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Papel de las actividades superóxido dismutasa y catalasa en la virulencia de *Photobacterium damselae* subsp. *piscicida*. Estrategias para la estimulación del estallido respiratorio en fagocitos de lenguados cultivados

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Memoria presentada por Dña. Patricia Díaz Rosales
para optar al grado de Doctora en Biología
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D. ANTONIO DE VICENTE MORENO, Director del Departamento de Microbiología de la Universidad de Málaga.

INFORMA QUE:

Dña. Patricia Díaz Rosales ha realizado en los laboratorios de este Departamento el trabajo experimental conducente a la elaboración de la presente memoria de Tesis Doctoral

Y para que así conste, expido el presente informe,

Málaga, 11 de Septiembre de 2006

Fdo. **Antonio de Vicente Moreno**

Esta Tesis ha sido realizada en el Departamento de Microbiología de la Universidad de Málaga, bajo la dirección del Dr. Miguel Ángel Moriñigo Gutiérrez y la Dra. Mª Carmen Balebona Accino. Durante la realización de este trabajo de investigación se ha llevado a cabo el aprendizaje de técnicas útiles para dicha tesis en los siguientes laboratorios :

- School of Biological Sciences, University of Aberdeen (Aberdeen, Escocia, Reino Unido), bajo la supervisión del Dr. C.J. Secombes (de Octubre a Diciembre de 2003).

- Departamento de Biología Celular, Facultad de Biología, Universidad de Murcia (Murcia, España), bajo la supervisión del Dr. J. Meseguer (de Septiembre a Diciembre de 2004).

- Laboratory of Microbiology, Agrotechnology and Food Sciences, University of Wageningen (Wageningen, Holanda), bajo la supervisión del Dr. H. Smidt (de Septiembre a Diciembre de 2005).

El Dr. Miguel Ángel Moriñigo Gutiérrez, Profesor Titular de Microbiología de la Universidad de Málaga, y la Dra. Mª Carmen Balebona Accino, Profesora Titular de Microbiología de la Universidad de Málaga, dan su conformidad a la Memoria de la Tesis titulada: **Papel de las actividades superóxido dismutasa y catalasa en la virulencia de *Photobacterium damselae* subsp. *piscicida*. Estrategias para la estimulación del estallido respiratorio en fagocitos de lenguados cultivados**, presentada por la Doctoranda Dña. Patricia Díaz Rosales para optar al Título de Doctor en Biología con Mención de Doctorado Europeo por la Universidad de Málaga.

En Málaga, a 11 de Septiembre de 2006.

Dr. Miguel Ángel Moriñigo Gutiérrez **Dra. Mª Carmen Balebona Accino**

Los ensayos que constituyen esta Tesis han sido subvencionados principalemente a través de diferentes proyectos del Ministerio de Ciencia y Tecnología (España), concretamente los proyectos con las referencias AGL2002-01488 y PETRI 95-0657 subvencionaron los trabajos realizados sobre la virulencia de *Photobacterium damselae* subsp. *piscicida*. Los experimentos realizados con *Porphyridium cruentum* fueron sufragados fundamentalmente con cargo al proyecto AGL2002-01488, así como los proyectos AGL2005-02655 y RNM-295 (Junta de Andalucía) que subvencionaron la parte relacionada con el cultivo de las algas. Por último, con cargo al proyecto AGL2005-07454-CO2-O2 se realizaron los ensayos con bacterias potencialmente probióticas.

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- Díaz-Rosales, P, Chabrillón, M, Moriñigo, MA & Balebona, MC (2003). Survival to exogenous hydrogen peroxide of *Photobacterium damselae* subsp. *piscicida* under different culture conditions. Journal of Fish Diseases 26, 305-308.

Congresos internacionales:

- Díaz-Rosales, P, Chabrillón, M, Smidt, H, Salinas, I, Arijo, S, Cuesta, A, Meseguer, J, Esteban, MA, Balebona, MC & Moriñigo, MA. Study of the intestinal microbiota of gilthead seabream (*Sparus aurata*, L.) and sole (*Solea senegalensis*, Kaup 1858) by DGGE. Society of Applied Microbiology. Summer conference "Living Together: polymicrobial communities". Edinburgh, Scotland, U.K. 2006.

- Díaz-Rosales, P, Rico, RM, Arijo, S, Chabrillón, M, Balebona, MC, Sáenz de Rodrigáñez, M, Alarcón, FJ & Moriñigo, MA. Effect of two probiotics on respiratory burst of phagocytes from sole (*Solea senegalensis*, Kaup 1858). Aquaculture Europe 2006. "Linking Tradition and Technology. Highest Quality for the Consumer". Florence, Italy. 2006.

- Balebona, MC, Díaz, P, Chabrillón, M, Zorrilla, I, Arijo, S & Martínez, E. Determination of superoxide dismutase and catalase activity in *Photobacterium damselae* subsp. *piscicida* under different culture conditions. 10th International Conference of the European Association of Fish Pathologists. Dublin, Ireland, U.K. 2001.

Congresos nacionales:

- Díaz-Rosales, P, León-Rubio, JM, Rico, RM, Decara, J, Balebona, MC, Abdala, R, Figueroa, FL & Moriñigo, MA. Efecto inmunoestimulante del alga *Porphyridium cruentum* sobre la respuesta inmune del lenguado (*Solea senegalensis*) tras su administración por vía oral frente a la infección por *Photobacterium damselae* subsp. *piscicida*. X Congreso Nacional de Acuicultura. Gandía. 2005.

- Díaz-Rosales, P, Martínez-Manzanares, E, Moriñigo, MA & Balebona, MC. Efecto inmunoestimulante del alga *Porphyridium cruentum* sobre el estallido respiratorio en fagocitos de lenguado (*Solea senegalensis*). V Congreso de Microbiología del Medio Acuático de la Sociedad Española de Microbiología. Tarragona. 2004.

- Díaz-Rosales, P, Arijo, S, Moriñigo, MA & Balebona, MC. Resistencia de *Photobacterium damselae* subsp. *piscicida* al estallido respiratorio de fagocitos de lenguado (*Solea senegalensis*). V Congreso de Microbiología del Medio Acuático de la Sociedad Española de Microbiología. Tarragona. 2004.

- Díaz-Rosales, P, Arijo, S, Chabrillón, M, Castán, J, Martínez-Manzanares, E & Balebona, MC. Papel de la superóxido dismutasa y de la catalasa en la virulencia de *Photobacterium damselae* subsp. *piscicida*. IV Congreso de Microbiología del Medio Acuático de la Sociedad Española de Microbiología. Sevilla. 2002.

A Raúl Díaz Rosales

Sabe esperar, aguarda que la marea fluya
–así en la costa un barco– sin que el partir te inquiete,
todo el que aguarda sabe que la victoria es suya;
porque la vida es larga y el arte es un juguete.
Y si la vida es corta
y no llega el mar a tu galera,
aguarda sin partir y siempre espera,
que el arte es largo y, además, no importa.

ANTONIO MACHADO

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RESUMEN

Photobacterium damselae subsp. *piscicida* es una bacteria gram negativa capaz de sobrevivir como patógeno intracelular en el interior de fagocitos de lenguado, gracias a la acción protectora de las actividades superóxido dismutasa y catalasa. Estas enzimas confieren resistencia al patógeno frente a los radicales reactivos de oxígeno producidos en el interior de los fagocitos durante el denominado estallido respiratorio. Por tanto, ambas actividades enzimáticas pueden ser consideradas importantes factores de virulencia de este patógeno, facilitando su invasión y el establecimiento de la enfermedad, la pseudotuberculosis. La estrategia desarrollada para la prevención de dicha enfermedad se ha enfocado hacia la búsqueda de microorganismos con capacidad estimulante del estallido respiratorio de fagocitos de lenguado. Los microorganismos ensayados fueron la microalga *Porphyridium cruentum* y dos bacterias potencialmente probióticas. Los resultados obtenidos son prometedores ya que tanto la microalga como una de las bacterias ensayadas –Pdp11– son capaces de estimular el estallido respiratorio y, de esta manera, contribuir a la resistencia a la enfermedad. Se abre, por tanto, un nuevo campo en la lucha contra la pseudotuberculosis: la aplicación de sustancias procedentes de algas, así como de componentes bacterianos, que pudieran ser considerados probióticos.

INTRODUCCIÓN

1. LA ACUICULTURA. EL CULTIVO DEL LENGUADO (*Solea senegalensis*, KAUP 1858)

El incremento demográfico mundial junto con el estancamiento de la actividad extractiva y cambios preferenciales en la dieta, en la que se valora cada vez más el pescado como un alimento muy saludable, son factores que han conducido a que la producción global de pesca no satisfaga las demandas crecientes del mercado. Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), nuestro planeta está habitado por alrededor de 6500 millones de personas, y se prevée que para el año 2050, la población mundial alcance la cifra de 9000 a 10000 millones. (FAO, 2005 <ftp://ftp.fao.org/docrep/fao/008/a0057e/A0057e01.pdf>). Esta situación preocupa a la hora de plantear cómo alimentar a la población mundial en un futuro próximo. En respuesta a esta situación surge la acuicultura como una buena alternativa para el abastecimiento de la población.

La acuicultura, según la definición de la FAO, es el cultivo de organismos acuáticos, incluyendo peces, moluscos, crustáceos y plantas acuáticas. Actualmente es uno de los sectores productores de alimento con mayor potencial de crecimiento y juega, por tanto, un papel esencial en el futuro de la alimentación humana.

En los últimos años la actividad productiva de la industria acuícola, tanto de agua marina como de agua dulce, ha experimentado un crecimiento exponencial muy importante, especialmente en lo que se refiere a la acuicultura marina intensiva de peces. Hasta ahora la acuicultura marina en países mediterráneos se ha centrado fundamentalmente en la producción de dos especies –dorada (*Sparus aurata*, L.) y lubina (*Dicentrarchus labrax*, L.)– lo que ha provocado una saturación en el mercado. En los últimos años, para incrementar las oportunidades de mercado, se han realizado investigaciones encaminadas a la búsqueda de nuevas especies potencialmente aptas para el cultivo. El lenguado senegalés (*Solea senegalensis*, Kaup 1858) es actualmente una de las especies cuyo cultivo se ha incrementado de forma significativa (JACUMAR, 2005) en el área atlántica y mediterránea (Dinis, 1999). El lenguado senegalés es una especie acuícola que desde la década de los setenta ha sido objeto de interés en

acuicultura, debido a su alto precio en el mercado, la posibilidad de reproducción en cautividad, los resultados obtenidos en el cultivo de las larvas y a que cuenta con un mercado potencial (Dinis y Reis, 1995; Magalhaes y Dinis, 1996).

Actualmente no es posible afirmar que esta especie esté consolidada a nivel de una producción industrial, ya que la incidencia de patologías limita la producción de engorde del lenguado. Se han realizado estudios sobre los microorganismos patógenos que afectan a esta especie (Rodríguez *et al.*, 1997; Zorrilla *et al.*, 1999; Magariños *et al.*, 2003; Arijo *et al.*, 2005), y aunque entre otros patógenos se han aislado *Vibrio harveyi* y *Tenacibaculum maritimum* (Zorrilla *et al.*, 1999; Cepeda y Santos, 2003), *Photobacterium damselae* subsp. *piscicida*, causante de la pasteurelosis, o pseudotuberculosis, es el principal patógeno responsable de importantes pérdidas en el cultivo del lenguado senegalés, llegando a ser el principal factor limitante en la producción de esta especie (Zorrilla *et al.*, 1999; Magariños *et al.,* 2003; Arijo *et al.*, 2005).

2. *Photobacterium damselae* subsp. *piscicida*

La bacteria marina *P. damselae* subsp. *piscicida* es el agente etiológico de la septicemia bacteriana denominada pseudotuberculosis (Kubota *et al.*, 1970) debido a que en los casos crónicos, los peces enfermos muestran gránulos blancos prominentes en los órganos internos, consistentes en una acumulación de células bacterianas. Esta enfermedad fue descrita por primera vez en poblaciones salvajes de perca (*Morone americanus*) y lubina estriada (*Morone saxatilis*) en Estados Unidos (Snieszko *et al.*, 1964). Sin embargo, actualmente los hospedadores naturales del patógeno incluyen una amplia variedad de especies piscícolas marinas, tanto de aguas cálidas como frías, provocando importantes pérdidas económicas en poblaciones salvajes y cultivadas de Japón, donde afecta principalmente a la seriola (*Seriola quinqueradiata*) (Kusuda y Salati, 1993), de Estados Unidos y de Europa, donde causa estragos en cultivos de dorada (*Sparus aurata*) (Ceshia *et al.*, 1991; Toranzo *et al.*, 1991), lubina (*Dicentrarchus labrax*) (Baudin-Laurencin *et al.*, 1991; Balebona *et al.*, 1992), lubina estriada (*Morone*

saxatilis) (Hawke *et al.*, 1987) y lenguado (*Solea senegalensis*), como se ha descrito recientemente (Zorrilla *et al.*, 1999; Magariños *et al.*, 2003; Arijo *et al.*, 2005).

2.1. CARACTERÍSTICAS MORFOLÓGICAS, BIOQUÍMICAS Y SEROLÓGICAS

P. damselae subsp. *piscicida* es una bacteria halófila, Gram negativa, de forma bacilar (0,8-1,3 x 1,4-4 μm de tamaño). Se caracteriza por su tinción bipolar y su pleomorfismo, dependiente de las condiciones de cultivo. Las características fenotípicas están resumidas en la Tabla 1.

Siempre se ha considerado que *P. damselae* subsp. *piscicida* constituía un taxón morfológico, bioquímico, fisiológico, fenotípico y serológicamente homogéneo (Magariños *et al.*, 1992). Sin embargo, la aplicación de nuevas técnicas moleculares para el análisis genético, como ribotipado y RAPD (*Random Amplification of Polymorphic DNA*) ha mostrado la existencia de dos linajes clonales o genogrupos en *P. damselae* subsp. *piscicida*, uno en cepas procedentes de Europa y otro en aislados de Japón (Magariños *et al*, 1997; Thyssen *et al.*, 1999; Magariños *et al.*, 2000; Kvitt *et al.*, 2002; Romalde, 2002; Juíz-Río *et al.*, 2005). En cualquier caso, se puede observar una homogeneidad dentro del linaje clonal entre cepas aisladas de especies piscícolas diferentes; confirmándose este hecho en los estudios genéticos realizados a cepas de este microorganismo aisladas a partir de lenguados cultivados en nuestro país, revelándose que estas cepas pertenecen al genogrupo europeo (Magariños *et al.*, 2003). Este hecho podría indicar una posible transmisión horizontal del patógeno entre peces cultivados en las mismas áreas (Magariños *et al.*, 2003).

Tabla 1 Características generales de *Photobacterium damselae* subsp. *piscicida*

Tinción de Gram	-	Tinción bipolar	+
Catalasa	+	Oxidasa	+
Movilidad	-	Rojo Metilo	+
Producción H_2S	-	O/F	+/+
Producción gas (glucosa)	-	Crecimiento TCBS	-
Crecimiento a:		Crecimiento en NaCl:	
4 ºC	-	0%	-
10 ºC	+	3%	+
20 ºC	+	6%	-
30 ºC	+	8%	-
37 ºC	-	Producción de ácidos:	
β galactosidasa (ONPG)	-	arabinosa	-
Gelatinasa	-	maltosa	-
Ureasa	-	sacarosa	-
Caseinasa	-	rhamnosa	-
Amilasa	-	amigdalina	-
Fosfolipasa	+	inositol	-
Lipasa (Tween 80)	+	manosa	+
Arginina dihidrolasa	+	manitol	-
Lisina descarboxilasa	-	sorbitol	-
Ornitina descarboxilasa	-	glicerol	-
Hidrólisis de esculina	-	lactosa	-
Voges-Proskauer	+	melobiosa	-
Indol	-	glucosa	+
Nitrato	-	galactosa	+
Citrato	-	fructosa	+

Resistencia a:	Sensibilidad a:	
Estreptomicina	Ampicilina	Novobiocina
Eritromicina	Cloranfenicol	Tetraciclina
Kanamicina	Oxitetraciclina	Ácido oxolínico
	Nitrofurantoína	Agente vibriostático O/129
	Trimetoprim-sulfametoxazol	

2.2. SINTOMATOLOGÍA DE LA PSEUDOTUBERCULOSIS

Los signos patológicos externos son, generalmente, poco llamativos y, por lo general, los peces afectados no suelen mostrar lesiones externas. En algunos casos de doradas enfermas se puede apreciar una pigmentación anormal en la piel así como leves zonas hemorrágicas en cabeza y branquias (Toranzo *et al.*, 1991). En lubina (*Dicentrarchus labrax*) se ha llegado a observar hinchazón en la cavidad abdominal (Balebona *et al.*, 1992), además de lesiones ulcerativas en la piel y extensas hemorragias, especialmente en la boca, ojos y musculatura (Fouz *et al.*, 2000).

Internamente los peces enfermos muestran septicemia hemorrágica y necrosis en la mayoría de los órganos, apareciendo los tubérculos típicos de la enfermedad. Estos no son sino acumulaciones de bacterias, fagocitos necróticos y granulomas. Histopatológicamente esas llamativas lesiones necróticas con grandes masas bacterianas, de forma extensiva, aguda y multifocal observadas en los órganos internos, sugieren que la enfermedad se desarrolla como un proceso septicémico agudo. Dichas lesiones granulomatosas aparecen como una reacción de las células epiteliales cuando la viabilidad de la bacteria decrece por medicación. Además de la necrosis y la existencia de granulomas en bazo, riñón e hígado, donde se puede detectar bacterias en los sinusoides y vasos hepáticos, es posible el desarrollo de una esplenomegalia (Toranzo *et al.*, 1991) debida a la infiltración de células sanguíneas junto con grupos de bacterias que tapan los capilares y espacios intersticiales, así como la aparición de zonas blanquecinas, o palidez, en bazo y riñón (Kubota *et al.*, 1970; Wolke, 1975; Tung *et al.*, 1985; Hawke *et al.*, 1987; Toranzo *et al.*, 1991; Balebona *et al.*, 1992; Noya *et al.*, 1995a).

En lenguados (*Solea senegalensis*) afectados por la pseudotuberculosis la pigmentación oscura en la piel y la hinchazón en la cavidad abdominal pueden ser dos síntomas externos que podemos detectar. Algunas muestras pueden también presentar exoftalmia hemorrágica, pequeñas úlceras en la piel y palidez branquial (Zorrilla *et al.*, 1999). En cuanto a los órganos internos se puede apreciar esplenomegalia, palidez en hígado y riñón, así como tubérculos de 1-2 mm de diámetro en el bazo.

2.3. MODO DE TRANSMISIÓN

P. damselae subsp. *piscicida* es una bacteria altamente patógena que no parece tener especificidad por el hospedador. Por tanto, la pseudotuberculosis puede ser un riesgo para especies piscícolas marinas en las que aún no se ha descrito. Algunos autores señalan la existencia de diferencias en la susceptibilidad a la pseudotuberculosis, en doradas y lubinas, basadas en la edad y el tamaño del pez (Noya *et al.*, 1995b). Esto podría deberse a la funcionalidad de macrófagos y neutrófilos que en doradas mayores de 20-30 g pueden fagocitar eficientemente y matar a las bacterias (Noya *et al.*, 1995b; Skarmeta *et al.*, 1995), en tanto que en doradas de menos de 1 g deben existir deficiencias en componentes del suero implicados en la fagocitosis y posterior muerte de *Photobacterium* por los fagocitos, haciéndolas más susceptibles a la infección.

El modo de transmisión y la ruta de infección implicadas en esta enfermedad aún se desconocen con detalle (Magariños *et al.*, 1995). Los datos existentes apuntan a que la pseudotuberculosis es una enfermedad de mayor prevalencia en los meses de verano (Frerichs y Roberts, 1989) con altas temperaturas del agua (mayores de 23ºC) y alta salinidad (20-30%) (Hawke *et al.*, 1987). En cuanto a la supervivencia en el agua, se ha demostrado que este patógeno sobrevive en ambientes acuáticos marinos como células viables pero no cultivables durante periodos prolongados (Magariños *et al.*, 1994) pero manteniendo prácticamente la misma infectividad potencial para los peces que las células viables y cultivables (Magariños *et al.*, 1994). Esto sugiere que el medio acuático podría constituir un reservorio y un vehículo de transmisión para este patógeno, contribuyendo el aumento de las temperaturas al desarrollo de la epizootia (Toranzo *et al.*, 1991; Magariños *et al.*, 2001).

Se han hecho diferentes estudios para valorar las distintas vías de entrada del patógeno. Por un lado, parece ser que la infección puede iniciarse por ingestión del patógeno (Magariños *et al.*, 1995). Por otro lado, evaluando el papel de la piel como puerta de entrada del microorganismo, se ha observado que *P. damselae* subsp. *piscicida* es resistente a la acción del mucus de piel de dorada y de lubina, aunque sensible al de rodaballo (Magariños *et al.*, 1995), lo que podría ser una razón por la que

las epidemias surgidas en Europa nunca hayan afectado al rodaballo, y sí a la dorada y a la lubina.

De momento, no se ha podido demostrar la existencia de un portador de esta enfermedad, ya que no se ha podido aislar la bacteria de peces supervivientes a una exposición experimental (Toranzo *et al.*, 1991), sin poder descartar, por otro lado, que la bacteria se encuentre en estos casos a una concentración por debajo de los límites de detección clásicos, o en estado viable no cultivable.

2.4. MECANISMOS DE VIRULENCIA

La virulencia de los microorganismos patógenos es un complejo proceso multifactorial. En el caso de *P. damselae* subsp. *piscicida* aún se desconocen muchos aspectos relativos a su virulencia, sobre todo a nivel molecular.

La capacidad de adherencia e invasión es esencial en los primeros estadios de la infección. Una vez en el interior del hospedador, la adhesión a los tejidos promueve la liberación de toxinas y precede a la penetración en las células diana por parte de los microorganismos. Aunque *P. damselae* subsp. *piscicida* presenta una débil adhesión a diferentes líneas celulares de peces, sí ha mostrado una elevada capacidad adhesiva a intestino de dorada, lubina y rodaballo (Magariños *et al.*, 1996a). Se ha detectado su capacidad para invadir líneas celulares de peces (Magariños *et al.*, 1996a; Elkamel y Thune, 2003) y permanecer viable, así como de proliferar en el interior de los macrófagos sin sufrir cambios morfológicos aparentes, liberándose microorganismos al medio que invaden células adyacentes (Magariños *et al.*, 1996a; Elkamel *et al.*, 2003). Este hecho puede ser relevante *in vivo* ya que garantiza el mantenimiento del patógeno durante cierto periodo de tiempo en el tejido infectado, lo que contribuye a un estado de infección crónica y de portador por parte del hospedador. Incluso Elkamel *et al.* (2003) concluyen en su estudio que *P. damselae* subsp. *piscicida* es un patógeno intracelular muy eficiente, que puede sobrevivir y multiplicarse en el interior de macrófagos de peces como la lubina.

La importancia como factores de virulencia de los productos extracelulares (ECPs) secretados por *P. damselae* subsp. *piscicida* está bien documentada (Balebona *et al.*,

1992; Magariños *et al.*, 1992; Noya *et al.*, 1995a y b; Romalde, 2002; Bakopoulos *et al.*, 2004). Los ECPs de *P. damselae* subsp. *piscicida* son fuertemente tóxicos por vía intraperitoneal (Noya *et al.*, 1995a; Bakopoulos *et al.*, 2004) y llegan a ser letales para diferentes especies piscícolas y para ratón (Magariños *et al.*, 1992). Las principales actividades demostradas son la hemolítica, fosfolipasa y citotóxica (Magariños *et al.*, 1992). Estudios histológicos han implicado estas actividades, en particular –las fosfolipasas– en la patogénesis de la pseudotuberculosis (Noya *et al.*, 1995b).

