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# ***Photobacterium damsela* subsp. *damsela*, an Emerging Pathogen Affecting New Cultured Marine Fish Species in Southern Spain**

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## **1. Introduction**

Aquaculture is the fastest growing food-producing sector, accounting almost 50% of the world food fish demand. Considering the projected population growth over the next two decades, it is estimated that at least an additional 40 million tonnes of aquatic food will be required by 2030 to maintain the current per capita consumption (NACA/FAO, 2001). Marine aquaculture production was 30.2 million tonnes in 2004, representing 50.9% of the global aquaculture production (FAO, 2004). By major groupings, fish is the top group whether by quantity or by value at 47.4% and 53.9%, respectively. However, according to the World Aquaculture Society (WAS, 2006), the future of this sector must be based on the increase of scientific and technical developments, on sustainable practices, and, mainly, on the diversification of the cultured fish species. For this reason, the European Union has designed an innovative plan to increase the culture of new fish and shellfish species, mainly marine, maintaining the production of other consolidated species (UE, 2010).

Marine fish farming is a very important activity of Spanish aquaculture industry. The main marine fish species intensively cultured are gilt-head seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), and turbot (*Scophthalmus maximus*), achieving production percentages of 47.91, 12.5 and 18.62%, respectively (MAPA, 2008). In last 7 years, several new marine fish species are being evaluated as potential candidates for aquaculture production. In Southern Spain, studies on the reproductive cycles, nutrition, growth, histology and immune system of species such as Senegalese sole (*Solea senegalensis*), redbanded seabream (*Pagrus auriga*), common seabream (*Pagrus pagrus*), white seabream (*Diplodus sargus*), and meagre (*Argyrosomus regius*) are ongoing (Cardenas & Calvo, 2003; Prieto et al., 2003; Ponce et al., 2004; Manchado et al., 2005; Fernandez-Trujillo et al., 2006; 2008; Martin-Antonio et al., 2007; Cardenas & Manchado, 2008). However, the intensive

culture of these new fish species has favoured the appearance of several outbreaks with varied mortality rates.

The development of a fish disease is the result of the interaction among pathogen, host and environment. Therefore, only multidisciplinary studies involving the virulence factors of the pathogenic microorganisms, aspects of the biology and immunology of the fish, as well as a better understanding of the environmental conditions affecting fish cultures, will allow the application of adequate measures to control and prevent the microbial diseases limiting the production of marine fish. According to Toranzo et al. (2005), several aspects would be raised regarding the infectious diseases caused by bacteria in marine fish: (i) only a relatively small number of pathogenic bacteria are responsible of important and significant economic losses in cultured fish; (ii) several classical diseases considered as typical of fresh water aquaculture are today important problems in marine culture; (iii) clinical signs (external and internal) provoked by each pathogen depend on the host species, fish age and stage of the disease; (iv) there is no correlation between external and internal signs of the disease; and (v) the severity of the disease and the mortality are higher in cultured fish than in wild fish populations, because to the lack of the stressful conditions that usually occur in the culture facilities.

In the present study, the description of the outbreaks and the characterization of the etiological agents involved are described in detail. From the results obtained, *Photobacterium damsela* subsp. *damsela* was the most frequently pathogenic bacteria implicated in these outbreaks. This microorganism has been recognized as a pathogen for a wide variety of aquatic animals, such as crustaceans, molluscs, fish and cetaceans. In addition, this bacterial pathogen has been reported to cause diseases in humans and, for this reason, it may be considered as an agent of zoonoses. We have revised the taxonomical position, and phenotypic and molecular characteristics of this microorganism. In addition, we describe the virulence properties and pathogenesis mechanisms of *P. damsela* subsp. *damsela*.

## **2. Microbiological study of newly cultured marine fish species in Southern Spain**

### **2.1 Macroscopic signs of disease in farmed fish**

Labella (2010), studying the microbial origin of diseases affecting new cultured marine fish species in Southern Spain, reported the occurrence of 9 epizootic outbreaks (from 2003 to 2006) affecting cultures of redbanded seabream (7 outbreaks), common seabream (3 outbreaks), white seabream (2 outbreaks), and meagre (1 outbreak). The mortality of these outbreaks varying between 5 and 94%, depending on season, affected fish species, and fish age (Table 1). Gross external and internal signs varied depending on the outbreak, being similar to those previously described for vibriosis in several fish species (Fouz et al., 1992; Balebona et al., 1998b). The main external signs were exophthalmia, dark skin pigmentation, and pale gills and eroded fins (Fig. 1), whilst in some specimens from outbreaks 3, 5 and 8 haemorrhagic areas and epidermic ulcers were observed. Internally, the predominant infection signs were the presence of a fatty liver, with or without petechiae, and abdominal swelling with ascitic liquid. Splenomegaly and visceral fat accumulation were also recorded (Fig. 1).

