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Research Article

Occurrence of Vibrio and Other Pathogenic Bacteria in *Mytilus galloprovincialis* and *Venus gallina* Harvested from the Marmara Sea

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Abstract: The objective of this research was to determine microbiological quality of the *Mytilus galloprovincialis* and *Venus gallina* harvested in the Marmara Sea (Gelibolu Region). The samples were examined for total aerobic mesophilic bacteria (TAMB), coliform group bacteria, *Escherichia coli*, *Staphylococcus aureus*, Salmonella spp., *Vibrio cholerae,* and *Vibrio parahaemolyticus*. The microbiological analysis showed 2.1 x 10^4-1.9 x 10^6 cfu/g TAMB, 2.9 x 10^2-8.2x10^3 cfu/g coliform group bacteria, 78-2.5 x 10^2 cfu/g *E. coli*, 3.1 x 10^2-1.1 x 10^3 cfu/g *S. aureus* for the *Mytilus galloprovincialis* - *Venus gallina* mussels samples respectively. Salmonella spp., *Vibrio cholerae* and *Vibrio parahaemolyticus* were not detected in any samples examined. In general, *Venus gallina* samples had higher TAMB, coliform group bacteria, *E. coli* and *S. aureus* counts than the *Mytilus galloprovincialis* samples. The study confirms the risk associated with the consumption of mussels and the need of proper storage and preparation conditions prior to consumption. In addition, in order to prevent the health risks associated with mussels, heat treatment has to be applied prior to consumption.

Key Words: Salmonella spp., *Vibrio cholerae, Vibrio parahaemolyticus, Venus gallina, Mytilus galloprovincialis*

Marmara Denizinde Avlanan Kum (*Venus gallina*) ve Kara Midyelerinde (*Mytilus galloprovincialis*) Vibrio Spp. ve Diğer Patojen Bakterilerin Varlığı

Özet: Bu araştırmada Marmara denizinden (Gelibolu Bölgesi) avlanan kum (*Venus gallina)* ve kara midyelerinin (*Mytilus galloprovincialis)* mikrobiyolojik kalitesi belirlenmiştir. Midyelerin toplam aerobik mezofilik bakteri, koliform grubu bakteri, *Escherichia coli*, *Staphylococcus aureus* sayıları ile Salmonella spp., *Vibrio cholerea* ve *Vibrio parahaemolyticus* varlığı incelenmiştir. Kara midyelerinin toplam canlı bakteri sayısı 2,1 x 10^4 kob/g, koliform grubu bakteri 2,9 x 10^2 kob/g, *E. coli* 78 kob/g, *S. aureus* 3,1 x 10^2 kob/g olarak tespit edilirken kum midyelerinin toplam aerobik mezofilik bakteri sayısı 1,9 x 10^6 kob/g, koliform grubu bakteri 8,2 x 10^3 kob/g, *E. coli* 2,5 x 10^2 kob/g, *S. aureus* 1,1 x 10^3 kob/g olarak belirlenmiştir. Örneklerin hiçbirinde Salmonella spp., *Vibrio cholerea* ve *Vibrio parahaemolyticus* varlığı tespit edilememiştir. Genel olarak kum midyelerinin mikrobiyolojik yükü kara midyelerinin mikrobiyolojik yükünden daha yüksektir. Midyelerin sağlık açısından risk oluşturmasını engellemek için tüketimden önce yeterli ısıl işlem uygulanmalıdır.

Anahtar Sözcükler: Salmonella spp., *Vibrio cholerae, Vibrio parahaemolyticus,* Kum midye *(Venus gallina),* Kara Midye *(Mytilus galloprovincialis)*

Introduction

The global importance of food safety is not fully appreciated by many public health authorities despite the constant increase in the prevalence of foodborne illnesses. The surveillance for foodborne illness has been stressed because of centralization of food production and increased international trade and tourism. The responsibility for food safety has expanded from individuals to industries and government, and thus these changes have created potentials for epidemiological outbreaks of foodborne diseases (1).

Fish and shellfish products are subjected to mandatory inspection, including microbiological analyses for bacterial pathogens like *Vibrio cholerae* and *Vibrio parahaemolyticus*, by importing countries (2).

Vibrio cholerae causes the disease cholera and is transmitted directly by the fecal oral route, and indirectly through sewage contaminated water supplies. Human to

human contamination is not common. They are frequently present in fish and shellfish caught in coastal waters. Foods commonly associated with *Vibrio parahaemolyticus* and outbreaks are fresh crab, shrimp, lobster, cooked and refrigerated seafoods, insufficient cooked foods and improper cooling of food after cooking (3).

