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Characterization of *Micrococcus luteus* and *Bacillus marisflavi* Recovered from Common Dentex (*Dentex dentex*) Larviculture System

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Abstract

In this manuscript, thirty yellow-pigmented Gram-positive bacteria were isolated from natural intestine microflora and from sea water around the marine cage of a rearing tank of common dentex (*Dentex dentex*), in the Aegean Sea on the Turkish coast and were characterized. Eighteen isolates were assigned to the species *Micrococcus luteus*, the other twelve to the species *Bacillus marisflavi*. Eight representative strains, six from *B. marisflavi* and two from *M. luteus*, were chosen for further 16S rDNA analyses. A pathogenicity assay for the isolated bacterial strains was carried out in rainbow trout and it evidenced absence of pathogenicity in the tested strains. The isolated strains were tested for in vitro antagonistic activity against *Listonella anguillarum*, a pathogen bacterium diffused in Mediterranean aquaculture and affecting various fish species. The isolated bacterial strains showed antagonistic activity against the pathogenic bacterium, suggesting a possible role of isolates as probiotics. In this study, for the first time, bacterial strains of the species *B. marisflavi*, known as an environmental species, were recovered in the gut microbiota of a healthy fish. The use of the isolates characterized in this study, mainly the yellow-pigmented bacterium, is suggested as possible probiotics to improve fish health, along with alternative methods of maintaining a healthy environment.

Keywords: *Micrococcus luteus*, *Bacillus marisflavi,* Bacterial characterization*, Dentex dentex.*

Introduction

Common dentex (*Dentex dentex*) is a sparid fish species which has been cultured in Mediterranean countries, including Turkey, since the early 2000's (Abellan, 1999; Firat *et al.* 2003). Aquaculture of this species has greatly developed due to the mass scientific studies on the morphology, physiology and aquaculture of this fish (Efthimiou *et al*., 1994; Fırat *et al*., 2003; Koumoundouros *et al,*. 2004). The main obstacle to further development are the disease problems, which are especially observed in the larval stages of this fish in culture systems (Rueda & Martinez, 2001).

Fish gut flora generally consists of a community of aerobic, facultative anaerobic and obligate anaerobic bacteria which are also present in the rearing system (Udey, 1978; Trust *et al.,* 1979). The increase in knowledge on the gut flora of cultured fish larvae allows to improve the detection of the potential source of pathogenic bacteria and eventually control disease outbursts, thus preventing economic losses, and it also contributes to a more efficient use of probiotics (Gomez-Gil *et al.,* 2000; Ganguly & Mukhopadhayay, 2010). Despite the fact that there are many studies on the gut flora of the larval stages of other sparids such as the gilt-head sea bream and sea bass (Grisez *et al.,* 1997; Savas *et al.,* 2005), there are insufficient data on the gut flora of the common dentex. In a previous study, we determined the larval and juvenile gut flora of common dentex cultured in Turkey (Akayli *et al.,* 2015). In that study, we recovered some yellow pigmented Gram-positive bacteria but we could identify them only at the genus level using conventional biochemical tests.

16S rRNA gene sequencing plays an important role in accurately identifying species in microbial communities (Woo *et al.,* 2009) together with biochemical profiles (Alsina & Blanch, 1994). This gene consists of conserved and varied nucleotide sequences used for determination based on sequencing approaches (Bintang *et al.,* 2014). Since the function of this gene has not changed over time, conserved gene sequence differences can be confidently used for bacterial definition at the species level (Janda & Abbott, 2007).

Due to the negative economical results of fish diseases, one of the main study area in aquaculture is their prevention by using consumer- and environment-friendly economical methods. Various bacterial groups present in fish digestive tract and their environment are beneficial to fish health (Gatesoupe, 1999; Gomez-Gil *et al.,* 2000; Spanggaard *et al.,* 2001) because they inhibit the colonization of potential pathogens due to their antagonistic affect (Verschuere *et al*., 2000; Irianto & Austin, 2002). *Bacillus* and *Micrococcus* species are among a wide

range of Gram-positive bacteria (*Carnobacterium, Enterococcus, Lactococcus, Lactobacillus* and *Streptococcus*) which have been evaluated as probiotics in aquaculture with successful results (Irianto & Austin, 2002).