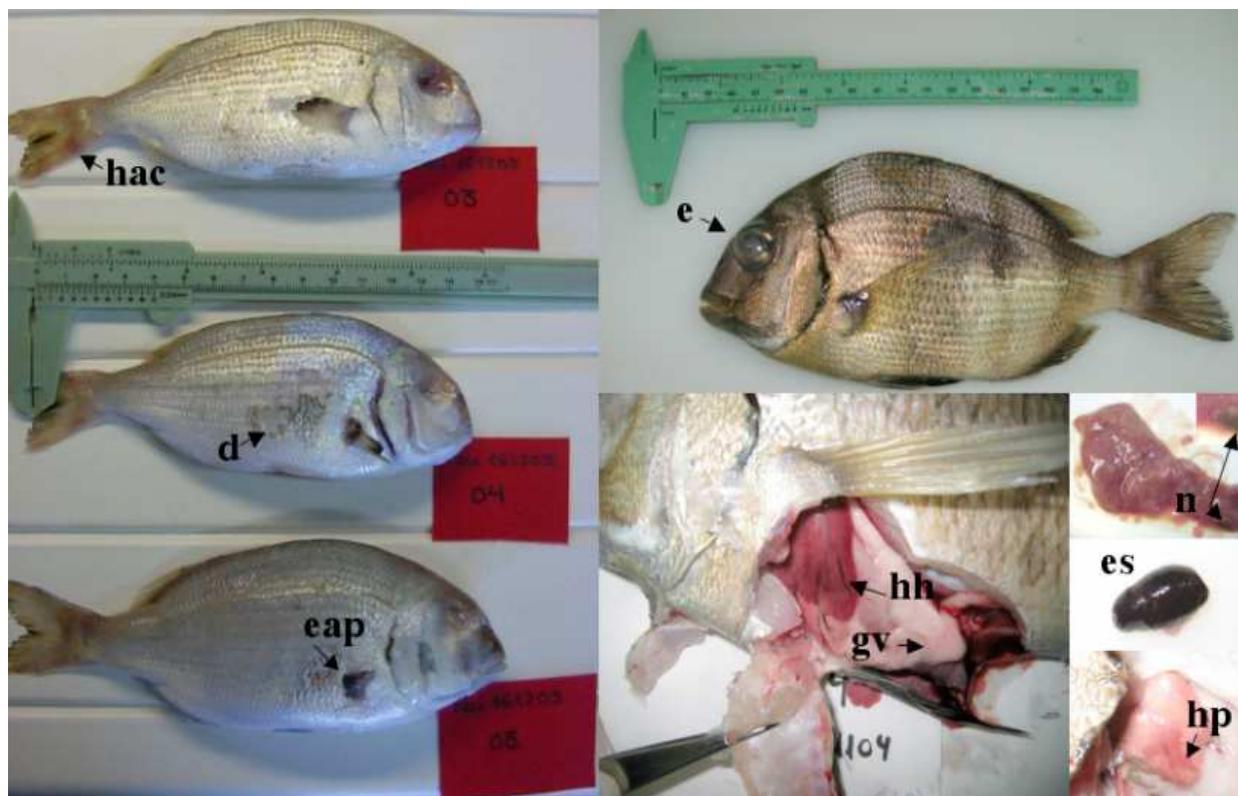


Fig. 1. Main external symptoms and pathological signs in the internal organs observed in affected fish. (hac) haemorrhages in caudal fin; (d) skin desquamation; (eap) fin erosion; (e) exophthalmia; (hh) haemorrhagic liver; (gv) visceral fat accumulation; (n) necrosis; (es) splenomegaly; (hp) petechiae.

## 2.2 Organs used for bacterial culture and its isolation

Affected or moribund specimens were killed with an overdose of MS-222 in seawater, and immediately processed for bacteriological analyses. Samples collected from the skin, eyes, brain, liver, spleen and kidney were seeded onto several routine bacteriological culture media for isolation of bacterial fish pathogens. The bacterial isolates were subjected to phenotypic characterization by using the tests specified in Table 2, according to Bergey's Manual of Systematic Bacteriology (Thyssen & Ollevier, 2005). All isolated bacteria were Gram-negative short rods or cocobacilli, motile, oxidase and catalase positives, glucose fermenters, and sensitive to vibriostatic agent pteridine (O/129, 150 µg). Bacterial identification was confirmed by the analysis of 16S rRNA gene sequences, amplified and sequenced as previously described by Labella et al. (2006). Table 1 shows the bacterial species identified and confirmed by 16S rDNA sequences.