The aim of the present study was to investigate the counts of potential pathogens of public health concern in mussels.

Materials and Methods

Sixty samples of *Mytilus galloprovincialis* (35 out of 60 samples) and *Venus gallina* (25 out of 60 samples) harvested from approved shellfish waters in the Marmara Sea (Gelibolu Region) were examined for the presence of total bacteria, coliforms, *Escherichia coli*, *Staphylococcus aureus*, Salmonella, *Vibrio cholerae* and *Vibrio parahaemolyticus*.

The mussel samples were purchased from local fisherman. Samples were taken from commercially packed containers and, after purchase, were introduced into sterile bags and transported to the laboratory in insulated coolers with frozen gel-packs to maintain a temperature at around 4 ^{0}C. All samples were examined within 2 h of collection.

Each sample package was scrubbed under tap water to remove debris, allowed to dry, disinfected with 70% ethanol, and opened aseptically using a sterile knife. Mussel inner content was homogenized for 1 min prior to analysis.

Total aerobic mesophilic bacteria, coliform group bacteria, *Escherichia coli*, *Staphylococcus aureus*, Salmonella, *Vibrio cholerae* and *Vibrio parahaemolyticus* counts of the mussel samples were determined according to the BAM (4) procedure and reported as cfu/g where appropriate.

Total aerobic mesophilic bacteria were determined on Plate Count Agar (PCA) and after incubation at 35 ^{0}C for 48 h. The number of coliform group bacteria was determined with Violet Red Bile Agar (VRBA) after the incubation at 35 ^{0}C for 24 h. The presence of *Escherichia coli* was examined by transferring 1 ml of each sample dilution to sterile petri dishes followed by pouring 10 ml of Violet Red Bile Agar (with 4-methylum belliferryl-β-D-glucuronide (VRB-MUG)), tempered to 48 ^{0}C, into plates. The plates were swirled, allowed to solidify, overlaid with 3 to 5 ml of VRB-MUG and then incubated at 37 ^{0}C for 24 to 48 h. The plates were examined for typical coliform colonies which were counted to obtain a presumptive coliform count. Isolates that were Gram-negative and which produced acid and gas in lactose broth were recorded as confirmed coliforms. These plates were also examined under long wave ultraviolet (UV) light for the presence of fluorescent colonies, indicating possible presence of *E. coli*. Those with positive fermentation and gas production in lactose broth were further characterized as *E. coli* using indole, methyl red, Voges-Proskauer and citrate (IMVIC) identification tests.

The presence of *Staphylacoccus aureus* was tested by surface plating on prepoured and dried Baird-Parker agar enriched with egg-yolk tellurite. The plates were incubated at 35 ^{0}C for 48 h after which day were examined for typical *S. aureus* colonies. Presumptive colonies were transferred to the slants for *S. aureus* confirmation by Gram staining, catalase reaction and coagulase test.

For the Salmonella, 25 g samples were enriched in Selenite Cystine Broth for 24 h at 35 ^{0}C. The cultures were streaked onto Bismuth Sulfite Agar and incubated at 35 ^{0}C for 24 h. The typical Salmonella colonies were subjected to subsequent biochemical tests by using Triple Sugar Iron and Lysine Iron Agar slants.

The mussel samples were washed, scrubbed free of dirt, and shucked with a sterile knife. Meat and liquor from 8 to 12 mussels were diluted with an equal volume of phosphate buffered peptone water and homogenized in a sterile Waring blender. The homogenates were then processed as seawater samples (incubation in APW and then plating onto TCBS agar). The suspected colony types (yellow and green) were picked out, streaked onto nutrient agar plus 2% NaCl to obtain pure cultures, screened for cytochrome oxidase, and examined for NaCl requirement (0%, 6% and 8%). Other morphological, biochemical and cultural tests carried out were Gram staining, catalase reaction, carbohydrates fermentation on triple sugar iron (TSI) agar plus 2% NaCl, growth at 4, 35 and 40 ^{0}C, Voges–Proskauer assay, citrate assay, indole assay, gelatinase, ONPG hydrolysis, aminoacids decarboxylase reaction. After that a representative sample of isolates (54%) selected on the basis of the morphology of colonies was submitted to phenotypic

