

Original Article

## Pathogenic bacteria load and safety of retail marine fish

Carga de bactérias patogênicas e segurança de peixes marinhos de varejo

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### Abstract

This study was conducted to determine the pathogenic bacteria load of 14 species of marine fish obtained from two suppliers (in Bitlis city, Turkey), which provide fish for fish markets, and to reveal the safety of the marine fish in terms of microbiological quality. The counts of total mesophilic aerobic bacteria (TMAB), *Staphylococcus aureus*, and *Escherichia coli*, the presence of *Salmonella* spp. and *Listeria monocytogenes* were determined in anchovy, horse mackerel, salmon, red mullet, gilthead seabream, bonito, pilchard, common sole, sand smelt, axillary seabream, seabass, Mediterranean horse mackerel, bluefish, and garpike. It was determined that common sole, axillary seabream, seabass, bluefish and Mediterranean horse mackerel obtained from both suppliers were unacceptable in terms of the counts of TMAB. Twenty-four samples exceeded the critical limit of *S. aureus* and all the samples were unacceptable according to the critical limit of *E. coli*. While *L. monocytogenes* was isolated from 50.0% of the samples, *Salmonella* spp. was isolated from 39.3% of the samples. These results showed that the pathogenic bacteria load of the analyzed marine fish was quite high and they were unsafe in terms of microbiological quality.

**Keywords:** marine fish, pathogenic bacteria, *S. aureus*, *Salmonella* spp., *L. monocytogenes*.

### Resumo

Este estudo foi realizado para determinar a carga de bactérias patogênicas de 14 espécies de peixes marinhos obtidos de dois fornecedores (na cidade de Bitlis, Turquia), que fornecem peixes para mercados de peixes, e para revelar a segurança do peixe marinho em termos de qualidade microbiológica. As contagens de bactérias aeróbias mesófilas totais (TMAB), *Staphylococcus aureus* e *Escherichia coli*, a presença de *Salmonella* spp. e *Listeria monocytogenes* foram determinados em anchova, carapau, salmão, salmonete, dourada, bonito, sardinha, linguado, cheirado, dourada-axilar, robalo, carapau-do-mediterrâneo, anchova e garpike. Foi determinado que o linguado, o dourada-axilar, o robalo, a anchova e o carapau-do-mediterrâneo obtidos de ambos os fornecedores eram inaceitáveis nas contagens do TMAB. Vinte e quatro amostras excederam o limite crítico de *S. aureus*, e todas as amostras foram inaceitáveis de acordo com o limite crítico de *E. coli*. Enquanto *L. monocytogenes* foi isolada em 50.0% das amostras, *Salmonella* spp. foi isolado de 39.3% das amostras. Esses resultados mostraram que a carga de bactérias patogênicas dos peixes marinhos analisados era bastante alta e eles eram inseguros em termos de qualidade microbiológica.

**Palavras-chave:** peixes marinhos, bactérias patogênicas, *S. aureus*, *Salmonella* spp., *L. monocytogenes*.

## 1. Introduction

Marine fish are important for the human diet due to its high nutrition value. However, it has also a great risk for pathogens in spite of this advantage. The number of pathogens is affected by microbial flora in the marine. Salt amount and temperature of the water, its pollution rate, catching methods and chilling situations are other factors that affect the microbial flora of products. Marine fish may contain many pathogens that cause foodborne diseases if they are caught from contaminated waters or people do not follow hygiene rules during its transportation and storage. Pathogenic bacteria in marine fish constitute majority of these pathogenic agents (Feldhusen, 2000).

Bacterial pathogens in marine fish are classified as three main groups. These are i) indigenous bacteria in natural

flora of water resources: virulent *Aeromonas hydrophila* strains, *Vibrio cholerae*, *Clostridium botulinum*, *Vibrio parahaemolyticus*, *Listeria monocytogenes* (*L. monocytogenes*) and *Vibrio vulnificus*; ii) nonindigenous bacteria occurring as a result of fecal contamination: few pathogenic *Yersinia enterocolitica* serotypes, *Campylobacter* spp., pathogenic *Escherichia coli* (*E. coli*), *Shigella* spp. and *Salmonella* spp.; and iii) contaminated bacteria: *Clostridium perfringens*, *Staphylococcus aureus* (*S. aureus*), *L. monocytogenes*, and toxigenic *Bacillus cereus* strains (Feldhusen, 2000).

Microflora of marine fish varies according to the genus, the water it lives in, season and development period. While the microorganisms in the skin, shell, gills, intestinal contents, and surrounding area cause first-

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Received: April 4, 2022 – Accepted: June 9, 2022



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degree contamination, secondary contamination occurs in processing, transportation, and marketing stages. Fish contain high moisture and protein and the pH values close to neutral; thus, microorganisms can develop in a very favorable media (Inal, 1992).

If the contaminated microorganism is a pathogen (disease-causing) and its count increases, it first causes economic losses due to the degradation of the product and then causes invasive infection at the consumer side. Moreover, if the pathogenic microorganism produces also toxin, more serious foodborne poisonings (intoxication and toxicoinfection) occur (Sahin and Basoglu, 2011).