Outbreak no. (date)	Affected species	Weight (g)±SD	Cumulative mortality (%)	Bacterial species
1 (December 2003)	<i>Pagrus auriga</i>	210 ± 80	22.0	<i>P. damsela</i> subsp. <i>damsela</i>
2 (April-June 2003)	<i>P. auriga</i>	277 ± 93	26.0	<i>P. damsela</i> subsp. <i>damsela</i>
2 (May-June 2003)	<i>Pagrus pagrus</i>	213 ± 60	26.5	<i>V. ichthyoenteri</i> <i>V. harveyi</i> <i>V. alginolyticus</i> <i>V. fischeri</i> <i>V. splendidus</i>
3 (July 2004)	<i>P. auriga</i>	35 ± 7	20.0	<i>V. harveyi</i>
3 (August 2004)	<i>P. pagrus</i>	300 ± 55	15.0	<i>V. harveyi</i> <i>V. alginolyticus</i> <i>V. splendidus</i>
4 (October-December 2004)	<i>P. auriga</i>	555 ± 137	28.0	<i>P. damsela</i> subsp. <i>damsela</i>
5 (February 2005)	<i>P. auriga</i>	627 ± 110	13.0	<i>V. splendidus</i>
5 (February 2005)	<i>P. pagrus</i>	311 ± 62	25.0	<i>V. splendidus</i> <i>V. ichthyoenteri</i>
6 (May 2005)	<i>Diplodus sargus</i>	2 ± 0.33	80.0	<i>P. damsela</i> subsp. <i>damsela</i> <i>V. fischeri</i>
7 (August 2005)	<i>D. sargus</i>	600 ± 70	94.0	<i>P. damsela</i> subsp. <i>damsela</i>
8 (May 2006)	<i>P. auriga</i>	60 ± 27	5.0	<i>V. ichthyoenteri</i> <i>V. harveyi</i>
9 (August 2006)	<i>Argyrosomus regius</i>	10 ± 0.3	80.0	<i>P. damsela</i> subsp. <i>damsela</i>

Table 1. Epizootic outbreaks affecting newly cultured marine fish species in Southern Spain and identification of the isolated bacterial strains.

Test	<i>P.</i> <i>damsela</i> subsp. <i>damsela</i>	<i>V.</i> <i>harveyi</i>	<i>V.</i> <i>alginolyticus</i>	<i>V.</i> <i>fischeri</i>	<i>V.</i> <i>splendidus</i>	<i>V.</i> <i>ichthyoenteri</i>
Growth in TCBS	G <sup>a</sup>	Y <sup>b</sup>	Y	G	Y	Y
Swarming	-	+	+	-	-	-
Arginine dehydrolase	+	V <sup>c</sup>	-	-	V	-
Lysine decarboxylase	V	+	+	+	V	+
Ornithine decarboxylase	-	+	+	-	V	+
Urease	V	+	-	+	-	-
Indole production	-	+	V	-	+	+
Acetoin production	V	-	-	-	-	-
Amylase	-	+	+	-	+	-
Gelatinase	-	+	+	-	+	-
Esculin hydrolysis	-	+	-	-	-	-
Nitrate reduction	+	+	+	+	+	-
Citrate utilization	-	+	-	+	+	+
Gas from glucose	+	-	-	-	-	-
Acids from:						
Lactose	-	-	-	V	-	-
Trehalose	V	+	+	-	V	+
Cellobiose	+	+	+	+	+	-
D-galactose	+	+	-	+	-	-
Growth in:						
0% NaCl	-	-	-	-	-	-
1% NaCl	+	+	+	+	V	V
6% NaCl	+	+	+	V	V	+
8% NaCl	-	+	+	-	-	-
Growth at:						
4°C	V	-	-	-	-	-
35°C	+	+	+	V	-	-

<sup>a</sup>Green colony; <sup>b</sup>Yellow colony; <sup>c</sup>Variable result.

Table 2. Phenotypic features of bacterial isolates collected from fish epizootic outbreaks.

### 2.3 Antimicrobial resistance

The antimicrobial resistance pattern to 14 antimicrobials routinely used in aquaculture practice was determined for selected strains of each identified bacterial pathogen. All the bacterial strains tested showed sensitivity to chloramphenicol, enrofloxacin, flumequine and nalidixic acid (Table 3). On the other hand, all the bacterial strains presented resistance to streptomycin, and a variable resistance pattern to the other 9 antimicrobials tested (Table 3). Labella (2010) reported three different resistotype profiles for the bacterial pathogens involved in the epizootic outbreaks. The resistotype I consisted of all the *Photobacterium damsela* subsp. *damsela* strains, and it is characterized for the sensitivity to trimethoprim-sulphamethaxazole, oxolinic acid, and nitrofurantoin. The resistotype II grouped the isolates included in the *Vibrio harveyi*, *V. splendidus*, *V. fischeri* and *V. alginolyticus* species. This resistotype possessed sensitivity to oxolinic acid, erythromycin, tetracycline, oxytetracycline, nitrofurantoin and novobiocin. Finally, the resistotype III included all the strains of *V. ichthyenteri*, and presented sensitivity to trimethoprim-sulphamethaxazole, tetracycline, oxytetracycline, novobiocin and amoxicillin (Table 3).

