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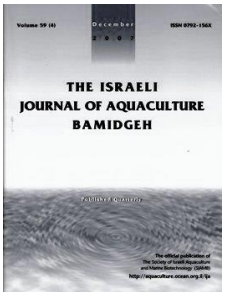

Interaction of Gut Flora and Bacterial Pathogens of Cultured Common Dentex (*Dentex dentex*)

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Abstract

This study was carried out to determine the population of gut flora and investigate the source of the pathogenic bacteria recovered from moribund dentex (*Dentex dentex*). Transmission electron microscopy (TEM) was used to monitor the translocation of bacterial pathogens which can enter the host through the gut. A total of 260 samples from larvae at different feeding stages were examined, 15 healthy and 50 moribund juvenile dentex were sampled over a 2-year period. Bacterial isolates (n=433) from healthy and moribund fish and their environments were identified by biochemical methods; 75% of them were identified as Gram-negative (mainly *Vibrio* spp.) and 25% as Gram-positive. *Vibrio scophthalmi* and other *Vibrio* species were recovered from moribund fish samples. Some bacteria were recovered from diseased fish reared in hatcheries, introduced mainly from sea water. Among anaerobic bacteria, *Eubacterium tarantellae* was recovered from larval gut flora while *Clostridium botulinum* was recovered from juvenile gut flora. TEM also showed that bacterial endocytosis first occurs when fish are fed granulated dry feeds and becomes more prevalent in the juvenile stages when many bacteria are present in the microvilli. TEM also revealed that the bacteria enter the larvae via the mouth and gills before the granulated feeding stage, after which they begin to penetrate the gut and finally also contaminate the marine rearing stage. The results of this study showed that the granulated feeding stage is critical for pathogen invasion in common dentex.

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Introduction

There have been significant developments in Mediterranean aquaculture due to the increase in aquaculture production, diversity of cultured fish species, and culture areas (Roncarati et al., 1996; Koumoundouros et al., 2004). Given suitable environmental conditions and due to its economic value, common dentex (*Dentex dentex*) is becoming increasingly important in Turkey (Fırat et al., 2003; Alpbaz, 2005). Most research has been conducted on the morphology, physiology, and aquaculture of this fish species of the Sparidae family (Efthimiou et al., 1994; Fırat et al., 2003, Koumoundouros et al., 2004) even though fish losses during larval and juvenile stages have been reported as the most serious problem for common dentex (Roncarati et al., 1996; Company et al., 1999).

During their life cycle, fish are in contact with a wide range of bacterial flora including pathogenic and opportunistic species that are able to colonize their external and internal body surfaces (Olafsen, 2001). Pathogenic bacteria may enter the fish host via the skin, gills or gastrointestinal tract. Fish gut flora generally consists of aerobic, facultative anaerobic, and obligate anaerobic bacteria (Udey 1978; Trust et al., 1979). Understanding the gut flora of cultured fish larvae makes it possible to detect the potential source of pathogenic bacteria and eventually control disease outbursts, prevent economic losses, and contribute to more efficient use of probiotics (Gomez-Gil et al., 2000; Ganguly and Mukhopadhayay, 2010). Although there has been much research on the gut flora of the larval stages of other sparids such as gilt-head sea bream and sea bass (Grisez et al., 1997; Savas et al., 2005; Ayaz and Karataş, 2010), no studies have been reported on the gut flora of common dentex.

In addition to monitoring gut bacterial population development, transmission electron microscopy (TEM) has been used to monitor adhesion and translocation of bacterial pathogens that enter the fish host via the gastrointestinal (GI) tract (Ringo et al., 2003). Two different mechanisms are involved in bacterial translocation in the GI tract, intracellular and paracellular. Intracellular translocation occurs by either endocytosis or penetration. Research with TEM has shown endocytosis of bacterial cells in the gut of marine fish larvae (Olafsen and Hansen, 1992) and juvenile fish (Ringo et al., 2004).

Like other cultured Sparids, common dentex is highly sensitive to pathogens (Rigos and Katharios, 2010). Some researchers (Company et al., 1999; Rigos and Katharios, 2010) have reported that when under stress dentex, as other fish species, is more sensitive to bacterial pathogens such as *Pasteurella* sp. and *V. alginolyticus* as well as some parasites (Rigos et al., 1998). *Vibrio harveyi*, *V. alginolyticus* and *Vibrio damselae* have been found to be infectious agents (Company et al. 1999) and *Aeromonas* spp., *V. nereis* and *V. costicola* have been found in diseased common dentex (Yiagnisis and Athanassopoulou 2011).

The aims of this study are: 1) to identify aerobic and anaerobic gut flora in the larval and juvenile stages of cultured common dentex; and 2) to identify the source, and origin of pathogenic bacteria recovered from moribund adults and determine the timing of the transition of pathogenic bacteria into the fish gut using TEM.