*Staphylococcus aureus* causes intoxications by forming toxins (Kutlu et al., 2011) and the enterotoxin produced by *S. aureus* bacteria leads to Staphylococcal poisoning. Since the cooking process does not destroy enterotoxin, food poisoning symptoms occur in the person consuming the food (Durgac, 2006). Since the proteins and their amino acids in fish are disintegrated into peptides with low molecular weight, the growth of *S. aureus* is provided. *S. aureus* is not found in freshly caught seafood; contamination only takes place after the seafood has been caught. Workers carry enterotoxigenic *S. aureus* via skin infections, alongside their throats and noses, hence allowing it to easily reach its way into seafood. The contamination could be the cause of a combination of cross-contamination, unhygienic or incorrect processing and incorrect storage conditions (Simon and Sanjeev, 2007).

*Escherichia coli*, which is a typical host microorganism found in the human intestinal tract, may also be an agent causing intestinal infections (Ivnitski et al., 1999). Therefore, it is often used as the indicator of the fecal contamination. *Escherichia coli* is the part of the natural microflora in unpolluted warm tropical waters (Feldhusen, 2000).

*Salmonella* spp., which causes foodborne gastrointestinal diseases across the world, is one of the most important microorganisms (USDA, 2015). Fish caught from offshore seas are not contaminated with *Salmonella* spp. under normal circumstances. *Salmonella* spp. can be found especially in the fresh fish caught from waters in polluted coastal areas. The contamination of *Salmonella* spp. in the marine depends on various factors. One such factor is pollution from nearby poultry farms (Feldhusen, 2000). Salmonellosis based on *Salmonella* spp. is still an important infection that affects human life in many countries. Fifty-nine percent of Salmonellosis outbreaks could be traced to a specific food vehicle between 1973 and 1987 (Ivnitski et al., 1999).

In 1929, *L. monocytogenes* was deemed as a pathogen in humans. In 1981 it was also accepted as a foodborne pathogen (Farber and Losos, 1988). *Listeria monocytogenes* causes foodborne infections including diarrhea, bacteremia, meningitis, brain abscess, endocarditis, osteomyelitis or pneumonia. Listeriosis based on *Listeria* spp. has a high mortality rate although its prevalence is between 2-10 per million (Kilinc, 2001; Midi et al., 2005; Weinstein and Ortiz, 2004). The bacterial load of the environment affects the bacterial flora of the fish and *Listeria* species are contaminated by contaminated waters (Ben Embarek, 1994).

There is no information from Bitlis, Turkey about the pathogenic bacteria load or how safe commercially-sold marine fish. Bitlis, which is an inland city, is located 20-24 hours away from all of the coastal cities (Istanbul, Samsun, Izmir and Iskenderun) which are the main suppliers of marine fish. If the cold chain damaged during transportation, it is inevitable that the existing microbial load in the marine fish will gradually increase and the hygienic quality of it will gradually deteriorate until it will reach to Bitlis. So, the aim of this study was to determine the load of some pathogenic bacteria (*S. aureus*, *E. coli*, *Salmonella* spp., *L. monocytogenes*) in 14 species of marine fish obtained from two suppliers, which provide fish for fish markets, and to reveal the safety of the marine fish in terms of microbiological quality.

## 2. Materials and Methods

### 2.1. Materials

Fourteen species of marine fish obtained from the two suppliers between January and February 2017 were used as the material. The suppliers in Bitlis transport the fish in the boxes containing crushed ice from Istanbul, Samsun, Izmir or Iskenderun to Bitlis for 20-24 hours.

Three packets of small fish (one packet of small fish contains several whole fishes and its total weight was 250-300 g) and 3 pieces of big fish (1 piece weighing 500-600 g) were taken based on the existence of the same fish species in both suppliers. A total of 84 samples (14 species X 2 suppliers X 3 packets or 3 pieces) were analyzed from the following species; anchovy (*Engraulis encrasicolus*), horse mackerel (*Trachurus trachurus*), salmon (*Salmo salar*), red mullet (*Mullus barbatus*), gilthead seabream (*Sparus aurata*), bonito (*Sarda sarda*), pilchard (*Sardina pilchardus*), common sole (*Pegusa lascaris*), sand smelt (*Atherina hepsetus*), axillary seabream (*Pagellus acarne*), seabass (*Dicentrarchus labrax*), Mediterranean horse mackerel (*Trachurus mediterraneus*), bluefish (*Pomatomus saltatrix*), and garpike (*Belone belone*). The fish were placed into sterile sample bags and then into a foam box filled with ice. Next they then were sent to the laboratory and analyzed there on the same day.

### 2.2. Methods

To find out whether or not the samples contained any pathogenic bacteria, counts were done for each of the total mesophilic aerobic bacteria (TMAB), *S. aureus*, *E. coli*, *Salmonella* spp. and *L. monocytogenes*. Each analysis was carried out twice. Colonies were counted using a colony counter.

#### 2.2.1. Sample preparation

Twenty-five gram samples was taken from each fish flesh and placed in sterile Stomacher bags (Seward Medical, London, UK). Two hundred twenty-five milliliters of 0.1% peptone water (Merck, 107228) was added onto each sample. The mixture was then homogenized using a 400 mL lab blender (Stomacher,

IUL Instrument, Spain) at an appropriate speed for 120 seconds. This was the first dilution. Serial dilutions (1:10, diluents in 0.1% peptone water (Merck, 1.07228)) to  $10^{-8}$  were then prepared from the first dilution (Andrews and Hammack, 2003) and later used to analyze TMAB, *S. aureus* and *E. coli*.