La implicación del material polisacarídico capsular ha sido claramente demostrada en la virulencia de *P. damselae* subsp. *piscicida* (Bonet *et al.*, 1994; Magariños *et al.*, 1996b; Romalde y Magariños, 1997; Acosta *et al.*, 2006). Aunque todas las cepas de *P. damselae* subsp. *piscicida* sintetizan una estructura externa adicional en un medio enriquecido en glucosa, sólo las cepas virulentas sintetizan constitutivamente una fina cápsula (Magariños *et al.*, 1996b) que les confiere resistencia a la inactivación por suero, e incrementa el grado de virulencia (Magariños *et al.*, 1996b; Acosta *et al.*, 2006), además de reducir la fagocitosis por parte de los macrófagos (Arijo *et al.*, 1998). Por lo tanto, la presencia de cápsula juega un importante papel en la patogénesis de *P. damselae* subsp. *piscicida*, como prueba el hecho de que las cepas no virulentas son eliminadas del pez en corto tiempo, aunque las cepas no virulentas, en las que se induce la síntesis de cápsula, presentan también resistencia al suero (Magariños *et al.*, 1997; Arijo *et al.*, 1998). Además, esta inducción de la expresión capsular en cepas no virulentas incrementa su resistencia a la acción bactericida del suero y disminuye su DL_{50} alrededor de 2-3 unidades logarítmicas (Magariños *et al.*, 1996b). De todas formas, *in vivo*, las condiciones limitantes en hierro hacen que *P. damselae* subsp. *piscicida* no presente una cápsula de tamaño importante (Acosta *et al.*, 2003), lo que podría suponer una mayor exposición de las adhesinas a la superficie, siendo esto un aspecto útil para la colonización (Magariños *et al.*, 1996b).

La capacidad de conseguir hierro es primordial para el crecimiento de bacterias patógenas en el interior del hospedador, siendo, por tanto, esencial para causar infección. Además, se ha constatado que este microorganismo muestra un elevado número de actividades de sus ECPs bajo condiciones limitantes de hierro (Bakopoulos

et al., 1997). En *P. damselae* subsp. *piscicida* se han descrito diferentes estrategias para conseguir hierro, un sistema de incorporación de hierro codificado a nivel cromosómico, consistente en un sideróforo química y biológicamente relacionado con la multocidina producida por *Pasteurella multocida* y, al menos, tres proteínas de membrana externa de alto peso molecular reguladas por hierro (Magariños *et al.*, 1994). También se ha descrito la capacidad de *P. damselae* subsp. *piscicida* de utilizar directamente el grupo hemo como única fuente de hierro y que la inyección intraperitoneal de hemina antes de la infección experimental incrementa la letalidad de este patógeno (Magariños *et al.*, 1994). Igualmente se ha demostrado que en rodaballo el sistema génico de transporte del grupo hemo se expresa *in vivo*, durante la infección de *P. damselae* subsp. *piscicida* (Juíz-Río, 2006). La base genética de estos sistemas de captación de hierro por sideróforos muestra una patente diversidad (Juíz-Río, 2006).

Se ha demostrado la relación entre la cápsula y la capacidad de adquirir hierro (do Vale *et al.*, 2001; Romalde, 2002), jugando los polisacáridos capsulares un papel secundario en la unión de la hemina. La expresión del material capsular es dependiente de la disponibilidad de hierro y de la fase de crecimiento, así las células crecidas bajo condiciones limitantes de hierro manifiestan menor cantidad de material capsular que las células suplementadas con hierro (do Vale *et al.*, 2001). Esto puede explicar la necesidad de la bacteria de expresar su sideróforo y/o receptores de hierro durante el tiempo que recorre el sistema circulatorio del hospedador. Una vez que el microorganismo alcanza los diferentes tejidos, la cantidad de material capsular probablemente se incrementa en respuesta a los mecanismos de defensa celular del hospedador. Además el papel del hierro en la expresión de actividades enzimáticas ha sido también descrito, y algunas enzimas proteolíticas, como la gelatinasa y caseinasa son sólo sintetizadas cuando las cepas son cultivadas bajo condiciones restrictivas de hierro (Magariños *et al.*, 1994; Romalde, 2002).

Recientemente se ha descrito que diferentes cepas virulentas de este patógeno producen una exotoxina, la AIP56, codificada plasmídicamente, y que tiene la capacidad de inducir apoptosis en leucocitos de lubina (do Vale *et al.*, 2005).

En resumen, los mecanismos de invasión y supervivencia de la bacteria *Photobacterium damselae* subsp. *piscicida* en el interior del hospedador aún no se conocen: mientras que unos autores confirman la presencia de bacterias intactas en el interior de las células del pez, sugiriendo la capacidad de la bacteria de sobrevivir como patógeno intracelular (Noya *et al.*, 1995b; López-Dóriga *et al.*, 2000); otros autores han observado que este patógeno es altamente susceptible a los radicales oxigénicos generados durante el estallido respiratorio en el interior de los fagocitos (Skarmeta *et al.*, 1995; Arijo *et al.*, 1998; Barnes *et al.*, 1999a).

Es obvio, por tanto, que la patogénesis de *P. damselae* subsp. *piscicida* es un proceso complejo y multifactorial, no entendido por completo. En esta Tesis Doctoral se intenta profundizar y aclarar, en la medida de lo posible, parte de ese proceso, si *P. damselae* subsp. *piscicida* es capaz, o no, de sobrevivir al estallido respiratorio generado en el interior de las células fagocíticas de lenguado senegalés, asimismo, se realiza un estudio de diferentes estrategias de prevención de esta enfermedad bacteriana, mediante la aplicación de inmunoestimulantes y probióticos al hospedador.

3. LAS ACTIVIDADES SUPERÓXIDO DISMUTASA Y CATALASA COMO FACTORES DE VIRULENCIA

La inactivación bacteriana en el interior de los fagocitos se efectúa mediante dos tipos de mecanismos: independientes de oxígeno, mediados por los constituyentes de los gránulos de los fagocitos (enzimas lisosomales, catepsinas, defensinas, lactoferrina, enzimas proteolíticas), y dependientes de oxígeno. En estos últimos se da la formación de compuestos oxigenados como peróxido de hidrógeno (H_2O_2), radicales como el anión superóxido ($O_2^{\cdot-}$) y radical hidroxilo (OH^-), productos que se forman durante el denominado estallido respiratorio que sigue a la activación de la enzima nicotinamida-adenín-dinucleótido-fosfato-hidrógeno (NADPH) oxidasa de la membrana tras la fagocitosis.

3.1. ESTALLIDO RESPIRATORIO

Tras la fagocitosis –proceso por el cual los fagocitos interiorizan a los microorganismos– los leucocitos liberan al interior de los fagosomas el contenido de sus gránulos citoplasmáticos, entre los que destacan diversos factores citotóxicos tales como metabolitos oxigenados y enzimas lisosomales, con el fin de matar y digerir a los microorganismos.

La producción de dichos metabolitos de oxígeno se lleva a cabo en el proceso conocido como estallido respiratorio, o explosión respiratoria, que se produce en los fagocitos ante la presencia de bacterias, experimentando un rápido incremento en el consumo de oxígeno. Actualmente, el término de estallido respiratorio se considera inadecuado ya que dicho incremento en el consumo de oxígeno no se debe a un incremento en la tasa respiratoria, sino que se produce en la superficie celular donde se usa el oxígeno extracelular para generar radicales reactivos de oxígeno, los denominados ROS (*reactive oxygen species*) ($O_2^{\cdot-}$, H_2O_2, OH^-). La presencia de dichos radicales libres se asocia al envejecimiento celular, sin embargo, su toxicidad ha encontrado utilidad en los fagocitos como mecanismo de defensa frente a bacterias debido a su gran actividad microbiocida.

El estallido respiratorio se desencadena por la estimulación de la membrana del fagocito. Tras dicha estimulación, la enzima NADPH oxidasa, presente en la membrana celular, es capaz de reducir el O_2 en anión superóxido ($O_2^{\cdot-}$) (Roos *et al.*, 2003). De forma secuencial, por la reducción univalente del O_2, se genera toda una serie de especies reactivas altamente tóxicas: los denominados radicales reactivos del oxígeno. Los primeros en producirse son el radical superóxido ($O_2^{\cdot-}$) y el peróxido de hidrógeno (H_2O_2) por acción de la superóxido dismutasa (SOD) sobre el $O_2^{\cdot-}$. El anión superóxido tiene un alto poder bactericida, así que es problable que este radical por sí solo sea capaz de eliminar microorganismos. El peróxido de hidrógeno puede reaccionar con el superóxido, generando radicales hidroxilo (OH^-) y oxígeno singleto ($^1O_2$), ambos altamente reactivos y tóxicos.

Por otro lado el anión superóxido puede también reaccionar con óxido de nitrógeno (NO), que es derivado de L-arginina y O_2 molecular, en una reacción catalizada por la

óxido nítrico sintasa (NOS) en la que se produce peroxinitrito, un intermediario del nitrógeno muy reactivo.

El oxígeno singleto puede ser convertido en un compuesto similar al ozono (O_3) en una reacción catalizada por la unión de anticuerpos con microorganismos o neutrófilos.

El peróxido de hidrógeno, junto con el cloruro, puede ser sustrato de la enzima mieloperoxidasa (MPO) generándose ácido hipoclórico (HClO), muy tóxico para la mayor parte de los microorganismos. El hipoclórico reacciona con aminas secundarias, formando cloraminas secundarias, que son igual de microbiocidas que el ácido, pero mucho más estables.

Por lo tanto, un gran número de reacciones químicas se produce en el pequeño espacio entre la bacteria ingerida y la membrana del fagosoma. Para compensar la carga electrónica debida a la reducción del oxígeno molecular en anión superóxido, se da un flujo de protones (H^+) o de otros cationes, como K^+. Si todos los electrones bombeados al interior del fagosoma fueran compensados por el flujo de protones, el pH del fagosoma permanecería neutro; sin embargo, se aumenta hasta 8, a pesar de la liberación de ácidos procedentes de los gránulos citoplasmáticos que se fusionan con el fagosoma. Esto indica que otros cationes, como el potasio (K^+), pueden entrar en el fagosoma en lugar de los protones (Reeves *et al.*, 2002). Si se da ese flujo de iones potasio, estos cationes mediarían la solubilización de proteasas que están unidas a la matriz de proteoglucano de los gránulos. Por tanto, el incremento del pH intrafagosomal alcanza los valores óptimos de la acción de proteasas, pudiendo afirmarse que la NADPH oxidasa, además de matar a los microorganismos por medio de sus radicales oxigénicos, actúa liberando proteasas lisosomales. De este modo, la NADPH oxidasa leucocitaria induce la muerte microbiana directa, vía productos oxidativos, e indirectamente, vía liberación de proteasas.

El estallido respiratorio conduce a la inactivación de proteínas y a la oxidación de ácidos nucleicos y otras moléculas esenciales, lo que representa una estrategia importante del sistema inmunitario en la lucha contra las infecciones. Para competir con los radicales libres generados, los microorganismos patógenos se han visto obligados a desarrollar estrategias en un doble frente: por un lado, la protección frente a los

radicales generados en su propio metabolismo aerobio y por otro, la defensa frente al contacto con estos radicales producidos en células fagocíticas. A esta resistencia frente a los ROS contribuyen enzimas antioxidantes tales como las superóxido dismutasas, catalasas y peroxidasas.

Estas enzimas representan un arma defensiva frente al ataque de células fagocíticas, contribuyendo al potencial virulento del microorganismo patógeno en su interacción con el hospedador.

3.2. LA ACTIVIDAD SUPERÓXIDO DISMUTASA

La superóxido dismutasa representa la primera línea de defensa de las células frente al estrés oxidativo. Cataliza la conversión de los radicales anión superóxido en peróxido de hidrógeno y oxígeno (ecuación 1).

(1) $$O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

La actividad SOD ha sido detectada en una amplia variedad de seres vivos, desde bacterias a humanos, implicada como defensa esencial frente a la toxicidad potencial del oxígeno. Cualquier célula que utilice el oxígeno tiene el potencial de producir anión superóxido y, por tanto, debe contener alguna forma de superóxido dismutasa (Fridovich, 1974).

Las superóxido dismutasas constituyen una familia de metaloenzimas que se clasifican en cuatro grupos según el metal que actúe de cofactor: FeSOD, MnSOD, CuZnSOD y NiSOD, esta última descrita recientemente en *Streptomyces* (Lynch y Kuramitsu, 2000).

3.3. LA ACTIVIDAD CATALASA

Las catalasas también forman parte de la defensa de las bacterias frente al estrés oxidativo. Catalizan la descomposición del peróxido de hidrógeno, transformándolo en agua y oxígeno (ecuación 2).

(2) $2H_2O_2 \longrightarrow 2H_2O + O_2$

Algunas catalasas tienen además actividad peroxidasa, un donador orgánico de electrones, o a veces un ión haluro, es empleado en la reducción del peróxido de hidrógeno (ecuación 3).

(3) $RH_2 + H_2O_2 \longrightarrow 2H_2O + R$

Las catalasas se pueden dividir en tres grupos (Loewen, 1997): catalasas monofuncionales con grupo hemo (FeCat), catalasas bifuncionales con grupo hemo (catalasas-peroxidasas) y pseudocatalasas sin grupo hemo (MnCat), estas últimas se denominan pseudocatalasas porque son resistentes a los inhibidores comunes para las catalasas, como la azida y el cianuro.

Las actividades superóxido dismutasa y catalasa protegen no sólo de forma directa eliminando aniones supéroxido y peróxido de hidrógeno, respectivamente, sino que también impiden la formación del radical hidroxilo OH·, la especie reactiva derivada del oxígeno con mayor poder oxidante.

De esta forma, el papel de dichas enzimas puede ser fundamental a la hora de proteger a bacterias patógenas durante el estallido respiratorio que sigue a la fagocitosis y por ello se asocian a mecanismos de virulencia (Tabla 2). Así, la habilidad de un organismo de infectar a su hospedador es debida, al menos en parte, a su resistencia frente a la producción de ROS por las células, principalmente monocitos/macrófagos y polimorfonucleados. Irónicamente la evolución ha seleccionado organismos que utilizan dichas células como diana, así que la posesión de enzimas como SOD y catalasa contribuirá a la resistencia frente al hospedador y, por tanto, al establecimiento de la infección.

Tabla 2 Ejemplos de microorganismos patógenos en los que las actividades SOD y catalasa juegan un papel importante en la virulencia

Patógeno	Factor de virulencia	Referencia
Listeria monocytogenes	FeSOD	Welch *et al.*, 1979
Shigella flexneri	FeSOD	Franzon *et al.*, 1990
Pseudomonas syringae	Catalasas (no determinadas)	Klotz y Hutcheson, 1992
Caulobacter crescentus	CuZnSOD	Schnell y Steinman, 1995
Aeromonas salmonicida	FeSOD	Barnes *et al.*, 1996
Pseudomonas aeruginosa	MnSOD	Polack *et al.*, 1996
Legionella pneumophila	CuZnSOD	St. John y Steinman, 1996
A. salmonicida subsp. *salmonicida*	MnSOD y FeCatalasa	Barnes *et al.*, 1999b
Mycobacterium tuberculosis	Catalasa-Peroxidasa	Manca *et al.*, 1999
Streptococcus pneumoniae	MnSOD	Yesilkaya *et al.*, 2000
Vibrio harveyi	Catalasa monofuncional	Vattanaviboon y Mongkolsuk, 2001
Salmonella enterica	CuZnSOD	Uzzau *et al.*, 2002
Vibrio shiloi	SOD (no determinada)	Banin *et al.*, 2003

3.4. LAS ACTIVIDADES SUPERÓXIDO DISMUTASA Y CATALASA EN *Photobacterium damselae* subsp. *piscicida*

Como ya se citó en el apartado 2.4., algunos autores confirman la presencia de bacterias intactas en el interior de células del pez, sugiriendo la habilidad de *P. damselae* subsp. *piscicida* de sobrevivir como patógeno intracelular (Noya *et al.*, 1995a; López-Dóriga *et al.*, 2000; Elkamel *et al.*, 2003), mientras que otros autores han observado que este patógeno es altamente susceptible a los radicales oxidativos generados durante el estallido respiratorio en los fagocitos (Skarmeta *et al.*, 1995; Arijo *et al.*, 1998; Barnes *et al.*, 1999a).

Para esclarecer este último punto es imprescindible profundizar en el estudio de las actividades superóxido dismutasa y catalasa manifestadas por *P. damselae* subsp. *piscicida*. Una mayor información sobre el papel de dichas actividades en la virulencia del patógeno podría contribuir a entender las interacciones entre *P. damselae* subsp. *piscicida* y su hospedador.

Barnes *et al.* (1999a) determinaron la existencia en *P. damselae* subsp. *piscicida* de una SOD con hierro en su centro activo, localizada en el espacio periplásmico, y de una catalasa, sin determinar, localizada en el citoplasma. La actividad SOD se veía reducida por el crecimiento en condiciones restrictivas de hierro, así como en bajas concentraciones de oxígeno, mientras que la catalasa era expresada constitutivamente, aunque en los geles se apreciaron diferencias en los niveles de la actividad de las diferentes cepas analizadas.

4. ESTIMULACIÓN DEL ESTALLIDO RESPIRATORIO POR DIFERENTES MICROORGANISMOS FRENTE A LA INFECCIÓN POR *Photobacterium damselae* subsp. *piscicida*

4.1. PREVENCIÓN Y TRATAMIENTO DE LA PSEUDOTUBERCULOSIS

El principal método utilizado en las piscifactorías para el control de la enfermedad son los agentes quimioterapéuticos: los antibióticos. Hasta finales de los ochenta eran muy efectivos en el tratamiento de esta infección, pero la aparición de cepas resistentes (Aoki *et al.*, 1981; Miranda y Zemelman, 2002; Radu *et al.*, 2003; Zorrilla *et al.*, 2003) y la contaminación del medio acuático (Kautsky *et al.*, 2000; Sivaram *et al.*, 2004) han llegado a constituir un grave problema en la acuicultura. La prevención de las enfermedades infecciosas es una alternativa conveniente, de ahí que el desarrollo de sustancias inmunoestimulantes, capaces de activar el sistema inmune del pez e inducir una mejor respuesta frente a los patógenos, suponga una buena alternativa al uso de antibióticos en acuicultura (Sakai, 1999). Además de la posible aparición de resistencias, otro inconveniente que explica la ineficacia del tratamiento con antibióticos es el hecho de que *P. damselae* subsp. *piscicida* puede pasar por un periodo intracelular

de parasitismo en el interior del macrófago durante la infección (Kusuda y Salati, 1993), lo que le evitaría estar en contacto con el antibiótico. Se comprueba, por tanto, que la inmunoprofilaxis sería la mejor vía para prevenir la pseudotuberculosis.

A lo largo de los últimos veinte años, ha salido a la luz una gran variedad de estudios que han analizado la eficacia de la inmunización mediante vacunación a la hora de prevenir la pseudotuberculosis (Romalde y Magariños, 1997). La mayoría de las vacunas probadas consistieron en células inactivadas por calor o por formalina (Fukuda y Kusuda, 1981; Kusuda y Hamaguchi, 1987; Kusuda y Hamaguchi, 1988; Hamaguchi y Kusuda, 1989). Aunque se alcanzó un cierto grado de protección, los mejores resultados fueron obtenidos empleando formulaciones basadas en los lipopolisacáridos y en las fracciones ribosomales de las bacterias (Fukuda y Kusuda, 1985; Kusuda *et al.*, 1988; Kawakami *et al.*, 1997). Sin embargo, estas formulaciones presentaron no sólo problemas de reproducibilidad, sino también dificultades en su producción a gran escala. La inmunización pasiva también ha sido evaluada (Fukuda y Kusuda, 1981), pero los resultados mostraron un tiempo muy corto de protección. Uno de los porcentajes de protección más altos frente a la pseudotuberculosis se obtuvo con una bacterina enriquecida con productos extracelulares (ECPs) (Magariños *et al.*, 1994, 1997, 1999). Esta vacuna en la actualidad está disponible comercialmente y ha sido empleada con éxito en varios países europeos, incluyendo España, Portugal y Grecia.

La investigación en busca de vacunas más efectivas se ha dirigido también hacia el uso de bacterias vivas atenuadas (Kusuda y Hamaguchi, 1988), cuya utilización todavía no está permitida, y el uso de proteínas de la envuelta celular (Magariños *et al.*, 1994) como antígenos protectores.

Tal y como mencionamos anteriormente, Magariños *et al.* (2000) demostraron por medio de la técnica RAPD la existencia de dos linajes clonales según su procedencia: uno que incluiría a las cepas de origen europeo y otro que englobaría a las de procedencia japonesa y norteamericana. Más recientemente, Juíz-Río *et al.* (2005), aplicando la técnica de hibridación subtractiva, concluyeron que este patógeno presenta una alta heterogeneidad genética. Sin embargo, *P. damselae* subsp. *piscicida* ha mostrado ser un microorganismo bioquímica y antigénicamente homogéneo, incluyendo

las cepas del patógeno aisladas de lenguados cultivados en España (Bakopoulos *et al.*, 1995; Magariños *et al.*, 1996c, 2003). Por lo tanto, la inmunización con vacunas comerciales, originalmente desarrolladas para otros peces cultivados tales como dorada y lubina (Romalde y Magariños, 1997; Magariños *et al.*, 1999), podría ser considerada como una medida efectiva para prevenir la pseudotuberculosis en lenguado. Es necesario reseñar, sin embargo, que el lenguado es una especie totalmente diferente a aquellas especies piscícolas en las que estas vacunas se vienen aplicando, y, por tanto, es necesaria una adecuación de su empleo en el lenguado. Hay descritos diseños vacunales divalentes que incluyen bacterina y ECPs inactivados por formol de *Vibrio harveyi* y *P. damselae* subsp. *piscicida* y que se han aplicado específicamente a lenguado obteniéndose resultados prometedores (Arijo *et al.*, 2005) aunque su efectividad es limitada en el tiempo. Por lo tanto, no hay que descartar otros aspectos de la profilaxis como es el empleo de los inmunoestimulantes y los probióticos.

Los inmunoestimulantes son más seguros que los antibióticos, y su rango de eficacia es más amplio que el de las vacunas, aunque su acción es de corta duración. Así, la estrategia más efectiva para prevenir y combatir posibles enfermedades infecciosas de peces puede ser el uso combinado de los métodos descritos (Sakai, 1999). De hecho, en la actualidad, el uso de inmunoestimulantes, junto a agentes quimioterapéuticos o vacunas, ha sido ampliamente aceptado por parte de los acuicultores. Sin embargo, es necesaria la búsqueda de nuevos agentes inmunoestimulantes que abaraten los costes de producción y resulten efectivos frente a los patógenos.

La aplicación de los probióticos en acuicultura surge también por la necesidad de contar con estrategias destinadas al control de enfermedades que afectan a las especies cultivadas.

4.2. INMUNOMODULACIÓN. INMUNOESTIMULACIÓN

La inmunomodulación es la capacidad que tienen determinadas sustancias y agentes de regular el sistema inmunitario, pudiéndose hablar de inmunoestimulación o inmunodepresión si se estimula o deprime dicho sistema, respectivamente. La principal

razón de la búsqueda de nuevos agentes inmunoestimulantes es el gran desarrollo de la acuicultura y el incremento de situaciones de estrés y enfermedades causadas por los cultivos intensivos, que suelen producir un incremento de la susceptibilidad a las infecciones. Se han llevado a cabo algunos estudios en los que este efecto negativo fue superado por el uso de inmunoestimulantes (Siwicki *et al.*, 1994; Anderson, 1996), ya que incrementan los mecanismos de defensa inespecíficos. Facilitan la función de las células fagocíticas e incrementa su actividad bactericida, siendo los mecanismos implicados los ya mencionados independientes y dependientes (estallido respiratorio) de oxígeno (Anderson *et al.*, 1992; Sakai, 1999).

El empleo de inmunoestimulantes tiene un valor principalmente preventivo, pueden ser capaces de compensar las limitaciones de los quimioterapéuticos y de las vacunas (Tabla 3). Los inmunoestimulantes son más seguros que los quimioterapéuticos y su rango de eficacia es más amplio que el de las vacunas (Sakai, 1999). Su principal inconveniente es la corta duración de su acción ya que estas sustancias actúan sobre el sistema inmunitario inespecífico el cual carece de memoria (Anderson, 1996; Sakai, 1999). Sakai (1999) afirma que, como regla general, la estrategia más efectiva para prevenir y combatir posibles enfermedades infecciosas de peces es el uso combinado de la vacunación y la administración de immunoestimulantes. De esta manera, con un conocimiento detallado de la eficacia y limitaciones, el inmunoestimulante puede llegar a ser una herramienta poderosa en el control de enfermedades en peces.

Aunque se han estudiado muchas sustancias naturales y sintéticas, con resultados que demuestran una potenciación del sistema inmune de peces y un incremento de la resistencia a la enfermedad, la búsqueda de nuevos inmunoestimulantes continúa hacia la mejora de las condiciones en los cultivos intensivos. Estos nuevos productos deben poseer dos características: proporcionar una estimulación general y ser económicamente asequibles. En los últimos años, los estudios destinados a tal fin se han centrado principalmente en el empleo de sustancias de origen natural cuyas ventajas principales respecto a las de origen sintético radican en el hecho de ser sustancias no tóxicas, biodegradables y biocompatibles con la salud humana.