Antimicrobials ( $\mu\text{g}/\text{disc}$ )	Resistance profiles		
	I	II	III
Chloramphenicol (30)	S <sup>a</sup>	S	S
Enrofloxacin (5)	S	S	S
Flumequine (30)	S	S	S
Nalidixic acid (30)	S	S	S
Streptomycin (10)	R <sup>b</sup>	R	R
Trimethoprim-sulphamethaxazole (1.25 + 23.75)	S	R	S
Oxolinic acid (10)	S	S	R
Erythromycin (15)	R	S	R
Tetracycline (30)	R	S	S
Oxytetracycline (30)	R	S	S
Nitrofurantoin (300)	S	S	R
Novobiocin (30)	R	S	S
Amoxicillin (25)	R	R	S
Ampicillin (10)	V <sup>c</sup>	R	R

<sup>a</sup>Sensitive; <sup>b</sup>Resistant; <sup>c</sup>Variable result.

Table 3. Antimicrobial resistance patterns of the bacterial isolates involved in epizootic outbreaks.

On the basis of its high prevalence in the epizootic outbreaks recorded, *P. damsela* subsp. *damsela* was considered as the main bacterial pathogen affecting new cultured marine fish species in Southern Spain (Garcia-Rosado et al., 2007).

## 3. Study of *Photobacterium damsela* subsp. *damsela*

### 3.1 Taxonomical position

The taxonomic status of *P. damsela* subsp. *damsela* within the family *Vibrionaceae* has changed repeatedly. *P. damsela* subsp. *damsela* was initially isolated as *Vibrio damsela* from skin ulcers of temperate-water damselfish (Love et al., 1981), and was recognized as

an opportunistic pathogen capable of causing disease in a variety of hosts, mainly fish and mammals. MacDonell & Colwell (1985) transferred this species to the new genus *Listonella* based on a review of phylogenetic relationships within the family by 5S rRNA sequence data. Later, Smith et al. (1991) transferred this species to the genus *Photobacterium* based on phenotypic data, which was further supported from the phylogenetic analysis carried out by Ruimy et al. (1994). Similarly, Gauthier et al. (1995) demonstrated that the fish pathogen *Pasteurella piscicida* was closely related to *P. damsela* on the basis of phylogenetic analysis of small-subunit rRNA sequences and DNA-DNA hybridization data. Accordingly, *P. damsela* was proposed to include two subspecies, *P. damsela* subsp. *damsela* and *P. damsela* subsp. *piscicida*. However, Thyssen et al. (1998) have showed that there is no phenotypic evidence that supports the inclusion of *P. damsela* subsp. *piscicida* as a subspecies of *P. damsela*. The distinctive diagnoses of both subspecies of *P. damsela* can be achieved by a multiplex PCR assay, which combines specific primers for 16S rRNA and urease C genes (Osorio et al., 2000b).

A new species, named *P. histaminum*, has been described by Okuzumi et al. (1994) as a halophylic potent histamine-producing bacterium. The new species has been distinguished from other members of the genus based on phenotypic characteristics, 16S rRNA gene sequence and DNA-DNA hybridization. A close physiological similarity between *P. damsela* subsp. *damsela* and *P. histaminum* has been reported (Dalgaard et al., 1997). Kimura et al. (2000) found that the type strain of *P. damsela* subsp. *damsela* has a histamine-producing ability as potent as *P. histaminum*. In addition, the levels of DNA relatedness between both species ranged from 80 to 88%. Regarding to the phenotypic differentiation of these organisms, the authors confirmed a biochemical profile identical for both species, except for the trehalose utilization. For these reasons, Kimura et al. (2000) proposed *P. histaminum* as a later subjective synonym of *P. damsela* subsp. *damsela*.

### 3.2 Phenotypic and molecular characteristics

According to Bergey's Manual of Systematic Bacteriology (Thyssen & Ollevier, 2005), *P. damsela* subsp. *damsela* belongs to the genus *Photobacterium* included in the family *Vibrionaceae*, displaying morphological characteristics typical of members of the family, appearing as coccobacilli. The flagellate organisms lack of a flagellar sheath, even *P. damsela* subsp. *piscicida* lacks flagella (Baumann & Baumann, 1981).