## 2.2.2. Microbiological media and enumeration

### 2.2.2.1. Enumeration of TMAB

Zero point one milliliter of each serial dilution was spread onto Plate Count Agar (PCA, Biomark B298), and incubated for 24–48 h at 37 °C. All colonies that developed afterwards were TMAB (Maturin and Peeler, 2001).

### 2.2.2.2. Enumeration of *E. coli*

Zero point one milliliter of each serial dilution was spread onto Violet Red Bile Agar Methylumbelliferyl- $\beta$ -D-glucuronide (VRBA MUG, Biolife, 4021862), and then incubated for 18 h at 37 °C. After incubation, dark red colonies that were 1–2 mm in diameter were checked under a UV lamp and the fluorescent colonies from these colonies were evaluated as *E. coli* (Feng et al., 2020).

### 2.2.2.3. Enumeration of *S. aureus*

Zero point one milliliter of each serial dilution was spread onto Baird Parker Agar Base (Merck, 1.05406) containing Egg Yolk Tellurite Emulsion (Merck, 103785) and incubated for 48 h at 35 °C. Black glossy colonies with transparent zones that were 1–1.5 mm in diameter and developed after incubation were regarded as *S. aureus* (Tallent et al., 2016).

### 2.2.2.4. Isolation of *Salmonella* spp.

ISO 6579 was used to isolate *Salmonella* spp. First, a pre-enrichment culture was prepared. A twenty-five gram sample was homogenized in 225 mL of buffered peptone water (Merck 1.07228) and incubated for 16–20 hours at 35–37 °C for non-selective pre-enrichment purpose. After incubation, 0.1 mL and 10 mL of the pre-enrichment culture were transferred to 10 mL of Rappaport Vassiliadis Soy (RVS) Broth (Merck 1.07700) and 100 mL of Selenite Cystine (SC) Broth (Merck 1.07709), respectively, for selective enrichment. The RVS Broth was incubated for 24 hours at 42/43 °C, while the SC Broth was incubated for 24 hours at 37 °C. Later, the selective enrichment culture was streaked onto Brilliant Green Phenol Red Lactose Sucrose Agar (Merck 1.10747) as well as onto Xylose Lysine Tergitol-4 (XLT-4) Agar (Merck 1.13919) to which XLT-4 Supplement (Liofilchem, 80410) was added. Both were then left to incubate aerobically for 24 hours at 37 °C. To confirm pink-red colored suspicious colonies surrounded by a bright red zone on Brilliant Green Phenol Red Agar and black suspicious colonies on XLT-4 Agar were indeed *Salmonella* spp., the culture was then streaked onto Triple Sugar Iron Agar (Merck 1.03915) and Lysine Iron Agar (Merck 1.11640) and incubated for 24 hours at 37 °C. It was then inoculated into Urea Broth (Merck 1.08483), and incubated again for 48 hours at 37 °C. Last, a *Salmonella*

Latex Test Kit (Oxoid FT0203A) was used for serological confirmation (Andrews et al., 2016).

### 2.2.2.5. Isolation of *L. monocytogenes*

*Listeria monocytogenes* was isolated based on method of FDA's Bacteriological Analytical Manual (Hitchins et al., 2016). First, a pre-enrichment culture was prepared. A twenty-five gram sample was homogenized in 225 mL of Buffered *Listeria* Enrichment Broth (LAB, LAB138) and incubated at 30 °C for 4 hours. After incubation, *Listeria* Enrichment Selective Supplement (LAB, LABX139) was added and incubated at the same temperature for additional 44 hours. Afterwards, the selective enrichment culture was streaked onto Palcam *Listeria*-Selective Agar (Merck, 1.11755) containing *Listeria* Palcam Antimicrobial Supplement (Biolife, 4240042) as well as Oxford Agar (Merck, 1.07004) containing Oxford Selective Supplement (Merck, 1.07006). In order to confirm that suspicious colonies growing on Palcam *Listeria*-Selective Agar, being olive green-gray colored, having sometimes black centers but always with black zones and suspicious blackish green brown colonies with black zones and sunken centers growing on Oxford Agar, were indeed *L. monocytogenes*, colonies were inoculated on Tryptone Soya Agar (Oxoid, CM013B) containing Yeast Extract (Merck, 1.03753) and incubated at 30–37 °C for 24–48 hours (Hitchins et al., 2016).

Suspected isolates which matched to all identification parameters (Gram staining, catalase activity, motility test, fermentation of maltose, rhamnose, mannitol, and xylose, hydrolyzation of esculin, reduction of nitrate) according to reference method were evaluated as *L. monocytogenes* CAMP, *S. aureus* and *Rhodococcus equi* tests were also applied to all suspected samples (Hitchins et al., 2016).

## 2.2.3. Counting TMAB, *E. coli* and *S. aureus*, and indicating the presence/the absence of *Salmonella* spp. and *L. monocytogenes*

The TMAB, *E. coli*, and *S. aureus* counts were indicated as a logarithm of colony-forming units per gram (log cfu/g) of the sample according to the number of colonies, dilution factor and cultivation amount (Bell et al., 2005). The results for *Salmonella* spp. and *L. monocytogenes* were expressed as present (+) / absent (–) in 25 g (European Union, 2005).