Tabla 3 Comparación de las características de quimioterapéuticos, vacunas e inmunoestimulantes (Sakai, 1999)

	QUIMIOTERAPÉUTICOS	VACUNAS	INMUNOESTIMULANTES
Cuándo	Terapéutico	Profiláctico	Profiláctico
Eficacia	Excelente	Excelente	Buena
Espectro de actividad	Medio	Limitado	Amplio
Duración	Corta	Larga	Corta

4.3. USO DE LAS ALGAS COMO INMUNOESTIMULANTES

En los últimos años se ha centrado la atención en organismos marinos como fuente de sustancias de interés terapéutico. En este sentido, la capacidad de las algas para producir metabolitos secundarios de interés farmacéutico, como antibióticos, antivirales, antitumorales y antiinflamatorios ha sido extensamente documentada (Scheuer, 1990; Faulkner, 1993; González del Val *et al.*, 2001). Sin embargo, los estudios enfocados hacia la detección de propiedades inmunomoduladoras de extractos procedentes de algas son todavía muy escasos (Blinkova *et al.*, 2001; Castro *et al.*, 2004, 2006). En esta Memoria nos hemos centrado en el estudio de la microalga roja *Porphyridium cruentum* como posible fuente de sustancias inmunoestimulantes para lenguados cultivados. El alga cumpliría con los requisitos que hoy día se plantean en la búsqueda de nuevas sustancias potencialmente inmunoestimulantes, ya que es una sustancia natural y su cultivo no suele ser costoso, tanto desde el punto de vista económico, como en cuanto a tiempo y esfuerzo necesarios.

Las algas tienen diferentes compuestos con efecto sobre el sistema inmunitario de los peces. Muchas algas son fuente importante de los denominados ácidos grasos poliinsaturados, PUFAs, esenciales como requerimiento dietético de muchos teleósteos (Bell *et al.*, 1985; Koven *et al.*, 2001). Además, algunos de ellos, como el ácido

araquidónico, están implicados en la síntesis de eicosanoide y, por tanto, en la producción de prostaglandinas, implicadas en los procesos de estrés a través de la modulación en la liberación del cortisol y, por consiguiente, de la inmunidad celular (Villalta *et al.*, 2005).

Otro de los componentes que encontramos en las algas son los carotenoides, como el β-caroteno, la astaxantina, la cataxantina o las xantofilinas. Amar *et al.* (2004) demostraron que el β-caroteno aislado del alga *Dunaliella salina* es capaz de modular, tras la administración oral, algunos de los mecanismos de defensa innata en trucha arcoiris (*Oncorhynchus mykiss*), como la actividad alternativa del complemento y la lisozima en el suero, así como la fagocitosis. Los carotenoides incrementan igualmente la actividad fagocítica y la producción de citoquinas (Bendich, 1989; Chew, 1993).

Las algas son también una fuente natural de vitaminas, algunas de las cuales tienen posibles efectos estimulantes sobre el sistema inmune de peces, como es el caso de la vitamina C (Hardie *et al.*, 1991; Cuesta *et al.*, 2002; Jeney y Jeney, 2002; Lin y Shiau, 2005), la vitamina E (Hardie *et al.*, 1990; Cuesta *et al.*, 2001) y otras del grupo B (Miles *et al.*, 2001).

La utilización de polisacáridos como inmunoestimulantes está ampliamente extendida en la acuicultura, pudiéndose adquirir comercialmente (Siwicki *et al.*, 1994; Cook *et al.*, 2003; Couso *et al.*, 2003; Bagni *et al.*, 2005), siendo los glucanos los más estudiados en peces (Kumar *et al.*, 2005). Los β-glucanos consisten en una serie de residuos de β-1,3-glucopiranosil derivados de levaduras y micelios de hongos. Estos azúcares parecen tener un potente efecto inmunoestimulante, fundamentalmente sobre los mecanismos de defensa inespecíficos, induciendo resistencia a infecciones. La mayor parte de estos estudios se centra en β-glucanos aislados de la levadura *Saccharomyces cerevisiae* (Santarém *et al.*, 1997; Castro *et al.*, 1999; Kumari y Sahoo, 2006; Marqués *et al.*, 2006).

Otro polisacárido potencialmente inmunoestimulante, procedente de diferentes macro-y micro- algas pardas, es el ácido algínico. El alginato es conocido en la acuicultura hace mucho tiempo, utilizándose en la fabricación de pienso como estabilizador de la estructura. Las propiedades inmunomoduladoras fueron determinadas

en extractos de *Phyophaecaetes*, como *Laminaria digitata* (Dalmo *et al.*, 1998; Gabrielsen y Austreng, 1998) y otros (Miles *et al.*, 2001; Peddie *et al.*, 2002; Skjermo y Bergh, 2004; Bagni *et al.*, 2005).

4.3.1. *Porphyridium cruentum*

Porphyridium cruentum es una microalga roja, perteneciente a la familia *Rodophyta*, orden *Porphyridiales*. Sus células se caracterizan por ser esféricas y sin pared celular. Acumula grandes cantidades de ácidos grasos, que llegan a alcanzar entre 9 y 14% del peso seco, especialmente el ácido araquidónico (36% del total de los ácidos grasos), y cantidades considerables de ácido eicosapentaenoico. El contenido proteico está en el rango del 28 al 39%, y los carbohidratos disponibles varían entre un 40 y un 57%. La biomasa contiene tocoferol, vitamina K y una gran cantidad de carotenos (Rebolloso *et al.*, 2000).

Una propiedad característica de *P. cruentum* es que sus células son capaces de excretar un polisacárido sulfatado, un heteropolímero acídico compuesto por xilosa, glucosa, galactosa y ésteres de sulfato (You y Barneu, 2004). Este polisacárido es de gran importancia dado que puede ser usado comercialmente como espesante, estabilizante y emulsionante (Arad *et al.*, 1985, 1988; Adda *et al.*, 1986).

El alga *P. cruentum* contiene sustancias presentes en otros microorganismos de los cuales se ha demostrado su efecto inmunoestimulante en fagocitos de peces. Estas sustancias incluyen ácido araquidónico (Koven *et al.*, 2001), carbohidratos (Kumar *et al.*, 2005), vitaminas (Hardie *et al.* 1990, 1991; Ortuño *et al.*, 1999, 2003; Jeney y Jeney, 2002), carotenoides (Amar *et al.*, 2004) y polisacáridos (Siwicki *et al.*, 1994; Santarém *et al.*, 1997; Castro *et al.*, 1999; Bagni *et al.*, 2000, 2005; Esteban *et al.*, 2001; Jeney y Jeney, 2002; Cook *et al.*, 2003; Couso *et al.*, 2003), pudiendo producir una respuesta inmune más general al poseer varias sustancias ya probadas como inmunoestimulantes (Ortuño *et al.*, 2002).

P. cruentum posee las principales características para poder ser considerada como un potencial inmunoestimulante. Su cultivo no es costoso, ni desde el punto de vista de esfuerzo necesario, ni en términos económicos. A nivel industrial es conveniente

simplificar la administración del inmunoestimulante, proporcionándola por vía oral, incluyéndola en el pienso y con el menor grado de manipulación posible. De hecho, durante los últimos años existen cada vez más trabajos en los que se ensayan organismos completos, como levaduras (Siwicki *et al.*, 1994; Ortuño *et al.*, 2002; Rodríguez *et al.*, 2003), hongos (Rodríguez *et al.*, 2004) y probióticos (Verschuere *et al.*, 2000; Irianto y Austin, 2003; Salinas *et al.*, 2005; Díaz-Rosales *et al.*, 2006). Sin embargo, a pesar de que son numerosos los estudios realizados con extractos o determinados compuestos derivados de algas (Koven *et al.*, 2001; Castro *et al.*, 2004; Skjermo y Bergh, 2004; Díaz-Rosales *et al.*, 2005; Hou y Chen, 2005; Villalta *et al.*, 2005), son escasos los estudios que plantean el empleo de algas completas (Blinkova *et al.*, 2001; Valente *et al.*, 2006).

4.4. EFECTO INMUNOESTIMULANTE DE BACTERIAS POTENCIALMENTE PROBIÓTICAS

La definición de probiótico ha ido cambiando a lo largo del tiempo, proponiéndose como probióticos a bacterias vivas o inactivadas o a alguno de sus componentes celulares que también pueden ejercer ciertos efectos beneficiosos (Ouwehand y Salminen, 1998; Isolauri *et al.*, 2002). Así, Salminen *et al.* (1999) han separado la definición de probiótico del alimento y de su característica de ser microorganismos vivos con lo que se ha dado paso al siguiente concepto: "un probiótico es cualquier preparación microbiana (no necesariamente viva), o los componentes de células microbianas, que tienen un efecto beneficioso en la salud del hospedador". Igualmente, Schrezenmeir y de Vrese (2001) hacen referencia a los probióticos como "una preparación o producto que contiene microorganismos definidos en número suficiente, capaces de alterar la microbiota, por implantación o colonización, en un compartimento del hospedador y por el que ejerce efectos beneficiosos sobre la salud del hospedador". Según la FAO, el término probiótico hace referencia a un complemento microbiano de la dieta que afecta beneficiosamente a la fisiología del hospedador mediante modulación de la inmunidad sistémica y local, además de mejorar el balance microbiano mediante la

prevención de la colonización gastrointestinal por bacterias no deseables. Los avances en el empleo de los probióticos en ganadería y medicina humana han conducido a considerar su aplicación también en la práctica acuícola ya que pueden ser una alternativa en la lucha contra la infección microbiana (Sakai, 1999). La investigación de la aplicación de los probióticos en la industria acuícola se ha incrementado en los últimos años por la demanda de una industria acuícola que, entre otros aspectos, intenta respetar el medio ambiente. Los probióticos surgen de la necesidad de contar con estrategias destinadas al biocontrol de enfermedades que afectan a las especies cultivadas en acuicultura. La mayoría de los trabajos realizados con probióticos en peces se ha centrado en el grado de protección del pez frente a enfermedades infecciosas por su capacidad para inhibir el crecimiento del patógeno. Actualmente muchos de los estudios que se están realizando para dilucidar los mecanismos responsables de los efectos de los probióticos se están centrando en las propiedades inmunomoduladoras de las cepas (Nikoskelainen *et al.*, 2003; Irianto y Austin, 2003; Panigrahi *et al.*, 2004; Salinas *et al.*, 2005, 2006; Díaz-Rosales, 2006). De hecho, muchos de los inmunoestimulantes probados en acuicultura son componentes de células microbianas, como los glucanos, lipopolisacáridos y muramil dipéptido (Anderson, 1992). Sin embargo, la mayoría de ellos están centrados en especies de agua dulce, siendo escasos, por el contrario, los llevados a cabo en especies marinas, y nulos los que se refieren a lenguado senegalés (Tabla 4).

Los animales acuáticos son muy diferentes a los terrestres por lo que el concepto de probiótico cambia a la hora de su aplicación en acuicultura (Verschuere *et al.*, 2000). La microbiota intestinal de las especies cultivadas interactúa de forma constante con el ambiente, el cual tiene una influencia mucho mayor sobre la salud de los peces que en el caso de los humanos o animales terrestres. Por lo tanto, debido a que existe un flujo continuo de agua pasando a través del tracto digestivo, la microbiota intestinal de los peces es dependiente del ambiente externo. De hecho, se han realizado estudios sobre la microbiota del pez y se ha visto que la variación es sustancial y que fluctúa diariamente (Spanggaard *et al.*, 2000). Por lo tanto, la mayoría de las bacterias son transitorias en el intestino del pez, con intrusiones continuas de bacterias procedentes del agua y de la comida. De esta manera, no sólo se habla de probiótico cuando se adiciona al alimento,

sino también cuando se añaden al medio, tanque o laguna de cultivo. Aquí el concepto de probiótico se amplía, denominándose biocontrol cuando el tratamiento es con microorganismos antagonistas al patógeno, o biorremediación cuando la calidad del agua es mejorada.

Verschuere *et al.* (2000) proponen una definición modificada para el término probiótico aplicada en acuicultura: "Complemento microbiano vivo que tiene un efecto beneficioso sobre el hospedador modificando la comunidad microbiana relacionada con el hospedador o con el ambiente, asegurando un uso mejorado del alimento o aumentando su valor nutricional, favoreciendo la respuesta del hospedador a las enfermedades, o mejorando la calidad del ambiente".

Entre los mecanismos propuestos para explicar el modo en el que los probióticos pueden interactuar con los patógenos tenemos: (1) Exclusión competitiva por la producción de compuestos antimicrobianos entre los que se han descrito bacteriocinas, lisozimas y proteasas (Austin *et al.*, 1995; Sugita *et al.*, 1997; Gatesoupe, 1999; Gram *et al.*, 1999; Verschuere *et al.*, 2000). Tan importante se ha considerado la capacidad de inhibir el crecimiento de bacterias patógenas de peces, que esta característica se ha convertido en uno de los criterios más empleados para la selección de potenciales probióticos en acuicultura; (2) Competición por los nutrientes y energía disponible (Smith y Davey, 1993; Pybus *et al.*, 1994; Gatesoupe *et al.*, 1997; Gram *et al.*, 1999) y (3) Interferencia adhesiva en el hospedador (Olsson *et al.*, 1992; Jöborn *et al.*, 1997; Nikoskelainen *et al.*, 2001; Chabrillón *et al.*, 2005a y b).

Tabla 4 Efectos beneficiosos de probióticos sobre el sistema inmune de peces

PROBIÓTICO	HOSPEDADOR	INCREMENTO RESPUESTA INMUNE	REFERENCIA
Bacteria Gram positiva, no identificada	*Oncorhynchus mykiss* (trucha arcoiris)	Número eritrocitos y leucocitos, actividad lisozima, fagocitosis	Irianto y Austin, 2003
Vibrio fluvialis	*Oncorhynchus mykiss*	Número eritrocitos y leucocitos, actividad lisozima, fagocitosis	Irianto y Austin, 2003
Aeromonas hydrophila	*Oncorhynchus mykiss*	Número eritrocitos y leucocitos, actividad lisozima, fagocitosis	Irianto y Austin, 2003
Carnobacterium	*Oncorhynchus mykiss*	Número eritrocitos y leucocitos, actividad lisozima, fagocitosis	Irianto y Austin, 2003
Lactobacillus rhamnosus	*Oncorhynchus mykiss*	Estallido respiratorio, actividad bactericida suero, niveles Ig suero	Nikoskelainen *et al.*, 2003
Bacillus	*Penaeus vannamei*	Índice inmune (hemograma, producción anión superóxido, actividad fenoloxidasa, actividad antibacteriana, concentración proteica plasma)	Gullian *et al.*, 2004
Lactobacillus rhamnosus	*Oncorhynchus mykiss*	Actividades lisozima y complemento suero, fagocitosis	Panigrahi *et al.*, 2004
Lactobacillus delbrüeckii subsp. *lactis*	*Sparus aurata* (dorada)	Fagocitosis, actividad citotóxica	Salinas *et al.*, 2005
Bacillus subtilis	*Sparus aurata*	Fagocitosis, actividad citotóxica	Salinas *et al.*, 2005
Aeromonas sobria	*Oncorhynchus mykiss*	Número leucocitos, fagocitosis, estallido respiratorio	Brunt y Austin, 2005
Alteromonadaceae, G. *Shewanella* (Pdp11)	*Sparus aurata*	Peroxidasa suero, actividad complemento, fagocitosis	Díaz-Rosales *et al.*, 2006
Alteromonadaceae, G. *Shewanella* (51M6)	*Sparus aurata*	Peroxidasa suero, actividad complemento, fagocitosis, actividad citotóxica	Díaz-Rosales *et al.*, 2006

La necesidad de mejorar la resistencia frente a las enfermedades, así como de aumentar la eficiencia en la alimentación y en el desarrollo del crecimiento son aspectos fundamentales en varios sectores de esta industria en el empeño de lograr una reducción de los costes de producción. La microbiota gastrointestinal desempeña una función importante en la nutrición y salud del organismo hospedador. En este sentido, los probióticos también pueden desempeñar un papel interesante, de esta forma en humanos y en la ganadería terrestre se han investigado distintas formas de alterar la microbiota gastrointestinal por el empleo de probióticos con vistas a lograr unos efectos favorables, tales como mejora del crecimiento, de la digestión, de la inmunidad y de la resistencia a la enfermedad del hospedador. Si bien en el campo de la acuicultura hay numerosos trabajos que han caracterizado la microbiota gastrointestinal de distintos peces cultivados, fundamentalmente salmónidos (Spanggaard *et al.*, 2000; Huber *et al.*, 2004, Jensen *et al.*, 2004; Burr *et al.*, 2005), estos estudios son nulos en lo que se refiere a algunos de los peces marinos más cultivados en nuestra área, así como en la valoración de los efectos que sobre la microbiota gastrointestinal de estos peces puede tener la aplicación de microorganismos probióticos. Este tipo de estudios son muy interesantes desde el punto de vista de la información que pueden aportar para una mejor aplicación de microorganismos en estrategias profilácticas y de biocontrol de enfermedades.

OBJETIVOS

El trabajo planteado en esta Memoria de Tesis Doctoral consiste en la profundización en el conocimiento de ciertos aspectos de la virulencia de *Photobacterium damselae* subsp. *piscicida*, así como la optimización de estrategias dirigidas a la prevención de la enfermedad que este patógeno causa. En base a ello, se han planteado los siguientes objetivos:

1. Estudio del papel de las actividades enzimáticas superóxido dismutasa y catalasa presentes en *Photobacterium damselae* subsp. *piscicida*, evaluando su contribución a la resistencia del patógeno frente a la acción bactericida de los fagocitos de lenguado.

2. Evaluación del posible efecto inmunoestimulante sobre el estallido respiratorio de fagocitos de lenguados cultivados que pueda ejercer la utilización de la microalga *Porphyridium cruentum* y de microorganismos probióticos.

MATERIAL Y MÉTODOS

La metodología, así como el material empleado, en la realización de los diferentes experimentos que conforman esta Tesis Doctoral, se detallan en cada uno de los artículos incluidos en la Sección de artículos.

RESULTADOS Y DISCUSIÓN

El primer objetivo abordado en esta Tesis Doctoral ha consistido en el estudio del papel de las actividades enzimáticas superóxido dismutasa y catalasa en la virulencia de *Photobacterium damselae* subsp. *piscicida*. En concreto, se ha evaluado la contribución de estas actividades en la resistencia del patógeno frente a la formación de radicales oxigénicos generados durante el estallido respiratorio de fagocitos de lenguado y, por tanto, su posible papel como factores de virulencia (artículos 1.1. y 1.2., Sección de artículos).

Previamente, Barnes *et al.* (1999a) demostraron que este microorganismo *in vitro* contiene suficiente actividad superóxido dismutasa para descomponer los aniones superóxido generados fotoquímicamente, y que la susceptibilidad de la bacteria viene dada por la acumulación de peróxido de hidrógeno, ya que al adicionar catalasa la supervivencia se incrementa. Estos hechos muestran la importancia del peróxido de hidrógeno en la inactivación de esta bacteria y, por consiguiente, la importancia de la actividad catalasa en la resistencia a este radical, cuyo papel no había sido determinado anteriormente. En el presente trabajo, los resultados obtenidos sobre el papel de la catalasa están desarrollados en los artículos 1.1. y 1.2. (Sección de artículos). El primer artículo evalúa el papel *in vitro* de dicha enzima antioxidante en la protección de *P. damselae* subsp. *piscicida* frente al peróxido de hidrógeno, demostrándose la relevancia de la actividad catalasa en la resistencia del patógeno frente a este radical oxigenado. Los resultados obtenidos muestran que la resistencia que confiere la actividad catalasa frente al peróxido de hidrógeno varía según la condición de cultivo a la que la bacteria se vea sometida. Así, cuando se ejerce un estrés oxidativo, por adición de peróxido de hidrógeno, esa resistencia se incrementa; en cambio, cuando las condiciones son limitantes de hierro, los porcentajes de supervivencia disminuyen. Además, según el grado de virulencia esa resistencia variará, siendo mayor en la cepa virulenta que en la no virulenta. Por otro lado, en el artículo 1.2. (Sección de artículos) se determinó la presencia de actividad catalasa en diferentes cepas de *P. damselae* subsp. *piscicida* y se cuantificó espectrofotométricamente dicha actividad en bacterias sometidas a diferentes condiciones de cultivo. Los resultados obtenidos señalan que los mayores porcentajes de supervivencia coinciden con las condiciones en las que los niveles de actividad catalasa detectados son también superiores. Así, la cepa virulenta $Lg_{h41/01}$ presenta mayor

resistencia al estallido respiratorio de fagocitos de lenguado que la no virulenta EPOY-8803-II, mientras que la adición de peróxido de hidrógeno a los cultivos incrementa la supervivencia de ambos. La ausencia de cápsula podría ser un factor que haría disminuir la supervivencia frente a los ROS, pero los bajos niveles de actividad catalasa obtenidos para EPOY-8803-II, sugieren que la falta de virulencia podría venir determinada, en gran parte, por la baja actividad enzimática de dicha proteína antioxidante, es decir, la catalasa ejerce un papel importante en la virulencia de *P. damselae* subsp. *piscicida*. En muchas bacterias ya se ha demostrado que la exposición previa a concentraciones subletales de un oxidante puede inducir la protección frente a concentraciones letales del mismo (Mongkolsuk *et al.*, 1996). Un ejemplo es el descrito por Barnes *et al.* (1999b) en el caso de *A. salmonicida*, la cual, tras los pulsos de peróxido de hidrógeno, es capaz de resistir la concentración de peróxido de 100 mM, letal en ausencia de pretratamiento con peróxido de hidrógeno. Es interesante señalar que para EPOY-8803-II las diferencias, en cuanto a los porcentajes de supervivencia, son significativas entre los cultivos crecidos hasta fase estacionaria y aquellos adicionados con peróxido, siendo estos últimos mayores. Sin embargo, para la cepa $Lg_{h41/01}$ no ocurre lo mismo, ya que no se aprecian diferencias significativas en la supervivencia de los cultivos con o sin peróxido.

Por otro lado, la supervivencia disminuye de forma significativa en ambas cepas cuando se someten a condiciones limitantes de hierro. No podemos olvidar que estamos ante una catalasa de tipo férrica (artículo 1.2., Sección de artículos), por lo que la carencia de este metal limita su síntesis, y, por tanto, su actividad.

Tanto superóxido dismutasa como catalasa son metaloenzimas, pudiendo poseer diferentes metales en su centro activo. En la bibliografía se han descrito microorganismos capaces de producir diferentes isoenzimas de superóxido dismutasa y catalasa inducibles según las condiciones de cultivo a las que el microorganismo es sometido, como altos niveles de oxígeno, bajos niveles de hierro o crecimiento hasta la fase estacionaria (Storz *et al.*, 1990; Privalle y Fridovich, 1992; Crockford *et al.*, 1995; Schnell y Steinman, 1995; Barnes *et al.*, 1996; Polack *et al.*, 1996; St. John y Steinman,

1996; Lynch y Kuramitsu, 2000; Yesilkaya *et al.*, 2000; Geslin *et al.*, 2001; Vattanaviboon y Mongkolsuk, 2001).

Sin embargo, aún es escasa la información sobre las actividades superóxido dismutasa y catalasa y la posible inducción de las diferentes isoformas en *P. damselae* subsp. *piscicida*. Así en este trabajo se determinó si este patógeno podría expresar diferentes isoenzimas de superóxido dismutasa y de catalasa cuando es cultivado bajo diferentes condiciones de cultivo. Los resultados obtenidos en este trabajo muestran que ninguna de las condiciones de cultivo ensayadas inducen la síntesis de diferentes isoformas de superóxido dismutasa o catalasa en ninguna de las cepas de *P. damselae* subsp. *piscicida*.

Todas y cada una de las cepas ensayadas presentan una sola banda de actividad superóxido dismutasa, banda de similar movilidad electroforética a la superóxido dismutasa férrica descrita por Barnes *et al.* (1999a). Incluso bajo condiciones de estrés oxidativo, tras la adición de peróxido de hidrógeno o del generador de radicales oxigénicos paraquat, no se induce una isoenzima distinta, estando descrito que condiciones aeróbicas inducen la síntesis de CuZnSOD y de MnSOD en *Escherichia coli* (Hassan y Fridovich, 1977; Privalle y Fridovich, 1992; Benov y Fridovich, 1994; Geslin *et al.*, 2001), así como de esta última en *A. salmonicida* (Barnes *et al.*, 1996; Barnes *et al.*, 1999b), *Pseudomonas aeruginosa* (Polack *et al.*, 1996) y *Streptococcus pneumoniae* (Yesilkaya *et al.*, 2000). Ni tan siquiera condiciones restrictivas de hierro que inducen la síntesis de MnSOD (Privalle y Fridovich, 1992; Barnes *et al.*, 1999b) inducen la síntesis de una isoenzima distinta. Aunque serían necesarios más estudios, la falta de inducción de una SOD nueva podría ser debida a la existencia de un único gen codificador de la superóxido dismutasa férrica, el *sodB* (Lynch y Kuramitsu, 2000).

