



Occurrence and characteristics of staphylococci and enterococci in retail fish used for human consumption in Turkey

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Abstract

A total of 163 (66.0 %) *Staphylococcus* isolates and 52 (21.0 %) *Enterococcus* isolates were isolated from 247 fish samples consisting of Atlantic salmon (*Salmo salar*), Atlantic bonito (*Sarda sarda*), Sea trout (*Salmo trutta*), and European seabass (*Dicentrarchus labrax*). The *Staphylococcus* isolates were identified as *S. aureus* (8.0 %), *S. intermedius* (6.7 %), *S. warneri* (32.0 %), *S. hemolyticus* (22.7 %), *S. saprophyticus* (7.4 %), *S. simulans* (5.5 %), *S. capitis* (5.5 %), *S. xylosus* (4.9 %), *S. epidermidis* (4.3 %), *S. schleiferi* (1.8 %) and *S. caprae* (1.2 %). The *Enterococcus* isolates were identified as *E. faecalis* (46.1 %), *E. avium* (25.0 %), *E. solitarius* (11.5 %), *E. gallinarium* (7.7 %), *E. casseliflavus* (6.0 %), *E. maladoratus* (1.9 %), and *E. flavescens* (1.9 %). The majority of *Staphylococcus* strains had biofilm formation (93.8 %), lipase production (89.6 %), slime production (84.0 %), hemolytic activity (69.9 %), DNase activity (63.1 %) and protease production (57.0 %). Biofilm formation, slime formation, DNase activity, proteolysis, and hemolysis were detected in 94.2 %, 90.3 %, 57.7 %, 36.5 %, and 3.8 % of *Enterococcus* strains, respectively. None of the *Enterococcus* species had lipolytic activity.

Keywords: *Staphylococcus*, *Enterococcus*, biofilm, slime, protease, lipase, DNase, hemolysin.

Türkiye'de tüketime sunulan perakende balıklarda stafilocok ve enterokok varlığı ve özellikleri

Öz

Araştırmamızda, Atlantik somonu (*Salmo salar*), palamut (*Sarda sarda*), deniz alabalığı (*Salmo trutta*) ve avrupa levreğinden (*Dicentrarchus labrax*) oluşan 247 balık örneğinden, toplam 163 (%66.0) *Staphylococcus* ve 52 (%21.0) *Enterococcus* izole edilmiştir. *Staphylococcus* izolatları, *S. aureus* (%8.0), *S. intermedius* (%6.7), *S. warneri* (%32.0), *S. hemolyticus* (%22.7), *S. saprophyticus* (%7.4), *S. simulans* (%5.5), *S. capitis* (%5.5), *S. xylosus* (%4.9), *S. epidermidis* (%4.3), *S. schleiferi* (%1.8) ve *S. caprae* (%1.2) olarak adlandırılmıştır. *Enterococcus* izolatları ise, *E. faecalis* (%46.1), *E. avium* (%25.0), *E. solitarius* (%11.5), *E. gallinarium* (%7.7), *E. casseliflavus* (%6.0), *E. maladoratus* (%1.9) ve *E. flavescens* (%1.9) olarak adlandırılmıştır. *Staphylococcus* izolatlarının çoğunda biyofilm oluşumu (%93.8), lipaz üretimi (%89.6), slime üretimi (%84.0), hemolitik aktivite (%69.9), DNaz aktivitesi (%63.1) ve proteaz üretimi (%57.0) tespit edilmiştir. *Enterococcus* izolatlarının sırasıyla %94,2'sinde, %90.3'ünde, %57.7'sinde, %36.5'inde ve %3.8'inde biyofilm oluşumu, slime oluşumu, DNaz aktivitesi, proteoliz ve hemoliz tespit edilmiştir. *Enterococcus* türlerinin hiçbirinde lipolitik aktivite belirlenmemiştir.

Anahtar Kelimeler: *Staphylococcus*, *Enterococcus*, biyofilm, slime, proteaz, lipaz, DNaz, hemolizin.