## 2.2.4. Statistical analysis

The data was analyzed using Statistical Package for the Social Sciences (IBM SPSS Statistics, Version 25.0). The results were expressed in mean  $\pm$  standard deviation. The one-way analysis of variance was used to establish whether or not there was any difference between means according to microbial load. As the data were parametric and homogeneity of variance was provided, the Tukey test was conducted to find the sources of the differences between the groups. A *P* value of  $< 0.05$  was accepted as a significant difference for each fish (Sumbuloglu and Sumbuloglu, 2002).

### 3. Results

Table 1 shows the variations in the microbiological load of marine fish obtained from two suppliers. The count of TMAB is the most important analysis method for the overall microbiological quality of the seafood. The highest number of TMAB ( $6.78 \pm 0.01$  log cfu/g) was found in *P. saltatrix* obtained from the first supplier ( $P < 0.05$ ) (Table 1).

The difference between the majority of the tested fish species in terms of the count of TMAB was significant ( $P < 0.05$ ). But, considering counts of TMAB in the some fish species, *S. salar* from the first supplier, *S. pilchardus* from the second supplier, *A. hepsetus* from the second supplier and *T. mediterraneus* from the second supplier; *T. trachurus* from the first supplier and *S. salar* from the second supplier; *M. barbatus* from the second supplier and *S. sarda* from the second supplier; *S. pilchardus* from the first supplier and *A. hepsetus* from the first supplier; *P. lascaris* from the first and second supplier were similar ( $P > 0.05$ ) (Table 1).

The highest number of *S. aureus* ( $4.42 \pm 0.01$  log cfu/g) was determined in *P. saltatrix* obtained from the first supplier ( $P < 0.05$ ) (Table 1). The difference between the majority of the tested fish species in terms of the count of *S. aureus* was significant ( $P < 0.05$ ). But, considering counts of *S. aureus* in the some fish species, *T. trachurus* from the first supplier and *A. hepsetus* from the first supplier; *S. salar* from the first and second supplier; *M. barbatus* from the second supplier and *S. sarda* from the second supplier; *S. pilchardus* from the first supplier and *D. labrax* from the first supplier; *P. lascaris* from the first supplier and *B. belone* from the first supplier; *T. trachurus* from the second supplier, *S. aurata* from the second supplier and *P. saltatrix* from the second supplier; *S. aurata* from the second supplier and *S. pilchardus* from the second supplier; *S. aurata* from the first supplier and *S. sarda* from the first supplier; *T. trachurus* from the second supplier, *P. lascaris* from the second supplier, *D. labrax* from the first supplier and *P. saltatrix* from the second supplier; *S. sarda* from the first supplier and *D. labrax* from the second supplier were similar ( $P > 0.05$ ) (Table 1).

The highest number of *E. coli* ( $5.52 \pm 0.01$  log cfu/g) was determined in *P. lascaris* obtained from the first supplier ( $P < 0.05$ ) (Table 1).

The difference between the majority of the tested fish species in terms of the count of *E. coli* was significant ( $P < 0.05$ ). But, considering counts of *E. coli* in the some fish species, *S. aurata* from the second supplier, *S. pilchardus* from the first supplier, *A. hepsetus* from the first supplier and *P. acarne* from the second supplier; *M. barbatus* from the second supplier, *S. sarda* from the first supplier and *T. mediterraneus* from the second supplier; *S. aurata* from the first supplier, *B. belone* from the first supplier and *B. belone* from the second supplier; *S. sarda* from the second supplier and *P. acarne* from the first supplier; *P. lascaris* from the first supplier and *P. saltatrix* from the first supplier; *S. sarda* from the first supplier and *P. lascaris* from the second supplier; *S. sarda* from the second supplier, *S. pilchardus* from the first supplier, *A. hepsetus* from the first supplier and *P. saltatrix* from the second supplier; *S. aurata* from the second supplier and *T. mediterraneus*

from the first supplier; *P. acarne* from the second supplier and *T. mediterraneus* from the first supplier were similar ( $P > 0.05$ ) (Table 1).

While *Salmonella* spp. was isolated from 39.3% of the samples, *L. monocytogenes* was isolated from 50.0% of the samples (Table 1).

### 4. Discussion

The Turkish Food Codex Regulation on Microbiological Criteria (Turkey, 2011) states no limit values for the microbiological values of fresh chilled fish, but only the limit value for histamine level. For the count of total TMAB, Turkish Seafood Regulation (Turkey, 1995) states the critical limit as 6-7 log cfu/g for frozen fish. No sample exceeded the limit of 7 log cfu/g in the present study.

For newly caught fish and fish products, the total bacterial load of 2.00-6.00 log cfu/g is considered as normal and most of the consumer safety standards state that, the total bacterial load of 6.00 log cfu/g is acceptable (Olafsdottir et al., 1997). *Pegusa lascaris* obtained from both suppliers as well as *D. labrax*, *P. saltatrix*, *T. mediterraneus* obtained from the first supplier exceeded the limit of 6.00 log cfu/g (Table 1).

However, the International Commission on Microbiological Specifications for Foods (ICMSF) (ICMSF, 1986) accepts the limit of TMAB in fresh fish as  $5.00 \times 10^5$  cfu/g (5.69 log cfu/g). The count of TMAB was between 5.69-6.00 log cfu/g in *S. sarda* obtained from the first supplier, *P. acarne* obtained from the both suppliers and *D. labrax* obtained from the second suppliers (Table 1).