Most importantly, antagonistic activity has been detected in the members of the genus *Bacillus* (Bernan *et al.,* 1997; Ganguly & Mukhopadhayay, 2010; Austin & Austin, 2012). Carotenoids are both necessary and sufficient to promote bacterial pathogenicity. In many cases, the microbial pigment contributes to disease pathogenesis and directly promotes immune suppression by interfering with host immune clearance mechanisms or exerting pro-inflammatory or cytotoxic properties (Liu *et al.,* 2005; Khaneja *et al.,* 2010). Some studies have been conducted on the probiotic use of pigmented Gram-positive bacteria against different fish pathogens (Lemos *et al.,* 1985; Nair & Simidu, 1987), but their antagonistic effect has not been investigated against *Listonella anguillarum*.

Alternative methods of maintaining healthy environments for aquacultured fish have been investigated (Koumoundouros *et al.,* 2004). Particularly, *L. anguillarum* is a common problem in Mediterranean aquaculture and affects many fish species. However, this organism developed antibiotic resistance and hence treatment of the disease became more complicated. The use of probiotics in aquaculture is becoming increasingly important to improve growth or survival of farmed aquatic species and provides protection from diseases (Gatesoupe, 1999; Gomez-Gil *et al.,* 2000).

The main aim of this study is the biochemical and molecular characterization of yellow-pigmented bacteria recovered from the rearing tank and indigenous gut microbiota of common dentex (*Dentex dentex*). Other purposes of this study are the investigation of the pathogenicity of these bacteria in rainbow trout and determination of their *in vitro* antagonistic effect against *Listonella anguillarum*.

Materials and Methods

Bacterial isolation and identification

Five sampling studies were done between 2009 and 2010 in a commercial land-based hatchery located in the Aegean Sea on the Turkish coast. Fish samples were examined aseptically, dissected under sterile conditions and bacterial inoculations were made from the rearing water and gut samples of common dentex larvae from different exogenous feeding stages (non-feeding, rotifer, *Artemia* spp. and artificial pellet feeds, respectively) (Muroga *et al.,* 1987). Briefly, water and gut samples were diluted at different proportions (1/10, 1/100, 1/1000 and 1/10000) with sterile phosphate buffer saline (PBS) which was prepared by using commercial PBS tablets (Medicago AB, Sweden) with a final pH 7.4 and spread onto various media (Marine Agar 2216 - MA [Difco], Plate Count Agar – PCA [Acumedia] and Tryriptic Soy Agar – TSA [HiMedia]). An extra 1.5 % NaCl was added to commercial formulation of PCA and TSA. After incubation at 22□ C for 2-5 days, bacterial colonies were grouped depending on their color, shape, margins and consistency differences. Especially yellow pigmented colonies were selected and standard morphological and biochemical methods such as Gram-staining, hanged drop motility test, oxidase and catalase activities etc. and API STAPH system was used for further identification of these strains. Gram-stained preparations were examined under light microscope for the determination of Gram characteristics and shape of the bacteria.

16S rRNA gene sequencing

A partial region of the 16S rRNA gene was amplified from genomic DNAs (extracted by using a commercial kit; Thermo-K0721, USA) with universal primer pair (27F: 5'-AGAGTTTGATCTGGCTCAG-3' and 1492R: 5'-ACCTTGTTACGACTT-3') developed by Lane (1991). PCR was performed using modified conditions and cycling profile reported by Eder *et al.* (1999). After purification, amplicons were sequenced based on a chain termination method (kit: Applied biosystems, USA) with ABI PRISM 3100. Chromatograms were monitored and analysed by Chromas Pro 1.7.6 (Technelysium, Australia). Two directional nucleotide sequence data were assembled with DNA Dragon software (1.1.9.1). Similarity was searched with BLASTN through the NCBI (Altschul *et al*., 1997). 16S rDNA sequences were subjected to CLUSTALW analysis using MEGA 6.0 (Tamura *et al.,* 2013). A similarity matrix was constructed with the neighbor-joining algorithm of Jones-Thornton-Taylor model (Tamura & Nei, 1993). A dendrogram was generated according to the cluster analysis using the UPGMA. 16S rDNA sequence data were deposited in Genbank by using Sequin 13.05 (Benson *et al.,* 2000).

