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KUZEY MARMARA DENİZİ'NDEN ELDE EDİLEN AVRUPA YASSI İSTİRİDYESİ (*OSTREA EDULIS*, L. 1758)'NİN DOĞAL BAKTERİYEL BİOTASININ TESPİTİ

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ÖZET

Bu çalışmada Kasım 2009'dan Nisan 2010 tarihine kadar Marmara Denizi'nin kuzey kıyısından toplanan 60 adet Avrupa yassı istiridyesi, *Ostrea edulis* örneklerinin doğal bakteriyel biotası araştırılmıştır. Doğal gelişme bölgelerinden mekanik istiridye direci kullanılarak, altmış istiridye kıyı şeridinin 500 m boyunca (10-15 m derinlik) toplanmıştır. Toplam 44 bakteri suşu izole edilmiş ve geleneksel bakteriyolojik yöntemler ve API 20E ticari hızlı tanımlama kiti kullanılarak tanımlanmıştır. Yapılan testler sonucunda, izolatların *Vibrio*, *Shewanella* ve *Citrobacter* cinslerinin birer üyesi olarak tanımlanmıştır. *Shewanella putrefaciens* sıklıkla izole edilen tür olmuştur. Bu çalışmada izole edilen diğer bakteri türleri ise *Vibrio fluvialis*, *V. vulnificus*, *V. alginolyticus* and *Citrobacter freundii* olmuştur. *S. putrefaciens* potansiyel sağlık riskine sahip olabilir ve gıda tüketimi yada kontamine suda yüzme yolu ile insanlara nakledilebilir. Bu nedenlerden dolayı, bakterinin istridye örneklerinden izolasyonu insan sağlığı için önemlidir.

Anahtar Kelimeler: Avrupa yassı istiridyesi, *Ostrea edulis*, API 20E, *Shewanella*, *Vibrio*, *Citrobacter*

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ABSTRACT
DETECTION OF NATURAL BACTERIAL BIOTA OF EUROPEAN FLAT OYSTER (*OSTREA EDULIS*, L. 1758) FROM NORTHERN SEA OF MARMARA

Natural bacterial biota of European flat oyster, 60 individual *Ostrea edulis* samples collected from the northern coast of the Marmara Sea, for each month from November 2009 to April 2010 were investigated in this study. Sixty oyster were harvested along 500 m of shallow (10-15 m depth) using a mechanical oyster dredge from natural growing areas. Totally 44 bacterial strains were isolated and identified by using conventional bacteriologic methods and API 20E commercial rapid identification kit. On the basis of the above mentioned tests, the isolates were identified as a member of the genera *Vibrio*, *Shewanella* and *Citrobacter*. *Shewanella putrefaciens* was mostly isolated species. The other isolated bacterial species were *Vibrio fluvialis*, *V. vulnificus*, *V. alginolyticus* and *Citrobacter freundii* in this study. *S. putrefaciens* could pose potential health risk and could be transmitted to humans via food consumption or swimming in contaminated water. For these reasons, its isolation from oysters samples is important for human health.

Keywords: European flat oyster, *Ostrea edulis*, API 20E, *Shewanella*, *Vibrio*, *Citrobacter*

INTRODUCTION

The European flat oyster, *Ostrea edulis*, a native uform of Europe, occurs naturally from Norway to Morocco in the North-Eastern Atlantic and also in the whole Mediterranean Basin (Jaziri, 1990). The species is cultured in many countries such as Spain, France and Ireland (Montes *et al*., 2003). *O. edulis* is the only economical oyster species found on the Turkish coastal waters (Uyan and Aral, 2000), however, although it was not reported commercial production of the species until this time, there are studies about this bivalve molluse that have been done for experimental aims such as larval methamorphosis (Uyan and Aral, 2000) and survival rates (Ateş, 1998). The microbiota of shellfish species such as oyster and mussel, has been examined according to the general public health hazard (Maugeri *et al*., 2000; Morris,