Erdem et al. (2010) found that the count of TMAB in anchovy (*E. encrasicolus*) samples sold at fish stalls in Trabzon, Turkey was  $2.3 \times 10^4$  cfu/g (4.36 log cfu/g) in the first sampling period (20/11/2008),  $1.6 \times 10^3$  cfu/g (3.20 log cfu/g) in the second sampling period (20/12/2008) and  $2.6 \times 10^3$  cfu/g (3.41 log cfu/g) in the third sampling period (27/12/2008). In the current study, the count of TMAB in *E. encrasicolus* samples was found to be lower than these values (Table 1).

The count of TMAB was found to be  $3.14 \pm 0.01$  log cfu/g in fresh bonito (*S. sarda*) samples and  $5.37 \pm 0.02$  log cfu/g in fresh anchovy (*E. encrasicolus*) samples in January 2015 (Corapci, 2018). These values are quite different from the values of the same fish species detected in the current study (Table 1).

The count of TMAB in *E. encrasicolus* obtained from the second supplier (Table 1) is compatible with a previous study reporting that the average count of TMAB was  $3.10 \pm 1.10$ - $3.42 \pm 1.96$  log cfu/g in anchovy (*E. encrasicolus*) samples sold at fish markets in three regions of Istanbul, Turkey (Mol and Tosun, 2011). The average count of TMAB was  $3.36 \pm 1.22$ - $4.04 \pm 1.21$  log cfu/g in horse mackerel (*T. trachurus*) in the same study (Mol and Tosun, 2011); whereas, it was  $4.62 \pm 0.01$ - $4.81 \pm 0.01$  log cfu/g in the current study (Table 1).

Bektas (2013) determined that the count of TMAB in gilthead seabream (*S. aurata*) samples taken from 4 different retail sale points in Isparta, Turkey was  $3.000 \pm 0.685$ - $5.810 \pm 0.469$  log cfu/g for 4 weeks. Mol and Tosun

**Table 1.** Microbial Load of Retail Marine Fish

Type of Fish (Species Name)	Supplier Number	TMAB (log cfu/g)	<i>S. aureus</i> (log cfu/g)	<i>E. coli</i> (log cfu/g)	<i>Salmonella</i> spp.	<i>L. monocytogenes</i>
<b>Anchovy</b> ( <i>Engraulis encrasicolus</i> )	1	2.83 ± 0.01 <sup>a*</sup>	2.44 ± 0.01 <sup>a</sup>	1.57 ± 0.01 <sup>a</sup>	-	+
	2	3.13 ± 0.01 <sup>b</sup>	1.24 ± 0.01 <sup>b</sup>	2.44 ± 0.01 <sup>b</sup>	+	-
<b>Horse Mackerel</b> ( <i>Trachurus trachurus</i> )	1	4.81 ± 0.01 <sup>c</sup>	3.08 ± 0.01 <sup>c</sup>	3.59 ± 0.01 <sup>c</sup>	+	-
	2	4.62 ± 0.01 <sup>d</sup>	3.62 ± 0.01 <sup>do</sup>	3.75 ± 0.01 <sup>d</sup>	+	+
<b>Salmon</b> ( <i>Salmo salar</i> )	1	5.37 ± 0.01 <sup>e</sup>	4.04 ± 0.01 <sup>e</sup>	3.98 ± 0.01 <sup>e</sup>	-	+
	2	4.79 ± 0.01 <sup>c</sup>	4.03 ± 0.01 <sup>e</sup>	3.40 ± 0.01 <sup>f</sup>	+	+
<b>Red Mullet</b> ( <i>Mullus barbatus</i> )	1	4.97 ± 0.01 <sup>f</sup>	3.92 ± 0.01 <sup>f</sup>	4.19 ± 0.01 <sup>g</sup>	-	+
	2	5.18 ± 0.01 <sup>g</sup>	3.79 ± 0.01 <sup>g</sup>	4.62 ± 0.01 <sup>h</sup>	+	+
<b>Gilthead Seabream</b> ( <i>Sparus aurata</i> )	1	4.37 ± 0.01 <sup>h</sup>	3.24 ± 0.01 <sup>h</sup>	3.47 ± 0.01 <sup>i</sup>	-	+
	2	5.25 ± 0.01 <sup>i</sup>	3.63 ± 0.01 <sup>dij</sup>	4.42 ± 0.01 <sup>jr</sup>	-	+
<b>Bonito</b> ( <i>Sarda sarda</i> )	1	5.80 ± 0.01 <sup>j</sup>	3.22 ± 0.01 <sup>hk</sup>	4.60 ± 0.01 <sup>hn</sup>	-	-
	2	5.16 ± 0.01 <sup>g</sup>	3.82 ± 0.01 <sup>gy</sup>	4.34 ± 0.01 <sup>kp</sup>	+	+
<b>Pilchard</b> ( <i>Sardina pilchardus</i> )	1	5.66 ± 0.01 <sup>k</sup>	3.52 ± 0.01 <sup>l</sup>	4.40 ± 0.01 <sup>jp</sup>	+	+
	2	5.37 ± 0.01 <sup>e</sup>	3.66 ± 0.01 <sup>in</sup>	4.71 ± 0.01 <sup>l</sup>	+	-
<b>Common Sole</b> ( <i>Pegusa lascaris</i> )	1	6.18 ± 0.01 <sup>l</sup>	2.92 ± 0.01 <sup>m</sup>	5.52 ± 0.01 <sup>m</sup>	+	-
	2	6.18 ± 0.01 <sup>l</sup>	3.58 ± 0.01 <sup>o</sup>	4.58 ± 0.01 <sup>n</sup>	+	-
<b>Sand Smelt</b> ( <i>Atherina hepsetus</i> )	1	5.67 ± 0.01 <sup>k</sup>	3.11 ± 0.01 <sup>c</sup>	4.40 ± 0.01 <sup>jp</sup>	+	-
	2	5.37 ± 0.01 <sup>e</sup>	4.12 ± 0.01 <sup>p</sup>	4.54 ± 0.01 <sup>q</sup>	-	+
<b>Axillary Seabream</b> ( <i>Pagellus acarne</i> )	1	5.74 ± 0.01 <sup>m</sup>	3.36 ± 0.01 <sup>q</sup>	4.31 ± 0.01 <sup>k</sup>	-	-
	2	5.88 ± 0.01 <sup>n</sup>	3.31 ± 0.01 <sup>r</sup>	4.42 ± 0.01 <sup>is</sup>	-	-
<b>Seabass</b> ( <i>Dicentrarchus labrax</i> )	1	6.07 ± 0.01 <sup>o</sup>	3.55 ± 0.01 <sup>lo</sup>	4.18 ± 0.01 <sup>g</sup>	-	-
	2	5.93 ± 0.01 <sup>p</sup>	3.84 ± 0.01 <sup>ky</sup>	4.51 ± 0.01 <sup>q</sup>	-	-
<b>Bluefish</b> ( <i>Pomatomus saltatrix</i> )	1	6.78 ± 0.01 <sup>q</sup>	4.42 ± 0.01 <sup>s</sup>	5.49 ± 0.01 <sup>m</sup>	-	-
	2	5.44 ± 0.01 <sup>r</sup>	3.60 ± 0.01 <sup>jdo</sup>	4.37 ± 0.01 <sup>op</sup>	-	-
<b>Mediterranean Horse Mackerel</b> ( <i>Trachurus mediterraneus</i> )	1	6.53 ± 0.01 <sup>s</sup>	4.22 ± 0.01 <sup>t</sup>	4.45 ± 0.01 <sup>rs</sup>	-	+
	2	5.35 ± 0.01 <sup>e</sup>	3.04 ± 0.01 <sup>u</sup>	4.62 ± 0.01 <sup>h</sup>	-	+
<b>Garpiki</b> ( <i>Belone belone</i> )	1	3.61 ± 0.01 <sup>t</sup>	2.92 ± 0.01 <sup>m</sup>	3.50 ± 0.01 <sup>i</sup>	-	-
	2	4.00 ± 0.01 <sup>u</sup>	3.45 ± 0.01 <sup>v</sup>	3.50 ± 0.01 <sup>i</sup>	-	+