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1. Introduction

Turkey has more than 8000 km of coastline in the Mediterranean, Aegean Sea, Marmara Sea and the Black Sea. In Turkey, 432. 246 tons of fish from seas and 37. 096 tons from inland waters were caught in 2011. In the same year, 187.343 tons of fish were aquacultured. In this year, about 168. 340 tons of total fishes were consumed in Ankara [1].

Retail stores are the endpoint of the seafood production chain. Although many bacterial species are not found in the normal flora of seafood, they are transmitted from food processing surfaces through cross-contamination [2], [3].

Although staphylococci are part of the normal microbiota of humans and animals, they are not members of the normal flora of fish. The presence of staphylococci in fish is an indication of poor hygiene of product handling personnel or disease in fish [4]. Humans are common carriers of *S. aureus* in the nose, throat, and skin infections and can easily be transferred to food during handling. Some strains of *Staphylococcus* can cause food poisoning by producing enterotoxins in food products [5]. Coagulase-negative staphylococci (CNS), which are generally considered as bacteria of low pathogenicity, have been found as causative agents of severe or even lethal infections in immunocompromised patients [6]. The virulence and pathogenicity of staphylococci are closely related to a wide variety of extracellular enzymes associated with their ability to colonize within the host and induce lysis of phagocytic cells. Examples of virulence factors for *S. aureus* are leukocidin, hyaluronidase, capsule, catalase, hemolysins, leukotoxin, proteases, lipases, deoxyribonucleases (DNase), and enterotoxins. Similar enzyme activities have been observed in CNS [7].

Enterococci are members of the normal flora of the gastrointestinal tract of humans and animals. They are also isolated from soil, water, plants, raw vegetables, and various foods, probably because of the contamination of the environment by human and animal feces [8]. Although regarded as harmless commensals, recently, enterococci have emerged as a cause of worldwide nosocomial infections in immunocompromised patients. Within the genus *Enterococcus*, *E. faecalis* is responsible for the majority of infections, followed by *E. faecium* [8].

In recent years, many virulence factors such as cytolysins, gelatinase, serine protease, hyaluronidase, aggregation agent, extracellular surface protein, cell wall adhesins and biofilm formation have been identified in enterococci [9]. Although isolates containing virulence factors have been identified in foods, no food-borne enterococcal infection has been reported yet [9].

Up to now, virulent staphylococci and enterococci originating from meat, milk and dairy samples [10], [11] as well as clinical samples [12], [13] have been reported. However, there is a lack of information on the characterization of staphylococci and enterococci from fish samples sold in the fish markets.

Therefore, the present study aimed to investigate the biofilm, slime, protease, lipase, DNase and hemolysins production of staphylococci and enterococci isolated from fish samples in Ankara, Turkey.

2. Material and Method

2.1. Sample Collection and Preparation

Two hundred forty seven fish samples (70 Atlantic salmon, 62 Atlantic bonito, 60 Seat trout, 55 European seabass) were bought from four different fish markets in Ankara, Turkey. Individual fish were collected in polyethylene bags and transported to the laboratory in an ice chest. They were immediately analyzed microbiologically for staphylococci and enterococci. Gill and intestine of each fish were inoculated into tryptic soy broth (TSB; Oxoid, Basingstoke, Hampshire, UK) for enrichment at 37 °C for 24-48 h.

2.2. Isolation and Identification of *Staphylococcus* spp.

From each sample, isolation and identification procedures were performed following the method of Gundogan et al., 2013 [11]. Coagulase activity of *Staphylococcus* spp. was determined by the method described by Quinn et al. [14].

2.3. Isolation and Identification of *Enterococcus* spp.

Isolation and identification of *Enterococcus* spp., also was done by following the method of Gündoğan et al. [11]. API 20 STREP (Bio Merieux SA, Marcy-l'Etoile, France) test kit was used to identify the isolates at the species level. *E. faecalis* CCM 254, *E. faecium* CCM 2518, and *Enterococcus durans* CCM 5612 were used as control strains.

2.3. Determination of Slime Formation

Production of slime from all isolates was studied by the cultivation of the isolates on CRA (Congo Red Agar, Oxoid). After incubation, bright black colonies were established as slime positive [10], [11].

