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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; Firat 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 (Grissez 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 (Al-sina & 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 (Verschuer et al., 2000; Irianto & Austin, 2002). *Bacillus* and *Micrococcus* species are among a wide
range of Gram-positive bacteria (*Carnobacterium, Ente-
rococcus, Lactococcus, Lactobacillus* and *Streptococcus*) which have been evaluated as probiotics in aquaculture with successful results (Irianto & Austin, 2002).

Most importantly, antagonistic activity has been de-
tected in the members of the genus *Bacillus* (Berman 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 environ-
ments for aquacultured fish have been investigated (Kou-
moundouro et al., 2004). Particularly, *L. anguillarum* is a common problem in Mediterranean aquaculture and affects many fish species. However, this organism de-
veloped 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
doctoral characterization of yellow-pigmented bacte-
ria recovered from the rearing tank and indigenous gut microbiota of common dentex (*Dentex dentex*). Other purposes of this study are the investigation of the patho-
genicity of these bacteria in rainbow trout and determina-
tion 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 ex-
amined aseptically, dissected under sterile conditions and
bacterial inoculations were made from the rearing water
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 (Mar-
ine Agar 2216 - MA [Difco], Plate Count Agar – PCA

[Acumedia] and Tryptic 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 determina-
tion 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'-ACCTTGTATCAGCTT-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, Austral-
ia). Two directional nucleotide sequence data were as-
sembled with DNA Dragon software (1.1.9.1). Similarity
was searched with BLASTN through the NCBI (Alts-
chul 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 gener-
ated 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 iso-
alted 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<sup>6</sup>, 10<sup>7</sup> and
10<sup>8</sup> 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 deter-
mination of pathogenicity.

**Antagonistic activity assays**

After identification of the bacteria, yellow pigment-
ed 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 ( Oncorhyncus mykiss) sampled from Turkey. Fresh cultures of L. anguillarum strains were streaked on 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 bacteria.

| 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-xylene                 | -                   | -                  |
| D-mannitol              | V                   | V                  |
| Degradation of           |                     |                    |
| Caecon                  | +                   | -                  |
| 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
**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.   | 100  |      |      |      |      |      |      |      |      |      |
| 2.   | 69.87| 100  |      |      |      |      |      |      |      |      |
| 3.   | 100  | 70.76| 100  |      |      |      |      |      |      |      |
| 4.   | 100  | 70.81| 100  | 100  |      |      |      |      |      |      |
| 5.   | 99.85| 70.67| 99.85| 99.85| 100  |      |      |      |      |      |
| 6.   | 99.92| 70.69| 99.92| 99.92| 99.78| 100  |      |      |      |      |
| 7.   | 100  | 70.81| 100  | 100  | 99.85| 99.92| 100  |      |      |      |
| 8.   | 99.92| 70.74| 99.92| 99.92| 99.78| 99.85| 99.92| 100  |      |      |
| 9.   | 70.29| 98.50| 69.76| 69.81| 69.67| 69.69| 69.81| 69.74| 100  |      |
| 10.  | 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 larvae system against 5 *L. anguillarum* strains.

| Antagonistic bacteria | $V_1$ | $V_2$ | $V_3$ | $V_4$ | $V_5$ |
|-----------------------|-------|-------|-------|-------|-------|
| *M. luteus*           | ++    | +     | ++    | +     | ++    |
| *B. marisflavi*       | +++   | ++    | +++   | ++    | +++   |

$V_i$: different *L. anguillarum* isolates

mean diameter measurements: +: > 1 to 10 mm, ++: > 10 to 20 mm, +++: > 20 to 30 mm
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 (Koumoundourovs 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 & Mukhopadhyay, 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 healthy 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 further studies on the fish health and welfare.
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