\*Lower case letters indicate the difference between lines in the same column ( $p < 0.05$ ). The difference between the mean values indicated by the same letter is insignificant ( $p > 0.05$ ). +It indicate the presence of the microorganism in 25 g sample. -It indicate the absence of the microorganism in 25 g sample.

(2011) found that the count of TMAB was  $3.97 \pm 1.16$ - $5.74 \pm 0.42$  log cfu/g in seabass (*D. labrax*) and  $3.72 \pm 0.21$ - $5.68 \pm 0.31$  log cfu/g in gilthead seabream (*S. aurata*). In the current study, the count of TMAB was  $4.37 \pm 0.01$  log cfu/g and  $5.25 \pm 0.01$  log cfu/g in *S. aurata* and  $6.07 \pm 0.01$  log cfu/g and  $5.93 \pm 0.01$  log cfu/g in *D. labrax* (Table 1).

Papadopoulos et al. (2003) found that the initial mesophylic bacteria load of fresh seabass (*D. labrax*) was 4 log cfu/g and evaluated the fish quality as "good". This

level was well below the TMAB value of the seabass (*D. labrax*) samples in the current study (Table 1).

Kaba et al. (2013) determined the count of TMAB as  $4.45 \pm 0.00$  cfu/g ( $0.65 \pm 0.00$  log cfu/g) in fresh bonito (*S. sarda*) samples analyzed before making the ball from smoked bonitos. The count of TMAB was  $4.48 \pm 0.29$  log cfu/g in fresh bonito (*S. sarda*) sold at the fish market in Giresun, Turkey, between September and December 2015 and stored at 4 °C (Kulcu, 2017). The count of TMAB was found at high

levels ( $5.16 \pm 0.01$  log cfu/g and  $5.80 \pm 0.01$  log cfu/g) in *S. sarda* in the current study ( $P < 0.05$ ) (Table 1).

The count of TMAB was reported to be  $11.4 \times 10^3$  cfu/g ( $4.06$  log cfu/g) in fresh pilchard (*S. pilchardus*) caught from Dardanelles, Turkey in 2010 (Ormanci and Arik Colakoglu, 2014). It was  $5.37 \pm 0.01$  log cfu/g and  $5.66 \pm 0.01$  log cfu/g in *S. pilchardus* tested in the current study ( $P < 0.05$ ) (Table 1).

Total viable count of fresh Mediterranean horse mackerel (*T. mediterraneus*) caught in the gulf of Chalkidiki (North Greece) found to be  $4.09$  log cfu/g and  $3.97$  log cfu/g, in December and in January, respectively (Tzikas et al., 2007). The count of TMAB contained in the same fish species was higher than this value in the current study ( $5.35 \pm 0.01$  log cfu/g and  $6.53 \pm 0.01$  log cfu/g) (Table 1).

