureus* via polymerase chain reaction (PCR)

DNA extraction

The same DNA extraction protocol as for *V. parahaemolyticus* was applied.

PCR protocol

Primers specific to 16S rRNA gene of *Staphylococcus* spp. and *nuc* gene of *S. aureus* were purchased from Eurofins Genomics (Germany) (Table 2). The reaction mixture for PCR had volume of 25 µl and contained 1 µl of the extracted DNA, 12.5 µl TopTaq Master Mix (QIAGEN, Germany), 0.2 µl of each primer (Eurofins Genomics, Germany) and 11.1 µl water free of nucleases. The polymerase chain reaction was performed in a thermocycler QB-96 (Quanta Biotech, USA) with the following programme: i) initial denaturation at 94°C for 5 min. followed by 30 cycles of ii) denaturation at 94°C for 1 min. iii) annealing at 55°C for 30 sec., iv) extension at 72°C for 1 min., v) final extension at 72°C for 7 min. The separation of the amplified DNA fragments was made by horizontal electrophoresis in 2% agarose gel TopVision Agarose (Thermo Scientific, USA) at 100 V for 1.30 hours. The gel was stained with peqGREEN (VWR International, Belgium) and visualized and documented with UV Transilluminator (ImageQuant 150, GE Healthcare). To determine the molecular weight Gene Ruler 100 bp DNA Ladder marker (Thermo Scientific, USA) was used.

Determining total bacterial count, total *Vibrio* spp. and *Staphylococcus* spp. count in mussels

A method for determining total bacterial count and number and species diversity of *Vibrio* spp. and *Staphylococcus* spp. in mussels by means of their colonial morphology onto selective agars was also used. For this purpose, ten-fold dilutions in 9 ml Maximum Recovery Diluent tubes (Merck, Germany) were made from the Stomacher bag prior to enrichment, and 0.1 ml cultures were made on Baird Parker agar (Merck, Germany) and TCBS agar (Himedia, India) after each dilution, as well as inoculation with 1 ml in empty Petri dishes, covered with molten and cooled Plate count agar (Himedia, India). After incubation at 30°C for 24-48 hours, the species of microorganisms were counted.

Statistical analysis

The results of the microbiological indicators were statistically processed by means of GraphPad InStat 3 (GraphPad Software, San Diego, CA) and presented as mean±SD. One-way ANOVA with Tukey post hoc test was performed to compare the significance of the differences between the wild and farmed mussels. The statistical significance was determined at $p<0.05$.

Results and discussion

Typical *V. parahaemolyticus* colonies were isolated from 2 (6%) of a total of 33 samples of frozen sea fish and from 20 (31%) of a total of 64 mussel samples. The species identification of *V. parahaemolyticus* via PCR confirmed all typical colonies in the TCBS agar with dark bluish-green colour (Figure 2). The expected amplicon value of 368 bp was established in both Atlantic cod (*Gadus morhua*) and Alaska pollock (*Gadus chalcogrammus*) isolates, as well as in all mussel isolates (Figure 3). Fish and mussels can be contaminated with pathogenic bacteria from the water they live in. Mussels feed by filtering sea water and, thus, are able to accumulate pathogenic microorganisms naturally occurring in it as

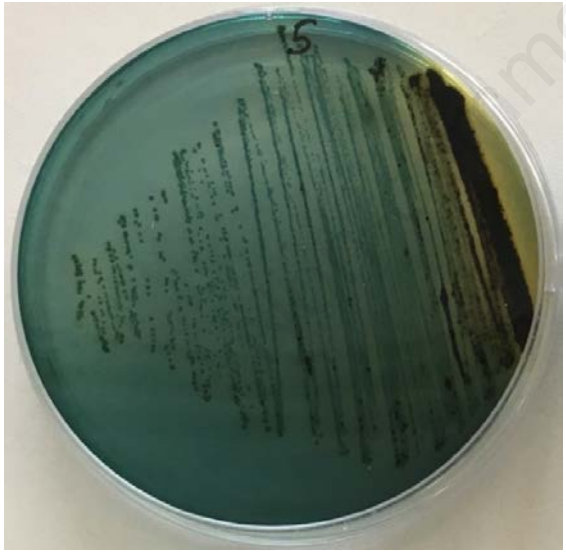

Figure 2. Pure culture of typical *Vibrio parahaemolyticus* colonies.