characterization by the mini automated API 20 E identification system ID 32 (Bio Merieux Sa, France) employing 1% sterile saline as inoculum diluent. The proportion of each species either in the water isolates, or in the mussel ones, is the mean value of the percentages of isolation of that species in the different samples examined. To enumerate the vibrios in seawater 1, 5 and 10 ml were filtered on 0.45 µm pore size filters and the filter disks were aseptically placed onto TCBS agar. After incubation for 2 days the emerging colonies of presumptive vibrios were counted according to the colony/forming units (cfu) method. Mean values for three replicate samples were determined and expressed as cfu/ml taking account of the dilution factor. For the vibrios quantitative research in mussels 0.1 ml of each mussel homogenate or appropriate decimal dilution of the homogenate (using a sterilized water sample from the collection area as diluent) was plated onto TCBS agar and after incubation for 2 days, the culturable vibrios were counted according to the colony forming units (cfu) method.

Results

Microbiological analysis results of the *Mytilus galloprovincialis* and *Venus gallina* (mussels) samples were summarized in Table 1 and Table 2.

The mean number of total aerobic mesophilic bacteria (TAMB) was determined as 2.1×10^4 cfu/g and 1.9×10^6 cfu/g in *Mytilus galloprovincialis* and *Venus gallina* samples, respectively.

Regarding coliform group bacteria counts in *Mytilus galloprovincialis*, the highest value was 8.0×10^2 cfu/g and lowest one was 5.8×10^1 cfu/g. The *E. coli* values ranged between <10 cfu/g and 5.9×10^2 cfu/g in the *Mytilus galloprovincialis.* Coliform bacteria counts were determined as 8.2×10^3 cfu/g in *Venus gallina*. The mean number of E. coli was determined as 2.5×10^2 cfu/g in Venus gallina.

S. aureus counts were determined as 3.1×10^2 cfu/g and 1.1×10^3 cfu/g in *Mytilus galloprovincialis* and *Venus gallina* samples, respectively. Mussel samples that have high levels of total aerobic mesophilic bacteria showed that coliform group bacteria, *E. coli* and *S. aureus* have increased.

Salmonella spp., *Vibro cholerae* and *Vibro parahaemolyticus* not detected in any samples examined.

Discussion

Two Vibrio species are examined separately as each is responsible for different disease syndromes, and their modes of action is different. There is a need for indicators of human-specific viral faecal contamination in order to improve the microbiological control of shellfish/seafoods.

It is well recognized that *Vibrio cholerae* is part of the natural bacterial flora of in aquatic environments. Diarrhea, a syndrome of cholera, results in loss of body fluids and minerals. In severe cholera cases, cardiovascular collapse and death may occur in a day's time. Cholera causes thousands of deaths each year, primarily in Asian countries (1). Organisms can be transmitted through contaminated water supplies and foods from those waters, particularly seafood. Humans are the only natural sources of the organism (5).

V. cholerae can be controlled by the use of purified water and by cooking seafood efficiently. *V. cholerae* does not multiply in water but can survive for up to two weeks. It is salt-tolerant, heat-sensitive, and destroyed by cooking.

Vibrio parahaemolyticus causes an illness characterized by severe abdominal pain, nausea, diarrhea, and vomiting. It is the most common foodborne illness in many Asian Countries where marine foods are frequently consumed (6). *V. parahaemolyticus* has been found in warm coastal waters of countries throughout the world (7). Most disease outbreaks occur during warm seasons. Growth of organisms occurs during handling of seafood. It does not grow under refrigeration. The organism is salt-tolerant, but it is very sensitive to heat and is destroyed by cooking.

Vibrio parahaemolyticus diseases are usually associated with the ingestion of raw or insufficiently cooked seafood, improper postharvest storage conditions or poor handling of seafood during preparation (8).

The most common halophilic *Vibrio* species isolated from both clinical and environmental samples were *Vibrio parahaemolyticus* (7). Environmental strains of *Vibrio parahaemolyticus* are typically not human pathogens. However, these strains cause disease in shrimps, oysters, mussels and other marine invertebrates (9) and in fishes (10). Refrigeration and proper cooking are important means of controlling *V. parahaemolyticus.* Consumption of raw fish and shellfish displays a risk for *V. parahaemolyticus* disease. After cooking, it is important

Table 1. Microbiological analysis results of *Mytilus galloprovincialis* (cfu/g).