The count of *S. aureus* was reported to be  $2.00 \times 10^2$  cfu/g ( $2.30$  log cfu/g) in fresh pilchard (*S. pilchardus*) (Ormanci and Arik Colakoglu, 2014). In the current study, the count of *S. aureus* was  $3.52 \pm 0.01$  log cfu/g and  $3.66 \pm 0.01$  log cfu/g in the same fish species ( $P < 0.05$ ) (Table 1).

Bektas (2013) determined that the count of *Staphylococcus* spp. was  $2.477 \pm 0.870$ – $5.892 \pm 1.243$  log cfu/g in gilthead seabream (*S. aurata*) samples. The count of *S. aureus* was  $3.24 \pm 0.01$  log cfu/g and  $3.63 \pm 0.01$  log cfu/g in *S. aurata* in the current study ( $P < 0.05$ ) (Table 1).

Many people carry *S. aureus* in their face, nose, and mouth. It is an opportunistic pathogenic bacterium which causes many infections in humans, especially with a weak immune system. *S. aureus* contaminates the hands, face and nose and then the seafood during the processes (catching, placement in the boxes, transporting, selling, etc.). Then, it grows and produces enterotoxin until the seafood becomes ready to eat (Kocatepe et al., 2013).

The presence of *S. aureus* is not seen in the native flora of marine fish caught from the unpolluted marine (Hernandez-Herrero et al., 1999). Some infections may occur when *S. aureus*, which is transmitted from workers to food and transmitted to consumers from food products prepared or stored under bad conditions, exceeds 5 log cfu/g (Varnam and Evans, 1991).

The count of *S. aureus* in frozen fish should not be more than the level of 3 log cfu/g (Turkey, 1995). The limit of *S. aureus* in fresh fish is also accepted as 1000 cfu/g (3 log cfu/g) by ICMSF (1986). Twenty-four samples exceeded this critical limit, except for *E. encrasicholus* obtained from the both suppliers as well as *P. lascaris*, *B. belone* obtained from the first supplier (Table 1).

The vegetative cells of *S. aureus* can be inactivated with heat treatment done for 2–50 min at 60 °C (Ash, 1997). However, the enterotoxin produced by *S. aureus* is heat resistant and therefore does not disintegrate when cooked (Azanza et al., 2001). A heat treatment at higher temperatures is required to inactivate staphylococcal enterotoxin. The norms of heat treatment vary from several hours at 80–100 °C to 5–10 min at 121 °C depending on the suspending medium and toxin type (Mossel et al., 1995).

Staphylococcal poisoning may occur with *S. aureus* bacteria producing enterotoxin. It was stated that the number of pathogens should reach  $10^5$  cells/g for *S. aureus* to form enterotoxins (Aytaç and Taban, 2011). Therefore, one can deduce that none of the fish samples in the study

was risky in terms of enterotoxins – meaning that *S. aureus* could form (Table 1).

*Escherichia coli* bacteria were not found in gilthead seabream (*S. aurata*) samples sold at four points of sale (Bektas, 2013). The count of *E. coli* was found to be  $3.47 \pm 0.01$  log cfu/g and  $4.42 \pm 0.01$  log cfu/g in *S. aurata* and  $4.18 \pm 0.01$  log cfu/g and  $4.51 \pm 0.01$  log cfu/g in *D. labrax* in the current study ( $P < 0.05$ ) (Table 1).

*Escherichia coli* is accepted as a hygiene indicator which shows whether or not fecal contamination is found in the product. And furthermore, it is an agent of the dangerous foodborne diseases (Sahin and Basoglu, 2011). The count of *E. coli* in frozen fish should not be higher than 0.95–1.08 log cfu/g (Turkey, 1995). But, the samples were approximately 2–3 times higher, except for *E. encrasicholus* obtained from the first supplier (Table 1).

The standard recommended by ICMSF (ICMSF, 1986) is  $< 3.0$  MPN/g for *E. coli* in fish. According to this determination, all samples were unacceptable in the current study (Table 1).

*Listeria monocytogenes* and *Salmonella* spp. both should be not found in 25 g of fish (Turkey, 1995; ICMSF, 1986). Bektas (2013) determined the average count of *Salmonella* spp. in gilthead seabream (*S. aurata*) samples sold at the two points of sale between  $3.477 \pm 0.733$  log cfu/g and  $5.810 \pm 0.595$  log cfu/g, but *Salmonella* spp. was not found in gilthead seabream (*S. aurata*) samples sold at the other two points of sale. In the current study, the development of *Salmonella* spp. was not also observed in *S. aurata*, *P. acarne*, *D. labrax*, *P. saltatrix*, *T. mediterraneus* and *B. belone* obtained from both suppliers (Table 1).

Although *Salmonella* isn't psychrotrophic or native in the aquatic environment, it has been isolated from fish, sometimes (Youssef et al., 1992). If fish or other seafood is caught from fecally contaminated water, *Salmonella* spp. is found naturally in these foods (Banwart, 1981). The cause behind the presence of *Salmonella* spp. in some fish may be human-based contamination due to unhygienic applications (use of ice obtained from contaminated water for chilling fish, dirty boxes where fish are kept, inadequate hand hygiene of personnel, etc.). Varnam and Evans (1991) reported that a minimum internal temperature of 74 °C is required for disintegrating *Salmonella* spp. in foods with high  $a_w$  like analyzed fish samples.