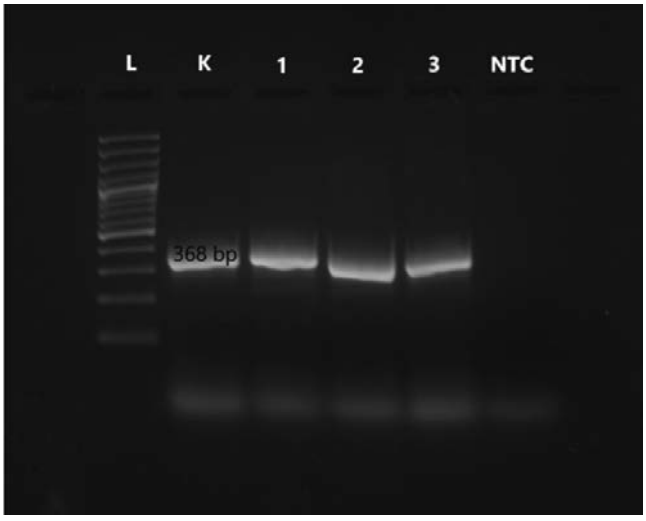

Figure 3. Identification of *Vibrio parahaemolyticus* via PCR. L, 100bp DNA Ladder; K, positive control; 1, 2 and 3 mussel isolates; NTC, negative control.

Table 2. Primers used for identification of *Staphylococcus* spp. and *Staphylococcus aureus*.

Gene	Primer	Oligonucleotide sequence (5'-3')	Product size (bp)	Source
16S rRNA	16s rRNA forw 16s rRNA rev	GTA GGT GGC AAG CGT TAT CC CGC ACA TCA GCG TCA G	228	Monday and Bohach (1999)
nuc	nuc forw nuc rev	GCG ATT GAT GGT GAT ACG GTT AGC CAA GCC TTG ACG AAC TAA AGC	279	Brakstad et al. (1992)

Vibrio spp. (Iwamoto *et al.,* 2010). Alive fish can be contaminated with pathogenic bacteria such as *Vibrio* spp., *Listeria monocytogenes* and *Aeromonas* spp., and simply the growth of these bacteria can be accepted as danger (Yucel and Balci, 2010). We used TCBS agar and *toxR* gene to isolate and identify *V. parahaemolyticus*, reliable for that purpose (Sujeewa *et al.,* 2009; Iwamoto *et al.,* 2010). By these methods we found two *V. parahaemolyticus* isolates in frozen fish, which confirmed the statement by Vasudevan *et al.* (2002) and Zhang *et al.* (2014) that this pathogen can survive at -18°C for a certain period. Although freezing inactivates a significant number of *V. parahaemolyticus* cells in fish, it cannot be accepted as a reliable method since reduction time depends on the initial *V. parahaemolyticus* count. According to Sanjeev and Stephen (1994), *V. parahaemolyticus* can survive in crab meat at -20°C up to 16 days and in lobster meat at -18°C up to 3 months. Unlike our results, Sanjeev and Stephen (1994) did not establish *V. parahaemolyticus* in frozen fish, but they isolated it in 4.4% of the cooked, picked and frozen crab meat samples. According to Letchumanan *et al.* (2015) and Di *et al.* (2017), *V. parahaemolyticus* is widespread in sea and estuarine water all over the world. The bacterial growth and count are directly related to water temperature and are the highest in the summer months and lower in the winter months. Mussel samples in our study were collected in the summer months, which is probably the reason for the relatively high number of mussels contaminated with *V. parahaemolyticus*. This is confirmed by Di Pinto *et al.* (2008), who isolated *V. parahaemolyticus* from 23 (16%) in a total of 144 mussels (*Mytilus galloprovincialis*) samples taken during the summer months from purification centers in Italy. This comes to prove that purification centers are not able to eliminate *V. parahaemolyticus* fully. In another study, Normanno *et al.* (2006) isolated *V. parahaemolyticus* from 47 (7.8%) in a total of 600 mussels (*Mytilus galloprovincialis*) samples taken from stores in Italy. The study of Henigman *et al.* (2011) showed that 24 (14.2%) of mussels (*Mytilus galloprovincialis*) samples of a total of 168 were contaminated with *V. parahaemolyticus*.