Pathogenicity assay

For the determination of the pathogenicity of isolated strains belonging to *B. marisflavi* and *M. luteus*, 3 experimental groups and a control group were created with 200 rainbow trout (mean weight 5-7 g) for each bacterial species. Bacterial suspensions of 10^6, 10^7 and 10^8 cells/ml were prepared with PBS (phosphate buffered saline) solution for each bacterial species and fish groups were immersed in these bacterial suspensions. Later, fish groups were reared for 30 days and were monitored for possible disease symptoms and mortalities for the determination of pathogenicity.

Antagonistic activity assays

After identification of the bacteria, yellow pigmented Gram-positive isolates were screened for antagonistic effect. *In vitro* antagonistic effect of these bacteria were determined with the Kirby-Bauer disc diffusion method

modified by Bhunia *et al.* (1988) on five different *Listonella anguillarum* strains that were recovered from the internal organs of diseased marine cultured gilt-head sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and fresh-water cultured rainbow trout (*Oncorhynchus mykiss*) sampled from Turkey. Fresh cultures of *L. anguillarum* strains were streaked onto Muller-Hinton agar and paper discs that were dipped into separate mixtures of *B. marisflavi* and *M. luteus* strains were placed on the agar surface. This assay was repeated three times for both bacterial species. An erythromycin disc was used as a positive control. Clear zones around discs were evaluated as positive results and their diameters were measured after incubation.

Results

In this study, a total of 30 yellow-pigmented Gram-positive isolates were recovered and identified based on their biochemical characteristics and 16S rRNA gene sequences. Eighteen were Gram-positive, non-motile, cocci-shaped tetrads, oxidase and catalase positive isolates, which were identified as *Micrococcus luteus*. Twelve were Gram-positive, motile, facultative anaerobe, gas-forming from glucose, catalase negative, oxidase-positive, spore-forming bacilli-shaped isolates, which were identified as *Bacillus marisflavi* (Table 1). These two bacterial species were especially recovered from the natural intestinal microflora of non-feeding larvae and other following larval stages. Also *M. luteus* was recovered from sea water around the marine cages.

Of the 30 yellow pigmented Gram-positive bacteria, six *B. marisflavi* (AKAYLI 09-14) and two *M. luteus* (AKAYLI 15 and 122) yielded a band of 1.5 kb, corresponding to a partial 16S rDNA region. After assembling analysis, crude nucleotide data obtained from chromatograms were ranged from 1.3 to 1.4 kb. All *B. marisflavi* strains showed significant nucleotide sequence homologies (except value < 0.05 and bit scores > 50) with the reference 16S rDNA sequence (accession number KC414706.1) of *B. marisflavi* (Table 2). Similarly, two *M. luteus* strains showed high levels of similarity (except value < 0.05 and bit scores > 50) with the reference sequence (KF733697.1) of *M. luteus* (Table 2). When compared to each other and their own reference species via CLUSTALW, similarity among bacteria ranged from 69.67 to 100%. The highest similarity percentage was detected among three *B. marisflavi* strains (AKAYLI 09, 10 and 13), and between one of them and the reference sequence; besides and interestingly, 98% similarity between *B. aquamaris* (NR_025241.1) and AKAYLI 09 was detected. The most genetically distant strains (69.67%) were determined as AKAYLI 11 and AKAYLI 15, belonging to *B. marisflavi* and *M. luteus*, respectively (Table 3). Deletions, insertions and SNPs in 16S rDNA were detected as distinguishing alterations for the two species (Fig. 1). Nucleotide data of these 8 strains were deposited under Genbank with accession numbers KJ541103, KJ560871, KJ560870, KJ560872, KJ560874, KJ560873, KM062059 and KM062060 (Table 2). An UPGMA dendrogram displayed monophyletic branching (Fig. 2). Gram-positive bacteria found in probiotic communities consist of two groups; group I and group II. While six *B. marisflavi* strains were grouped together with their own reference genome KC414706.1 in group I, two *M. luteus* were clustered in group II with the reference strain KF733697.1.