2.4. Determination of Quantitative Biofilm Formation

Biofilm-forming ability was measured by the determination of adhesion to polystyrene microtiter plates according to the protocol of Christensen et al. [15]. The adherence ability of the tested strains was classified into four categories based on the OD: “ $OD \leq OD_c$: non-adherent, $OD_c < OD \leq 2XOD_c$: weakly adherent, $2XOD_c < OD \leq 4XOD_c$: moderately adherent, $4XOD_c < OD$: strongly adherent”. All tests were carried out three times and the results were averaged.

2.5. Determination of Hemolysin Production, DNase, Protease and Lipase Activity

Hemolysin activity was determined on blood agar base (BAB, Oxoid) containing 5% defibrinized sheep blood. After 48 h at 37°C incubation, hemolytic activity was determined by evaluating the patency zones around the colonies [9]. DNase agar (Oxoid) was used to determine DNase activity. After overnight incubation, the isolates were considered DNase positive with the formation of clear colored areas around the colonies when 1 N HCl was poured into the plates [10], [11]. For determining proteolysis, the isolates were inoculated on skimmed milk agar (SMA, Oxoid). The plates were incubated for 10 days at +20°C. After incubation, a clear zone of casein hydrolysis was observed directly on SMA [16]. Nutrient Agar (Oxoid) containing 1% tributyrin (Fluka, Buchs, Germany) was

used to study lipolytic activity under psychrotrophic conditions. The isolates were inoculated on tributyrin agar plates and incubated for 10 days at +20°C. The presence of clear zones was taken as an indication of positive lipase activity [16].

2.6. Statistical Analysis

The Chi-square (χ^2) tests were used to determine statistically significant differences in the prevalence of *Staphylococcus* and *Enterococcus* in fish samples. P values of less than 0.05 were considered significant.

3. Results and Discussion

Staphylococci and enterococci are inhabitants of soil, water, plants and the wide range animals. That means they could enter into the food chain and contribute to disease and spoilage [10, 11]. The occurrence, origin, and species distribution of

Staphylococcus spp. and *Enterococcus* spp. in fish samples marketed in Ankara are shown in Table 1 and Table 2.

Table 1. Prevalence of *Staphylococcus* spp. and *Enterococcus* spp. in fish samples

Fishes	No. of tested samples	No. of Staph. Isolate (%)	No. of Ent. Isolate (%)
Freshwater fish			
Atlantic salmon	70	49 (70%)	15 (21%)
European seabass	55	36 (65%)	12 (22%)
Total	125	85 (68%)	27 (22%)
Seawater fish			
Sea trout	60	41 (68%)	12 (20%)
Atlantic bonito	62	37 (60%)	13 (21%)
Total	122	78 (64%)	25 (21%)
Overall Total	247	163 (66%)	52 (21%)

Table 2. Origin of isolates and species distribution of the staphylococci and enterococci

Isolates	Freshwater fish species				Seawater fish species				Total (%)
	Atlantic Salmon		European seabass		Sea trout		Atlantic bonito		
	Gill	Intestine	Gill	Intestine	Gill	Intestine	Gill	Intestine	
<i>S. aureus</i>	4	2	4	-	1	-	2	-	13 (8%)
<i>S. intermedius</i>	-	-	3	-	3	-	5	-	11 (7%)
<i>S. warnerii</i>	15	2	11	-	13	4	6	1	52 (32%)
<i>S. hemolyticus</i>	7	2	3	2	9	1	11	2	37 (23%)
<i>S. saprophyticus</i>	2	-	4	1	4	-	1	-	12 (7%)
<i>S. simulans</i>	2	2	-	1	-	3	-	9	9 (7%)
<i>S. capitis</i>	7	1	-	-	-	1	-	9	9 (6%)
<i>S. xyloso</i>	2	-	1	-	-	2	1	8	8 (5%)
<i>S. epidermidis</i>	-	1	4	1	1	-	-	7	7 (4%)
<i>S. schleiferi</i>	-	-	1	-	1	-	-	3	3 (2%)
<i>S. caprae</i>	-	-	-	-	-	1	1	2	2 (1%)
Total	39	10	31	5	34	7	32	5	163
<i>E. faecalis</i>	3	2	4	3	9	-	3	-	24 (46%)
<i>E. avium</i>	1	-	5	-	3	-	2	2	13 (25%)
<i>E. solitarius</i>	2	1	-	-	-	-	2	1	6 (12%)
<i>E. gallinarium</i>	-	1	-	-	-	-	1	2	4 (8%)
<i>E. casseliflavus</i>	1	2	-	-	-	-	-	-	3 (6%)
<i>E. maladoratus</i>	1	-	-	-	-	-	-	-	1 (2%)
<i>E. flavescens</i>	1	-	-	-	-	-	-	-	1 (2%)
Total	9	6	9	3	12	-	8	5	52