*Listeria monocytogenes* was determined in *S. salar*, *M. barbatus*, *S. aurata* and *T. mediterraneus* obtained from both suppliers (Table 1).

The incidence of *L. monocytogenes* in some aquatic products has been reported in different researches (Rodassuarez et al., 2006; Rodrigues et al., 2015). Ben Embarek (1994) stated that the prevalence of *L. monocytogenes* in fresh fish and seafood varied from 4–12% in temperate areas and was lower (0–2%) in tropical areas. Fifty percent prevalence for *L. monocytogenes* (Table 1) determined in the current study was not similar to Ben Embarek (1994)'s study.

The presence of *Listeria* spp. was not observed in anchovy (*E. encrasicholus*) (Erdem et al., 2010). *Listeria monocytogenes* was not detected in *E. encrasicholus* obtained from the second supplier, but it was isolated in *E. encrasicholus* sample obtained from the first supplier in the current

study. The development of *L. monocytogenes* was not also observed in *P. lascaris*, *P. acarne*, *D. labrax* and *P. saltatrix* obtained from both suppliers (Table 1).

The growth of all analyzed species of microorganisms was seen in *T. trachurus*, *S. salar*, *M. barbatus*, and *S. sarda* obtained from the second supplier and *S. pilchardus* obtained from the first supplier (Table 1). The quantity of *T. trachurus*, *S. pilchardus* and *S. sarda* caught from the marines in Turkey was 8065.6 tons, 23425.7 tons and 7577.6 tons, respectively according to the fisheries statistics of Turkey Statistical Institute (TSI) in 2017. According to TSI, as of 2017, *S. pilchardus*, *T. trachurus* and *S. sarda* were among three most commonly caught and commercially sold fish- ranking at, third, fifth, and sixth places (TSI, 2018).

In mentioned studies, the fresh marine fish samples were obtained usually from the fish markets in the coastal cities or were analyzed immediately after they were caught (Papadopoulos et al., 2003; Tzikas et al., 2007; Erdem et al., 2010; Mol and Tosun, 2011; Bektas, 2013; Kaba et al., 2013; Ormanci and Arik Colakoglu, 2014; Kulcu, 2017; Corapci, 2018). However, it takes at least 20-24 hours for marine fish to reach Bitlis. During this period, every application that causes contamination increases the microbial load of marine fish.

The transport temperature and the hygienic quality of the transport boxes of fish, the ice used for cooling, the fish stalls in the markets, and the personnel's hands are very important. Inadequate hygiene practices cause pathogenic bacteria to contaminate fish.

Enterotoxigenic *E. coli* and *S. aureus* detected in seafood (Ayulo et al., 1994) and fish have been reported to be isolated from the hands and nasal mucosa of workers (Acco et al., 2003). Fish are contaminated with microorganisms in many ways, primarily unhygienic treatments, storage conditions and cross-contamination (Jablonski and Bohach, 2001; Huang et al., 2001).

The total heterotrophic plate count of ice samples taken from different fish markets was determined to be  $1.05 \pm 0.98-2.95 \pm 3.15$  log cfu/mL at 37 °C and  $1.38 \pm 1.35-3.00 \pm 3.25$  log cfu/mL at 5 °C (Economou et al., 2017).

The average count of TMAB was  $4.8 \times 10^2-8.5 \times 10^2$  cfu/cm<sup>2</sup> in the morning samples and  $6.3 \times 10^2-7.5 \times 10^2$  cfu/cm<sup>2</sup> in the evening samples in groups with the development of microorganisms in the study investigating the microbiological quality of the fish stalls. While the coliform group bacteria did not grow in the morning samples, the average coliform count was  $1.8 \times 10^2-2.2 \times 10^2$  cfu/cm<sup>2</sup> in the evening samples (Kocatepe et al., 2011).

The studies conducted by Acco et al. (2003), Economou et al. (2017), and Kocatepe et al. (2011) showed clearly some contamination ways to seafood and fish.

In conclusion, the results of the current study showed the rate of contamination by four pathogens in marine fish sold in Bitlis for the first time. The samples of common sole, axillary seabream, seabass, bluefish and Mediterranean horse mackerel obtained from both suppliers were unacceptable according to the counts of TMAB. Twenty-four samples exceeded the critical limit of *S. aureus*. All samples were unacceptable according to the critical limit of *E. coli*. While *L. monocytogenes* was isolated from 50.0% of the samples, *Salmonella* spp. was isolated from 39.3%

of the samples. These results showed that the pathogenic bacteria load of the analyzed marine fish was quite high and they were unsafe in terms of microbiological quality.

The study demonstrated that the conditions of appropriate cooling, transporting, processing, and storage in marine fish were very important. Risk assessment and risk reduction are necessary to reduce foodborne disease based on contaminated fish in Bitlis. In order to kill the pathogenic bacteria, the marine fish should be cooked well. An understanding of the impacts of *S. aureus*, *E. coli*, *Salmonella* spp. and *L. monocytogenes* and other pathogenic bacteria levels in marine fish are necessary to develop control measures to reduce the risk of infection caused by these microorganisms. Risk management should be taken into account throughout the whole food chain from the processing of raw material to consumption and should be applied in the context of proper food safety infrastructures such as regulation application and the systems of food product tracing and traceability.

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