Samples	Total Aerobic Mesophilic Bacteria	Coliform Group Bacteria	*E. coli*	*S. aureus*	Salmonella	*V. cholerae*	*V. parahaemolyticus*
1	1.0×10^5	2.1×10^3	5.9×10^2	6.3×10^2	-	-	-
2	2.4×10^4	1.3×10^2	5.9×10^1	5.4×10^2	-	-	-
3	1.5×10^4	8.0×10^2	9.6×10^1	3.0×10^2	-	-	-
4	4.4×10^4	3.0×10^2	6.3×10^1	8.5×10^1	-	-	-
5	1.4×10^4	2.0×10^2	2.8×10^1	1.6×10^2	-	-	-
6	2.0×10^4	9.6×10^1	<10	2.6×10^2	-	-	-
7	3.1×10^4	2.8×10^2	1.4×10^2	1.8×10^2	-	-	-
8	1.0×10^4	4.5×10^2	1.1×10^2	7.8×10^1	-	-	-
9	7.0×10^4	8.5×10^1	1.5×10^1	2.6×10^2	-	-	-
10	1.0×10^4	5.6×10^2	1.6×10^2	2.5×10^2	-	-	-
11	2.7×10^4	1.6×10^2	3.9×10^1	3.9×10^2	-	-	-
12	1.0×10^4	2.3×10^2	5.2×10^1	6.7×10^1	-	-	-
13	1.0×10^4	2.9×10^2	4.1×10^1	8.5×10^2	-	-	-
14	1.1×10^4	8.9×10^1	2.4×10^1	6.3×10^2	-	-	-
15	1.0×10^4	2.8×10^2	1.2×10^2	5.7×10^2	-	-	-
16	1.2×10^4	9.2×10^1	2.7×10^1	1.2×10^2	-	-	-
17	1.4×10^4	1.1×10^2	3.2×10^1	9.3×10^1	-	-	-
18	1.7×10^4	1.6×10^2	4.6×10^1	2.7×10^2	-	-	-
19	1.7×10^4	7.5×10^1	1.9×10^1	5.1×10^2	-	-	-
20	1.0×10^4	6.9×10^1	3.1×10^1	5.3×10^1	-	-	-
21	2.3×10^4	4.8×10^2	1.6×10^2	9.2×10^2	-	-	-
22	1.8×10^4	1.2×10^2	3.2×10^1	5.8×10^2	-	-	-
23	2.4×10^4	3.0×10^2	5.6×10^1	6.1×10^2	-	-	-
24	1.7×10^4	3.7×10^2	8.8×10^1	2.5×10^2	-	-	-
25	1.8×10^4	5.6×10^2	1.3×10^2	1.9×10^2	-	-	-
26	1.0×10^4	7.8×10^1	1.8×10^1	1.6×10^2	-	-	-
27	1.0×10^4	9.4×10^1	1.7×10^1	9.2×10^1	-	-	-
28	8.3×10^4	4.9×10^2	1.9×10^2	6.7×10^2	-	-	-
29	1.0×10^4	3.8×10^2	5.6×10^1	1.7×10^2	-	-	-
30	1.1×10^4	5.0×10^2	1.4×10^2	1.8×10^2	-	-	-
31	8.3×10^3	7.4×10^1	1.2×10^1	1.1×10^2	-	-	-
32	5.6×10^3	5.8×10^1	2.2×10^1	9.1×10^1	-	-	-
33	2.0×10^3	2.0×10^2	7.1×10^1	8.6×10^1	-	-	-
34	3.2×10^3	9.6×10^1	<10	1.5×10^2	-	-	-
35	6.1×10^3	8.5×10^1	2.8×10^1	1.3×10^2	-	-	-
Average	2.1×10^4	2.9×10^2	7.8×10^1	3.1×10^2			

- not detected

Table 2. Microbiological analysis results of the *Venus gallina* (cfu/g).