Labella (2010) carried out a wide study on the phenotypic characteristics of *P. damsela* subsp. *damsela*, resulting positive the following traits: motility, catalase, cytochrome oxidase, growth at the range of 20-35°C and in 1-6% NaCl, arginine dehydrolase, nitrate reduction, and fermentation of melibiose and maltose. *P. damsela* subsp. *damsela* uses the following compounds as unique carbon source: D-galactose,  $\alpha$ -D-glucose, raffinose, turanose, D-ribose, N-acetylgalactosamine, glycogen, methyl  $\alpha$ -D-glucoside, dextrin, D-glucose 6 phosphate, glycerol, sorbitol, succinate, D-L-lactic acid, glycil L-glutamic acid, tween 40, tween 80, L-glutamic acid, glycil L-aspartic acid, inosine, L-serine, L-aspartic acid, L-asparagine, L-alanine, uridine, L-alanyl glycine and thymine. On the other hand, *P. damsela* subsp. *damsela* strains showed negative results for ornithine decarboxylase, production of H<sub>2</sub>S, indole production, alginase, and fermentation of D-mannitol, D-sorbitol, inositol, erytritol, D-adonitol, D-arabitol, dulcitol, raffinose, L-rhamnose and L-arabinose. This subspecies is unable to use D-fucose,  $\alpha$ -D-lactose, L-arabinose, gentibiose, melibiose, L-rhamnose, D-mannitol, adonitol, myo-inositol, erytritol, xylitol, arabinitol, acetate, L-

glutamate, formate, D-gluconic acid, propionic acid, L-leucine, D-alanine, L-proline, L-threonine, L-ornithine, L-histidine,  $\alpha$ -ceto glutaric acid, aconytic acid and  $\beta$ -hydroxyphenylacetic acid.

The fatty acid profile of *P. damsela* subsp. *damsela* contains high concentrations of C<sub>16:1</sub>, C<sub>16:0</sub>, and C<sub>18:1</sub> fatty acids, and in lesser extent C<sub>12:0</sub>, C<sub>14:0</sub>, C<sub>14:1</sub>, C<sub>15:0</sub>, C<sub>17:0</sub>, C<sub>17:1</sub>, and C<sub>18:0</sub> (Nogi et al., 1998). The electrophoretic analyses carried out revealed similar band pattern for *P. damsela* strains, sharing four major outer membrane proteins (OMP), with molecular masses of 20, 30, 42 and 53 kDa (Magariños et al., 1992). An OMP of 37 kDa (OMP-PD) forms a trimeric structure of approximately 110 kDa that conform an ion channel and acts as a porin in *P. damsela* (Gribun et al., 2004). These results have been confirmed by Western blot performed with anti-OMP polyclonal serum against the monomeric form of OMP-PD. As in the case of the OMP, all *P. damsela* strains showed the same silver-stained lipopolysaccharide (LPS) profile obtained by proteinase K digested whole cell lysates. This profile had a ladder like pattern, typical of smooth type LPS (S-LPS), with low amounts of 2-keto-3-deoxyoctonate (KDO) (Kuwaie et al., 1982).

The G+C content of the genomic DNA of *P. damsela* subsp. *damsela* is 40.6-41.4 mol% (Thyssen & Ollevier, 2005). The DNA relatedness of *P. damsela* subsp. *damsela* and other classical *Photobacterium* species, demonstrated by DNA-DNA hybridization, varied between 12 and 37% with *P. leiognathi*, 21 and 30% with *P. phosphoreum*, 19.5% with *P. profundum* and 28% with *P. augustum* (Nogi et al., 1998; Kimura et al., 2000). Plasmids are present in most *P. damsela* subsp. *damsela* strains tested, with sizes ranging from 3.0 kb to higher than 190 kb (Pedersen et al., 1997).

Several studies have demonstrated that strains of *P. damsela* subsp. *damsela* showed a high heterogeneity in biochemical and serological characteristics (Smith et al., 1991; Fouz et al., 1992; Pedersen et al., 1997; 2009; Labella et al., 2006). Botella et al. (2002) established that 11 biochemical features were variable among the 33 *P. damsela* subsp. *damsela* strains tested: acetoin production, luminescence, gas from glucose, lysine decarboxylase, growth at 4° and 40°C, urease, and utilization of sucrose, D-mannose, D-cellobiose and D-gluconate. Labella et al. (2009) obtained that *P. damsela* subsp. *damsela* strains showed variability for the following tests: acetoin production,  $\beta$ -galactosidase, lysine decarboxylase, growth at 4° and 40°C, esculin hydrolysis, acid from mannitol, sorbitol and amygdalin, citrate utilization and assimilation of D-mannose, maltose, malate and N-acetylglucosamine. This variability led the authors to establish 8 different biotypes or phenotypic profiles among the 17 strains tested, isolated from cultured marine fish.

However, the genetic variation of the strains of *P. damsela* subsp. *damsela* has received less attention. Botella et al. (2002), using the amplified fragment length polymorphism (AFLP) technique, demonstrated a high genetic variability among the *P. damsela* subsp. *damsela* strains isolated from gilthead seabream and European seabass cultured in the same geographical area and collected in a short time period (2 years). In fact, the 33 tested strains yielded 24 AFLP profiles, with almost every strain showing a different band pattern.