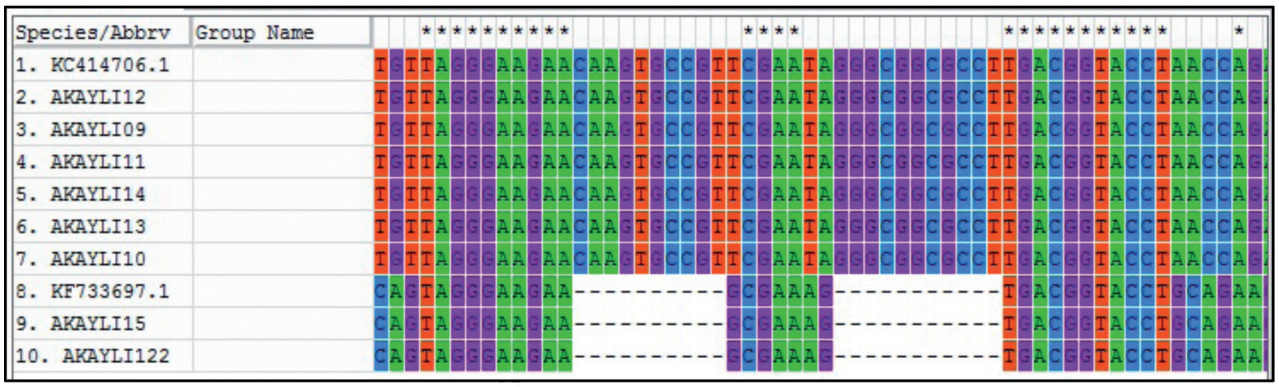
Bacterial strains of *B. marisflavi* and *M. luteus* were determined as non-pathogenic in rainbow trout because they did not cause any important clinical symptoms nor mortality during 30 days investigation after the bacterial challenge.

As a result of the antagonistic activity assays, it was determined that both *B. marisflavi* and *M. luteus* strains isolated in this study showed *in vitro* antagonistic activity against *L. anguillarum* and produced inhibition zones of various diameters (5–30 mm) around the paper discs.

Table 1. General phenotypic and biochemical characteristics of the yellow pigmented Gram-positive bacter.

Characteristics	*Bacillus marisflavi*	*Micrococcus luteus*
Gram staining	+	+
Catalase	+	+
Oxidase	-	+
Motility by flagella	+	-
Gliding motility	-	-
O/F	O	O
VP	+	-
Indole	-	-
Arginine	V	V
Ornithine	-	-
Lysine	-	-
MR	+	-
β-galactosidase	+	-
Acid production from		
D-glucose	+	-
L-arabinose	-	-
D-xylose	-	-
D-mannitole	-	V
Degradation of		
Casein	+	-
Gelatin	+	+
Starch	+	ND
Aesculin	-	+
Utilization of citrate	+	-
Urease	V	V
Nitrate reduction	+	-
Growth in		
2% NaCl	+	+
5% NaCl	+	+
7% NaCl	-	+

+: positive -: negative V: variable ND: not detected O: oxidative



*point out conserved nucleotides; - imply neither deletion nor no sequencing information

Fig. 1: Multiple alignment obtained from CLUSTALW analysis of 10 bacterial strains.

Table 2. BLASTN analysis of 16S rDNA sequences of 8 strains used in this study.

