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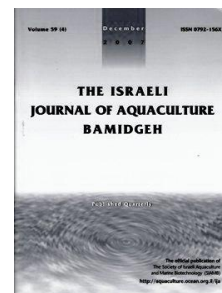
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A Study on Probiotic Bacteria Isolated from Common Dentex (*Dentex dentex*) Larvae and their antagonistic effect on *Photobacterium damsela* subsp. *damsela*

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Short running title: Probiotic bacteria against *P. damsela* subsp. *damsela*

Abstract

Bacterial species that can be considered as probiotic are gradually gaining attention and contributing to the success of aquaculture. The aim of this study was to determine the indigenous dominant probiotic bacterial species of common dentex (*Dentex dentex*) larvae and their effectiveness against the putative fish pathogen *Photobacterium damsela* subsp. *damsela* at different feeding stages. In 2009-2010 samples of larvae and tank water were collected at 5 different occasions. After inoculations of samples, bacterial isolates were identified with standard biochemical methods. The antagonistic effects of the identified probiotic bacteria against *P. damsela* subsp. *damsela* isolated from different mariculture fishes were determined by using disc diffusion method. *Bacillus cereus*, *B. macquariensis*, and other *Bacillus* sp., *Micrococcus luteus*, *Flavobacterium flevense* and *Flavobacterium* sp. were identified as candidate probiotic species and their antagonistic effect against different *P. damsela* isolates was identified. The results of this study showed that *B. cereus* and *M. luteus* are more effective against *Photobacterium damsela* subsp. *damsela*. As a result, the identification of the candidate probiotic species and the widespread use of probiotics may improve the success of common dentex culture in the future.

Introduction

Common dentex (*Dentex dentex* L., 1758) is a promising Sparid fish species for Mediterranean aquaculture (Koumoundouros et al., 2004). However, high mortality rates during the larval stages inhibits the culture (Efthimiou et al., 1994; Rueda and Martinez, 2001).

Probiotics are cultured antioxidants or live microbial feed supplements (Fuller, 1986) and the probiotic treatments may be considered as methods of biological bio treatments (Gatesoupe, 1999; Ganguly, 2010). Microbial management of culture water with probiotics and stimulation of the immune system can have important influences on the growth of sparid larvae (Yufera, 2011). Because of potential antibiotic resistance and

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growing reluctance of the use of antibiotics, probiotics in general and especially in larviculture is becoming increasingly popular (Gatesoupe, 1999; Gomez-Gil et al., 2000).

Bacterial antagonism is a common phenomenon in nature (Nayak and Mukherjee, 2011). Some produce inhibitory compounds, particularly in the digestive tract, that are responsible for inhibiting the colonization of potential pathogens in fish (Irianto and Austin, 2002). Isolation of putative probiotic bacteria from the indigenous microbiota of fish or their rearing environment (Gullian et al., 2004) may have desirable probiotic effects (Verschuere et al., 2000). Some known probiotic bacteria are *Bacillus*, *Vibrio*, *Micrococcus*, *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Xanthomonas* and *Achromobacter* genera (Gauthier et al., 1975; Austin, 1989, Bernan et al., 1997).

Vibrio species are abundant in the marine environment such as *Photobacterium damsela* subsp. *damsela* (syn: *Vibrio damsela*) cause vibriosis, a particularly severe disease and great economic losses in aquaculture (Austin and Austin, 2012). Additionally, it is a zoonotic bacteria that has also been isolated from human wounds (Love et al., 1981). The aim of this study is to determine the dominant probiotic bacterial species of common dentex larvae production systems and to determine the antagonistic activity of these against *P. damsela* subsp. *damsela*.

Materials and Methods

Sample collection. Samples of common dentex larvae and their respective tank water were collected at 5 different occasions in 2009-2010 from a land-based commercial farm. A total of 100 larvae at different feeding stages (no feeding, rotifer, *Artemia* spp. and artificial pellet weaning) were collected. They were first disinfected with benzalconium chloride and then homogenized in phosphate buffered saline (PBS) (Grisez et al., 1997). Water samples were collected in sterile glass tubes and all samples were brought to the laboratory within half an hour.