Takahashi et al. (2008) established, using ribotyping, AFLP and pulsed-field gel electrophoresis (PFGE), that *P. damsela* subsp. *damsela* clinical isolates causing fatal cases in humans had similar genotypes, but they were not clearly distinguishable from environmental isolates (including isolates from fish). Nevertheless, the phenotypic profiles of the clinical isolates were clearly distinct from those showed by environmental isolates. The authors explained the inconsistency between the results obtained from genotypic and

phenotypic analysis arguing that the divergence of clinical strains from environmental ones is a recent event, and these phenotypic differences are so small that they are not detected by whole genome typing techniques such as PFGE and AFLP. However, sequencing analysis of the *gyrB*, *toxR* and *ompU* genes showed larger differences between clinical and environmental isolates. Similar results were obtained by Labella (2010) comparing these clinical strains with fish isolates by repetitive extragenic palindromic (REP)-PCR.

Labella et al. (2009) compared three PCR-based techniques [random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC)-PCR, and REP-PCR] for the analysis of genetic variability within *P. damsela* subsp. *damsela* strains isolated from several fish species in outbreaks occurred in different geographical locations. All the PCR-based typing methods supported the high variability within *P. damsela* subsp. *damsela*, the strains being discriminated into 8-14 genetic groups, depending on the method employed. In addition, no concordance among the genetic assignation of the strains by the different PCR methods was obtained. These results suggest, as concluded also by Botella et al. (2002), that different clonal variants of *P. damsela* subsp. *damsela* potentially pathogenic for several fish species exist, and even can be involved in a single outbreak. On the other hand, and similarly to previous results (Botella et al., 2002), a relationship between the genetic profiles and the origin of isolation or the host fish species could not be established.

### 3.3 Pathogenicity and virulence factors

*P. damsela* subsp. *damsela* has been recognized as a bacterial pathogen in a wide variety of aquatic animals including fish, molluscs and crustaceans (Vera et al., 1991; Fouz et al., 1992; Company et al., 1999; Sung et al., 2001; Lozano-Leon et al., 2003; Labella et al., 2006; Wang & Cheng, 2006; Vaseeharan et al., 2007; Han et al. 2009; Kanchanopas-Barnette et al., 2009). This microorganism is an autochthonous inhabitant of aquatic ecosystems, which may survive in seawater and sediment for a long time, maintaining its infectivity and pathogenic properties (Ghinsberg et al., 1995; Fouz et al., 1998; 2000). In addition, *P. damsela* subsp. *damsela* may be a primary pathogen for mammals, including humans (Morris et al., 1982; Clarridge & Zigelboim-Daum, 1985; Fujioka et al., 1988; Perez-Tirse et al., 1993; Yuen et al., 1993; Shin et al., 1996; Fraser et al., 1997; Tang & Wong, 1999; Goodell et al., 2004; Yamame et al., 2004). A comparatively small number of bacterial species belonging to the family *Vibrionaceae* causes diseases in both aquatic animals and humans. However, the fact that an organism provokes disease in an aquatic animal does not necessarily mean that this is the source for human infections (Austin, 2010). Indeed, the origin of some of these bacteria may be the waters in which the aquatic animals are found, and the transmission to humans may be via wound or may be food/water-borne.

The extracellular products (ECPs) are produced by bacterial pathogens to facilitate the uptake of nutrients from the surrounding environment and/or for the successful penetration and survival of pathogens inside the host (Sakai, 1985; Bakopoulos et al., 2003). Main ECP components related to virulence include proteases, haemolysins, and siderophore-mediated iron sequestering systems (Norqvist et al., 1990; Toranzo & Barja, 1993; Balebona et al., 1998a; Rodkhum et al., 2005; Wang et al., 2007). These mechanisms can provoke host tissue destruction and haemorrhages, playing an important role in colonization, invasiveness and dissemination of the bacterial pathogen within the host (Finkelstein et al., 1992; Silva et al., 2003). However, only a few studies have been carried out on the role of ECPs in the pathogenesis of *P. damsela* subsp. *damsela*.