Typical colonies for coagulase-positive staphylococci of all 33 sea fish samples

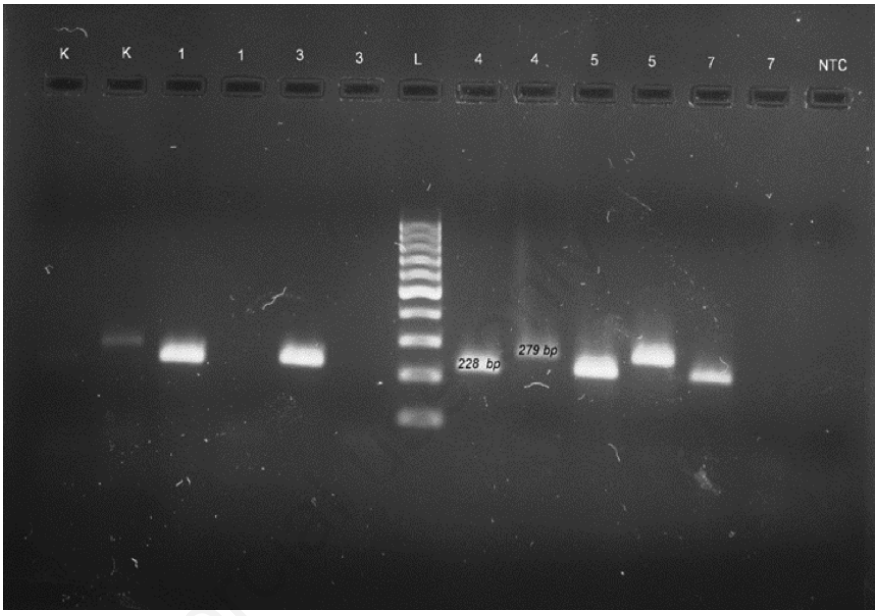

Figure 4. Identification of *Staphylococcus aureus* via PCR. K – positive control; 1 – *G. chalcogrammus* isolate; 3 – *G. chalcogrammus* isolate; L – 100 bp DNA Ladder; 4 – *M. hubbsi* isolate; 5 – *G. morhua* isolate; 7 – *G. chalcogrammus* isolate; NTC – negative control.

Table 3. *Staphylococcus* spp. and *Staphylococcus aureus* isolates from frozen fish.

Fish	Number of samples	Number of positive samples for *Staphylococcus* spp.	Number of positive samples for *S. aureus*
Baird's slickhead (*Alepocephalus bairdii*)	2	2	0
Atlantic cod (*Gadus morhua*)	4	3	1
Patagonian grenadier (*Macrruronus magellanicus*)	3	3	0
Argentine hake (*Merluccius hubbsi*)	5	3	1
Alaska pollock (*Gadus chalcogrammus*)	19	19	0
Total	33	30	2

Table 4. Microbiological indicators of mussels (log cfu/g meat).

	Wild mussels Mean±SD	Farmed mussels Mean±SD	Significance (p)
Total bacterial count	6.54±6.18	6.69±6.28	*
Vibrio spp.	5.90±5.84	5.83±5.76	ISD
Vibrio cholerae	5.14±5.13	5.38±5.37	ISD
Vibrio parahaemoliticus	4.45±4.69	4.99±4.89	ISD
Vibrio vulnificus	5.42±5.53	5.80±5.51	ISD
Staphylococcus spp.	5.54±5.82	5.37±5.24	ISD
Staphylococcus aureus	4.50±4.76	3.14±3.46	ISD