In our study, 163 staphylococci and 52 enterococci isolates were obtained from 247 samples of Atlantic salmon, Atlantic bonito, Sea trout, and European seabass which are the most popular fishes consumed in Turkey. We found that there was not a significant difference in the staphylococci and enterococci contamination levels among freshwater fish and seawater fish ($P > 0.05$). It can be seen that both freshwater and seawater fish sold in the fish markets have high contamination with *Staphylococcus* spp. *Staphylococcus* spp. was observed in 70.0 %, 68.3 %, 65.4 % and 59.7 % of the Atlantic salmon, Sea trout, European seabass, and Atlantic bonito, respectively. High incidences of *Staphylococcus* spp. in fish samples have been reported by some researchers. Mhango et al. [17] reported 86 % from frozen tilapia, Hammad et al. [4] reported 92 % from ready-to eat raw fish (sashimi), and Boari et al. [18] reported 94.4 % from tilapia fresh fillets. According to our results, the most isolated CPS species were *S. aureus*, and *S. intermedius*. The CNS isolates were identified as *S. warneri*, *S. hemolyticus*, *S. saprophyticus*,

S. simulans, *S. capitis*, *S. xyloso*, *S. epidermidis*, *S. schleiferi* and *S. caprae*. Human nares and fingers are the main sources of *S. aureus*. Meanwhile, the incidence of CNS isolates (85.3 %) found in this study is much higher than those CPS isolates (14.7 %) ($P < 0.05$). Likewise, Boari et al. [18], Himelbloom and Crapo [19], and Grigoryan et al. [20] reported that CNS species comprised 75-80 % of the staphylococci isolates in salmon and tilapia fishes.

Enterococci live as part of the natural flora in the intestinal tract of animals and humans. They are considered as suitable indicators of fecal pollution in an aquatic environment [21]. Enterococci are isolated from many foods, including meat and dairy products [6], [10], [11]. In previous studies, *Enterococcus* spp. were isolated from clinical sources [21], [22], seawater [23], well water [6], and river water [21]. In the present study, the majority of the *Enterococcus* spp. was isolated from European seabass (21.8 %), followed by Atlantic salmon (21.4 %), Atlantic

bonito (21.0 %), and Sea trout (20.0 %). *Enterococcus* isolates were identified at species level as *E. faecalis*, *E. avium*, *E. solitarius*, *E. gallinarium*, *E. casseliflavus*, *E. maladoratus* and *E. flavescens*. The species identified in this study were similar to the reported by Hammad et al. [4] who showed that 96 enterococcal isolates recovered from 90 samples of retail ready-to-eat raw fish (sashimi) were *E. faecalis* (32.2 %), *E. faecium* (7.2 %), *E. casseliflavus* (7.2 %) and *E. gallinarium* (3.1 %). The high incidence of *E. faecalis* in the present study contrasted with previous reports by Al Bulushi et al. [24] and Valenzuela et al. [25], in which *E. faecium* was the most common species. There may be several reasons for these variations, such as differences in geographic location and season and differences in fish species studied. Fish samples were obtained from a wide variety of sources and vendors with different storage conditions, which is

thought to have resulted in different results. According to Mol and Saglam [26], fish boxes are generally laid on the floor, and this is a major cause of bacterial contamination in Turkish fish markets. Furthermore, the transportation of fish from seaside cities to Ankara will take at least 5 hours. During the transportation, sprinkling of fish with contaminated water, packing it with contaminated ice, coupled with unhygienic handling may explain the high prevalence of bacteria in fish in the markets.