De igual modo, todas las cepas, bajo todas las condiciones de cultivo ensayadas, presentan una sola banda de actividad catalasa de similar movilidad electroforética a la ya descrita por Barnes *et al.* (1999a). Esta enzima fue caracterizada, mediante el uso de inhibidores, no pudiendo ser detectada en geles tras la exposición a la azida sódica, y viéndose reducida ligeramente tras el tratamiento con cianuro potásico, por lo que estamos ante una catalasa férrica, ya que las catalasas con manganeso en su centro

activo retienen su actividad tras tratamiento con azida y cianuro, y son inhibidas con cloruro de mercurio (Kono y Fridovich, 1983; Allgood y Perry, 1986; Barnes *et al.*, 1999b).

A pesar de que ninguna de las condiciones de cultivo ensayadas inducen la síntesis de más de un tipo de isoenzima superóxido dismutasa o catalasa, sí se aprecian diferencias en la intensidad de las bandas detectadas por electroforesis nativa, así como en los niveles de actividad, tras su cuantificación espectrofotométricamente. Estos resultados concuerdan con los obtenidos por Barnes *et al.* (1999a) que detectaron diferencias en cultivos sometidos a diferentes concentraciones de hierro y distintos niveles de oxígeno. Los niveles más bajos de actividad superóxido dismutasa y catalasa se obtienen en cultivos sometidos a condiciones limitantes de hierro, hecho atribuible a la naturaleza férrica de ambas enzimas. Es interesante señalar que, tanto para la actividad superóxido dismutasa, como catalasa, bajo condiciones restrictivas de hierro, la cepa virulenta presenta mayores niveles de actividad. Esto nos indica la importancia de la presencia de mecanismos de captación de hierro tanto para la expresión de SOD como de catalasa. De esta forma, los microorganismos capaces de obtener hierro a partir del hospedador serían capaces de expresar niveles más elevados de las enzimas antioxidantes y podrían descomponer los radicales superóxido y peróxido de hidrógeno generados por dicho hospedador. Sin embargo, si bien en otras especies bacterianas como *Listeria monocytogenes* (Welch *et al.*, 1979), *Shigella flexneri* (Franzon *et al.*, 1990) o *A. salmonicida* (Barnes *et al.*, 1999b) se ha demostrado la importancia de SOD como enzima antioxidante y su contribución a la patogénesis, en el caso de *P. damselae* subsp. *piscicida* este papel no está tan claro. Pero la cepa EPOY-8803-II, que no es virulenta para lenguados, tiene niveles de actividad cercanos a los de la cepa virulenta. Estos resultados pueden atribuirse a unos niveles deficientes de otras actividades antioxidantes tales como catalasa, lo que haría que se dieran oxidaciones como consecuencia de la acumulación de otros radicales derivados de la descomposición de superóxido. Otra posible explicación a la no virulencia de la cepa EPOY-8803-II es su carencia de cápsula, que la haría más susceptible al reconocimiento por el sistema inmune del hospedador.

Una vez determinada *in vitro* la importancia tanto de SOD (Barnes *et al.*, 1999a), como de catalasa (artículo 1.2., Sección de artículos), se analizó el papel de estas actividades *in vivo*, mediante incubación de la bacteria con fagocitos. Como se ha mencionado anteriormente, la interacción, el modo de invadir y sobrevivir en el interior del hospedador, entre los fagocitos de lenguado y *P. damselae* subsp. *piscicida* aún no se conocen bien. Mientras que algunos autores señalan la presencia de células de *P. damselae* subsp. *piscicida* intactas en el interior de células de dorada, sugiriendo la capacidad de la bacteria para sobrevivir como patógeno intracelular en dorada (Noya *et al.*, 1995b; López-Dóriga *et al.*, 2000), incluso de multiplicarse en el interior de macrófagos del pez (Kubota *et al.*, 1970; Hawke *et al.*, 1987; Noya *et al.*, 1995a; Elkamel *et al.*, 2003), otros han observado que este patógeno es altamente susceptible a los radicales oxidativos generados durante el estallido respiratorio en los fagocitos de trucha, lubina y dorada (Skarmeta *et al.*, 1995). Barnes *et al.* (1999a) confirmaron que *P. damselae* subsp. *piscicida* es incapaz de responder al ataque oxidativo generado durante el estallido respiratorio, ya que en dicho trabajo las cepas ensayadas mostraron alta susceptibilidad a radicales de oxígeno generados *in vitro*.

Los resultados incluidos en el artículo 1.2. (Sección de artículos) muestran que *P. damselae* subsp. *piscicida* es capaz de sobrevivir al menos cinco horas en contacto con fagocitos de lenguado, siendo los porcentajes de supervivencia mayores en la cepa virulenta (62%) que en la no virulenta (19%), y correspondiendo las condiciones de cultivo con aquellas en las que la actividad catalasa muestra también un incremento, lo que indica un importante papel de la catalasa en la supervivencia bacteriana. Estos resultados sugieren que la inactivación bacteriana podría ser debida a la acumulación de peróxido de hidrógeno, precursor del radical hidroxilo. Mientras que Barnes *et al.* (1999a) mostraron que tanto la cepa virulenta como la no virulenta eran susceptibles a los radicales generados fotoquímicamente, en este trabajo se demuestra que la cepa no virulenta EPOY-8803-II, es significativamente más susceptible que la virulenta, $Lg_{h41/01}$, al estallido respiratorio de fagocitos de lenguado. Además de los niveles bajos de actividad catalasa, esa susceptibilidad podría también ser causada por la ausencia de cápsula, la cápsula podría proteger a las bacterias de los radicales oxigenados o prevenir la activación de los fagocitos (Miller y Britigan, 1997; Arijo *et al.*, 1998).

Finalmente, se ha constatado la importancia del hierro en la resistencia de la bacteria *P. damselae* subsp. *piscicida*, tal y como se ha descrito en otros microorganismos (Miller y Britigan, 1997; Weinberg, 2000). *P. damselae* subsp. *piscicida* es más susceptible a los fagocitos de lenguado cuando las células bacterianas son cultivadas bajo condiciones limitantes de hierro. La bacteria requiere hierro para su crecimiento, replicación y síntesis de enzimas tales como superóxido dismutasa y catalasa, habiéndose descrito la presencia de un sideróforo en *P. damselae* subsp. *piscicida* (Magariños *et al.*, 1994; Naka *et al.*, 2005). Sin embargo, a pesar de su capacidad para obtener hierro, varios autores han observado que las células cultivadas en condiciones limitantes de hierro, reducen su material capsular (do Vale *et al.*, 2001). Cabría pensar que estas células con cápsula reducida, serían más susceptibles a la fagocitosis y al estrés oxidativo. Los resultados obtenidos (artículos 1.1. y 1.2., Sección de artículos) indican que el hierro juega un importante papel en la supervivencia de *P. damselae* subsp. *piscicida* en contacto con los fagocitos, sugiriéndose que ello es atribuible a la contribución del material capsular, o a la síntesis de SOD y catalasa. En conclusión, *P. damselae* subsp. *piscicida* es capaz de sobrevivir en contacto con fagocitos de lenguado, siendo los porcentajes de supervivencia mayores en la cepa virulenta que en la no virulenta. El hecho de que los niveles de catalasa también se vean incrementados sugiere un posible papel de la enzima catalasa en la supervivencia bacteriana.

Una vez determinados los papeles de las actividades superóxido dismutasa y catalasa en la protección de *P. damselae* subsp. *piscicida* frente al estallido respiratorio de fagocitos de lenguado, el siguiente problema a abordar fue la búsqueda de estrategias para la prevención de la enfermedad que dicha bacteria produce. Como ya hemos mencionado anteriormente, una de las líneas a desarrollar en la prevención y tratamiento de la pseudotuberculosis es la aplicación de inmunoestimulantes, que incrementan la respuesta inmune del hospedador, frente a una infección. El parámetro inmunológico en el que se ha centrado esta Memoria es el estallido respiratorio en fagocitos de lenguado y como posibles immunoestimulantes, dos tipos de microorganismos: una micralga roja, *Porphyridium cruentum* y dos bacterias potencialmente probióticas, cuyas actividades

inmunoestimulantes han sido demostradas en fagocitos de mamíferos (Morris *et al.*, 2000) y de dorada (Díaz-Rosales *et al.*, 2006; Salinas *et al.*, 2006), respectivamente.

En primer lugar se evaluó la posible actividad inmunoestimulante del estallido respiratorio de fagocitos de lenguado ejercido por *P. cruentum*. Para ello se realizaron experimentos *in vitro* con los extractos acuosos y etanólicos obtenidos a partir del alga, ya que existen numerosos datos acerca de la capacidad de estimulación de diferentes extractos algales sobre el sistema inmune de peces (Fujiki *et al.*, 1992; Castro *et al.*, 2004; Díaz-Rosales *et al.*, 2005; Hou y Chen, 2005; Castro *et al.*, 2006). Los resultados obtenidos (artículo 2.1., Sección de artículos) muestran que ninguno de los dos extractos estimula la producción del anión superóxido, mientras que el control positivo que se ensayó, β-glucano comercial extraído del alga *Euglena gracilis*, sí es capaz de incrementar el estallido respiratorio tras 30 min en contacto con los fagocitos pero sólo a la concentración más alta ensayada (10 mg ml^{-1}). Estos resultados concuerdan con los descritos por Castro *et al.* (1999), que observó incremento del estallido respiratorio en fagocitos de rodaballo (*Psetta maxima*) y dorada tras ensayar diferentes concentraciones de β-glucanos procedentes de hongos y levaduras. Por otro lado, Castro *et al*. (2004) encontraron grandes variaciones en las capacidades inmunoestimulantes de los extractos algales dependiendo, no sólo del origen, sino también de las concentraciones ensayadas y de los tiempos de incubación.

P. cruentum presenta varias ventajas en su cultivo, pues tiene un crecimiento rápido y con bajo coste, lo que hace que sea muy fácil trabajar con ella. Además, el hecho de ser una sustancia natural permite considerarla *a priori* como biocompatible, biodegradable y segura para el medio ambiente y la salud humana, características que permiten considerarla una buena sustancia inmunoestimulante. Por ello, y una vez ensayados *in vitro* los extractos acuosos y etanólicos de la microalga, se realizó un experimento *in vivo* en el que se administró por vía oral el alga completa (artículo 2.1., Sección de artículos). Hasta ahora, los trabajos realizados administrando microorganismos completos a peces han sido, principalmente, con bacterias, consideradas probióticas (Verschuere *et al.*, 2000; Nikoskelainen *et al.*, 2001; Irianto y Austin, 2003; Salinas *et al.*, 2005; Balcázar *et al.*, 2006; Díaz-Rosales *et al.*, 2006; Salinas

et al., 2006) pero son muy escasos los estudios sobre algas completas (Blinkova *et al.*, 2001; Valente *et al.*, 2006). Por otro lado, *P. cruentum* acumula grandes cantidades de lípidos, como ácido araquidónico o eicosapentanoico (Kinsella *et al.*, 1990; Koven *et al.*, 2001); carbohidratos (Fujiki *et al.*, 1992; Santarém *et al.*, 1997; Bagni *et al.*, 2000; Morris *et al.*, 2000; Esteban *et al.*, 2001; Jeney y Jeney, 2002; Cook *et al.*, 2003; Couso *et al.*, 2003; Castro *et al.*, 2004; Bagni *et al.,* 2005; Kumar *et al.*, 2005); carotenos (Tachinaba *et al.*, 1997; Amar *et al.*, 2004); vitaminas (Hardie *et al.*, 1990, 1991; Ortuño *et al.*, 1999; Jeney y Jeney, 2002). Debido al hecho de contener diferentes sustancias inmunoestimulantes, su uso podría generar una respuesta inmune más general como ya ha sido propuesta para otros microorganismos como levaduras (Ortuño *et al.*, 2002; Rodríguez *et al.*, 2003).

Así, durante cuatro semanas, se alimentaron tres grupos de lenguados con pienso suplementado con la microalga liofilizada, pienso normal, o con un inmunoestimulante comercial, Sanostim™. Además, para evaluar el posible efecto sinérgico del inmunoestimulante con una vacuna, tras dos semanas del inicio del experimento, un conjunto de peces fue inoculado intraperitonealmente con una bacterina compuesta por células de *P. damselae* subsp. *piscicida* inactivadas con formol. Los resultados obtenidos muestran que tras cuatro semanas de administración del alga como suplemento en la dieta normal de los peces, la producción de anión superóxido se incrementa en aquellos lenguados que han sido inmunizados con la vacuna. Ese incremento es estadísticamente significativo, no sólo respecto a los peces alimentados con una dieta normal, sino también respecto a los peces alimentados con el inmunoestimulante comercial. La acción conjunta de un inmunoestimulante y una vacuna ha sido descrita por numerosos autores que concluyen que la combinación de vacunación y administración de inmunoestimulante incrementa la potencia de la vacuna (Jeney y Anderson, 1993; Røsrstad *et al.*, 1993; Aakre *et al.*, 1994; Sakai *et al.*, 1995; Baulny *et al.*, 1996; Sakai, 1999).

Seguidamente, y una vez demostrado el efecto inmunoestimulante del alga, se valoró el efecto inmunoestimulante de la fracción polisacarídica extracelular de *P.*

cruentum sobre el estallido respiratorio de fagocitos de lenguado, tanto *in vitro* como *in vivo* (artículo 2.2., Sección de artículos).

Los resultados obtenidos indican que *in vitro*, después de treinta minutos de contacto con las células del pez, ninguna de las concentraciones ensayadas del polisacárido extracelular de *P. cruentum* estimula el estallido respiratorio de fagocitos de lenguado. Estos resultados contrastan con los obtenidos por Castro *et al.* (2004, 2006) quienes sugieren que la estimulación del estallido respiratorio en fagocitos de rodaballo (*Psetta maxima*), en presencia de extractos de algas, es debida a los polisacáridos algales. Por otro lado, los resultados obtenidos por Castro *et al.* (2004) indican que la capacidad inmunoestimulante varía en gran medida según la especie de alga. Por lo que la falta de estimulación del estallido respiratorio *in vitro* por parte de los polisacáridos de *P. cruentum* puede ser debida, por un lado, a que simplemente no tengan capacidad inmunoestimulante, la concentración a la que se encuentra sea baja, o el tiempo de contacto con los fagocitos sea insuficiente. Sin embargo, el control positivo que contenía β-glucano comercial sí induce un incremento en el estallido respiratorio, aunque únicamente a la concentración mayor ensayada (10 mg ml^{-1}).

Adicionalmente, los lenguados se inocularon intraperitonealmente con 500 μg de la fracción extracelular polisacarídica. Como en el trabajo anterior, posteriormente se inmunizaron con una bacterina compuesta por células de *P. damselae* subsp. *piscicida* inactivadas con formol. Se tomaron muestras a las 24 h y a los 7 días. Los resultados obtenidos indican que a la concentración y tiempos ensayados, 1 y 7 días tras la inoculación de la fracción polisacarídica, no se produce un incremento en el estallido respiratorio de fagocitos de lenguado, ni siquiera en los peces inmunizados. Además, se observa que 24 h post-inoculación, el estallido respiratorio se reduce en los fagocitos de los peces inoculados con la fracción polisacarídica o con la bacterina. Esta disminución del estallido respiratorio podría ser atribuida a una inmunosupresión causada por estrés tras la manipulación (Thompson *et al.*, 1993; Pulsford *et al.*, 1995), desapareciendo este efecto tras siete días del inicio del experimento.

En resumen, se puede concluir que la fracción polisacarídica de *P. cruentum* en las condiciones descritas no incrementó el estallido respiratorio de fagocitos de lenguado.

Estos resultados sugieren que la estimulación observada tras la administración por vía oral del alga completa liofilizada sería debida a otro de los componentes del alga, con propiedades inmunoestimulantes. Por otro lado, la acción inmunoestimulante del polisacárido puede estar dirigida a otro parámetro inmunológico, no necesariamente al estallido respiratorio, así existen numerosos trabajos que describen la capacidad inmunoestimulante de polisacáridos en peces, incrementando la actividad lisozima en el suero, la actividad del complemento, la actividad citotóxica de fagocitos o la actividad fagocítica, entre otras (Santarém *et al.*, 1997; Esteban *et al.*, 2001; Chang *et al.*, 2003; Bagni *et al.*, 2005; Kumari y Sahoo, 2006).

Otra de las estrategias desarrolladas en esta Memoria para el tratamiento de la pseudotuberculosis fue el empleo de bacterias potencialmente probióticas. En el trabajo realizado (artículo 2.3., Sección de artículos) las bacterias potencialmente probióticas fueron administradas por vía oral, suplementándose el pienso con el que eran alimentados los lenguados. En este caso, además de evaluar el estallido respiratorio de fagocitos de riñón, se llevó a cabo una infección experimental con *P. damselae* subsp. *piscicida*, para determinar el grado de protección que pudieran aportar los probióticos. Al mismo tiempo, se estudió la microbiota intestinal, con objeto de detectar posibles cambios que produjeran los probióticos incorporados en la dieta.

Las bacterias seleccionadas para este experimento fueron las cepas Pdp11 y Pdp13, aisladas de piel de dorada (Chabrillón, 2003). Su identificación nos lleva a situarlas dentro de la familia *Alteromonadaceae*, género *Shewanella*. Es el primer ensayo *in vivo* que se realiza con Pdp13, en cambio con la cepa Pdp11 ya existen varios trabajos publicados, así Chabrillón *et al.* (2005a) estudiaron la interacción con el patógeno *Vibrio harveyi*, mostrando la capacidad de Pdp11 de adherirse al mucus intestinal de dorada, el efecto antagonista frente a una cepa patógena de *V. harveyi*, la capacidad de inhibir la unión del patógeno y, por último, de conferir protección frente una infección experimental. También se han publicado resultados obtenidos con Pdp11 y la bacteria patógena *P. damselae* subsp. *piscicida*, así Chabrillón *et al.* (2005b) demostraron el efecto antagonista de Pdp11 frente a una cepa de *P. damselae* subsp. *piscicida* y la inhibición de la unión de dicho patógeno al mucus intestinal. Estos resultados obtenidos

permiten considerar a la cepa Pdp11 como una buena candidata para ser usada como probiótico.

En cuanto a la posible estimulación del estallido respiratorio se ha observado que mientras la producción de anión superóxido por parte de los fagocitos aislados de peces alimentados con la cepa Pdp11 se ve incrementada significativamente transcurridos dos meses desde el inicio del experimento, los peces alimentados con la cepa Pdp13 no muestran esta respuesta. El efecto de la cepa Pdp13 se observa tras la infección experimental, ya que el grupo de peces alimentados con esta cepa alcanza un porcentaje de supervivencia más elevado tras la inoculación del patógeno *P. damselae* subsp. *piscicida*.

Aunque existen numerosos trabajos en los que se muestra la inducción del estallido respiratorio por probióticos (Nikoskelainen *et al.*, 2003; Gullian *et al.*, 2004; Aubin *et al.*, 2005; Brunt y Austin, 2005), el que el potencial probiótico Pdp13 no haya incrementado la producción de anión superóxido, no significa que no sea capaz de inducir algún otro parámetro inmunológico. De hecho, el porcentaje de supervivencia, tras la infección experimental, sí se ve incrementado. Varios autores han mostrado el efecto de probióticos sobre parámetros inmunológicos diferentes al estallido respiratorio, como las actividades fagocítica (Irianto y Austin, 2003; Panigrahi *et al.*, 2004; Brunt y Austin, 2005; Salinas *et al.*, 2005; Díaz-Rosales *et al.*, 2006), del complemento (Panigrahi *et al.*, 2004; Díaz-Rosales *et al.*, 2006), de la lisozima (Irianto y Austin, 2003; Panigrahi *et al.*, 2004) o la citotóxica (Salinas *et al.*, 2005; Díaz-Rosales *et al.*, 2006). Además ha sido descrito el efecto de los probióticos no sólo sobre la respuesta inmune inespecífica, sino también sobre la específica, incrementando los niveles de inmunoglobulinas en el suero (Nikoskelainen *et al.*, 2003; Aubin *et al.*, 2005). Por estas razones, la cepa Pdp13 puede ser considerado también como probiótico, aunque sería necesaria la evaluación de otros parámetros inmunológicos.

Por otro lado, las bacterias probióticas tienen que ser administradas a una dosis óptima, que dependerá del tamaño del pez y de la cepa (Nikoskelainen *et al.*, 2003), por esta razón puede que el efecto de la cepa Pdp11 sea diferente al de Pdp13, y que los

resultados obtenidos con Pdp11 y lenguado sean diferentes a los obtenidos previamente con Pdp11 y dorada (Díaz-Rosales *et al.*, 2006).

Por último, además del efecto inmunológico de los probióticos sobre la respuesta inmune de lenguado, se han evaluado posibles cambios en la microbiota intestinal provocados por los probióticos adicionados a la dieta. A pesar del gran número de trabajos publicados que estudian las comunidades microbianas de peces (Spanggaard *et al.*, 2000; Holben *et al.*, 2002; Sandaa *et al.*, 2003; Al-Harbi y Naim Uddin, 2004; Hjelm *et al.*, 2004; Huber *et al.*, 2004; Jensen *et al.*, 2004), ninguno evalúa los posibles cambios en esa microbiota tras la administración de probióticos.

En este trabajo se ha empleado la técnica de electroforesis en geles de gradiente desnaturalizante (DGGE) (Muyzer *et al.*, 1993) para estudiar la microbiota intestinal de lenguados alimentados con probióticos. Se ensayaron dos pares de cebadores universales (Nübel *et al.*, 1996; Jensen *et al.*, 2004), y tras el análisis del patrón de bandas por el coeficiente de Pearson, el de Nübel *et al.* (1996) fue seleccionado como el mejor para el estudio de las comunidades con las que se estaba trabajando.

El patrón de bandas obtenido fue muy simple, con pocas bandas predominantes, lo que podría concordar con lo descrito por Muyzer *et al.* (1993) quienes afirman que comunidades con pocas especies dominantes producirán patrones más simples y que las especies menos abundantes no estarán representadas adecuadamente en dicho patrón. Además la técnica presenta limitaciones, y es posible que algunas bandas no representen especies individuales, como sería en teoría, sino que grupos de especies pueden tener el mismo contenido relativo de G+C, comigrando (Simpson *et al.*, 1999; Temmerman *et al.*, 2003). Estas limitaciones conducirían a un descenso en el número de bandas presentes, pudiendo tener una influencia en la aparente diversidad, así como en los valores de similitud (McCraken *et al.*, 2001).

Los resultados obtenidos no demuestran que los probióticos induzcan cambios significativos en la microbiota intestinal, ya que las bandas que aparecen en los grupos que reciben el probiótico también están presentes en los grupos control.

Por otro lado, no puede confirmarse que las bandas presentes correspondan a la cepa Pdp11 o a Pdp13, atendiendo simplemente a la movilidad electroforética, por tanto,

harían falta estudios de identificación filogenética, secuenciación de los productos de PCR. Lo que sí podemos afirmar es que ninguna de las cepas es capaz, a la dosis (10^9 ufc g^{-1}) y tiempo ensayados (dos meses), de inducir cambios significativos en la microbiota intestinal. De acuerdo con Ouwehand *et al.* (2002), para considerar una bacteria como probiótico no es necesario que induzca cambios en la microbiota intestinal, para ejercer un efecto local o durante el tránsito a través del sistema gastrointestinal, de hecho, la variación en la microbiota en peces es sustancial y fluctúa diariamente (Spanggaard *et al.*, 2000; Al-Harbi y Naim Uddin, 2004; Panigrahi *et al.*, 2004).

CONCLUSIONES

Tras los estudios realizados sobre el papel de las actividades superóxido dismutasa y catalasa en la virulencia del patógeno *Photobacterium damselae* subsp. *piscicida*, así como de las estrategias desarrolladas en el control de dicho patógeno, se obtienen las siguientes conclusiones:

1. *Photobacterium damselae* subsp. *piscicida* sintetiza una sola isoenzima con actividad superóxido dismutasa, caracterizada por la presencia de hierro en su centro activo.

2. *Photobacterium damselae* subsp. *piscicida* contiene una sola isoenzima con actividad catalasa, con hierro en su centro activo.

3. La actividad catalasa es de gran importancia en la resistencia de *P. damselae* subsp. *piscicida* frente al peróxido de hidrógeno, pues cuando las células bacterianas presentan niveles elevados de esta actividad, resisten más eficientemente a estos radicales reactivos de oxígeno.