Materials and Methods

Fish. In a two year study, samples of fish in the larval and juvenile stages were taken from off-shore marine cages in the Aegean Sea in Turkey, and a land-based hatchery unit that uses filtered marine water. In this study, a total of 260 common dentex larvae (0.001–0.0059 g), 15 healthy juvenile, and 50 moribund juvenile samples (100-250 g) were collected and studied.

Isolation and identification of culturable and non-culturable intestinal microbiota: In this study, aerobic and anaerobic bacteria from the gut flora of dentex larvae reared in a hatchery, and juvenile *D. dentex* reared in marine cages, were investigated, as well as samples of water taken from the hatchery tank water, the supplied marine water, as well as *Artemia salina* tank water.

260 common dentex larvae were sampled at three different feeding regimes: 1) not fed, 2) fed various live foods, and 3) fed granulated dry feeds, to determine the population of larval gut flora. Larvae were disinfected externally by immersion in benzalkonium chloride (0.1% w/v), prepared in 1.5% (w/v) saline for a few seconds and washed three times in sterile saline (Grisez et al., 1997). Subsequently, 10 larvae were transferred to 2 ml of sterile saline in a test tube and homogenized by vortex. The volume was adjusted to 10ml with saline and the resulting homogenate was serially diluted tenfold with sterile saline. Then 100 µl of each dilution was spread onto Trypticase Soy Agar (TSA), Plate Count Agar (PCA) and Marine Agar (MA) to culture aerobic bacteria, and incubated at 22 °C for five days. Bacterial isolates were sub-cultured to obtain pure colonies and were then identified using biochemical and phenotypic tests (Buller, 2004; Austin and Austin, 2012) with API 20E, API STREP and API STAPH test strips.

Juvenile fish samples were dissected and a total of 1 g of juvenile fish gut was homogenized in 9 ml of sterile seawater in a test tube. The homogenate was diluted as described above and cultured

on various bacteriological media. In order to determine the amount of anaerobic bacteria present in the larvae and juvenile gut flora, 100 µl of each dilution of larval samples was spread onto Reinforced Clostridial Agar (RCA) placed into a jar, and incubated under anaerobic conditions at 20 °C for five days to isolate anaerobic bacteria. API 20A test strips were used for identification of these bacteria.

Transmission electron microscopy. TEM was used to determine bacterial transition stages into the fish gut. Midgut samples of nine larval and four juvenile samples were used at this stage of the study, after their gut flora isolations were made. Midgut samples (1 mm^2) were then fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7) containing 3.5 % sucrose at 4ºC for two hours. The samples were transferred to a cavity slide, placed on ice, and the tail was removed under microscope to enable infiltration of the fixative. Specimens were post-fixed in 2% osmium tetroxide in the same buffer for two hours at 3°C, serially dehydrated in an ethanol series followed by propylene oxide, and finally embedded in Epon. Ultrathin sections were cut with a Leica ultramicrotome and contrasted with alcoholic uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). The sections were examined at 60 kV with a JEOL 100 CX II transmission electron microscope.

Adult fish sampling and bacterial examination. Fifty fish samples weighing between 100-250 g were examined. Generally, darkening of the skin, abdominal distension, fin rot, common ulcerative skin lesions, and hemorrhages around the cranial region were observed externally in diseased fish. Internally, hemorrhages in the abdominal muscles, sloughing of the liver and hemorrhages, liquefactive necrosis in the kidneys, accumulation of a yellowish fluid in the intestines as well as splenomegaly were observed.

Fish samples were anesthetized with 2-phenoxyethanol (1.5 ml/l) and dissected under aseptic conditions. Bacterial isolations were made from the internal organs (liver, kidney, intestines, and spleen), and blood samples of diseased fish were examined by spreading onto TSA (supplemented with 2 % NaCl), MA and TCBS (Cholera medium) and incubated at 22-24 °C for 48 hours. Bacterial isolates were identified according to the routine laboratory methods (Austin and Austin, 2012) and an API 20E rapid identification kit.

Results

Gut flora. A total of 350 bacterial isolates were recovered. Most of the isolates corresponded to Gram-negative microorganisms (75 %) and the rest to Gram-positives (25 %). Identification of dominant bacteria recovered from dentex gut flora, and hatchery water are listed in Table 1.