Our results show that most of the *Staphylococcus* spp., and *Enterococcus* spp. isolated from fish samples have the ability to produce biofilm, slime, protease, lipase, DNase, and hemolysins (Table 3). However, there are no comparable studies on these properties produced by staphylococci and enterococci isolates from fish samples.

Table 3. The production of dnase, slime, biofilm, hemolysins, protease and lipase among *Staphylococcus* and *Enterococcus*

Species	DNase n (%)	Slime n (%)	Biofilm n (%)	β -Hemolysis n (%)	Proteolysis n (%)	Lipolysis n (%)
<i>S. aureus</i> (13)	11 (85%)	13 (100%)	11 (85%)	12 (100%)	13 (100%)	13 (100%)
<i>S. intermedius</i> (11)	4 (36%)	11 (100%)	9 (82%)	9 (82%)	11 (100%)	11 (100%)
<i>S. warnerii</i> (52)	31 (60%)	48 (92%)	52 (100%)	41 (79%)	32 (62%)	47 (90%)
<i>S. hemolyticus</i> (37)	26 (70%)	30 (81%)	33 (89%)	30 (81%)	9 (24%)	31 (84%)
<i>S. saprophyticus</i> (12)	9 (75%)	9 (75%)	12 (100%)	3 (25%)	12 (100%)	12 (100%)
<i>S. simulans</i> (9)	7 (78%)	6 (67%)	9 (100%)	3 (33%)	9 (24%)	8 (89%)
<i>S. capitis</i> (9)	-	5 (56%)	8 (88%)	5 (56%)	2 (22%)	8 (89%)
<i>S. xyloso</i> (8)	5 (63%)	6 (75%)	7 (88%)	1 (12%)	4 (50%)	7 (88%)
<i>S. epidermidis</i> (7)	5 (71%)	5 (71%)	7 (100%)	7 (100%)	-	4 (57%)
<i>S. schleiferi</i> (3)	3 (100%)	2 (67%)	3 (100%)	3 (100%)	-	3 (100%)
<i>S. caprae</i> (2)	2 (100%)	2 (100%)	2 (100%)	-	1 (50%)	2 (100%)
Total (163)	103 (63%)	137 (84%)	153 (94%)	114 (70%)	93 (57%)	146 (90%)
<i>E. faecalis</i> (24)	13 (54%)	24 (100%)	24 (100%)	-	9 (38%)	-
<i>E. avium</i> (13)	9 (69%)	11 (85%)	13 (100%)	1 (8%)	1 (8%)	-
<i>E. solitarius</i> (6)	5 (83%)	6 (100%)	6 (100%)	1 (17%)	4 (68%)	-
<i>E. gallinarium</i> (4)	3 (75%)	3 (75%)	4 (100%)	-	2 (50%)	-
<i>E. casseliflavus</i> (3)	-	3 (100%)	2 (67%)	-	3 (100%)	-
<i>E. maladoratus</i> (1)	-	-	-	-	-	-
<i>E. flavescens</i> (1)	-	-	-	-	-	-
Total (52)	30 (58%)	47 (90%)	49 (94%)	2 (4%)	19 (37%)	-

Microorganisms in food are known to form biofilms on the surface of many equipments in food and food processing plants. Foods such as fish, meat, and poultry can be contaminated with biofilm-forming bacteria through contact with contaminated surfaces [3]. In our study, the rate of CRA and MP methods positiveness was for CPS 100 % and 83.3 %, for CNS 81.3 % and 95.7 %, respectively. The incidence of slime-producing *S. aureus* strains (100 %) in the present study was higher than the values of 5.1 % reported by Citak et al. [27], 37.2 % by Ciftci et al. [28], 53 % by Gundogan et al [11], and 70.8 % by Gundogan et al. [10]. In this study, 43.3 % and 9.5 % of *Staphylococcus* isolates were classified as moderate and strong biofilm producers, respectively (data not shown). Gundogan et al. [10] found in their study in 2012 that 39% and 18.3% of *Staphylococcus* isolates produced moderate and strong biofilms, respectively. Biofilm formation (94.2 %) and slime formation (90.3 %) were also found in *Enterococcus* spp. According to our results, 50.0 % and 23.1 % of *Enterococcus* isolates were

moderate and strong biofilm producers, respectively (data not shown). Likewise, Necidová et al. [29] showed that 33% of *E. faecium* and 28 % of *E. faecalis* isolates isolated from raw milk and cream samples were positive for biofilm production. In contrast, Gundogan et al. [11] reported that biofilm production by the MP method was not detected in *Enterococcus* isolates. These findings are not consistent with the results of our study. However, some studies have shown that the nutrient content of the growth medium affects slime/biofilm formation [11]. However, it is very important to effectively implement hygiene protocols in fish markets to prevent contamination of fish products and prevent biofilm formation.