ISD – insignificant difference, >0.05; * <0.05

were selected and identified via PCR. A total of 30 isolates (91%) were confirmed as *Staphylococcus* spp., of these 2 (7%) were identified as *S. aureus* (Table 3). The expected amplicon size was 228 bp for *Staphylococcus* spp. and 279 bp for *S. aureus*. The *S. aureus* amplicon was found in two Argentine hake (*Merluccius hubbsi*) and Atlantic cod (*Gadus morhua*) isolates (Figure 4). Although *S. aureus* is widespread, food remains to be the most important source of infection for humans (Wu *et al.,* 2019). Seafoods are rich in protein, which is decomposed to low molecular peptides and amino acids maintaining the growth of *S. aureus*. Food causing staphylococcal intoxication are canned, smoked, and salty products, frozen fish products, boiled fish paste and fish sausages that inhibit the growth of competing bacteria (Simon and Sanjeev 2007). *S. aureus* is not part of the natural fish flora, where it is found because of contamination from workers, equipment, and environment from catching to processing. Contamination with microorganisms happens through the water used during processing, workers, inadequate cleaning procedures (Murugadas *et al.,* 2017), inadequate and unhygienic treatment, inadequate storage, and cross contamination (Simon and Sanjeev, 2007). Zarei *et al.* (2012) isolated *S. aureus* from 3 (15%) of a total of 20 samples of frozen fish fillets. Simon and Sanjeev (2007) recorded *S. aureus* in 6 (33%) of a total of 18 frozen fish samples in quantities from $0.72x10^3$ to $2.4x10^3$. Unlike that study, we found occurrence of *S. aureus* in 2 (6%) of 33 frozen fish samples. Wu and Su (2014) confirmed the findings in these studies that *S. aureus* can survive in frozen fish stored at -20°C.

The results from microbiological assays of mussels (Table 4) showed that regardless of their origin, mussels were highly contaminated with microorganisms exceeding the recommended values of $5x10^5$ cfu/g (*i.e.* 5.7 log cfu/g) for total bacterial count (ICMSF, 1986). Significant differences ($p<0.05$) were found in the total bacterial contamination between wild mussels (6.54 log cfu/g) and farmed mussels (6.69 log cfu/g). Cappello *et al.* (2015) also found higher values of the total bacterial count (7.34 log cfu/g) in mussels (*Mytilus galloprovincialis*) caught in the Lake Faro located on the north-eastern tip of Sicily (Messina, Italy). The total bacterial count in mussels depends on the bacteria count in the water they dwell in. This emphasizes the ability of *Mytilus galloprovincialis* to accumulate bacteria from the surrounding environment. The *Vibrio* spp., *V. parahaemolyticus*, *Staphylococcus* spp. and *S. aureus* count did not show any significant differences ($p>0.05$) between wild and farmed mussels either. The International commission on microbiological specifications of foods recommends a plan for sample taking and limits of *V. parahaemolyticus* in fresh and frozen bivalve mollusks consisting of the indicators n=10, c=1, $m=10^2$ cfu/g and $M=10^3$ cfu/g (ICMSF, 1986). Our results about *V. parahaemolyticus* from wild and farmed mussels exceed these recommended limits. The study by Lamon *et al.* (2019) showed lower amount (2.04 log cfu/g) of *Vibrio* spp. in 34 mussel samples collected from two class B harvesting areas located in Sardinia (Italy). Unlike our results, Yilmaz *et al.* (2005) did not find *V. parahaemolyticus* in 35 mussels (*Mytilus galloprovincialis*) samples harvested from approved shellfish waters in the Marmara Sea. We established in wild mussels that the average *S. aureus* count amounts to 4.50 log cfu/g, while in farmed mussels it was 3.14 log cfu/g. Yilmaz *et al.* (2005) found that the average *S. aureus* count in 35 mussels (*Mytilus galloprovincialis*) samples was 2.49 log cfu/g. It has been established that mussels with great microorganism count showed greater *S. aureus* count, too.

There are differences in the microbial contamination of seafood, which depends on the place of catch, such as near or far from the seashore or rivers and lakes. Health risk when consuming fish products from unpolluted seawater is small. Potential risks to consumer health from aquaculture products vary depending on the method of cultivation and include pathogenic bacteria and viruses, trematodes, drug residues and pesticides, and toxic metals (Okocha *et al.,* 2018). Adequate heat treatment kills pathogens, although seafood is often consumed raw or prepared in a way that does not kill microorganisms (Iwamoto *et al.,* 2010). In order to reduce the incidence of food poisoning when consuming raw bivalve molluscs, it is advisable to monitor for pathogenic bacteria potentially dangerous to human health such as *V. parahaemolyticus*. In this way, consumers will be informed about the possible dangers of consuming these products raw or insufficiently heat-treated (Normanno *et al.,* 2006).

Conclusions

Vibrio parahaemolyticus and *Staphylococcus aureus* are isolated from frozen sea fish and fresh mussels, which is a risk to consumer health. Enhanced monitoring of these pathogens in fish and mussels is needed to avoid risks to human health. The total bacterial count in mussels exceeds the recommended limit, which is why it is necessary to improve and strictly observe hygiene during processing and storage.

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