Table 1. Bacteria recovered from the gut flora of dentex larvae in different feeding stages and hatchery water

Feeding regime	*Aerobic bacteria*	*Anaerobic bacteria*
No feed	*Micrococcus sp., Vibrio alginolyticus*	*Eubacterium tarantellae*
Rotifer	*Flavobacterium psycrophilum, Vibrio sp., Micrococcus luteus, Bacillus spp.*	*Eu. tarantellae*
Artemia + Rotifer	*Vibrio alginolyticus, Vibrio splendidus biotype I,. Vibrio sp., Flavobacterium sp., Micrococcus luteus*	*Eu. tarantellae*
Enriched Artemia	*V. harveyi, V. vulnificus, V. splendidus biyotip I, V. parahaemolyticus, Flavobacterium xantham, Photobacterium phosphoreum, Brukholderia pseudomallei, Micrococcus luteus, Streptococcus sp., Bacteriodes sp.*	*Eu. tarantellae*
Artemia + Granulated feed	*V. splendidus, V. furnissii, V. proteolyticus, Moritellla viscosa, Bacillus* sp.*, Acinetobacter sp., Burkholderi pseudomallei*	*Eu. tarantellae*
Granulated feed	*V. alginolyticus, V. vulnificus, V. splendidus, Flavobacterium psychrophilum, F. hibernium, Tenacibaculum maritimum, Staphylococcus aureus, Micrococcus luteus*	*Eu. tarantellae*
Hatchery water	*V. alginolyticus, V. vulnificus, V. splendidus, V. scopthalmi, Acinetobacter sp., Moraxella sp., Moritella viscosa*	*Eu. tarantellae*

Bacterial isolation in moribund fish samples. From 50 moribund fish samples with the above mentioned disease symptoms, 60 bacterial strains were isolated and characterized. The most common were *Vibrio* species such as *V. scophthalmi, V. alginolyticus* and *V. vulnificus* (75%). Gram-positive fish pathogens constituted the remaining 25%, of which *Micrococcus luteus* and *Streptococcus* sp. were identified in mixed infections. Bacteria recovered from the internal organs of infected fish are listed in Table 2.

Table 2. Bacteria recovered from gut flora of healthy juvenile dentex, internal organs of moribund fish samples and sea water

	Fish		
	Healthy juvenile Dentex (n=100)	*Moribund fish samples (n=60)*	*Sea water (n=30)*
Aerobic bacteria	*V. scophthalmi* *Vibrio sp.* *Listonella pelagia* *Moritella marina* *Pseudomonas sp.* *A. sal.*subsp.*achromogenes* *Flavobacterium sp.* *Staphylococcus spp.*, *Photobacterium leiognathi* *Burkholderia sp.* *Hafnia alvei* *Bacillus spp.*	*V. scophthalmi* *V. alginolyticus,* *V. vulnificus* *V. mediterranei* *V. splendidus I* *T. maritimum* *Flavobacterium sp.* *Micrococcus luteus* *Streptococcus sp.* *Bacillus sp.*	*V. damsela* *V. mediterranei* *V. penaeicida* *Vibrio* sp. *Aeromonas* spp. *Micrococcus sp.* *Flavobacterium sp.* *Streptococcus sp.* *Staphylococus sp.*
Anaerobic bacteria	*Clostridium botulinum*		

Transmission electron microscopy. Microvilli began to develop in the rotifer feeding regime (Fig 1a). An increase in the number of microvilli was detected in the *Artemia* feeding regime (Fig 1b). Bacterial transition was not detected in either of these two stages. Very small cocci-shaped bacteria entered the fish gut by endocytosis in the granulated feeding stage (Fig 1c). In the TEM analysis of marine juvenile samples, it was observed that many bacteria were present among the microvilli and a dense bacterial transition from the microvilli to the fish gut was observed (Fig 1d).