Microbial deterioration of fish results from various enzyme activities of microorganisms, resulting in products unsuitable for consumption. Apart from endogenous proteases, several microorganisms growing on muscle secrete a wide variety of enzyme, particularly protease. Storing foods at inappropriate temperatures causes quality and shelf life problems [30]. In our

study, it is important to note that all of the CPS and 49.6 % of the CNS displayed proteolytic activity under psychrotrophic conditions. Lipolytic activity of CPS (100 %) and CNS (87.8 %) were also high incidences. The fact that 36.5% of isolated *Enterococcus* spp. in our study showed proteolytic activity at +20°C, supports the knowledge that these bacteria play an important role in the proteolysis of foods. In our study, lipolytic activity could not be determined in any of the *Enterococcus* isolates. Many studies have been conducted to investigate protease and lipase production by enterococci and staphylococci, and it has been found that these bacteria have high levels of protease and lipase activities when grown in meat and dairy products [10], [11]. Peter et al. [13] also revealed that the frequent occurrence of protease and lipase production among enterococci from water and clinical isolates.

DNase is a virulence enzyme that breaks down DNA. In the present study, the rate of the DNase positiveness was 62.5 % for CPS, 63.3 % for CNS and 84.6 % for *S. aureus*. Citak et al. [27] showed that 93.6 % of *S. aureus* isolates isolated from raw milk had DNase activity while Batish et al. [31] reported an incidence of 36 %. We found that 57.7 % of *Enterococcus* spp. had also DNase activity. The prevalence of DNase-producing *E. faecalis* strains (54.2 %) in this study was much higher than those obtained from other studies [11]. Researchers indicated that only 5 % of *E. faecalis* strains had DNase activity. On the other hand, Barbosa et al. [9] and Peter et al. [13] reported that enterococci with DNase enzyme were not found in food and clinical samples.

There are several studies stating that hemolysin producing staphylococci and enterococci were shown to be virulent in animal and human infections, and were associated with increased severity of infection [12]. In the present study, it was determined that 87.5 % of CPS and 66.9 % of CNS have beta hemolysis. These values were similar to the rates of 100 % reported by Ebrahimi and Akhavan [32] but higher than the rates of 75 % reported by Gundogan et al. [10], 58.9 % by Turkyilmaz and Kaya [33] and 40 % by Gundogan et al. [11]. Furthermore, the incidence of hemolysin production among *S. aureus* isolates (100 %) in our study seems to be much higher than that reported for clinical strains by Ali-Vehmas et al. [34] (24 %). In this study, hemolysis was observed in only 6.7 % of *E. avium* and 16.7 % of *E. solitarius* isolates. More recently, hemolysis was reported in 7 % *E. faecalis* and 13 % *E. faecium* isolated from meat and meat products [11]. Many studies have shown that enterococcal isolates isolated from foods had lower hemolytic activity compared to the human clinical isolates. Ike et al. [12] reported that 60 % and 17 %, of *E. faecalis* isolates isolated from clinical and nonclinical sources, respectively, exhibited hemolytic activity. Peter et al. [13] studied the virulence factors in enterococci isolates from water, chicken and human clinical specimens. They indicated that β -hemolysis was significantly higher in clinical isolates than in other sources.

4. Conclusions and Recommendations

This study confirmed the presence of virulent staphylococci and enterococci in raw fish and emphasize the need for urgent action by the regulatory agencies to improve the hygiene status of retail fish markets in Turkey. Our results also highlight the presence of staphylococci and enterococci in fish that consumed undercooked may pose a health risk, particularly for susceptible populations.

5. Acknowledge

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