Samples	Total Aerobic Mesophilic Bacteria	Coliform Group Bacteria	*E. coli*	*S. aureus*	Salmonella	*V. cholerae*	*V. parahaemolyticus*
1	3.4×10^6	1.1×10^3	9.3×10^1	3.0×10^3	-	-	-
2	2.8×10^5	1.8×10^2	5.8×10^1	9.5×10^1	-	-	-
3	2.7×10^4	1.1×10^4	1.3×10^2	8.2×10^1	-	-	-
4	9.0×10^4	1.2×10^4	1.8×10^2	2.0×10^3	-	-	-
5	1.2×10^5	1.1×10^4	1.7×10^2	9.3×10^1	-	-	-
6	1.8×10^5	1.7×10^4	8.5×10^1	5.0×10^3	-	-	-
7	1.2×10^5	2.3×10^3	9.9×10^1	7.8×10^1	-	-	-
8	4.5×10^4	1.1×10^4	7.1×10^2	6.2×10^1	-	-	-
9	6.0×10^4	1.1×10^4	2.3×10^2	1.6×10^3	-	-	-
10	1.2×10^4	9.0×10^2	-	5.1×10^1	-	-	-
11	5.4×10^4	7.5×10^2	-	7.6×10^1	-	-	-
12	3.7×10^6	1.5×10^4	7.0×10^2	9.1×10^1	-	-	-
13	1.9×10^6	1.2×10^4	6.0×10^2	9.0×10^1	-	-	-
14	7.5×10^4	1.3×10^4	9.0×10^2	8.6×10^1	-	-	-
15	1.5×10^5	1.8×10^4	8.3×10^2	7.7×10^1	-	-	-
16	6.6×10^6	1.5×10^4	9.6×10^2	1.5×10^2	-	-	-
17	2.5×10^5	2.1×10^3	7.4×10^1	1.6×10^2	-	-	-
18	4.0×10^3	1.1×10^3	8.7×10^1	1.7×10^3	-	-	-
19	1.0×10^3	1.6×10^2	4.8×10^1	1.0×10^2	-	-	-
20	1.4×10^5	1.1×10^4	4.4×10^2	2.1×10^3	-	-	-
21	1.6×10^6	3.1×10^4	3.1×10^2	3.4×10^3	-	-	-
22	2.6×10^7	3.6×10^3	8.6×10^1	5.0×10^3	-	-	-
23	4.8×10^6	1.1×10^3	8.1×10^1	2.0×10^3	-	-	-
24	6.0×10^4	1.2×10^3	7.7×10^1	1.0×10^2	-	-	-
25	3.8×10^4	1.3×10^3	9.1×10^1	5.0×10^2	-	-	-
Average	1.9×10^6	8.2×10^3	2.5×10^2	1.1×10^3			

- not detected

to avoid cross-contamination between raw and cooked seafood.

Wong et al. (11) examined 686 samples of seafood imported from Hong Kong, Indonesia, Thailand and Vietnam for *Vibrio parahaemolyticus*, of which it was recovered from 315 (45.9%) samples. Wilson and Moore (12) could not detect Vibrio spp. from 433 shellfish samples in Northern Ireland. The results of the present study were agreement with Wilson and Moore (12). In another research, 26 out of 200 fresh seafood samples contained Vibrio spp., and the highest percentage of contamination was found in mussels (13). *Vibrio cholerae* and *Vibrio parahaemolyticus* were not isolated from any sample. These results were in disagreement with Croci et al. (14), Ripabelli et al. (15), Lee et al. (1), Karaçam et al. (16), Matte et al. (17), Shih et al. (18) and Berry et al., (19).

Turkish Seafood products governmental guideline (20) states that faecal coliforms and *E. coli* are limited as 300 MPN/100g, 230 MPN/100g respectively, and there should not be any Salmonella spp. and *Vibrio parahaemolyticus*. In general, the results of the present study are in accordance with the Turkish Seafood products guidelines, regarding to Salmonella and *Vibrio* spp. presence and none of the samples contained Salmonella spp. and *Vibrio parahaemolyticus*. Shellfish contamination from sewage-polluted waters is a serious and continuous problem: Salmonella have been shown to survive for over a month in the aqueous-sediment microcosm (21). Salmonella spp. were also frequently isolated from coastal waters and shellfish. D'Aoust et al. (22) did not detect Salmonella in fish and mussels. Salmonella spp. were not isolated from any samples. Our results are in disagreement with Wilson and Moore (12), who reported 8% Salmonella spp. isolation from 433 shellfish collected from authorized harvesting beds in Northern Ireland.

The mean number of total aerobic mesophilic bacteria (TAMB) was determined as 2.1×10^4 cfu/g and 1.9×10^6 cfu/g in *Mytilus galloprovincialis* and *Venus gallina* samples, respectively. In another study on the mussel samples sold in Samsun, Turkey, an average of 1.4×10^2-1.5×10^3 cfu/g total aerobic mesophilic bacteria count and 5–50 cfu/100g *E. coli* were determined while no *Vibrio parahaemolyticus* was found in any samples (23). The results were in agreement with Özçakmak and Arıcı (23).

In conclusion, no Salmonella spp., *Vibrio cholerae* and *Vibrio parahaemolyticus* were detected in analyzed samples. However, in order to prevent possible adverse effects of microorganisms living in polluted waters, necessary hygienic measurements in all production steps (harvesting, transporting, processing, etc.) should be taken. In addition, heat treatment during cooking process should be efficiently done in order to minimize foodborne diseases.

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