4. El hierro juega un importante papel en la supervivencia de *P. damselae* subsp. *piscicida* en presencia de radicales oxidantes ya que bajo condiciones limitantes de este metal se detectan niveles más bajos de actividad tanto superóxido dismutasa como catalasa, así como mayor susceptibilidad al peróxido de hidrógeno.

5. *Photobacterium damselae* subsp. *piscicida* es capaz de sobrevivir como patógeno intracelular en el interior de fagocitos de lenguado durante, al menos, 5 h.

6. La administración por vía oral de la microalga roja *Porphyridium cruentum*, en combinación con la inoculación intraperitoneal de una bacterina frente a *P. damselae* subsp. *piscicida*, incrementa el estallido respiratorio de los fagocitos de lenguado.

7. La administración oral de las cepas de *Shewanella* Pdp11 y Pdp13, propuestas como probióticos, incrementa el estallido respiratorio de los fagocitos de

lenguado y confiere protección frente a la infección experimental con *P. damselae* subsp. *piscicida*, respectivamente.

8. La técnica DGGE no ha permitido detectar posibles cambios que se hayan podido efectuar en la microbiota intestinal de lenguado, tras la administración oral de las cepas *Shewanella* Pdp11 y Pdp13.

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Role of superoxide dismutase and catalase activities in *Photobacterium damselae* subsp. *piscicida* virulence. Strategies for respiratory burst activity stimulation in sole phagocytes

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ABSTRACT

Photobacterium damselae subsp. *piscicida* is a gram negative bacterium, capable to survive as intracellular pathogen within sole phagocytes, thanks to the protective action of superoxide dismutase and catalase activities. These enzymes confer the pathogen resistance against oxygen reactive radicals produced within phagocytes during the respiratory burst. Therefore, both of these enzymes can be considered important virulence factors for *P. damselae* subsp. *piscicida*, facilitating its invasion and disease establishment. Research on disease prevention has been focused on the use of microorganisms capable to stimulate the respiratory burst activity of sole phagocytes. Assayed microorganisms include the microalga *Porphyridium cruentum* and two potential probiotic bacteria strains. Results obtained are promising, since the microalga and one strain of the assayed bacteria, Pdp11, are capable to stimulate the respiratory burst activity and, therefore, confer resistance against the disease. A new research field is opened in the fight against pseudotuberculosis, applying substances from algae or bacterial cells that may be considered as probiotics.

INTRODUCTION

1. AQUACULTURE. THE CULTURE OF SOLE (*Solea senegalensis*, Kaup, 1858)

According to Food and Agriculture Organization of the United Nations (FAO) the Earth is inhabited by nearly 6.5 billion people and the population will increase to between 9 and 10 billion by 2050. This concerns, particularly people planning on how to feed the future world (FAO, 2005 <ftp://ftp.fao.org/docrep/fao/008/a0057e/A0057e01.pdf>).

Fish is one of the most widely used low-cost protein sources in many parts of the world. However, it is clear that the availability of fish harvested from capture fisheries to support the growing demand for fish protein will be inadequate. Thus, the world needs to turn to producing fish, i.e. aquaculture. FAO defines aquaculture as the culture of aquatic organisms, including fish, mollusks, crustaceans and aquatic plants. Nowadays aquaculture industry plays an essential role in feeding future world.

In southern European countries, aquaculture production is concentrated on shore based cultivation of gilthead seabream (*Sparus aurata*, L.) and seabass (*Dicentrarchus labrax*, L.). Due to high production, markets have begun to be saturated. Investigation of potential new species for aquaculture is one of the strategies to increase market opportunities. Senegalese sole (*Solea senegalensis*, Kaup 1858) is a common high-value flatfish in Southern Europe, is well adapted to warm climates and is commonly raised in the extensive earthen ponds along the south coasts of Portugal and Spain (Dinis *et al.*, 1999; JACUMAR, 2005).

Regardless of the potential economic importance of the culture of this fish species (Dinis *et al.*, 1999; Imsland *et al.*, 2003) data about the susceptibility of captive Senegalese sole to fish pathogens are still scarce. The control and prevention of infectious diseases is a major goal in farmed sole. Several authors have reported different diseases and pathogenic microorganisms (Rodríguez *et al.*, 1997; Zorrilla *et al.*, 1999; Magariños *et al.*, 2003; Arijo *et al.*, 2005). Although, some studies reported *Vibrio harveyi* and *Tenacibaculum maritimum* isolation from diseased fish (Zorrilla *et al.*, 1999; Cepeda and Santos, 2003), pseudotuberculosis, caused by *Photobacterium damselae*

subsp. *piscicida,* is the disease responsible for higher mortalities (Zorrilla *et al.*, 1999), becoming the main limiting factor for sole production.

2. *Photobacterium damselae* subsp. *piscicida*

Photobacterium damselae subsp. *piscicida,* an obligate halophilic bacterium, is the causal agent of psedotuberculosis (Kubota *et al.*, 1970), because in chronic cases, affected fish show prominent white tubercles in several internal organs. This disease was first described in wild populations of white perch (*Morone americanus*) and stripped bass (*Morone saxatilis*) in the United States (Snieszko *et al.*, 1964). Currently natural hosts of the pathogen include a wide variety of marine fish. This disease has great economic impact in Japan, where affects mainly yellowtail (*Seriola quinqueradiata*) cultures (Kusuda and Salati, 1993), in the United States and in the European Mediterranean area, causing important losses in gilthead seabream (*Sparus aurata*, L.) (Ceshia *et al.*, 1991; Toranzo *et al.*, 1991), stripped seabass (Hauwke *et al.*, 1987), seabass (*Dicentrarchus labrax*, L.) (Baudin-Laurencin *et al.*, 1991; Balebona *et al.*, 1992) and, recently, in sole (*Solea senegalensis*, Kaup) (Zorrilla *et al.*, 1999; Magariños *et al.*, 2003; Arijo *et al.*, 2005).

External pathological signs of the pseudotuberculosis are usually inconspicuous, surface lesions being usually absent in affected fish. Occasionally, diseased gilthead seabream and seabass display abnormal skin pigmentation or slight haemorrhagic areas in the head and gills and swelling of the abdominal cavity (Toranzo *et al.*, 1991; Balebona *et al.* 1992; Fouz *et al.*, 2000).

Internally, diseased fish show haemorrhagic septicaemia and necrosis in the majority of the organs, displaying the typical tubercles consisting in accumulation of bacteria, necrotic phagocytes and granulomes. Moreover the necrosis and the existence of granulomata in spleen, kidney and liver, where bacteria are visible in the sinusoids and within the hepatic vessels, have been reported (Kubota *et al.*, 1970; Wolke, 1975; Tung *et al.*, 1985; Hawke *et al.*, 1987; Toranzo *et al.*, 1991; Balebona *et al.*, 1992; Noya *et al.*, 1995a).

With regard to *Solea senegalensis*, diseased fish show no apparent lesions except for dark skin pigmentation and swelling in the abdominal cavity. In some cases, haemorrhagic exophthalmia, small ulcers on the skin and gill paleness have also been observed. Internally, affected specimens show paleness of liver and kidney, and the typical white tubercles of 1-2 mm in diameter in the spleen (Zorrilla *et al.*, 1999).

2.1. TRANSMISSION MODE

P. damselae subsp. *piscicida* is a highly pathogenic bacterium apparently without host specificity. Therefore, pseudotuberculosis could be a risk for new fish species where the disease has not been described yet. Noya *et al.* (1995b) reported that the resistance in seabream and seabass is related to the size and age of the fish. This maybe due to macrophage and neutrophyl functionality, since efficient phagocytosis and killing of the bacteria have been observed in older seabream (Noya *et al.*, 1995b; Skarmeta *et al.*, 1995). Also, it is possible that some deficiencies in serum components may have some influence on phagocytosis and killing of *P. damselae* subsp. *piscicida* by phagocytes, making younger seabream more sensitive to infection.

The transmission route of infection involved in these diseases is still uncertain (Magariños *et al.*, 1995). Some authors suggest that *Photobacterium* may survive in the aquatic environment as unculturable viable cells and an increase in water temperature and salinity (20-30%) could contribute to the development of the epizootic outbreak (Hawke *et al.*, 1987; Toranzo *et al.*, 1991; Magariños *et al.*, 2001).

The pathways of entry may vary depending on the host. With some fish, infection may follow ingestion of the pathogen (Magariños *et al.*, 1995). Studies to determine the importance of skin as a portal of entry for *P. damselae* subsp. *piscicida* have been carried out (Magariños *et al.*, 1995), and the results obtained show that all isolates are sensitive to the antibacterial action of turbot (*Scophthalmus maximus*) mucus but resistant to gilthead seabream and seabass skin mucus. These observations may explain the fact that all *P. damselae* subsp. *piscicida* outbreaks described in Europe affected seabream and seabass, but never turbot.

2.2. VIRULENCE MECHANISMS

Several virulence mechanisms have been described in *P. damselae* subsp. *piscicida* that include production of extracellular products (ECPs) with haemolytic, phospholipase and cytotoxic activities (Balebona *et al.*, 1992; Magariños *et al.*, 1992; Noya *et al.*, 1995a and b; Romalde, 2002; Bakopoulos *et al.*, 2004). In addition, virulent strains constitutively synthesize a thin layer of capsular material that confers resistance to serum killing and decreases macrophage phagocytosis (Magariños *et al.*, 1996b; Arijo *et al.*, 1998; Acosta *et al.*, 2006).

A close relationship has been observed between capsule production and iron availability. Thus, do Vale *et al*. (2001) observed that cells grown under iron-limiting conditions always have significantly lower amounts of capsular material. This thinner capsule probably results in a better exposure of the adhesins and iron receptors at the bacterial surface during passage through circulatory system. Once the microorganism reaches the different host tissues, the amount of capsular material probably increases in response to host cellular defence mechanisms such as phagocytosis

3. SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES AS VIRULENCE FACTORS

Bacterial inactivation within phagocytes takes place by two mechanisms: oxygen independent mechanisms through phagocyte granule constituents (lysosomal enzymes, catepsines, defensines, lactoferrine, proteolytic enzymes) and oxygen dependent mechanisms. In the latter mechanisms oxygenic compounds such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical (OH^-) takes place. These products are generated during the called respiratory burst, due to the activation after phagocytosis of nicotinamide-adenin-dinucleotide-phosphate-hydrogen (NADPH) oxidase located in the membranes.

3.1. RESPIRATORY BURST

After phagocytosis, a process that involves the ingestion of microorganisms, phagocytic cells liberate the content of their lysosomes and a phago-lysosome is formed. Lysosomes contain several cytotoxic factors, such as oxygen metabolites and hydrolytic enzymes capable to kill and digest microorganisms.

Production of these oxygen metabolites occurs during the respiratory burst, within phagocytes in the presence of bacteria, increasing oxygen rate consumption. Extracellular oxygen is used to generate reactive oxygen species (ROS), $O_2^{\cdot-}$, H_2O_2, OH^- in the cellular surface. The presence of these free radicals is associated with cellular ageing; however, their toxicity is useful as a defence mechanism against bacteria, due to their great microbiocidal activity.

The respiratory burst starts by stimulation of the NADPH oxidase located in the phagocytic membrane. This enzymatic activity is able to reduce O_2 in superoxide anion ($O_2^{\cdot-}$) (Roos *et al.*, 2003). Sequentially, by univalent reduction of O_2, highly toxigenic reactive species, are generated. Within the phagosome, superoxide is, spontaneously or by superoxide dismutase (SOD), converted to hydrogen peroxide (H_2O_2), which may then react with superoxide to generate hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$), both highly reactive and toxic compounds. Superoxide can also react with nitrogen oxide (NO), generated by inducible NO synthase (NOS), to yield peroxynitrite, a very reactive nitrogen intermediate. There are even indications that single oxygen may be converted to a ozone-like (O_3) compound in a reaction catalyzed by antibodies bound to microbes or neutrophils. H_2O_2 may also, together with chloride, be used as a substrate by myeloperoxidase released from granules to generate hypochlorous acid, a very toxic compound for almost all microbes. Subsequently, the short-lived hypochlorous acid can react with secondary amines to form secondary chloramines, which are as microbiocidal as hypochlorous acid but more stable.

The respiratory burst, due to the generation of a great amount of free radicals highly toxic, inactivating proteins and oxidizing nucleic acids and other essential molecules, represents an immune system strategy in the fight against infections. Pathogenic microorganisms have had to develop a fight against free radicals, in two

sides, on one hand, protection against free radicals that are generated by their own metabolism, and, on the other hand, defence against the contact with phagocytic cells. Enzymes, such as superoxide dismutases, catalases and peroxidases, contribute to the resistance against ROS.

These enzymes are considered as virulence factors; they are defensive weapons against phagocytic cell attack and, therefore, contribute to the virulence of pathogens.

3.2. SUPEROXIDE DISMUTASE ACTIVITY

Superoxide dismutase is the first defence line of the cells against oxidative stress. This enzyme catalyzes the conversion of superoxide anion radicals to hydrogen peroxide and oxygen (equation 4).

$$(4) \qquad O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide dismutase activity has been detected in a wide variety of living organisms, from bacteria to humans. Any cell that utilizes oxygen has the capacity to produce superoxide anion ($O_2^{\cdot-}$) and so should contain some form of superoxide dismutase (Fridovich, 1974).

Superoxide dismutases constitute a family of metalloenzymes, classified into four groups, depending on the metal cofactor: FeSOD, MnSOD, CuZnSOD and NiSOD, this latter described recently in *Streptomyces* (Lynch and Kuramitsu, 2000).

3.3. CATALASE ACTIVITY

Catalases participate in the decomposition of hydrogen peroxide into water (H_2O) and oxygen (O_2) (equation 5).

$$(5) \qquad 2H_2O_2 \longrightarrow 2H_2O + O_2$$

Some catalases have also peroxidase activity and in this case an organic electron donor, or sometimes an halide ion, is employed in the reduction of hydrogen peroxide (equation 6).

(6) $$RH_2 + H_2O_2 \longrightarrow 2H_2O + R$$

Catalases have been divided into three groups (Loewen, 1997): monofunctional catalase with heme group (FeCat); bifunctional catalase with heme group (catalase-peroxidase) and pseudocatalase without heme group (MnCat), referred to as pseudocatalase because they are not inhibited by the common catalytic inhibitors, azide and cyanide.

Superoxide dismutase and catalase activities protect the cells also by preventing the generation of hydroxyl radical ($OH^{\cdot}$), a more toxic reactive species.

The role of these enzymes can be essential to protect pathogenic bacteria during respiratory burst after phagocytosis, therefore superoxide dismutase and catalase are related to virulence mechanisms (Table 5). The ability of many microorganisms to infect the host depends on their resistance to ROS production by cells, mainly monocytes, macrophages and polymorphonuclears. Ironically, evolution has selected organisms that use these cells as targets, so presence of SOD and catalase in bacteria will contribute to resistance against host and, therefore, infection establishment.

Table 5 Examples of pathogens in which a role in the virulence has been demonstrated for SOD and catalase activities

Pathogen	Virulence factor	Reference
Listeria monocytogenes	FeSOD	Welch *et al.*, 1979
Shigella flexneri	FeSOD	Franzon *et al.*, 1990
Pseudomonas syringae	Catalases (not determined)	Klotz and Hutcheson, 1992
Caulobacter crescentus	CuZnSOD	Schnell and Steinman, 1995
Aeromonas salmonicida	FeSOD	Barnes *et al.*, 1996
Pseudomonas aeruginosa	MnSOD	Polack *et al.*, 1996
Legionella pneumophila	CuZnSOD	St. John and Steinman, 1996
Aeromonas salmonicida subsp. *salmonicida*	MnSOD and FeCatalase	Barnes *et al.*, 1999b
Mycobacterium tuberculosis	Catalase-Peroxidase	Manca *et al.*, 1999
Streptococcus pneumoniae	MnSOD	Yesilkaya *et al.*, 2000
Vibrio harveyi	Monofunctional catalase	Vattanaviboon and Mongkolsuk, 2001
Salmonella enterica	CuZnSOD	Uzzau *et al.*, 2002
Vibrio shiloi	SOD (not determined)	Banin *et al.*, 2003

3.4. SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES IN *Photobacterium damselae* subsp. *piscicida*

Processes involved in the invasion and survival of *P. damselae* subsp. *piscicida* inside the host are still unknown and while authors have reported the presence of intact bacteria inside fish cells, suggesting the ability of the bacterium to survive as an intracellular pathogen (Noya *et al.*, 1995a; López-Dóriga *et al.*, 2000), others have observed that this pathogen is highly susceptible to oxidative radicals generated during the macrophage respiratory burst (Skarmeta *et al.*, 1995; Barnes *et al.*, 1999a). Due to the protective role attributed to SOD and catalase activities in a variety of bacterial

pathogens, study of these activities in *P. damselae* subsp. *piscicida* could contribute to the understanding of the interactions between this bacterium and its host.

Barnes *et al.* (1999a) determined the existence of a periplasmic FeSOD and a citoplasmic catalase, not characterized. The SOD activity is repressed under iron restricted or low oxygen conditions. Catalase activity is constituvely expressed, although there are differences in intensity gel bands depending on strains. In this PhD. Thesis we have tried to deep more in the study of the role of superoxide dismutase and catalase activities in *P. damseale* subsp. *piscicida* virulence.

4. STIMULATION OF RESPIRATORY BURST ACTIVITY BY DIFFERENT MICROORGANISMS AFTER *Photobacterium damselae* subsp. *piscicida* INFECTION

4.1. PREVENTION AND TREATMENT OF PSEUDOTUBERCULOSIS

Over the last decade, incidence of drug-resistant strains, carrying a transferable R-plasmid have increased, making treatment with antimicrobial chemotherapeutics less successful. Moreover, a period of intracellular parasitism within macrophages has been described for *P. damselae* subsp. *piscicida* (Kusuda and Salati, 1993); this finding can explain the ineffectiveness of chemotherapy in the treatment of some disease outbreaks. Therefore, immunoprophylaxis has become the best way to prevent pseudotuberculosis.

Throughout the last 20 years, there have been a variety of studies analyzing the effectiveness of immunization in preventing pseudotuberculosis (Romalde and Magariños, 1997). Most vaccine formulations tested consisted of heat- or formalin-killed cells (Fukuda and Kusuda, 1981; Kusuda and Hamaguchi, 1987; Kusuda and Hamaguchi, 1988; Hamaguchi and Kusuda, 1989). Best results were obtained using formulations based on lipopolysaccharides (LPS) and ribosomal fractions of the bacteria (Fukuda and Sukuda, 1985; Kusuda *et al.*, 1988; Kawakami *et al.*, 1997). However, these formulations presented not only problems of reproducibility, but also difficulties on large scale production. Passive immunization has also been evaluated (Fukuda and Kusuda, 1981),

but the results showed short-term protection. The best protection against pseudotuberculosis was obtained with an ECP-enriched bacterin (Magariños *et al.*, 1994, 1997, 1999). This vaccine is currently commercially available and has been successfully employed in several European countries including Spain, Portugal and Greece. In addition, a divalent vaccine with bacterin and formaline-inactivated ECPs of *V. harveyi* and *P. damselae* subsp. *piscicida* has shown promising results in sole (Arijo *et al.*, 2005).

Future trends in vaccine formulations against *P. damselae* subsp. *piscicida* include the use of proteins from cellular envelope (Magariños *et al.*, 1994) and iron-regulated OMPs as protective antigens.

The combination of vaccination and immunostimulants appears as the most effective strategy to prevent and fight against infectious diseases in fish (Sakai, 1999). At present, the use of immunostimulants, in addition to chemotherapeutic agents and vaccines, has been widely accepted by fish farmers. However it is necessary search for new immunostimulant agents effective against pathogens and with reduced production costs.

Finally, probiotics, microbial cells orally administered capable to induce positive effects on host health represent another alternative to combat diseases affecting farmed fish.

4.2. IMMUNOMODULATION. IMMUNOSTIMULATION

Immunomodulation is the ability of certain substances to regulate the immune system, may be immunostimulation or immunodepression, stimulating or depressing the immune system, respectively. The main reason to search for new immunostimulant agents is the great development of the aquaculture and the increase of stress situations and diseases caused by intensive cultures. Some studies have reported that the negative effects associated to immunodepression were overcome by immunostimulants, since the immunostimulants increase resistance against infectious diseases, by enhancing non-specific defence mechanisms. Immunostimulants facilitate phagocytic cells function and

increase their bactericidal activity, oxygen dependent and independent mechanisms being involved (Siwicki et al., 1994; Anderson, 1996).

Immunostimulants may be able to compensate some chemotherapeutic and vaccine limitations. Immunostimulants are safer than chemotherapeutics and their range of efficacy is wider than vaccination. However, we cannot expect the marked or long-term effects observed with vaccines to occur with immunostimulants because these substances act on non-specific immune system, without a memory component (Anderson, 1996; Sakai, 1999). Sakai (1999) suggests that the most effective strategy to prevent and combate possible infectious diseases in fish, is the combination of immuostimulants and vaccines. Thus, with a detailed understanding of the efficacy and limitations of immunostimulants, they may become powerful tools to control fish diseases.

4.3. USE OF ALGAE AS IMMUNOSTIMULANTS

During last years searching for therapeutically active substances has focused on marine organisms. Ability of algae to produce secondary metabolites, such as antibiotics, antivirals, antitumorals and antiinflammatories, with potential pharmaceutical interest, has been well documented (Scheuer, 1990; Faulkner, 1993; González del Val *et al.*, 2001). However, studies on immunomodulatory properties of extracts and whole cells from algae are still scarce. In this Thesis we have studied the red microalga *Porphyridium cruentum* as a possible source of immunostimulants in cultured sole.

A great number of algae constitute an important source of polyunsaturated fatty acids (PUFAs), which are the essential dietary requirements for many marine teleosts (Bell *et al.*, 1985; Koven *et al.*, 2001). Some of these fatty acids, such as the arachidonic acid, AA (20:4(n-6)) participate in eicosanoid synthesis, therefore, in prostaglandin production, involved in stress through modulation of cortisol release and, therefore in cellular immunity (Villalta *et al.*, 2005).

Algae also contain carotenoids, β-carotene, astaxanthin, cataxanthin or xanthofilins. Amar *et al.* (2004) demonstrated that β-carotene, isolated from *Dunaliella*

salina, is able to modulate, after oral administration, some components of the innate defence mechanisms in rainbow trout (*Oncorhynchus mykiss*) such as the complement alternative way, serum lysozyme and phagocytosis. The carotenoids increase the phagocytic activity and citoquine production (Bendich, 1989; Chew, 1993).

Algae are also a natural source of vitamins, some of them have possible immunostimulant effects on fish immune system, such as vitamin C (Hardie *et al.*, 1991; Cuesta *et al.*, 2002; Jeney and Jeney, 2002; Lin and Shiau, 2005), vitamin E (Hardie *et al.*, 1990; Cuesta *et al.*, 2001) and vitamins B (Miles *et al.*, 2001).

The use of polysaccharides as immunostimulants is widely extended in aquaculture (Kumar *et al.*, 2005), the most studied in fish are the glucans. β-glucans are polyglucoses that consist in series of residues of β-1,3-glucopiranosyl derived from yeast and fungus micellium. These sugars seem to have a potent immunostimulant effect, mainly on unspecific defence mechanisms, inducing resistance against infections. Numerous studies confirm the use of β,1-3,1-6 glucans from yeasts and fungus cell walls as immunostimulants in aquaculture. Greater part of these studies has focused on β-glucans from the yeast specie *Saccharomyces cerevisiae* (Santarém *et al.*, 1997; Castro *et al.*, 1999; Kumari and Sahoo, 2006; Marqués *et al.*, 2006).

The use of these polysaccharides as immunostimulants in aquaculture industry is widely extended and there are commercial products available (Siwicki *et al.*, 1994; Cook *et al.*, 2003; Couso *et al.*, 2003; Bagni *et al.*, 2005).

Another potential immunostimulant polysaccharide derived from brown macroalgae and microalgae, is the alginic acid. The alginate is known in aquaculture as stabilizer of the structure of pellet diets. The immunostimulant properties were determined in phyophaecaetes extracts of species such as *Laminaria digitata* (Dalmo *et al.*, 1998; Gabrielsen and Austreng, 1998) and others (Miles *et al.*, 2001; Peddie *et al.*, 2002; Skjermo and Bergh, 2004; Bagni *et al.*, 2005).

4.3.1. *Porphyridium cruentum*

Porphyridium cruentum is a red microalga belonging to *Rodophyta* family and *Porphyridiales* order, with spherical cells that lack of cell wall. This alga accumulates large amounts of fatty acids (9-14% dry weight), specially araquidonic acid (36%) and noticeable amounts of eicosapentaenoic acid. The protein content ranges from 28 to 39%, and available carbohydrates vary between 40 and 57%. The biomass contents tocopherol, vitamin K and a large amount of carotenes (Rebolloso *et al.*, 2000).