Strain/GenBank no	Associated Organism	Accession no	E value	Bit scores	Identity (%)
AKAYLI 09/ KJ541103	*B. marisflavi*	KC414706.1	0.0	2584	100
AKAYLI 10/ KJ560871	*B. marisflavi*	KC414706.1	0.0	2582	100
AKAYLI 11/ KJ560870	*B. marisflavi*	KC414706.1	0.0	2571	99
AKAYLI 12/ KJ560872	*B. marisflavi*	KC414706.1	0.0	2579	99
AKAYLI 13/ KJ560874	*B. marisflavi*	KC414706.1	0.0	2582	100
AKAYLI 14/ KJ560873	*B. marisflavi*	KC414706.1	0.0	2577	99
AKAYLI 15/ KM062059	*M. luteus*	KF733697.1	0.0	2560	99
AKAYLI 122/ KM062060	*M. luteus*	KF733697.1	0.0	2340	99

Table 3. Similarity matrix of 16S rDNA sequences in 10 bacterial strains.

	1	2	3	4	5	6	7	8	9	10
1. KC414706.1	100									
2. KF733697.1	69.87	100								
3. AKAYLI 09	100	70.76	100							
4. AKAYLI 10	100	70.81	100	100						
5. AKAYLI 11	99.85	70.67	99.85	99.85	100					
6. AKAYLI 12	99.92	70.69	99.92	99.92	99.78	100				
7. AKAYLI 13	100	70.81	100	100	99.85	99.92	100			
8. AKAYLI 14	99.92	70.74	99.92	99.92	99.78	99.85	99.92	100		
9. AKAYLI 15	70.29	98.50	69.76	69.81	69.67	69.69	69.81	69.74	100	
10. AKAYLI 122	71.32	99.29	71.32	71.32	71.17	71.25	71.32	71.25	97.65	100

Table 4. Antagonistic activity of Gram-positive yellow pigmented bacteria isolated from dentex larvaea system against 5 *L. anguillarum* strains.

Antagonistic bacteria	V_1	V_2	V_3	V_4	V_5
M. luteus	++	+	++	+	++
B. marisflavi	+++	++	+++	++	+++

V_1 – V_5: different *L. anguillarum* isolates
mean diameter measurements: +: > 1 to 10 mm, ++ : > 10 to 20 mm, +++ : > 20 to 30 mm

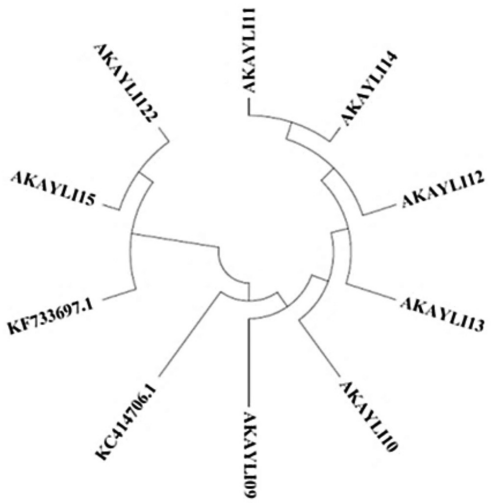


Fig. 2: Dendrogram, constructed by UPGMA analysis, including 8 bacterial strains together with their references

However, *B. marisflavi* showed a greater inhibitory activity against *L. anguillarum* (Table 4) than *M. luteus* isolates in general.

Discussion

Because of high mortality rate during the larval stages (Rueda & Martinez, 2001), alternative methods need to be developed to maintain a healthy microbial environment in the larval rearing tanks (Koumoundouros *et al.,* 2004). Identification of bacterial communities present in the fish gut microbiota and the rearing environment can provide useful information for the improvement of the success of the aquaculture operations and fish welfare (Gatesoupe, 1999; Gomez-Gil *et al.,* 2000; Spanggaard *et al.,* 2001). Here we report the identification and characterization of yellow-pigmented Gram-positive bacteria that are commonly and abundantly recovered from the gut microbiota and the rearing environment of cultured common dentex in Turkey.