2003; La Valley *et al*., 2008) since these species are filter feeding organisms so they are able to accumulate pathogenic microorganisms at concentrations that are 100-fold higher than those in the surrounding water and they may cause serious problems in humans through their consumptions (Pujalte *et al*., 1999; Morris, 2003). Methods for examination of seafood safety have focused on fecal contamination, enteric pathogens such as *Salmonella* spp. and pathogenic species of *Vibrio* including *Vibrio parahaemolyticus* (Campbell and Wright, 2003; Daniels *et al*., 2000). However, except of the above mentioned studies, researches focused on the natural bacterial biota of different oyster species mainly Pacific oyster, *Crassostrea gigas* and Eastern oyster, *C. virginica* were carried out (Pujalte *et al*., 1999; Parveen *et al*., 2008). In Turkey, although there are a few studies about indicator and some pathogenic bacteria of clams such as striped venus, *Chamelea gallina* and wedge clam, *Donax trunculus* from the northern coast of the Sea of Marmara (Altuğ *et al*., 2008) and freshwater mussel, *Unio elongatulus eucirrus*, collected from the Koçkale and Pertek regions of Keban Dam Lake, Elazığ (Şeker and Sarıeyyupoglu, 2003), there is no research which has been focused on the bacterial content of European flat oyster, *O. edulis* until this study.

The goal of the present study was to asses the natural bacterial biota of European flat oyster, *Ostrea edulis* samples (60) taken from the northern coast of the Marmara Sea during the period of November 2009 to April 2010.

MATERIALS AND METHODS

Study Area and Sample Collection

A total of 60 individual *O. edulis* samples were harvested along 500 m of shallow (10-15 m depth) using a mechanical oyster dredge in two sites near Tekirdag (Kumbağ and Yeniçiftlik) on the northern coast of the Marmara Sea, for each month from November 2009 to April 2010. They were immediately transferred to our faculty laboratory at the cold conditions. Under sterile conditions, the exterior surfaces of the oyster valves were rinsed with 0.2 µm filter-sterilized sea water to remove surface attached material and the valves of the samples were opened with a sterile knife.

Bacteriology

All together gills, gonads and mantles of oyster samples were homogenized in 5 ml sterile saline solution . Ten-fold serial dilutions of the homogenates were then made with sterile saline. The samples (100 µl of the each dilution) were spread on to Sea Water Agar (SWA) (trypton 5 g, yeast extract 3 g, glycerol 3 ml, agar 15 g, filtered sea water 1000 ml, pH 7.5) and Thiosulfate Citrate Bile salt Sucrose (TCBS) agar (supplemented with 1.5% NaCl; TCBS) (Merck) (Bowman, 2005). The Petri dishes were incubated at 28±2°C for 72 and 48 hours, respectively (Bowman, 2005). After incubation, the plates were examined for bacterial colony growth. The dominant colonies were selected for further purification on SWA and TCBS. The bacterial isolates grown on the plates were identified according to Bergey's Manual of Systematic Bacteriology (Bowman, 2005; Farmer and Janda, 2005; Frederiksen, 2005). Motility, Gram-staining, cell morphology, the cytochrome oxidase and catalase tests, gas production from D-glucose, O/F test, susceptibility of the vibriostatic compound O/129 (10 and 150 µg/disc) and ampiciline (10 µg) for differentiation of different *Vibrio* species were performed. The pure cultures were examined by the following biochemical tests; growth in 0, 3, 6, 8 and 10% NaCl, growth at 4, 20, 30, 35, 40°C and swarming, amylase production; metil red test; acid production from lactose, D-galactose, xylose, D-fructose, D-mannose. Other tests were carried out using API 20E commercial rapid identification kits (Biomeriéux SA, Marcy-I'Etoile, France) with some modifications for salinity (1.5% NaCl as diluent) and temperature. The kits were incubated for 24 h and/or 48 h at 28±2°C according to the manufacturer's instructions. The results were read through the APILAB PLUS software.

RESULTS

A total of 51 phenotipic features were examined for the 44 isolates. The results of the biochemical investigation of isolates included in this study are given in Table 1. All the strains tested were motile, Gram-negative, no gas was produced from D-glucose. They grew in 3% of NaCl and at 4, 20 and 30°C. On the basis of the biochemical characterictics and API 20E test results, the 44 isolates were identified as *Shewanella putrefaciens* (31 strains), *V. fluvialis* (7 strains), *V. vulnificus* (3 strains), *V. alginolyticus* (2

strains) and *Citrobacter freundii* (1 strain) members of the genera *Vibrio*, *Shewanella* and *Citrobacter* (Table 1, 2).