Isolation and identification of marine bacteria. To isolate marine bacteria, intestine homogenates of the larvae and water samples were diluted separately at different ratios (1/10, 1/100, 1/1.000 and 1/10.000) and were inoculated onto Marine Agar, PCA (Plate Count Agar) and TSA (Tryptic Soy Agar) supplemented with 1% NaCl. Isolated bacterial strains were identified with standard biochemical methods (Holt et al., 1994; Austin and Austin, 2012) and API kits (API STAPH, API 20E). The pathogenic bacterium *P. damsela* subsp. *damsela* used for this study were previously isolated from diseased gilt-head sea bream cultured in the same region.

Antagonistic effect. The antagonistic effect of the identified probiotic bacteria against different *P. damsela* subsp. *damsela* isolates was determined by Kirby-Bauer disc diffusion method modified by Bhunia et al. (1988). The candidate probiotic bacterial isolates and the control bacterial isolates were freshly cultured in TSB (Tryptic Soy Broth). Washed cells of *P. damsela* subsp. *damsela* at concentration of 10^6 cells/ml (560 μ l) were added into 7 ml TSA and then mixed. This inoculum was transferred on to plates. Candidate probiotic bacterial cells were added to paper discs that were placed on the medium and incubated for 48 h. Later the zone diameters were measured. All experiments were conducted in triplicates for each candidate probiotic species and a total of 18 *in-vitro* antagonistic effect tests were carried out.

Results

Bacterial isolates. 40 bacterial isolates were recovered and identified from the fish larvae and water samples. The biochemical properties of candidate probiotic bacteria and the *P. damsela* subsp. *damsela* used in this study are shown on Table 1.

Fifteen Gram-positive, *Bacillus* shaped, endospore-forming and halophilic isolates were identified as *Bacillus* sp. After the additional tests, 8 of them were identified as *B. cereus*, 5 of them as *B. macquariensis* and 2 isolates as *Bacillus* sp.. Fifteen Gram-positive isolates non-motile, cocci shaped tetrads were identified as *M. luteus*. Ten Gram-negative oxidative isolates were identified as *Flavobacterium* sp., and 6 of them as *F. flevense* and 4 of as *Flavobacterium* sp.

Table 1. Biochemical properties of candidate probiotic bacteria and the pathogenic bacterium *P. damsela* subsp. *damsela*

Characteristics	<i>Bacillus cereus</i>	<i>Bacillus macquariensis</i>	<i>Bacillus</i> sp.	<i>Flavobacterium flevense</i>	<i>Flavobacterium</i> sp.	<i>Micrococcus luteus</i>	<i>P. damsela</i> subsp. <i>damsela</i>
Gram staining	+	+	+	+	+	+	-
Catalase	+	+	+	+	+	+	+
Oxidase	ND	ND	-	+	+	+	+
Motility by flagella	+	-	+	-	-	-	+
Gliding motility	-	-	-	+	+	-	-
O/F	O	F	F	O	F	O	F
VP	+	+	+	-	-	-	+
Indole	-	-	ND	-	-	-	-
Arginine	-	-	-	-	-	V	+
Ornithine	-	-	-	-	-	-	-
Lysine	-	-	-	-	-	-	-
MR	+	-	ND	ND	-	-	+
β -galactosidase	+	-	-	+	+	-	-
Acid production from							
D-glucose	+	+	+	+	V	-	+
L-arabinose	V	+	-	-	-	-	-
D-xylose	-	-	+	-	-	-	-
D-mannitole	-	+	+	-	+	V	-
Degradation of							
Casein	V	+	+	-	-	-	-
Gelatin	-	-	-	-	+	+	-
Starch	+	+	-	-	ND	ND	+
Aesculin	-	-	+	+	+	+	-
Utilization of citrate	+	+	+	-	-	-	-
Urease	V	-	-	-	V	V	+
Nitrate reduction	+	-	+	-	ND	-	+
Growth in							
2% NaCl	+	+	+	+	+	+	+
5% NaCl	+	-	+	-	-	+	+
7% NaCl	+	-	-	-	-	+	-

Antagonistic activity against P. damsela subsp. *damsela*. Through the disc diffusion procedure candidate bacteria showing an antagonistic effect against *P. damsela* subsp. *damsela* by producing clear inhibition zones on TSA (Fig. 1) were determined. *Bacillus cereus*, that produced an inhibition zone with a diameter of 28 mm was the most effective, while *M. luteus* (22 mm) and *Flavobacterium flevense* (20 mm) showed a moderate antagonistic effect against *P. damsela* subsp. *damsela*. *B. macquariensis* (15 mm), *Bacillus* sp. (15 mm) and *Flavobacterium* sp. (13 mm) were found to be relatively less effective.