P. cruentum cells are capable to excrete a sulphated polysaccharide, an acidic heteropolymer composed of xylose, glucose, galactose and sulphate esters (You and Barneu, 2004). This polysaccharide is commercially used as thickener, stabilizer and emulsifier (Arad *et al.*, 1985, 1988; Adda *et al.*, 1986).

Substances such as araquidonic acid (Koven *et al.*, 2001), carbohydrates (Kumar *et al.*, 2005), vitamins (Hardie *et al.*, 1990, 1991; Ortuño *et al.*, 1999, 2003; Jeney and Jeney, 2002), carotenoids (Amar *et al.*, 2004) and polysaccharides (Siwicki *et al.*, 1994; Santarém *et al.*, 1997; Castro *et al.*, 1999; Bagni *et al.*, 2000, 2005; Esteban *et al.*, 2001; Jeney and Jeney, 2002; Cook *et al.*, 2003; Couso *et al.*, 2003) present in different organisms, have been demonstrated their immunostimulant effects on fish. All of these compounds have been determined in *P. cruentum*.

In addition, the fact that *P. cruentum* culture is not costly makes this alga a good candidate as a source of immunostimulant active substances. However, the polysaccharide extraction is a laborious process; moreover intraperitoneal administration is not advisable due to stress by handling. For this reason it is convenient to simplify the immunostimulant administration, providing whole and oral, supplementing feed. Last years the number of works, that study whole organisms is increasing, such as yeasts (Siwicki *et al.*, 1994; Ortuño *et al.*, 2002; Rodríguez *et al.*, 2003), fungus (Rodríguez *et al.*, 2004) and probiotics (Verschuere *et al.*, 2000; Irianto and Austin, 2003; Salinas *et al.*, 2005; Díaz-Rosales *et al.*, 2006). However, in spite of the large number of studies carried out with extracts or compounds derived from algae (Koven *et al.*, 2001; Castro *et al.*, 2004; Skjermo and Bergh, 2004; Díaz-Rosales *et al.*,

2005; Hou and Chen, 2005; Villalta *et al.*, 2005), the works using whole alga cells are still scarce (Blinkova *et al.*, 2001; Valente *et al.*, 2006).

As described above, the combination of vaccination and immunostimulant administration could increase vaccine potency. Thus, one objective is to evaluate a possible fish immunostimulation by administration of the alga *P. cruentum* jointly with a vaccine formulation, evaluating a possible synergetic effect of both prophylactic methods against *P. damselae* subsp. *piscicida* infection.

4.4. IMMUNOSTIMULANT EFFECT OF POTENTIAL PROBIOTIC BACTERIA

Definition of probiotics has changed along time. Thus, Salminen *et al.* (1999) consider probiotics as "every microbiane preparation (not alive necessary) or microbial cellular compounds that have beneficial effect on host health". According to FAO probiotics consist of a microbial complement that affects beneficially to host physiology by modulation of local and systemic immunity, moreover to improve microbial balance by prevention of gastrointestinal colonization by non desired bacteria. The advances in probiotic employment in cattle and human medicine have lead to consider their application in aquaculture, as an alternative in the fight against microbial infection (Sakai, 1999).

Aquatic animals are very different to terrestrial animals, therefore probiotic concept changes in aquaculture application (Verschuere *et al.*, 2000). In aquatic animals there is a constant interaction between the intestinal microbiota and environment. For this reason, in aquaculture systems the immediate environment has larger influence on the health status than in the case of terrestrial animals or humans. Due to the existence of a continuous flux of water trough digestive tract, the fish intestinal microbiota is highly dependent on external environment.

Verschuere *et al.* (2000) proposed a modified definition: "as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment".

Different mechanisms have been proposed to explain the types of interactions between probiotics and pathogens: (1) Competitive exclussion, production of antimicrobial compounds such as bacteriocines, lysozimes and proteases (Austin *et al.*, 1995; Sugita *et al.*, 1997; Gatesoupe, 1999; Gram *et al.*, 1999; Verschuere *et al.*, 2000); (2) competition for available energy and nutrients (Smith and Davey, 1993; Pybus *et al.*, 1994; Gatesoupe *et al.*, 1997; Gram *et al.*, 1999); (3) Adhesive interference in the host (Olsson *et al.*, 1992; Jöborn *et al.*, 1997; Nikoskelainen *et al.*, 2001; Chabrillón *et al.*, 2005a and b). Recently, research have focused also on the immunomodulatory properties of probiotic microorganisms (Irianto and Austin, 2003; Nikoskelainen *et al.*, 2003; Panigrahi *et al.*, 2004; Salinas *et al.*, 2005, 2006; Díaz-Rosales, 2006) (Table 6).

The gastrointestinal microbiota plays an important role in host nutrition and health. Therefore, probiotics could perform an interesting function, modifying gastrointestinal microbiota to induce favourable effects, such as improvement of growth, digestion, immunity or resistance against a pathogen. There are numerous authors that have characterized gastrointestinal microbiota of different farmed fish, fundamentally salmonids (Spanggaard *et al.*, 2000; Huber *et al.*, 2004, Jensen *et al.*, 2004; Burr *et al.*, 2005), however studies on gastrointestinal microbiota characterization or evaluation of possible effects of probiotics are null in fish from Mediterranean area. These studies are very interesting since could provide information for a better application of microorganisms in prophylactic strategies and diseases biocontrol.

Table 6 Beneficial effects of probiotics on fish immune system

PROBIOTIC	HOST	INCREASE OF IMMUNE RESPONSE	REFERENCE
Bacteria Gram positive, not identified	*Oncorhynchus mykiss* (rainbow trout)	Erythrocytes and leucocytes number, lysozyme activity, phagocytosis	Irianto and Austin, 2003
Vibrio fluvialis	*Oncorhynchus mykiss*	Erythrocytes and leucocytes number, lysozyme activity, phagocytosis	Irianto and Austin, 2003
Aeromonas hydrophila	*Oncorhynchus mykiss*	Erythrocytes and leucocytes number, lysozyme activity, phagocytosis	Irianto and Austin, 2003
Carnobacterium	*Oncorhynchus mykiss*	Erythrocytes and leucocytes number, lysozyme activity, phagocytosis	Irianto and Austin, 2003
Lactobacillus rhamnosus	*Oncorhynchus mykiss*	Respiratory burst, serum bactericidal activity, Ig serum levels	Nikoskelainen *et al.*, 2003
Bacillus	*Penaeus vannamei*	Immune index (hemograme, superoxide anion production, phenoloxidase activity, antibacterial activity, plasma protein concentration)	Gullian *et al.*, 2004
Lactobacillus rhamnosus	*Oncorhynchus mykiss*	Lysozyme, serum complement and phagocytic activities	Panigrahi *et al.*, 2004
Lactobacillus delbrüeckii subsp. *lactis*	*Sparus aurata* (gilthead seabream)	Phagocytosis, cytotoxic activity	Salinas *et al.*, 2005
Bacillus subtilis	*Sparus aurata*	Phagocytosis, cytotoxic activity	Salinas *et al.*, 2005
Aeromonas sobria	*Oncorhynchus mykiss*	Leucocytes number, phagocytosis, respiratory burst	Brunt and Austin, 2005
Alteromonadaceae, G. *Shewanella* (Pdp11)	*Sparus aurata*	Serum peroxidase, complement activity, phagocytosis	Díaz-Rosales *et al.*, 2006
Alteromonadaceae, G. *Shewanella* (51M6)	*Sparus aurata*	Serum peroxidase, complement activity, phagocytosis, cytotoxic activity	Díaz-Rosales *et al.*, 2006

A IMS

The work presented in this Thesis contributes to the knowledege of *Photobacterium damselae* subsp. *piscicida* virulence, and develops new strategies for prevention of the disease caused by this pathogen. The aims proposed are the following:

1. Study of the role of superoxide dimutase and catalase enzymatic activities in *Photobacterium damselae* subsp. *piscicida*, evaluating the potential resistance of the pathogen against bactericidal action of sole phagocytes.

2. Evaluation of possible immunostimulant effect on respiratory burst activity of sole phagocytes, that could be exert the use of the microalgae *Porphyridium cruentum* and probiotics microorganisms.

MATERIALS AND METHODS

Materials and methodology followed to carry out the experiments included in this PhD. Thesis are detailed in the papers attached in Article section.

RESULTS AND DISCUSSION

The first objective of this PhD. Thesis consisted in the study of the contribution of superoxide dismutase and catalase activities to *Photobacterium damselae* subsp. *piscicida* resistance against oxygenic radicals generated during respiratory burst in sole phagocytes and, therefore, their possible role as virulence factors (articles 1.1. and 1.2., Article section).

Previously, Barnes *et al.* (1999a) demonstrated *in vitro* that this microorganism contains enough superoxide dismutase activity to disproportionate photochemically generated superoxide anions, and that the bacterial susceptibility is due to the action of hydrogen peroxide. In fact, the addition of exogenous catalase to the medium protected the bacteria from inactivation by superoxide anions. The role of catalase and superoxide dismutase activities in *P. damselae* subsp. *piscicida* virulence has been studied in this work and results are included in articles 1.1. and 1.2. (Article section).

In vitro role of catalase activity in *P. damselae* subsp. *piscicida* protection against exogenous hydrogen peroxide has been evaluated in the first article. Results obtained show that resistance to hydrogen peroxide increases when bacterial cultures are pulsed with hydrogen peroxide. On the contrary, survival percentages decrease when bacteria grow under iron-limiting conditions. Quantification of catalase activity in a set of *P. damselae* subsp. *piscicida* strains shows that highest survival percentages are present in bacterial cultures with higher catalase activity levels. Moreover, resistance to oxidative stress varied depending on the virulence, being higher in more virulent strains. Thus, the virulent strain $Lg_{h41/01}$ shows higher resistance to respiratory burst of sole phagocytes than the non virulent strain EPOY-8803-II, and hydrogen peroxide addition into cultures increases survival. The lack of cell capsule could contribute to the decrease of survival against reactive oxygen species, but the low levels of catalase activity obtained in EPOY-8803-II, suggest that its absence of virulence could be determined, in great part, by low catalase activity. Therefore, catalase plays an important role in *P. damselae* subsp. *piscicida* virulence. It has been demonstrated, in a great number of bacteria, that previous exposition to an oxidant in sublethal concentrations can induce protection against lethal concentrations of the oxidant (Mongkolsuk *et al.*, 1996). Barnes *et al.* (1999b) reported that after being pulsed with hydrogen peroxide *A. salmonicida* is able to

resist 100 mM of peroxide, a lethal concentration without peroxide pretreatment. In the case of *P. damselae* subsp. *piscicida*, it is interesting to note that a significant increase in survival rates of the non-virulent strain was observed when cultures were pulsed with hydrogen peroxide compared to cells cultured until stationary phase. In contrast, this increase has not been observed for the virulent strain, which always showed higher survival regardless of the growth phase, or the pulse with hydrogen peroxide.

On the other hand, culture under iron-restricted conditions results in a significant decrease in survival of both virulent and avirulent strains. The fact that this bacterial species contains a ferric catalase (article 1.2., Article section), whose activity is reduced under iron limiting conditions, may explain this result.

Superoxide dismutase and catalase are metalloenzymes that can possess different metals in their active centre. Microorganisms capable to produce different superoxide dismutase and catalase isoenzymes, depending on culture conditions, have been described. These culture conditions include oxygen levels, low iron levels or growth until stationary phase (Storz *et al.*, 1990; Privalle and Fridovich, 1992; Crockford *et al.*, 1995; Schnell and Steinman, 1995; Barnes *et al.*, 1996; Polack *et al.*, 1996; St. John and Steinman, 1996; Lynch and Kuramitsu, 2000; Yesilkaya *et al.*, 2000; Geslin *et al.*, 2001; Vattanaviboon and Mongkolsuk, 2001).

Results obtained in the present work show that *P. damselae* subsp. *piscicida* does not synthetize a new form of superoxide dismutase or catalase when cultured under assayed conditions. Thus, all strains show only one band of superoxide dismutase activity, with similar electrophoretic mobility to ferric superoxide dismutase described by Barnes *et al.* (1999a). Even oxidative stress, by hydrogen peroxide or paraquat (an oxygenic radical generator) addition, did not induce the synthesis of a different isoenzyme, unlike CuZnSOD and MnSOD described in *Escherichia coli* (Hassan and Fridovich, 1977; Privalle and Fridovich, 1992; Benov and Fridovich, 1994; Geslin *et al.*, 2001), MnSOD in *A. salmonicida* (Barnes *et al.*, 1996; Barnes *et al.*, 1999b), *Pseudomonas aeruginosa* (Polack *et al.*, 1996) and *Streptococcus pneumoniae* (Yesilkaya *et al.*, 2000). Neither restrictive iron conditions induce MnSOD synthesis (Privalle and Fridovich, 1992; Barnes *et al.*, 1999b). Although further studies would be

necessary, this lack of a new superoxide dismutase induction could be due to the presence of only one sod gene, *sodB*, encoding ferric superoxide dismutase (Lynch and Kuramitsu, 2000).

In the same way all strains, under all assayed culture conditions, show only one band of catalase activity, with similar electrophoretic mobility to the band described by Barnes *et al.* (1999a). Treatment of catalase gels with inhibitors indicates that this bacterium contains an iron-cofactored enzyme, because catalases with manganese retain its activity after treatment with azida and cyanide, but they are inhibited with mercuric chloride (Kono and Fridovich, 1983; Allgood and Perry, 1986; Barnes *et al.*, 1999b).

Despite none assayed culture condition induced synthesis of more than one superoxide dismutase or catalase isoenzyme, differences in the intensity of the bands and activity levels, after spectrophotometrically quantification, are observed. These results are in agreement with those obtained by Barnes *et al.* (1999a), who also detected differences in cultures carried out under iron replete and depleted conditions and high- and low- aerated broths. The quantification of both superoxide dismutase and catalase activities carried out in this study corroborated that different band intensities corresponded to variations in the levels of activity. The lowest levels of superoxide dismutase activity and catalase are detected in bacteria grown under iron-restricted conditions, attributable to the ferric nature of *P. damselae* subsp. *piscicida* superoxide dismutase and catalase. Under iron-limiting conditions the virulent strain shows higher activity levels of superoxide dismutase and catalase, than the non virulent strain. This result indicates the relevant role of iron uptake mechanisms for superoxide dismutase and catalase activity. Thus, a microorganism capable to obtain iron from the host would be able to express higher levels of antioxidant enzymes and could decompose superoxide and hydrogen peroxide radicals generated by the host. The importance of superoxide dismutase as antioxidant enzyme and its contribution to bacterial virulence have been demonstrated in several bacterial species such as *Listeria monocytogenes* (Welch *et al.*, 1979), *Shigella flexneri* (Franzon *et al.*, 1990) or *A. salmonicida* (Barnes *et al.*, 1999a), however its role in *P. damselae* subsp. *piscicida* is not very clear and in the

present work we have observed that the non virulent strain, EPOY-8803-II, shows activity levels similar to the virulent strain.

As described before, information concerning mechanisms involved in the invasion and survival of *P. damselae* subsp. *piscicida* inside the host is scarce and results regarding interaction of this pathogen with phagocytes have been contradictory. For this reason, pathogen survival after contact with sole phagocytes was evaluated. Results included in article 1.2. (Article section) show that *P. damselae* subsp. *piscicida* is able to survive inside sole phagocytes at least for five hours, survival rates being higher for the virulent strain (62%) than the non virulent strain (19%). Also, higher survival rates were observed in cultures with higher catalase activity. These results suggest that bacterial inactivation could be due to the accumulation of hydrogen peroxide, the precursor of hydroxyl radicals, after decomposition of superoxide radicals by bacterial superoxide dismutase. Both virulent and non-virulent strains assayed by Barnes *et al.* (1999a) showed high susceptibility to cell-free generated superoxide radicals, in contrast, we have observed that a non-virulent strain, EPOY-8803-II, is significantly more susceptible to killing by sole phagocytes than a virulent strain ($Lg_{h41/01}$). Besides the lower catalase activity present in the non-virulent strain, the lack of a capsule in cells of EPOY-8803-II could contribute to the high inactivation rates observed. Thus, the capsule could protect bacterial cells from oxidative radicals or even prevent activation of phagocytes (Miller and Britigan, 1997; Arijo *et al.*, 1998).

Finally, the important role of iron in microbial infections has been pointed out by several authors (Miller and Britigan, 1997; Weinberg, 2000). *P. damselae* subsp. *piscicida* is more susceptible to killing by sole phagocytes when bacterial cells have been cultured under iron-depleted conditions. Bacteria require iron for growth and replication and synthesize SOD and catalase to deal with oxidizing anions. *P. damselae* subsp. *piscicida* posses a high-affinity iron uptake system, a syderophore (Magariños *et al.* 1994; Naka *et al.*, 2005). However, despite its ability to obtain iron from high-affinity systems, several authors have reported that cells grown under iron-limited conditions have reduced amounts of capsular material covering the cells (do Vale *et al.*, 2001). These cells with reduced capsule would be more susceptible to phagocytosis and

oxidative stress. Results obtained (articles 1.1. and 1.2., Article section) show that iron plays an important role in survival of *P. damselae* subsp. *piscicida* in contact with sole phagocytes; whether this is attributable to its contribution to capsular material or SOD and catalase synthesis by the bacterium needs to be investigated. In conclusion, *P. damselae* subsp. *piscicida* is able to survive in contact with sole phagocytes, survival rates being higher for a virulent strain. The increased levels of catalase activity detected in the virulent strain indicate a possible role for this enzyme in bacterial survival.

Once determined the role of superoxide dismutase and catalase activities in *P. damselae* subsp. *piscicida* protection against respiratory burst of sole phagocyes, the following topic to study was the search for prevention tools such as immunostimulant application. This work has focused on the respiratory burst activity of sole phagocytes to evaluate the possible immunostimulant effect of two types of microorganisms: a red microalga, *Porphyridium cruentum*, and two potentially probiotic bacteria strains, whose immunostimulant activity has been reported in mammals (Morris *et al.*, 2000) and gilthead seabream (Díaz-Rosales *et al.*, 2006; Salinas *et al.*, 2006) phagocytes, respectively.

Evaluation of the potential stimulant activity of aqueous and ethanolic extracts from *P. cruentum* indicated that none of them stimulated superoxide anion production, while the positive control, commercial β-glucan from alga *Euglena gracilis*, is able to increase respiratory burst after 30 min in contact with phagocytes, but only at the highest concentration assayed (10 mg ml^{-1}). These results are in agreement with data reported by Castro *et al.* (1999), who observed increases of the respiratory burst activity of head kidney phagocytes of turbot (*Psetta maxima*) phagocytes and gilthead seabream with different concentrations of β-glucans obtained from fungus and yeasts. On the other hand, Castro *et al.* (2004) found great variations in the stimulative capacities of algal extracts depending not only on their origin, but on the concentrations used and time of incubation.

P. cruentum culture has several advantages, such as fast growth and low cost. On the other hand *P. cruentum* could be a natural immunostimulant, which is biocompatible, biodegradable and safe for the environment and human health. For this

reason, once aqueous and ethanolic extracts were assayed *in vitro*, oral administration of whole alga cells was performed. In fish, as in other aquatic organisms, administration of whole microorganisms has focused mainly on bacterial species, such as probiotics (Verschuere *et al.*, 2000; Nikoskelainen *et al.*, 2001; Irianto and Austin, 2003; Salinas *et al.*, 2005; Balcázar *et al.*, 2006; Díaz-Rosales *et al.*, 2006; Salinas *et al.*, 2006), but studies about whole algae are still very scarce. On the other hand, *P. cruentum* acumulates large amounts of lipids, specially arachidonic acid and noticeable amounts of eicosapentaenoic acid (Kinsella *et al.*, 1990; Koven *et al.*, 2001); carbohydrates (Fujiki *et al.*, 1992; Santarém *et al.*, 1997; Bagni *et al.*, 2000; Morris *et al.*, 2000; Esteban *et al.*, 2001; Jeney and Jeney, 2002; Cook *et al.*, 2003; Couso *et al.*, 2003; Castro *et al.*, 2004; Bagni *et al.,* 2005; Kumar *et al.*, 2005); carotenes (Tachinaba *et al.*, 1997; Amar *et al.*, 2004); vitamins (Hardie *et al.*, 1990, 1991; Ortuño *et al.*, 1999; Jeney and Jeney, 2002). Due to the fact that this alga contains different immunostimulant substances, its use could generate a more general immune response as has been proposed for other microorganisms such as yeasts (Ortuño *et al.*, 2002; Rodríguez *et al.*, 2003).

Thus, three groups of sole specimens received daily for four weeks one of the different diets assayed on a daily basis: commercial diet supplemented with lyophilized alga, diet consisting on non-supplemented commercial diet (control group) or diet composed of commercial diet containing immunostimulant, Sanostim™. Besides, to evaluate the possible synergic effect of immunostimulant with a vaccine, two weeks after the beginning of the feeding trial, a group of fish per treatment were intraperitoneally inoculated with a bacterin of *P. damselae* subsp. *piscicida*, a formalin-killed aqueous vaccine. Results obtained show that after four weeks of algal administration, the superoxide anion production increases in immunized fish. This increase is statistically significant, not only compared to fish fed with normal diet, but also compared to fish fed with commercial immunostimulant. The combined action of immunostimulant and vaccine has been described by numerous authors, who concluded that combination of vaccination and immunostimulant administration increases vaccine potency (Jeney and Anderson, 1993; Røsrstad *et al.*, 1993; Aakre *et al.*, 1994; Sakai *et al.*, 1995; Baulny *et al.*, 1996; Sakai, 1999).

Once immunostimulant effect of algal cells was demonstrated, the potential effect of the extracellular polysaccharidic fraction of *P. cruentum* on the respiratory burst activity of sole phagocytes was evaluated (article 2.2., Article section).

Results obtained indicate that *in vitro* none of the assayed concentrations of the extracellular polysaccharide from *P. cruentum* stimulate the respiratory burst of sole phagocytes, after 30 min contact with fish cells. These results are not in agreement with the data obtained by Castro *et al.* (2004, 2006), who suggest that stimulation of respiratory burst activity in turbot (*Psetta maxima*) phagocytes incubated with algal extracts, is due to algal polysaccharides. On the other hand, according to Castro *et al.* (2004) the modulatory ability of the respiratory burst activity of fish phagocytes varies greatly among algal species. Therefore, the non stimulation of respiratory burst *in vitro* by *P. cruentum* polysaccharides could be due to the absence of immunostimulant activity in this fraction, presence in low concentration or short incubation time with phagocytes. Whereas, the positive control, a commercial β-glucan, induced an increase in the respiratory burst, activity when applied at 10 mg ml^{-1}.

Additionally, fish were intraperitoneally inoculated with 500 μg of extracellular polysaccharidic fraction. Later, fish were immunized with a bacterin, composed by *P. damselae* subsp. *piscicida* formol-inactivated cells. Sampling time was carried out at 24 h and seven days post-vaccination. Results obtained indicate that the concentration and time assayed, 1 and 7 days after polysaccharidic fraction inoculation, do not produce an increase in the respiratory burst activity of sole phagocytes, not even immunized fish. Moreover, 24 h post-inoculation, the respiratory burst decreases in phagocytes from fish inoculated with polysaccharidic fraction or with the bacterin. This decrease may be due to an immunosupression by stress after handling (Thompson *et al.*, 1993; Pulsford *et al.*, 1995) as it is not observed after 7 days of the inoculation.

To sum up, the polysaccharidic fraction of *P. cruentum*, in assayed conditions, does not enhance the respiratory burst activity in sole phagocytes. These results suggest that stimulation observed after oral administration of alga cells would be due to other compounds with immunostimulant properties. However, we cannot rule out the possibility that the polysaccharide stimulate another immunological parameter. Thus, a

great number of works point out the immunostimulant capacity of polysaccharides in fish, increasing serum lysozyme, complement, cytotoxic or phagocytic activities (Santarém *et al.*, 1997; Esteban *et al.*, 2001; Chang *et al.*, 2003; Bagni *et al.*, 2005; Kumari and Sahoo, 2006).

Results obtained after oral administration of potential probiotic bacteria are included in article 2.3. (Article section). In this work, the respiratory burst activity was evaluated and, in order to determine the protection degree that probiotics could provide, a challenge with *P. damselae* subsp. *piscicida* was carried out. On the other hand, intestinal microbiota of fish fed with probiotics was studied in order to detect possible changes due to feeding treatment.