S. putrefaciens was the most frequently recovered species (51.6%) from oyster samples. These strains (31) were non-fermentative, pink-orange coloured, resistance to the vibriostatic agents (10 and 150 µg/disc). They produced H_2S. Most isolates (3/31) were not able to grow with 0% NaCl. All the strains grew on MacConkey agar supplemented with 1.5% NaCl. *Vibrio* strains were also isolated in all oyster samples. The strains were Gram-negative, D-glucose fermentative, O/129 vibriostatic agents (10 and 150 µg/disc) sensitive. H_2S production–negative were classified within the genus *Vibrio*. The strains were detected in 12 of 60 (20%) oyster samples. The suspected *Vibrio* strains were distinguished according to the forming of yellow or green colonies on TCBS. *V. fluvialis* was the mostly recovered *Vibrio* species. The strains represented 11.6% of the 44 isolates. They were fermentative and produced yellow colonies on TCBS. All the strains were unable to grow in 10% NaCl. *V. vulnificus* was the secondly isolated *Vibrio* species (5%) from oyster samples. The strains were distinguished of as biotype 1, the production of indole-positive and they produced green colonies on TCBS (Noguerola and Blanch, 2008). The strains did not grow in the nutrient broth supplemented with 8% NaCl. *V. alginolyticus* was also recovered in oyster samples but was not abundant (3.3%) in oysters. The isolates produced yellow colonies on TCBS and they were unable to grow without NaCl. *C. freundii* was the only strain which did not produce cytochrome-oxidase enzyme. The strain was able to grow without NaCl. *C. freundii* grew on MacConkey agar, however the colony formation of the bacteria was not detected on TCBS.

Table 1. The biochemical test results of isolated bacterial species from *O. edulis* samples.

Tablo 1. *O. edulis* örneklerinden izole edilen bakteriyel türlerin biyokimyasal test sonuçları.

Tests	*S. putrefaciens* (31)	*V. fluvialis* (7)	*V. vulnificus* (3)	*V. alginolyticus* (2)	*C. freundii* (1)
Motility	100	100	100	100	100
Gram-staining	-	-	-	-	-
Cell morphology	Rod	Rod	Rod	Rod	Rod
Cytochrome oxidase	100	100	100	100	0
Catalase	100	100	100	100	100
O/F	NF	F	F	F	F
MR	0	43	67	100	100
Amylase production	87	100	100	100	0
Gas production from D-glucose	0	0	0	0	0
Swarming	0	0	0	100	0
Acid production from: Lactose	71	100	100	100	100
D-Galactose	87	100	100	100	100
Xylose	65	100	100	100	100
D-Fructose	68	100	100	100	100
D-Mannose	61	100	100	100	100
Growth in: %0 NaCl	10	0	0	0	100

%3 NaCl	100	100	100	100	100
%6 NaCl	94	100	100	100	100
%8 NaCl	13	71	0	100	100
%10 NaCl	10	14	0	0	100
Growth in: 4°C	100	100	100	100	100
20°C	100	100	100	100	100
30°C	100	100	100	100	100
35°C	90	100	67	100	100
40°C	55	86	67	100	100
TCBS	100, G	100, Y	100, G	100, Y	0
Mac Conkey (1.5% NaCl)	100	100	100	100	100
Susceptibility of the vibriostatic compound: O/129 (10 µg/ $disc^{-1}$)	100, R	100, S	100, S	100, S	100, R
O/129 (150 µg/$disc^{-1}$)	100, R	100, S	100, S	100, S	100, R
Susceptibility of ampiciline (10 µg/ $disc^{-1}$)	19, R	100, S	100, S	100, S	100, R

F: Facultative, NF: Non-facultative, Y: Yellow, G: Green, S: Sensitive, R: Resistance

Table 2. The API 20E profiles of the strains*.
Tablo 2. Suşların API 20E profilleri.

Tests	*S. putrefaciens* (31)	*V. fluvialis* (7)	*V. vulnificus* (3)	*V. alginolyticus* (2)	*C. freundii* (1)
ONPG	0	100	100	0	100
ADH	0	57	0	0	100
LDC	0	0	0	0	0
ODC	0	0	0	0	0
CIT	45	14	0	50	100
H_2S	100	14	0	0	100
URE	0	0	0	0	0
TDA	0	0	0	0	0
IND	0	57	100	100	0
VP	0	0	0	0	0
GEL	74	29	100	50	0
GLU	0	86	100	100	100
MAN	0	86	67	100	100
INO	0	0	0	0	0
SOR	0	0	0	0	100
RHA	0	0	0	0	100
SAC	0	71	0	100	100
MEL	0	14	67	0	100
AMY	0	14	0	0	0
ARA	0	0	0	0	100
NO_2	100	42	100	50	100
N_2	0	57	0	50	0

*The results of the kits applied for some strains of these strains were read after 24 hours of incubation.