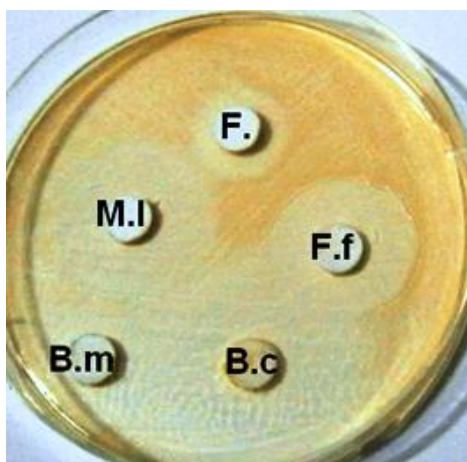


Fig. 1. Inhibition zones of candidate probiotic bacteria against *P. damsela* subsp. *damsela* on TSA. (F.: *Flavobacterium* sp. F.f: *F. flevense*, M.l: *M. luteus*, Bc: *Bacillus cereus*, B.m: *B. macquariensis*)

Discussion

Probiotic application in aquaculture against diseases induced by pathogenic bacteria is a relatively recent technique with a potential natural inhibitory mechanism (Fuller, 1986;

Gatesoupe, 1999; Verschuere et al., 2000; Irianto and Austin, 2002) Most candidate probiotic microorganism selection studies carried out have focused on in vitro antagonism tests, which confirm the production of inhibitory compounds against pathogenic microorganisms (Gomez-Gil et al., 2000; Slierendrecht and Gram, 2001; Chabrillon et al., 2006). Here we report that *Bacillus cereus*, *B. macquariensis*, *Bacillus* sp., *Micrococcus luteus*, *Flavobacterium flevense* and *Flavobacterium* sp. isolated from common dentex larval culture systems have antagonistic effects against *P. damsela* subsp. *damselae*.

The organisms selected in the present study were identified as *Bacillus*, *Micrococcus* and *Flavobacterium* that are the marine microorganisms with a potential antagonistic effect (Gauthier et al., 1975; Austin, 1989, Bernan et al., 1997). Besides, these bacteria are present in the larval intestine and in the rearing water as described by other researchers (Slierendrecht and Gram, 2001; Irianto and Austin, 2002).

Photobacterium damsela contains two subspecies (*P. damsela* subsp. *damselae* and *P. damsela* subsp. *piscicida*) both of which are pathogenic to fish (Austin and Austin, 2012). Chabrillon et al. (2005) studied the antagonistic effect of the bacteria isolated from sole against *P. piscicida* in gilt-head sea breams. Yet apparently no such studies were reported on *P. damsela* subsp. *damselae*. Only one probiotic trial study was nevertheless conducted against this pathogen in shrimp with *B. subtilis* (Vaseeharan and Ramasamy, 2003). This study appears therefore to be the first to report the inhibitory activity of intestinal microflora of *Dentex dentex* against *P. damsela* subsp. *damselae*. According to Sanders et al. (2003), members of the genus *Bacillus* produce a large number of antimicrobials such as *Bacillus cereus*, *B. macquariensis* and *Bacillus* sp that were also identified in our study.

M. luteus, a member of the microbial flora of the fresh-water ecosystem that was previously used as a probiotic agent against Aeromonad infections in rainbow trout (Austin et al., 1992) and tilapia (Abd El-Rahman et al., 2009) produced a strong antibiotic effect against *Listonella anguillarum* and *V. harveyi* (Chabrillon et al., 2006) but a considerably lower antagonistic effect against *P. damsela* subsp. *piscicida* (Chabrillon et al., 2005).

Flavobacterial species that are mostly pigmented are members of the marine and freshwater ecosystems can also be found on fish skin, gills and intestinal flora (Austin and Austin, 2012). This concurs with our findings nevertheless the results presented here indicated that *Flavobacterium flevense* and *Flavobacterium* sp. showed a relatively lower antagonistic effect against *P. damsela* subsp. *damselae*.

This study is a preliminary step in the investigation of effective probiotic bacterial species against *P. damsela* subsp. *damselae*. Further investigations regarding probiotic suitability and in vivo pathogenicity tests of these bacteria should be carried out before they can be used in aquaculture.

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