In this study, 30 yellow pigmented Gram-positive bacterial isolates were recovered from the intestine of common dentex larvae and tank water. Biochemical tests showed that 60% of these yellow pigmented bacteria were *M. luteus* and 40% were *B. marisflavi*. Yoon *et al.* (2003) compared rDNA sequences of one *B. marisflavi* and one *B. aquamaris* strains to that of other *Bacillus* species. They detected similarity of less than 97% and reported that these two strains belonged to different species. Wieser *et al.* (2002) characterized nine yellow pigmented bacterial strains and analysed their 16S rRNA gene sequences. They reported that all isolates belonged to *M. luteus* and that the minimum homology value among them and the *M. luteus* reference (DSM20030T) was 97.5 %. Despite the hypothesis that sequence similarity within the same species can be minimum 97.5% (Stackebrandt & Goebel, 1994), according to recent limited data, 98 % similarity between *B. aquamaris* (NR_025241.1) and AKAYLI 09 was detected. As the information in the gene sequencing database increased, distinction between *B. marisflavi* and *B. aquamaris* would be enlightened more accurately and a lower similarity between these two species can be detected.

Micrococcus luteus is a natural yellow-pigmented Gram-positive bacterial member of the aquatic environment and also found in fish intestinal microbiota (Jayanth *et al.,* 2001; Chabrillon *et al.,* 2005; Abd El-Rahman *et al.*, 2009). There are published data on the association of this organism with fish diseases (Austin & Stobie, 1992) and non-pathogenic strains of this species were used as probiotic against *Aeromonas salmonicida* in rainbow trout (Irianto & Austin, 2002), *Aeromonas hydrophila* in tilapia (Abd El-Rahman *et al.,* 2009; Osman *et al*., 2010), *V. harveyi* in Senegal sole (*Solea senegalensis*) (Chabrillon *et al.,* 2005) and *L. anguillarum* in gilt-head sea bream (*Sparus aurata*) (Chabrillon *et al.,* 2006). Similarly, *M. luteus* isolates used in this study were recovered from the gut microbiota of healthy common dentex and they were determined as non-pathogenic to the rainbow trout in the pathogenicity assays. Also they showed an *in vitro* antagonistic effect against *L. anguillarum.*

Members of the genus *Bacillus* are usually found in the intestinal microbiota of fresh water and marine fish (Gatesoupe, 1999; Ghosh *et al.,* 2002). Furthermore, many non-pigmented *Bacillus* strains (for example *B. subtilis*) were selected in the probiotic research due to their antibiotic effectiveness against fish pathogens (Vaseeharan & Ramasamy, 2003). Some *Bacillus* species contain carotenoid pigments and, among them, *B. marisflavi* was identified in marine waters (Yoon *et al.,* 2003; Khaneja *et al.,* 2010). Despite it was detected in marine water samples, This study is the first record for the presence of *B. marisflavi* in the intestinal microflora of a marine fish species. *B. marisflavi* isolates which are determined to be non-pathogenic for rainbow trout also showed an *in vitro* antagonistic effect on *L. anguillarum*, which indicates the production of antimicrobials by *B. marisflavi*. It is likely the same result of a pathogen-inhibiting mechanism as that was previously reported for other *Bacillus* species (Bernan *et al.,* 1997; Ganguly & Mukhopadhayay, 2010; Austin & Austin, 2012).

As a result, in this study, yellow pigmented Gram-positive bacteria that are commonly and abundantly found in the gut microbiota of the healhty common dentex and larviculture system were identified by using biochemical and molecular methods. Furthermore, they were determined to be non-pathogenic for rainbow trout and their antagonistic affect against *L. anguillarum* was revealed. Thus, being non-pathogenic and having an inhibitory mechanism against *L. anguillarum*, *B. marisflavi* and *M. luteus* can be regarded as probiotic candidate species and maybe used in the furhter studies on the fish health and welfare.

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