Labella et al. (2010), studying the pathogenicity of *P. damsela* subsp. *damsela* strains isolated from cultured fish, demonstrated that the intraperitoneal inoculation of ECPs from virulent strains (mean LD<sub>50</sub> of about  $1 \times 10^5$  CFU) was lethal for redbanded seabream at 2 to 4 h post-inoculation, whilst ECP samples from a non-virulent strain (LD<sub>50</sub> >  $10^8$  CFU) did not produced toxic effects in fish after a 7d post-inoculation period. The inoculation of heated ECPs (100°C, 10 min) to fish did not produce deaths, which suggests that the active toxic fraction present in the ECPs is secreted and thermolabile, and it is not associated with the thermoresistant bacterial lipopolysaccharide content. Similar results have been reported for several fish pathogens (Lamas et al., 1994), including *P. damsela* subsp. *damsela* (Fouz et al., 1995). Fish inoculated with heated ECPs (and also with ECPs from non-virulent strains) showed enlarged lymphohaematopoietic organs (Fig. 2), suggesting a stimulation of immune response with cellular accumulation, as also reported for *Aeromonas hydrophila* ECPs (Rey et al., 2009).

*P. damsela* subsp. *damsela* ECPs displayed cytotoxic activity for different fish and mammalian cell lines (Wang et al., 1998; Labella et al., 2010). The cytotoxicity was limited to ECPs from virulent strains, and it was totally lost on heated ECP samples, which suggests the presence of thermolabile cytotoxic components in the raw ECPs (Labella et al., 2010).

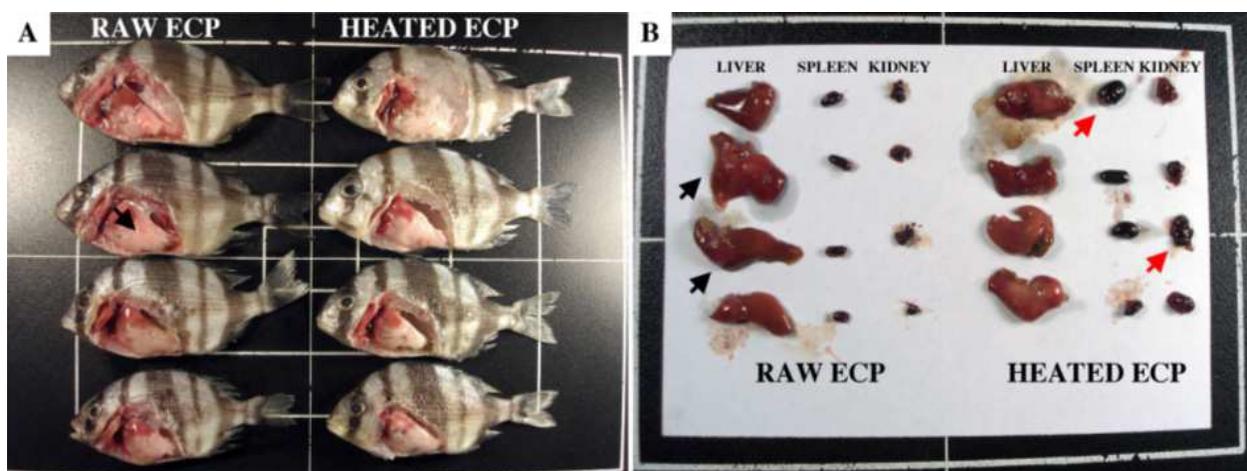


Fig. 2. Effect of the *P. damsela* subsp. *damsela* ECPs (raw and thermally treated at 100°C for 10 min) on redbanded seabream (*P. auriga*) (10 g weight). (A) Arrows indicate the presence of visceral haemorrhages. (B) Black arrows indicate signs of hepatomegaly, and red arrows show the inflammation of haematopoietic organs (spleen and head kidney).

The main virulence factor characterized in *P. damsela* subsp. *damsela* is the damselysin, a thermolabile extracellular cytotoxin of 69 kDa, which is a phospholipase D active against the sphingomyelin of the sheep erythrocyte membrane (Kreger, 1984; Kothary & Kreger, 1985; Cutter & Kreger, 1990). The damselysin also presents haemolytic activity against several erythrocyte types, including fish (Kreger et al., 1987). Classically, damselysin production has been related to the pathogenicity of *P. damsela* subsp. *damsela* in diverse animal models (Kothary & Kreger, 1985; Fouz et al., 1993), although Osorio et al. (2000a) demonstrated that the presence of this toxin is not a requisite for the virulence of this bacterial pathogen. Labella et al. (2010) found that 75% of virulent *P. damsela* subsp. *damsela* strains showed phospholipase activity in their ECPs, but the specific 567 bp PCR amplicon corresponding to the phospholipase D (*dly*) gene was detected in only two

strains (12.5%). Interestingly, the phospholipase activity in the *dly* + strains remained unaltered after thermal treatment, which differs from the behaviour described for phospholipase toxins in *Vibrionaceae* (Songer, 1987).

Two types of bacterial phospholipases have been described, the extracellular phospholipases (A2, C or D), which are considered as virulence factors (Schmiel & Miller, 1999), and the phospholipases associated with the outer membrane (A type), whose role in pathogenesis had not been established (Dekker, 2000; Snijder & Dijkstra, 2000). Besides the phospholipase D activity associated to damselysin, other phospholipases (extracellular and/or A type) seem to be present in *P. damsela* subsp. *damsela* strains, although they are not directly related to the pathogenic properties of the strains (Labella et al., 2010).