The selected bacterial strains, Pdp11 and Pdp13, isolated from gilthead seabream skin (Chabrillón, 2003), belong to *Alteromonadaceae* family, *Shewanella* genus. Although this is the first assay *in vivo* with strain Pdp13, several works have been carried out previously with strain Pdp11. Thus, Chabrillón *et al.* (2005a) studied interactions with the pathogen *Vibrio harveyi*, showing Pdp11 capacity to adhere to gilthead seabream intestinal mucus, the antagonist effect against a pathogenic strain of *V. harveyi*, the capacity to inhibit the pathogen union and confer protection against an experimental infection. With regard to interactions between strain Pdp11 and *P. damselae* subsp. *piscicida*, Chabrillón *et al.* (2005b) demonstrated the antagonistic effect of Pdp11 against one strain of *P. damselae* subsp. *piscicida* and the inhibition of the adhesion to intestinal mucus of this pathogen. These results lead the authors to consider the Pdp11 strain as a good candidate to be used as probiotic.

In the present work, it has been observed that superoxide anion production is significantly increased in fish fed with strain Pdp11 after two months from the beginning of the feeding trial, but fish fed with strain Pdp13 do not show modify phagocyte respiratory burst. However, fish fed with strain Pdp13 showed higher survival percentages after inoculation of the pathogen *P. damselae* subsp. *piscicida*.

Although there are numerous works in which respiratory burst activity induction by probiotics is demonstrated (Nikoskelainen *et al.*, 2003; Gullian *et al.*, 2004; Aubin *et al.*, 2005; Brunt and Austin, 2005) the fact that potential probiotic strain Pdp13 does not

increase superoxide anion production, does not mean that this bacterial strains cannot stimulate another immunological parameter. In fact, survival rates, after experimental infection, increased in fish fed with strain Pdp13. Several authors have shown that probiotics may stimulate different immunological parameters, such as phagocytic activity (Irianto and Austin, 2003; Panigrahi *et al.*, 2004; Brunt and Austin, 2005; Salinas *et al.*, 2005; Díaz-Rosales *et al.*, 2006), complement activity (Panigrahi *et al.*, 2004; Díaz-Rosales *et al.*, 2006), lysozyme activity (Irianto and Austin, 2003; Panigrahi *et al.*, 2004) or cytotoxic activity (Salinas *et al.*, 2005; Díaz-Rosales *et al.*, 2006). Moreover, effects of probiotics have been described on specific immune response, increasing serum immunoglobulins levels (Nikoskelainen *et al.*, 2003; Aubin *et al.*, 2005). For these reasons, strain Pdp13 can be considered as a probiotic also, although evaluation of other immunological parameters would be necessary.

On the other hand, probiotic bacteria have to be administered at optimal doses, depending on fish size and bacterial strain (Nikoskelainen *et al.*, 2003), for this reason Pdp11 effect is different to Pdp13 and the results obtained with Pdp11 and sole are different to results previously obtained with Pdp11 and gilthead seabream (Díaz-Rosales *et al.*, 2006).

Finally, apart from the immunological effects of probiotics in sole immune response, possible changes in intestinal microbiota due to probiotics were evaluated. In spite of the great number of published works concerning microbial communities in fish (Spanggaard *et al.*, 2000; Holben *et al.*, 2002; Sandaa *et al.*, 2003; Al-Harbi and Naim Uddin, 2004; Hjelm *et al.*, 2004; Huber *et al.*, 2004; Jensen *et al.*, 2004), none of them try to evaluate possible shifts in microbiota after probiotic administration.

In this work DGGE, Denaturing Gradient Gel Electrophoresis, (Muyzer *et al.*, 1993), was used to study the intestinal microbiota of soles fed with probiotics. Two sets of primers were evaluated (Nübel *et al.*, 1996; Jensen *et al.*, 2004). Primers described by Nübel *et al.* (1996) being selected as the best to study of bacterial communities in the present work after analysis of banding pattern by Pearson coefficient.

The band pattern obtained was very simple, with few predominant bands, results alike those described by Muyzer *et al.* (1993), who reported that communities with a few

dominant species will produce simpler patterns and the less abundant species may not adequately represent in the community pattern. Moreover, the technique has limitations, and it is possible that some of these bands may not be individual species, as theoretically is defined, but rather groups which have the same relative G+C content and have comigrated (Simpson *et al.*, 1999; Temmerman *et al.*, 2003). These limitations may account in part for the decreased band number and may also have influenced the apparent diversity and similarity values (McCraken *et al.*, 2001).

The obtained results do not demonstrate that probiotics induce significant shifts in intestinal microbiota, since bands that appear in groups fed with probiotics are also present in control groups.

On the other hand, it is not possible to confirm that observed bands correspond to Pdp11 or Pdp13, strains only attending to electrophoretic mobility, therefore, phylogenetic identification or PCR products sequencing studies will be necessary. After this work it is possible to affirm that Pdp11 and Pdp13 strains are not capable, at doses (10^9 ufc g^{-1}) and time assayed (two months), to induce significant shifts in intestinal microbiota. However to exert a local effect during transit through gastrointestinal system it is not necessary colonization to induce shifts on intestinal microbiota (Ouwehand *et al.*, 2002). In fact, variation of fish microbiota is substantial and fluctuates daily (Spanggaard *et al.*, 2000; Al-Harbi and Naim Uddin, 2004; Panigrahi *et al.*, 2004).

CONCLUSIONS

Studies carried out on the role of superoxide dismutase and catalase activities in the virulence of *Photobacterium damselae* subsp. *piscicida*, and the use of microorganisms for the control of this pathogen, have yielded the following conclusions:

1. *Photobacterium damselae* subsp. *piscicida* synthetizes only one isoenzyme with superoxide dismutase activity, characterized by the presence of iron in its active centre.

2. *Photobacterium damselae* subsp. *piscicida* contains only one isoenzyme with catalase activity, with iron in its active centre.

3. Catalase activity plays an important role in *P. damselae* subsp. *piscicida* resistance against hydrogen peroxide. Thus, bacterial cells with higher contents of this activity, are able to resist efficiently to hydrogen peroxide.

4. Iron plays a significant role in *P. damselae* subsp. *piscicida* survival in the presence of oxidant radicals, because under iron limiting conditions, lower levels of superoxide dismutase and catalase activities are detected, and higher susceptibility to hydrogen peroxide.

5. *Photobacterium damselae* subsp. *piscicida* is able to survive as intracellular pathogen within sole phagocytes, at least for 5 h.

6. Oral administration of red microalga *Porphyridium cruentum*, in combination with intraperitoneal inoculation of a bacterin containing *P. damselae* subsp. *piscicida* cells, increases respiratory burst activity.

7. Oral administration of *Shewanella* strains Pdp11 and Pdp13, proposed as probiotics, increases respiratory burst activity and confers protection against experimental infection with *P. damselae* subsp. *piscicida*, respectively.

8. The technique DGGE has not allowed to detect possible shifts of sole intestinal microbiota after oral administration of *Shewanella* strains Pdp11 and Pdp13.

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Article section

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1.2. Díaz-Rosales, P, Chabrillón, M, Arijo, S, Martínez-Manzanares, E, Moriñigo, MA, Balebona & MC. Superoxide dismutase and catalase activities in *Photobacterium damselae* ssp. *piscicida*. Journal of Fish Diseases 2006; 29, 355–364.

2.1. Díaz-Rosales, P, Felices, C, Chabrillón, M, Abdala, RT, Figueroa, FL, Balebona, MC & Moriñigo, MA. Effect of dietary administration of *Porphyridium cruentum* on the respiratory burst activity of sole (*Solea senegalensis*, Kaup 1858) phagocytes. Send to: Fish & Shellfish Immunology.

2.2. Díaz-Rosales, P, Abdala, RT, Decara, J, Arijo, A, Figueroa, FL, Moriñigo, MA & Balebona, MC. Effect of the extracellular polysaccharidic fraction from the red microalga *Porphyridium cruentum* on the respiratory burst activity of sole (*Solea senegalensis*, Kaup 1858) phagocytes. Manuscript in preparation.

2.3. Díaz-Rosales, P, Chabrillón, M, Smidt, H, Arijo, A, León-Rubio, JM, Rico, RM, Alarcón, FJ, Sáenz de Rodrigáñez, MA, Balebona, MC & Moriñigo, MA. Effect of dietary administration of probiotics on respiratory burst activity of phagocytes and intestinal microbiota of Senegalese sole (*Solea senegalensis*, Kaup 1858). Manuscript in preparation.

ARTÍCULO 1.1.

ARTICLE 1.1.

Journal of Fish Diseases 2003, **26,** 305–308

Short communication

Survival against exogenous hydrogen peroxide of *Photobacterium damselae* subsp. *piscicida* under different culture conditions

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Keywords: culture, hydrogen peroxide, *Photobacterium damselae* subsp. *piscicida*, survival.

Photobacterium damselae subsp. *piscicida* is a fish pathogen responsible for important losses in aquaculture world-wide. Several studies on its virulence mechanisms have been carried out and outer membrane proteins involved in the acquisition of iron or production of extracellular products have been suggested as the main determinants of its virulence for fish (Magariños, Santos, Romalde, Rivas, Barja & Toranzo 1992; Magariños, Romalde, Lemos, Barja & Toranzo 1994). However, the actual methods of invasion and survival inside the host are still unknown and while some authors have reported the presence of intact bacteria inside fish cells, suggesting the ability of the bacterium to survive as an intracellular pathogen (Noya, Magariños, Toranzo & Lamas 1995; López-Dóriga, Barnes, dos Santos & Ellis 2000), others have observed that this pathogen is highly susceptible to oxidative radicals generated during the macrophage respiratory burst (Skarmeta, Bandín, Santos & Toranzo 1995; Barnes, Balebona, Horne & Ellis 1999a).

Reactive oxygen species (ROS) such as hydrogen peroxide and superoxide are generated during the macrophage respiratory burst in response to microbial infection. Bacterial pathogens must overcome the toxic effects of ROS to establish infections. Production of superoxide dismutase and catalase enzymes, which decompose superoxide and peroxide radicals, respectively, have been reported to contribute to the virulence of a number of pathogens (Franzon, Arondel & Sansonetti 1990; Lefebre & Valvano 2001; Uzzau, Bossi & Figueroa-Bossi 2002). Thus, the ability of catalase to decompose peroxide radicals increases survival of bacteria in the presence of peroxide.

In addition, increased levels of catalase activity when bacteria are cultured under certain conditions, such as the presence of peroxide radicals or until the stationary phase, have been reported (Stortz, Tartaglia & Ames 1990; Loewen 1997). Moreover, the fact that most catalases are iron-cofactored suggests that growth under different iron concentrations may have some effect on this enzyme activity.

Catalase activity has been reported in *P. damselae* subsp. *piscicida* (Barnes *et al.* 1999a), however, the role of this enzyme in the protection against peroxide has not yet been determined. For this reason, the resistance to peroxide radicals of *P. damselae* subsp. *piscicida* cells grown under iron limited and replete conditions, and pulsed with hydrogen peroxide, has been evaluated in this study.

Two strains of *P. damselae* subsp. *piscicida* have been included in this study. The virulent strain (Lg41/01) ($LD_{50} = 2.2 \times 10^4$ CFU g^{-1}) was isolated from diseased sole, *Solea senegalensis* Kaup, showing typical signs of pseudotuberculosis, and the non-virulent strain (Epoy) ($LD_{50} > 1.0 \times 10^8$ CFU g^{-1}; Magariños, Bonet, Romalde, Martínez, Congregado & Toranzo 1996) kindly supplied by Dr K. Muroga (Faculty of Applied

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Biological Science, Hiroshima University, Japan). Isolates were cultured in 250-mL flasks containing 100 mL of tryptic soya broth supplemented with 2% NaCl (TSBS) at 22° C until the early stationary phase (O.D. 600 nm = 1.0). The effect of iron concentration on the cultures was evaluated in cells grown in TSBS supplemented with 2,2-dipyridyl (100 μM) or ferric chloride (100 μM) according to the methodology described by Barnes *et al.* (1999a). Bacterial survival against peroxide after a potential induction of catalase by hydrogen peroxide was tested according to Barnes, Bowden, Horne & Ellis (1999b) by adding 20 μM hydrogen peroxide to mid-exponential phase cultures and 2 mM hydrogen peroxide to early stationary phase cultures.

Cells were harvested, washed and resuspended in phosphate-buffered saline (PBS) to a density of 10^9 CFU mL^{-1} (O.D. 600 nm = 1.00). Aliquots of 100 μL were used to inoculate 9.9 mL PBS containing hydrogen peroxide at concentrations of 0, 0.05, 0.1, 0.5, 1 and 10 mM. Samples were incubated for 1 h at 22° C and surviving bacteria were enumerated by viable counts on tryptic soya agar with 2% NaCl (TSAS) plates. The survival of H_2O_2-treated bacteria was expressed as the percentage of colony forming units recovered compared with untreated samples. An ANOVA test was performed to compare the results of the experiments.

Previous studies with *P. damselae* subsp. *piscicida* exposed to photochemically generated superoxide radicals show that bacterial inactivation is overcome when catalase is added to the medium (Barnes *et al.* 1999b), thus indicating the important effect of hydrogen peroxide on the inactivation of this bacterium. Results obtained in this study indicate that *P. damselae* subsp. *piscicida* shows increased survival when exposed to peroxide radicals when cells have previously been in contact with hydrogen peroxide. Both the virulent and non-virulent strains were inactivated after 1 h incubation with 10 mM H_2O_2, however, when decreasing concentrations of peroxide were used, a higher degree of resistance to peroxide was observed in the virulent strain compared with the non-virulent strain (Fig. 1).

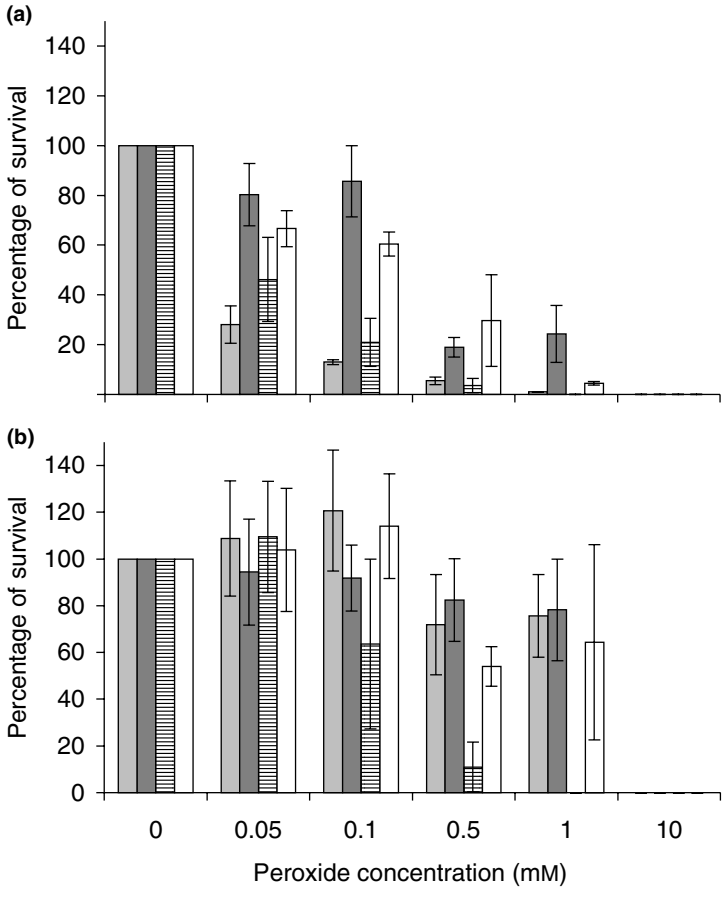

Figure 1 Survival of *Photobacterium damselae* subsp. *piscicida*, strains Epoy (a) and Lg41/01 (b) to exogenous peroxide. (□)Stationary phase cultures; (■) cultures treated at the mid-exponential phase with 20 μM peroxide followed by 2 mM peroxide in the early stationary phase; (▤) cells grown in TSBS with 100 μM 2,2-dypiridyl; (□) cells grown in TSBS with 100 μM ferric chloride.

A significant ($P < 0.05$) increase in the survival rates of the non-virulent strain was observed when cultures were pulsed with hydrogen peroxide compared with cells cultured until the stationary phase. In contrast, this increase has not been observed for the virulent strain, which always showed higher survival regardless of the growth phase or the pulse with hydrogen peroxide. Peroxide induction of catalase and increased cell survival have been reported for several bacterial pathogens (Loewen, Switala & Triggs-Raine 1985; Barnes *et al.* 1999b; Vattanaviboon & Mongkolsuk 2001). Results obtained in this study suggest that peroxide-decomposing enzymes induced in the strain Epoy only by peroxide treatment could protect these cells from oxidation, whilst decreasing survival rates observed in cells grown in other conditions could be attributable to lower levels of catalase and peroxidase activities. In contrast, the high survival rates observed in the virulent strain in stationary phase cultures, and in cells cultured in the presence of iron or pulsed with hydrogen peroxide suggest the presence of higher levels of catalase activity in the cells grown under these conditions, although a possible relationship with virulence remains to be demonstrated. Furthermore, the presence of a capsule in the virulent strain may have an important role in the protection of *P. damselae* subsp. *piscicida* cells against peroxide. This capsule would partially contribute to the increased survival of the virulent strain compared with strain Epoy, a non-capsulated strain (Magariños *et al.* 1996).

When bacteria were cultured under iron limited conditions, a significant decrease ($P < 0.05$) in survival was observed for both strains compared with cells grown under iron replete conditions or pulsed with peroxide. The decrease in bacterial survival in cultures grown under iron limited conditions suggests the presence of an iron-cofactored catalase in *P. damselae* subsp. *piscicida*. In this way, the ability to obtain iron from the host would determine the ability to cope with the radicals generated during the respiratory burst. It should also be noted that decomposition of superoxide anions primarily generated during the phagocytic respiratory burst depends on the activity of a ferric superoxide dismutase in *P. damselae* subsp. *piscicida* (Barnes *et al.* 1999a). Additional studies to demonstrate the presence of iron as a cofactor in the catalase, and the sensitivity of *P. damselae* subsp. *piscicida* to the radicals generated during the macrophage respiratory burst, are in progress.

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ARTÍCULO 1.2.

ARTICLE 1.2.

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Superoxide dismutase and catalase activities in *Photobacterium damselae* ssp. *piscicida*

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Abstract

The ability of a set of *Photobacterium damselae* ssp. *piscicida* strains isolated from different fish species to produce different superoxide dismutase (SOD) and catalase enzymes was determined. Unlike other bacterial pathogens, *P. damselae* ssp. *piscicida* is not able to produce different isoforms of SOD or catalase containing different metal cofactors when cultured under oxidative stress induced by hydrogen peroxide or methyl viologen, or under iron depleted conditions. However, iron content of the growth medium influenced the levels of SOD and catalase activity in cells, these levels decreasing with iron availability of the medium. Comparison of virulent and non-virulent strains of *P. damselae* ssp. *piscicida* showed similar contents of SOD, but higher levels of catalase were detected in cells of the virulent strain. Incubation of bacteria with sole, *Solea senegalensis* (Kaup), phagocytes has shown that survival rates range from 19% to 62%, these rates being higher for the virulent strain. The increased levels of catalase activity detected in the virulent strain indicates a possible role for this enzyme in bacterial survival.

Keywords: catalase, phagocyte, *Photobacterium damselae* subsp, *Solea senegalensis*, superoxide dismutase, virulence.

Introduction

Photobacterium damselae ssp. *piscicida* is a pathogen responsible for important losses in fish aquaculture worldwide. The importance of extracellular products, the presence of iron uptake mechanisms and the capsular material as virulence factors in *P. damselae* ssp. *piscicida* are well documented (Magariños, Romalde, Bandín, Fouz & Toranzo 1992; Magariños, Pazos, Santos, Romalde & Toranzo 1994; Magariños, Romalde, Lemos, Barja & Toranzo 1995; Arijo, Borrego, Zorrilla, Balebona & Moriñigo 1998). However, information concerning mechanisms involved in the invasion and survival inside the host is scarce and results regarding interaction of *P. damselae* ssp. *piscicida* with phagocytes have been contradictory. While some authors have reported the presence of intact bacteria inside fish cells, suggesting the ability of *P. damselae* to survive as an intracellular pathogen (Kubota, Kimura & Egusa 1970; Nelson, Kawahara, Kawai & Kusuda 1981; Kusuda & Salati 1993; Noya, Magariños & Lamas 1995a; Noya, Magariños, Toranzo & Lamas 1995b), others have observed that this pathogen is highly susceptible to oxidative radicals generated during the macrophage respiratory burst (Skarmeta, Bandín, Santos & Toranzo 1995; Arijo *et al.* 1998).

The reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$), are produced by phagocytes in response to microbial infection. ROS constitute an important component of the innate active defence response against invading microorganisms by fish phagocytic cells. Therefore, bacterial pathogens must overcome the toxic effects of ROS to establish infections. Microorganisms have evolved systems to protect themselves from these highly toxic radicals. One of these protective pathways involves the production of detoxifying enzymes such as

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superoxide dismutases (SODs) and catalases. Production of SOD and catalase enzymes, which decompose superoxide and peroxide radicals, respectively, have been reported to contribute to the virulence of a great number of pathogens (Franzon, Arondel & Sansonetti 1990; Lynch & Kuramitsu 2000; Lefebre & Valvano 2001; Uzzau, Bossi & Figueroa-Bossi 2002).

Superoxide dismutases are a family of metalloenzymes including four types depending on the metal cofactor, copper-zinc (Cu/Zn-SOD), manganese (Mn-SOD), iron (Fe-SOD) and nickel (Ni-SOD) (Lynch & Kuramitsu 2000). Three types of catalase have been described: monofunctional catalases, bifunctional catalases or catalase/peroxidase and pseudocatalases or non-haeme catalases, with manganese as a metal cofactor (Loewen 1997).

Microorganisms produce different SOD and catalase isozymes inducible under certain culture conditions such as high oxygen tension, low levels of iron or stationary growth phase (Crockford, Davis & Williams 1995; Schnell & Steinman 1995; Barnes, Horne & Ellis 1996; Polack, Dacheux, Delic-Attree, Toussaint & Vignais 1996; St John & Steinman 1996; Lynch & Kuramitsu 2000; Vattanaviboon & Mongkolsuk 2001). However, information on the SOD and catalase activities of *P. damselae* ssp. *piscicida* is scarce.

The aim of this work was to determine whether *P. damselae* ssp. *piscicida* can express different SOD and catalase activities when cultured under different conditions, and whether these enzymatic activities may protect the bacterium *in vitro* from oxygen radicals generated during the macrophage respiratory burst.

Materials and methods

Bacteria

Strains of *P. damselae* ssp. *piscicida* used in this study are listed in Table 1. Strains B180, $D_{26/98}$, Pp8H, R45, R46, B51 and $Lg_{h41/01}$ were isolated in our laboratory (Department of Microbiology, Faculty of Sciences, University of Málaga, Spain). Strains MT 1415, MT 1375, MT 1376 and MT 1379 were kindly provided by Dr A.C. Barnes (Marine Laboratory, Aberdeen, UK); strain DI-21S by Dr A.E. Toranzo (Department of Microbiology and Parasitology, Faculty of Chemistry, University of Santiago de Compostela, Spain) and EPOY-8803-II by Dr K. Muroga (Faculty of Applied Biological Sciences, Hiroshima University, Hiroshima, Japan). Strains 17911 and 29690 were obtained from the American Type Culture Collection (ATCC).

Virulence assays were carried out with two selected strains: $Lg_{h41/01}$ and EPOY-8803-II. Assays to determine the lethal dose 50% (LD_{50}) for sole, *Solea senegalensis* (Kaup), were carried out following the methodology described by Santos (1991). Groups of five fish (10–15 g body weight) maintained in tanks at 24 °C, were intraperitoneally inoculated with 0.1 mL of serial bacterial dilutions containing 10^3–10^8 cfu. The same number of fish was inoculated with phosphate-buffered saline (PBS) and used as a control. Inoculated fish were observed daily for 14 days, and all mortalities were recorded. Mortalities were considered to be due to the inoculation when the bacterial strain was isolated in pure culture from internal organs of dead fish. Lethal dose 50% (LD_{50}) represents the

Table 1 *Photobacterium damselae* subsp. *piscicida* strains used in this study

Strain	Host	Source
17911	*Roccus americanus*	ATCC
29690	*Seriola quinqueradiata*	ATCC
B51	*Dicentrarchus labrax*	UMA, Spain
B180	*Sparus aurata*	UMA, Spain
$D_{26/98}$	*S. aurata*	UMA, Spain
Pp8H	*S. aurata*	UMA, Spain
R45	*S. aurata*	UMA, Spain
R46	*S. aurata*	UMA, Spain
DI-21S	*S. aurata*	USC, Spain
EPOY-8803-II	*Epinephelus akaara*	Japan
$Lg_{h41/01}$	*Solea senegalensis*	UMA, Spain
MT1415	*D. labrax*	Marine Laboratory, Aberdeen, UK
MT1375	*D. labrax*	Marine Laboratory, Aberdeen, UK
MT1376	*S. aurata*	Marine Laboratory, Aberdeen, UK
MT1379	*S. aurata*	Marine Laboratory, Aberdeen, UK

ATCC, American Type Culture Collection; UMA, University of Málaga; USC, University of Santiago de Compostela.

number of bacteria needed to kill 50% of the inoculated fish (Reed & Müench 1938). Strain $Lg_{h41/01}$ with an $LD_{50} = 2.8 \times 10^4$ cfu g^{-1} fish was considered virulent for sole and strain EPOY-8803-II with $LD_{50} > 7.7 \times 10^6$ cfu g^{-1} fish was considered non-virulent.