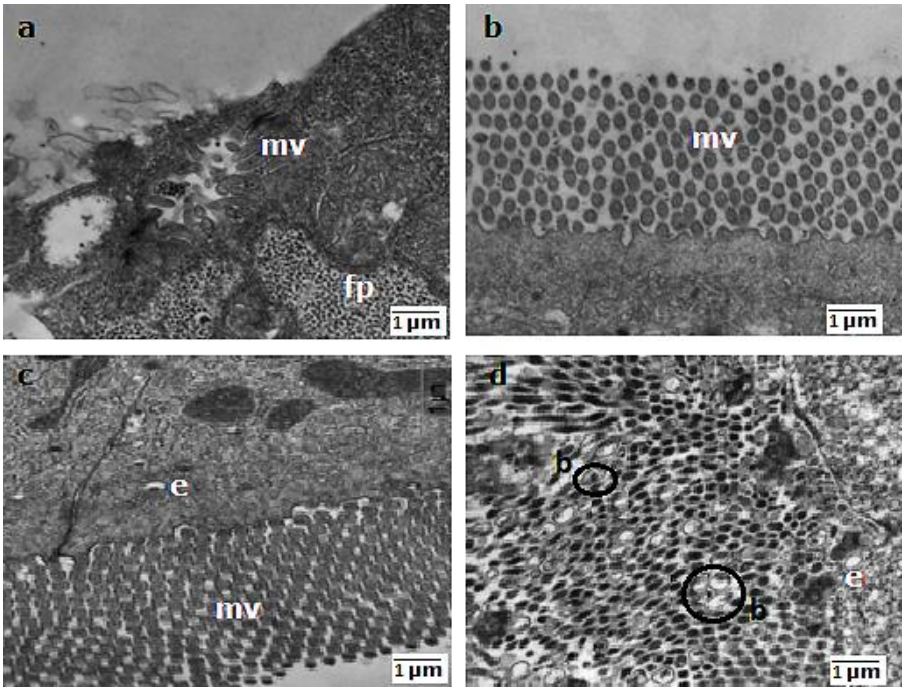

Fig.1. Transelectron microscopic view of larval and juvenile fish gut and bacterial translocation
a-rotifer feeding stage;
b-Artemia feeding stage;
c-granulated feeding stage; d-juvenile fish gut
mv: microvilli; fp: food particles; e: endocytosis of bacteria; b: bacteria

Discussion

Successful larval rearing of marine fish species depends on management of complex interactions between the host and pathogenic or environmental bacterial communities that colonize the gastrointestinal tract and the rearing systems (Hansen, and Olafsen, 1999; Olafsen, 2001). In this study, various bacterial isolates were recovered from healthy and moribund fish samples, hatchery water and sea water. The isolates were in the main Gram-negative (mainly *Vibrio* spp.) species. It was determined that some of the pathogenic bacteria originate in hatcheries via the live feed but some pathogens originate from the marine environment.

Vibrio species were predominant at all life stages of healthy common dentex samples, moribund samples, and water. The bacterial examination primarily identified *V. scophthalmi* in moribund fish samples. Other *Vibrio* species such as *V. alginolyticus, V. splendidus, V. vulnificus and V. mediterranei* were also detected. The gut flora of common dentex in this study was similar to that of other fish species cultured in the Mediterranean region (Grisez et al., 1997; Savas et al., 2005; Ayaz, 2006). *V. scophthalmi* (Sitja-Bobadilla et al., 2007), *V. alginolyticus* (Company et al., 1999; Rigos and Katharios, 2010) and *V. splendidus* were isolated from reared common dentex (Company et al., 1999; Rigos and Katharios, 2010). In our study we isolated these bacteria from normal gut

flora of common dentex larvae during the endogenous feeding stage, which suggests a possible bacterium transition from the broodstock to larvae. *V. vulnificus* was isolated in this study. It was identified in normal gut flora of common dentex larvae in the Artemia feeding regime and contributed to mixed bacterial infections. *V. mediterranei* were found in the marine environment but not in moribund samples (Company et al. 1999). In contrast, in our study we found *V. mediterranei* in moribund fish samples and sea water. Our results found that most bacterial species originated and were transferred from marine water to the fish thus causing infections.

Tenacibaculum maritimum, one of the main pathogens of European sea bass cultured in the Mediterranean, was also recovered from cultured moribund common dentex (Mancuso et al., 2005). This study detected *T. maritimum* in the granulated feeding stage of the hatchery and contributed to various infections of juvenile fish. *Aeromonas* species were present in the marine environment, causing diseases in marine fish including common dentex (Yiagnisis and Athanassopoulou, 2011). Although Aeromonads were detected in the marine water and the gut flora of healthy juveniles, *A. sal.* subsp. *achromogenes* was not recovered from the moribund samples. This study shows that common dentex is a carrier of obligate fish parasites.

There is very little information on diseases caused by Gram-positive organisms (Company et al, 1999) in common dentex. For the first time, Gram-positive bacteria such as *Micrococcus luteus*, *Streptococcus* sp. and *Bacillus* sp. were detected in mixed infections. These results show that *Bacillus* sp. and *Streptococci* enter the hatchery system via live food. Gram-positive anaerobic bacteria cause diseases in fish (Trust et al., 2011). In this study, *Eu. tarantellae* were recovered from larval gut flora in the hatchery and *Cl. botulinum* were recovered from juvenile gut flora in the marine stage.

Bacteria are transferred via the gastrointestinal tract in herring (*Clupea harengus*) larvae at an earlier larval stage (Hansen and Olafsen 1999). However, in this study, endocytosis was observed later, during the dry feed stage. Mass bacterial transition and endocytosis was observed in juvenile common dentex in the marine rearing stage for juvenile Arctic charr, *Salvelinus alpinus* (Ringo et al. 2003). This dense bacterial transition observed in the common dentex is thought to be evidence of waterborne pathogenic bacterial transition into this fish species via the gastrointestinal tract.

In conclusion, the dry feed feeding stage in hatcheries is critical for pathogen invasion, based on the observed increase in the bacterial transition. At this stage, optimum culturing conditions for fish and optimum storage conditions for feeds should be provided and the beginning of this stage may be a very appropriate time to start applying probiotics.

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