Several authors have pointed out that the pathogenicity of some bacterial fish pathogens was related to their ability to haemolyse the host erythrocytes (Borrego et al., 1991; Fouz et al., 1993; Grizzle & Kiryu, 1993; Pedersen et al., 2009). The haemolytic activity in *Vibrionaceae* can be related to extracellular enzymatic activities, such as phospholipases in *V. parahaemolyticus*, *V. mimicus*, *V. harveyi* and *P. damsela* subsp. *piscicida* (Shinoda et al., 1991; Lee et al., 2002; Zhong et al., 2006; Naka et al., 2007) and phospholipase D in *P. damsela* subsp. *damsela* (Kreger et al., 1987), or to the direct action of haemolysins that provoke the pore-structure formation on the erythrocyte membrane (Iida & Honda, 1997; Zhang & Austin, 2005). Labella et al. (2010) reported that all *P. damsela* subsp. *damsela* strains tested produced haemolysis of fish and/or sheep erythrocytes. This ability was exclusively associated with bacterial cultures in 81.25% of the strains. These results could suggest that the haemolysin is associated with the bacterial core or it is an extracellular haemolysin whose activity is inhibited by the enzymatic content of ECPs, as has been described for VTH haemolysin of *V. tubiashii* (Hasegawa & Hase, 2009). As in the case of phospholipases, a correlation between the haemolytic activity of *P. damsela* subsp. *damsela* strains and their virulence properties could not also be established (Labella et al., 2010).

Several extracellular bacterial proteases, mainly metalloproteases and serine-proteases such as vibriolysins, are considered as virulence factors in numerous bacterial pathogens (Ishihara et al., 2002; Miyoshi et al., 2002; Farto et al., 2006). These proteases provoke tissue damages and degradation of host tissues, favouring the colonization and invasion of pathogens into the host (Miyoshi & Shinoda, 2000). In addition, proteases enable the evasion of the bacteria from several fish defence mechanisms (Vivas et al., 2004). A limited number of enzymatic activities has been detected in *P. damsela* subsp. *damsela* ECPs, including phosphatases, esterases, amylases and glycosidases, but their proteolytic activity was very low, lacking caseinase and gelatinase activities (Toranzo & Barja, 1993; Fouz et al., 1993; Labella et al., 2010). Nevertheless, none of these enzymatic activities could be related with the degree of toxicity, both *in vivo* and *in vitro*, presented by the ECPs (Labella et al., 2010), in contrast to results reported for other fish pathogens such as *Aeromonas* (Esteve et al., 1995).

In short, the presence of phospholipases (including damselysin), haemolysins or other enzymatic activities in the ECPs is not directly related to the pathogenicity of *P. damsela* subsp. *damsela*. Labella et al. (2010) hypothesized that another unknown type of toxin, different to the damselysin, could be involved in the toxicity of *P. damsela* subsp. *damsela* ECPs. A neurotoxin possessing an acetylcholine-esterase activity (ictiotoxin) has been described in strains of several species of *Vibrionaceae*, including *P. damsela* subsp. *damsela* (Balebona et al. 1998a; Perez et al. 1998), and may be responsible for several clinical signs observed by these authors.

#### 4. Conclusions

In recent years, *Photobacterium damsela* subsp. *damsela* has been repeatedly isolated from epizootic outbreaks affecting several cultured fish species. In addition, this bacterial pathogen has been reported to cause diseases in humans, and for this reason, it may be considered as an agent of zoonoses. The unique virulence factor characterized in *P. damsela* subsp. *damsela* is the damselysin, a thermolabile extracellular cytotoxin, which is a phospholipase D and presents haemolytic activity against different erythrocytes types. However, recent results obtained by our research team demonstrate there is no correlation between the presence of the *dly* gene and the pathogenicity of *P. damsela* subsp. *damsela*, therefore, other virulence factors may be involved in the pathological damages that this microorganism caused in infected fish.

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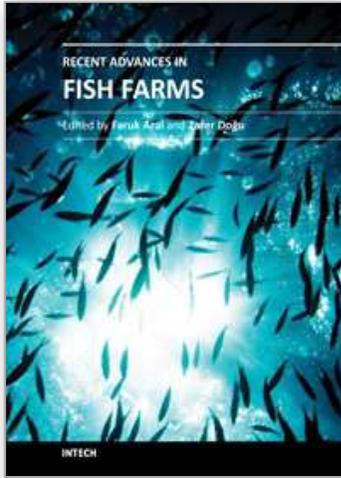
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### **Recent Advances in Fish Farms**

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