DISCUSSION

The detection of Gram-negative bacteria, especially zoonotic species such as *V. parahaemolyticus*, *V. vulnificus* have been investigated from different bivalve molluscs in marine environment and it was reported that the most isolated bacterial species are included in the genera *Vibrio*/*Photobacterium*, *Flavobacterium*/*Cytophage* (Pujalte *et al*., 1999; Vasconcelos and Lee, 1972; Sousa *et al*., 2004). The occurence of *Escherichia coli*, *Salmonella* spp. in the clams, bivalve molluscs (*C. gallina* and *D. trunculus*) from marine environments has been documented by Altuğ et al. (Altuğ *et a*l., 2008). However, this study firstly reports the identification of different Gram-negative bacterial species such as *S. putrefaciens*, *V. fluvialis*, *V. vulnificus*, *V. alginolyticus* and *C. freundii* from the native European flat oyster, *O. edulis*, another one bivalve mollusc species, in Turkish coastal marine zones.

S. putrefaciens is a common cause of food spoilage (Leblanc *et al*., 2000). The species is rod-shaped, cytochrome-oxidase positive, indole-negative and usually produces H_2S on Kligler or triple sugar iron agar (TSI) (Venkateswaran *et al*., 1999). *S. putrefaciens* is isolated from human and non-human sources. It was reported that this species has been associated with ear and skin infections, leg ulcers, bacteraemia in humans. However, the infections causing this bacterium are rare and mostly associated with the immunosuppressed host (Richards *et al*., 2008; Papanaoum *et al*., 1998). The *S. putrefaciens* isolates have been also reported from various non-human sources including sea water, soil, bottlenose dolphin, *Tursiops truncatus*, fish such as *Dicentrarchus labrax*, *Siganus rivulatus*, bivalve mollucs like zebra mussel, *Dreissena polymorpha* (Buck *et al*., 2006; Richards *et al*., 2008; Korun *et a*l., 2009; Grizzle and Brunner, 2009). The species was also isolated from different oyster species such as slipper, *Crassostrea iradelei* (Najiah *et al*., 2008) and *O.* edulis (Pujalte *et al*., 1999). In our study, we isolated *S. putrefaciens* from the bacterial content of oyster samples. However, this bacterium could pose potential health risk and could be transmitted to human via consumption or swimming in contaminated water (Najiah *et al*., 2008).

There are many resarch studies focused on the microbiota of oysters and these studies have been reported different *Vibrio* species such as *V. paraha-*

emolyticus, *V. campelii*, *V. splendidus*, *V. harveyi*, *V. pelagius*, *V. tubiashii*, *V. mediterranei* and *V. lentus* sp. (Pujalte *et al*., 1999; Daniels *et al*., 2000; Macián *et al*., 2001; Williams *et al*., 2008). However, we found three different *Vibrio* species, *V. fluvialis, V. vulnificus* and *V. alginolyticus* from the oyster samples. *V. fluvialis* has been reported to cause sporodic infections and outbreaks of diarrhoea in humans and has also been isolated from both marine and estuarine environments (Chakraborty *et al*., 2006). Another *Vibrio* strain, *V. alginolyticus* is a part of the normal marine flora and it was reported that this species could reach concentrations in the shellfish that is sufficient to cause disease in humans during warm periods. This bacterium has been found to associate with wound infections, otitis media and otitis externa (Ripabelli *et al*., 2003). *V. vulnificus* inhabits microbiota of marine and estuarine vertebrates and invertebrates. The species has been isolated from clams, oysters, crabs, mussels and also benthic fishes. It is a zoonotic bacterium and the infections causing *V. vulnificus* usually result from the consumption of raw shellfish (Shehane and Sizemore, 2002; Parvathi *et al*., 2005). Addition to the above mentioned Gram-negative, cytochrome-oxidase positive bacterial strains, *Vibrio* species isolated from oyster samples, *C. freundii* was also obtained from *O. edulis* in the present study. Shellfish species including oysters filter suspended particles from their surrounding water and they accumulate different microorganisms in their body tissues (Jones *et al*., 1993). Consumption of raw oysters by humans may pose a vector for pathogenic bacterial species such as *V. vulnificus* and *S. putrefaciens* for disease transmisions from oysters to humans. Regardless of the results of survey of the natural bacterial biota of European flat oyster, *O. edulis*, this study could represent the presence of important bacterial species related to human health. In conclusion, after being digested raw oysters, these bacteria species may cause health problems. The present study reports basic information on bacterial biota of *O. edulis* in Turkey. However the detailed studies related to microorganisms isolated from *O. edulis* which may or may not be hazardous for human beings and resistance of these microorganisms to antibiotics using widely in mariculture in Turkey need to be investigated.

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