Bacterial growth conditions

Bacteria were stored at −80 °C in tryptic soy broth (TSB; Oxoid Ltd., Basingstoke, UK) containing 2% NaCl and 20% glycerol. Bacteria were cultured on tryptic soy agar (TSA; Oxoid) containing 2% NaCl and incubated at 22 °C for 48 h. One colony was used to inoculate 5 mL TSBs and incubated for 18 h at 22 °C with shaking. Aliquots (25 μL) of these cultures were used to inoculate 250 mL TSBs which was incubated at 22 °C with shaking. The incubation time varied depending on the culture condition and strain to be assayed.

Different growth conditions were assayed to determine the potential induction of SOD and catalase activities. Thus, 250-mL culture flasks were supplemented with an iron chelant, dipyridyl (100 μM), $FeCl_3 \cdot 6H_2O$ (100 μM) or $MnSO_4 \cdot 2H_2O$ (250 μM) to determine the influence of iron and manganese availability on enzymatic activity. In order to induce oxidative stress, methyl viologen (0.2 mM), which generates superoxide radicals, was added to mid-exponential cultures, which were then incubated for 8 h before centrifugation. The potential induction of enzymatic activities by hydrogen peroxide was tested in cultures after the addition of two pulses of hydrogen peroxide, one of 20 μM in the mid-exponential phase, and another of 2 mM in the early stationary phase. Cells were harvested after 1-h incubation. The influence of the growth phase was investigated with bacteria harvested from mid-exponential ($OD_{600} = 0.4$–0.6) and early stationary ($OD_{600} = 1$–1.2) phase cultures.

Preparation of crude extracts

Bacteria were harvested from cultures grown as described above by centrifugation at 2000 ***g*** for 20 min at 4 °C and washing twice in 25 mM potassium phosphate buffer containing 1 mM disodium ethylene diamine tetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, MO, USA), pH 7.2 and 0.5 mM phenyl methylsulphonyl fluoride (Sigma) followed by re-suspension in 1 mL of the same buffer. Suspensions were sonicated on ice for 120 s (four pulses of 30 s with 15 s cooling between bursts). Lysates were clarified twice by centrifugation at 10 000 ***g*** for 20 min at 4 °C. Supernatants were assayed for the detection of SOD and catalase and quantification of enzymatic activity on acrylamide gels. Total protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

Polyacrylamide gel electrophoresis

Electrophoresis was performed in non-denaturing discontinuous polyacrylamide mini-gels using the Bio-Rad Mini Protean II System (Bio-Rad Laboratories, Richmond, CA, USA) with a 10% acrylamide/bis separating gel (1.5 M Tris–HCl, pH 8.8) and a 4% acrylamide/bis stacking gel (0.5 M Tris–HCl, pH 8.3). The extracts in the sample buffer were applied to the gel at a concentration of 20–24 μg protein per lane. Gels were then stained for SOD or catalase and peroxidase activities.

Detection and quantification of SOD activity

Superoxide dismutase activity was visualized on gels by nitroblue tetrazolium (NBT; Sigma) negative staining (Beauchamp & Fridovich 1971). Briefly, gels were washed in distilled water, soaked in a solution of 2.45 mM NBT for 20 min, followed by 10-min incubation in darkness in a solution containing 50 mM potassium phosphate buffer (pH 7.2), 0.028 mM riboflavin (Sigma) and 28 mM tetramethylethylenediamine (TEMED; Sigma). Gels were illuminated on a light box to develop a dark background with achromatic bands corresponding to SOD activity, due to inhibition of the photochemical reduction of NBT to formazan blue.

The method employed to quantify SOD activity is based on the ability of SOD to inhibit the reduction of NBT by superoxide (Winterbourn, Hawkins, Brian & Correll 1975; Worthington Enzyme Manual 1993). One unit is defined as the amount of enzyme causing half the maximum inhibition of NBT reduction. Different volumes of extracts were added to cuvettes containing 0.2 mL of a solution of 0.1 M EDTA, 0.3 mM sodium cyanide (NaCN; Sigma) and 0.1 mL of 1.5 mM NBT. Then, 0.05 mL of 0.12 mM riboflavin was added at zero time and at timed intervals. All cuvettes were incubated in a light box for 12 min and absorbance at 560 nm was read at timed

intervals by a spectrophotometer (Hitachi U-2000: Hitachi, Tokyo, Japan). The amount of enzyme resulting in 50% of maximum inhibition of NBT reduction was determined.

Detection, characterization and quantification of catalase activity

Catalase activity was visualized on non-denaturing acrylamide gels following the methodology of Woodbury, Spencer & Stahmann (1971). After electrophoresis, gels were washed three times in distilled water for 20 min and soaked in a solution of 0.015% H_2O_2 (30%) (Merck, Darmstadt, Germany). Then, the activity was visualized by transferring the gels to a solution of 1% (w/v) ferric chloride (Panreac Quimica, Barcelona, Spain) and 1% (w/v) potassium ferricyanide (Sigma). Regions corresponding to catalase activity were identified as clear yellow bands on a dark green background.

The metal cofactor of the catalase produced by *P. damselae* ssp. *piscicida* was determined by enzymatic inhibition studies according to Barnes, Bowden, Horne & Ellis (1999b). Lysates of *P. damselae* ssp. *piscicida* strains were incubated for 1 h with either 100 and 50 mM potassium cyanide (KCN; Sigma), 1 and 0.5 mM mercuric chloride ($HgCl_2$; Sigma), 25 and 12.5 mM sodium azide (NaN_3; Sigma) or 50 mM phosphate buffer as control. Equal volumes of treated extracts were electrophoresed and gels stained for catalase activity (Woodbury *et al.* 1971). Catalases with manganese as metal cofactor are resistant against sodium azide and potassium cyanide and sensitive to mercuric chloride (Kono & Fridovich 1983; Allgood & Perry 1986; Barnes *et al.* 1999b). Control wells inoculated with extracts of *Escherichia coli* (ATCC 13706) containing a ferric catalase retained the activity after treatment with mercuric chloride but not with sodium azide.

Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide. One unit of catalase was defined as the activity causing the hydrolysis of 1 μmol of hydrogen peroxide per minute (Aebi 1984). Briefly, bacterial extracts were diluted (1:100) in 50 mM potassium phosphate buffer, pH 7.0 and the absorbance of the sample containing 660 μL of lysate and 340 μL of H_2O_2 was measured against a blank with buffer. The decrease in absorbance at 240 nm (Hitachi U-2000) was monitored during a 10-min period.

Bactericidal activity of sole phagocytes

Monolayers of sole phagocytes were prepared following the methodology of Secombes (1990). Briefly, the kidneys of 100–300 g sole were dissected and pressed through a 100 μm nylon mesh with L-15 medium (Gibco, Gaithersburg, MD, USA) containing 2% fetal calf serum (FCS; Sigma), 1% penicillin/streptomycin (Sigma), 0.1% gentamicin sulphate (50 mg mL^{-1} distilled water; Sigma) and 10 U mL^{-1} sodium heparin. The resultant suspension was layered onto a 30–51% (v/v) Percoll (Amersham Pharmacia, Piscataway, NJ, USA) density gradient and the band of cells lying at the 30–51% interface was collected. The cell suspension was washed and adjusted to 10^7 cells mL^{-1} in L-15 medium with antibiotics. The viability was determined by the exclusion test with trypan blue (Sigma) (0.5% in PBS). A volume of 100 μL per well was added to 96-well microtitre plates. Monolayers were maintained at 22 °C overnight until bactericidal assays were performed.

Bacterial culture conditions to determine the ability to resist the bactericidal activity of phagocytes included growth until stationary phase, addition of two hydrogen peroxide pulses and growth in replete or reduced iron medium as previously described. The bacterial concentration was adjusted to 1 OD_{600}, corresponding to 10^8 bacteria per mL. The methodology employed to test bacterial survival after contact with phagocytes was according to Secombes (1990).

Phagocyte monolayers were washed twice with L-15 and the cells were then supplemented with 100 μL L-15, 5% FCS per well. Bacterial suspensions (20 μL) were added to triplicate wells containing macrophages. The microtitre plate was shaken and centrifuged at 150 ***g*** for 5 min to bring the bacteria into contact with cells and subsequently incubated at 22 °C for 0 and 5 h. At the end of the incubation period, the supernatants were removed and the killing stopped by lysing the phagocytes with 50 μL of cold sterile distilled water. Subsequently, 100 μL of TSBs was added to support the growth of the surviving bacteria for 18–20 h at 22 °C.

The number of surviving bacteria was quantified colorimetrically following the methodology of Peck (1985) as modified by Graham, Jeffries & Secombes (1988). Briefly, 10 μL of 3 [4,5-di-

methylthiazoyl-2-yl] 2,5-diphenyltetrazolium bromide (MTT, Sigma) (5 mg mL^{-1} distilled water) was added to the wells, plates were shaken and absorbance at 550 nm was read after 15-min incubation on a multiscan spectrophotometer (Microplate Reader 2001; Whittaker Bioproducts Inc., Walkersville, MD, USA). The percentage of surviving bacteria was calculated by dividing the absorbance obtained from the wells incubated with bacteria for 5 h by the values obtained from wells incubated with bacteria for 0 h.

Statistical analysis

Quantification of enzymatic activities was carried out in three independent experiments. Fish experiments were performed in triplicate, data corresponding to measurements were carried out with phagocytes from three different fish and three replicate wells for each fish. An ANOVA test was performed to compare the results obtained. $P < 0.05$ was considered significant.

Results

All the extracts of the strains of *P. damselae* ssp. *piscicida* included in this study produced similar SOD and catalase activity bands (Fig. 1). Thus, a single band with identical mobility in native polyacrylamide gel electrophoresis gels was observed for all isolates and culture conditions assayed (Fig. 2).

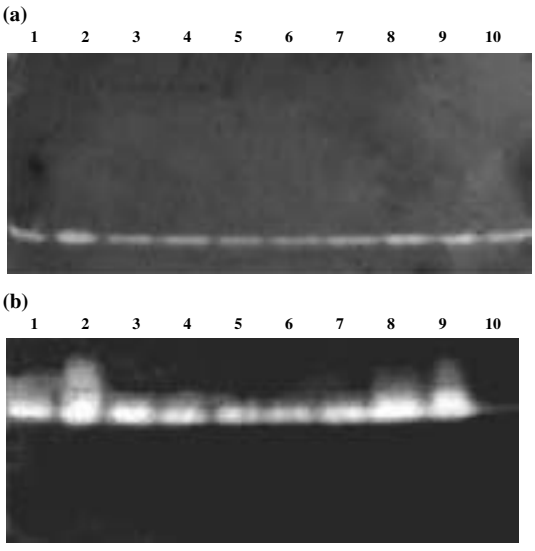

Figure 1 Detection of superoxide dismutase (a) and catalase activity (b) in extracts of different strains of *Photobacterium damselae* subsp. *piscicida* grown until stationary phase. Lane 1: ATCC 17911; 2: B51; 3: B180; 4: DI-21S; 5: $D_{26/98}$; 6: Pp8H; 7: R45; 8: $Lg_{h41/01}$; 9: MT 1415 and 10: MT 1379.

Similar protein concentrations were loaded in the gel lanes. However, differences in the intensity of the SOD and catalase bands were observed. Thus, SOD and catalase activity bands showed lower intensity in the extracts from cultures carried out under iron-limiting conditions, whilst increased intensity of SOD bands was observed in extracts from cultures under iron-supplemented conditions and in the presence of the cytoplasmic superoxide radical generator, methyl viologen (Fig. 2).

Two isolates with different degrees of virulence for sole were selected for further characterization: one virulent, $Lg_{h41/01}$ ($LD_{50} = 2.8 \times 10^4$ cfu g^{-1} fish) and one non-virulent, EPOY-8803-II ($LD_{50} > 7 \times 10^6$ cfu g^{-1} fish).

Cultures carried out until the early stationary phase of the non-virulent isolate contained significantly ($P < 0.05$) lower amounts of SOD than cultures of the virulent strain. However, when iron was added to the growth broth, EPOY-8803-II contained significantly higher amounts of SOD (Fig. 3).

There was no significant hydrogen peroxide induction of SOD in any of the strains, and indeed a decrease in activity in strain $Lg_{h41/01}$ was detected (Fig. 3). In contrast, cells of both strains cultured under iron limiting or replete conditions contained significantly different amounts of SOD activity. In all the cases, growth under iron-limiting conditions resulted in a significant decrease in SOD activity compared with iron replete conditions, this decrease being more important in the non-virulent strain than in the virulent strain.

Unlike SOD, catalase activity in cultures of the non-virulent strain was lower than in the virulent strain (Fig. 4). Moreover, whilst no significant differences were observed in catalase contents of $Lg_{h41/01}$ cultures grown until stationary phase and those pulsed with hydrogen peroxide, strain EPOY-8803-II showed a considerably greater amount of catalase activity when cultures were pulsed with hydrogen peroxide. A significant decrease of activity was also observed for cultures of both strains carried out under iron-limiting conditions compared with iron-overloaded broths.

Catalase activity could not be detected in the gels following exposure to 100 mM sodium azide and treatment with potassium cyanide resulted in a slight reduction of activity, suggesting that this bacterium contains an iron-cofactored enzyme.

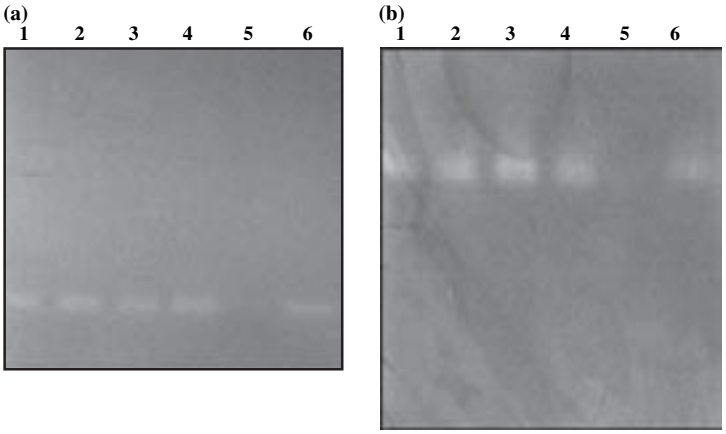

Figure 2 Detection of superoxide dismutase (a) and catalase activity (b) in extracts of *Photobacterium damselae* subsp. *piscicida* (strain EPOY-8803-II) grown under different conditions. Lane 1: growth until exponential phase; 2: stationary growth phase; 3: exposure to hydrogen peroxide (20 μM H_2O_2 mid-exponential phase and 2 mM H_2O_2 early stationary phase); 4: addition of methyl viologen (0.2 mM) to the culture medium; 5: addition of 2,2′-dipyridyl (100 μM) to the culture medium; 6: addition of $FeCl_3 \cdot 6H_2O$ (100 μM) to the culture medium.

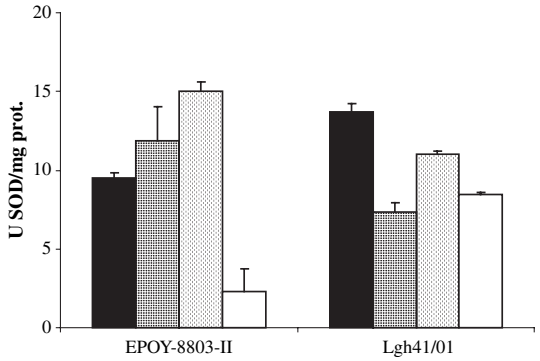

Figure 3 Superoxide dismutase activity (U mg^{-1} protein) of *Photobacterium damselae* subsp. *piscicida* strains grown under different culture conditions. (■) Growth until stationary phase; (▦) exposure to hydrogen peroxide (20 μM mid-exponential phase and 2 mM early stationary phase); (▨) culture supplemented with $FeCl_3 \cdot 6H_2O$ 100 μM and (□) culture supplemented with the iron chelant 2,2′-dipyridyl 100 μM. Data represent the mean (±SD) of three independent determinations.

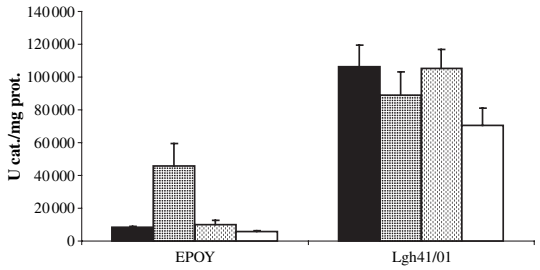

Figure 4 Catalase activity (U mg^{-1} protein) of *Photobacterium damselae* subsp. *piscicida* strains grown under different culture conditions. (■) Growth until stationary phase; (▦) exposure to hydrogen peroxide (20 μM mid-exponential phase and 2 mM early stationary phase); (▨) culture supplemented with $FeCl_3 \cdot 6H_2O$ 100 μM and (□) culture supplemented with the iron chelant 2,2′-dipyridyl 100 μM. Data represent the mean (±SD) of three independent determinations.

In order to determine the influence of the levels of SOD and catalase activity on the resistance to the bactericidal activity of sole phagocytes, killing assays were carried out with a virulent and non-virulent strain of *P. damselae* ssp. *piscicida*. The percentages of surviving bacteria after 5 h contact with sole phagocytes are shown in Fig. 5. It can be observed that survival of the virulent strain in contact with phagocytes was significantly higher ($P < 0.05$) in all cases compared with the non-virulent strain. Despite this different survival rate, both strains showed a similar behaviour depending on the bacterial culture condition with highest rates corresponding in both cases to growth in iron-replete broths and lowest to growth under iron-limiting conditions. In addition, a significant increase in the survival percentages was observed

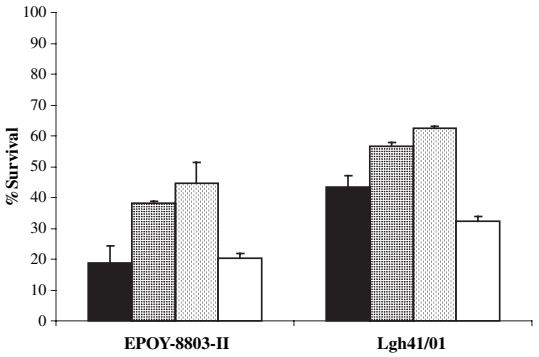

Figure 5 Survival percentage of *Photobacterium damselae* subsp. *piscicida* after 5 h in contact with sole phagocytes. (■) Growth until stationary phase; (▦) exposure to hydrogen peroxide (20 μM mid-exponential phase and 2 mM early stationary phase); (▨) culture supplemented with $FeCl_3 \cdot 6H_2O$ 100 μM and (□) culture supplemented with 2,2′-dipyridyl 100 μM. Data represent the mean (±SD) of nine wells containing phagocytes from three fish specimens.

in both strains pulsed with hydrogen peroxide compared with stationary phase cultures.

Discussion

Enzymes such as SOD and catalase, which neutralize ROS produced during aerobic metabolism or during respiratory burst in fish phagocytes are important virulence factors in many pathogens (Barnes *et al.* 1996, 1999b; Yesilkaya, Kadioglu, Gingles, Alexander, Mitchell & Andrew 2000; Vattanaviboon & Mongkolsuk 2001; Uzzau *et al.* 2002; Banin, Vassilakos, Orr, Martínez & Rosenberg 2003). In this study, all the strains of *P. damselae* ssp. *piscicida* assayed showed a single band of SOD activity with identical mobility on acrylamide gels. A unique band similar in all the strains was also observed on catalase activity gels. Similarly, Barnes, Balebona, Horne & Ellis (1999a), in a study that included a collection of *P. damselae* ssp. *piscicida* strains isolated from gilthead seabream, *Sparus aurata* (L.), reported only one SOD located in the periplasmic space and one cytoplasmic catalase.

Several studies have reported that microorganisms contain different SOD and catalase isozymes inducible under certain growth conditions (Storz, Tartaglia, Farr & Ames 1990; Privalle & Fridovich 1992; Barnes *et al.* 1996; Yesilkaya *et al.* 2000; Geslin, Llanos, Prieur & Jeanthon 2001; Vattanaviboon & Mongkolsuk 2001). However, culture conditions assayed in this work have not induced new SOD or catalase isozymes in *P. damselae* ssp. *piscicida*. Mn-SOD activity has been reported to be modulated by oxidative stress and iron-limiting conditions (Privalle & Fridovich 1992; Barnes *et al.* 1999b) but in the case of *P. damselae* ssp. *piscicida* neither production of intracellular superoxide by methyl viologen nor culture under iron-restricted conditions induced the production of a different type of SOD. Although further studies are necessary, this lack of induction of a new SOD could be due to the presence of only one *sod* gene, i.e. *sod* B encoding Fe-SOD (Lynch & Kuramitsu 2000).

In contrast, differences in the intensity of the bands were observed in extracts obtained under different culture conditions for both SOD and catalase activities. As the amount of protein loaded in the electrophoretic lanes was similar in all cases, the different intensities suggest variations in the levels of activity in the extracts depending on the culture condition. These results are in agreement with those obtained by Barnes, Balebona, Horne & Ellis (1999a), who also detected differences in cultures carried out under iron replete and depleted conditions and high- and low-aerated broths.

The quantification of both SOD and catalase activities carried out in this study corroborated that different band intensities corresponded to variations in the levels of activity. The lowest levels of SOD activity were detected when bacteria were grown under iron-restricted conditions. The ferric nature of *P. damselae* ssp. *piscicida* SOD described by Barnes *et al.* (1999a) could explain this lower activity in the presence of an iron chelant.

Iron also influenced the levels of catalase activity in *P. damselae* ssp. *piscicida*. The role of iron as cofactor in this enzyme has been demonstrated with inhibition studies. Thus, catalase activity could not be detected in the gels following exposure to sodium azide and it was slightly reduced after treatment with potassium cyanide. These results suggest that the enzyme is an iron cofactored catalase, as Mn-containing catalases retain activity after treatment with azide and cyanide and are inhibited by mercuric chloride (Kono & Fridovich 1983; Allgood & Perry 1986; Barnes *et al.* 1999b). This ferric nature of the catalase may explain the lower catalase activity observed in cultures with added iron chelant and lower survival with H_2O_2 observed by Díaz-Rosales, Chabrillón, Moriñigo & Balebona (2003).

Lower survival of *P. damselae* ssp. *piscicida* in sole phagocytes has been observed for strain EPOY-8803-II compared with the virulent strain. Contradictory results have been reported on the ability of *P. damselae* ssp. *piscicida* to survive inside macrophages from several fish species. In a study using macrophages from sea bass, gilthead sea bream and rainbow trout, Skarmeta *et al.* (1995) concluded that head kidney macrophages from these fish species were able to kill the pathogen. However, Noya *et al.* (1995b) reported that whilst bacteria within granulocytes and macrophages from large gilthead sea bream were morphologically altered, bacteria inside small fish remained unaffected. In addition, data on the ability of *P. damselae* to survive inside fish macrophages have been reported by several authors who observed that bacteria can multiply inside fish macrophages (Kubota *et al.* 1970; Hawke, Plakas, Minton, McPherson, Zinder & Guarino 1987; Noya *et al.* 1995a; Elkamel, Hawke, Henk & Thune 2003).

Multiplication of *P. damselae* ssp. *piscicida* inside several fish cell lines has also been reported. Elkamel & Thune (2003) observed that the bacteria multiply in EPC, CCO, and FHM cells and López-Dóriga, Barnes, dos Santos & Ellis (2000) using EPC cells observed that both virulent and avirulent isolates were able to adhere to and invade cells.

Results obtained from this study show that *P. damselae* ssp. *piscicida* is able to survive inside sole phagocytes at least for 5 h, the survival rates being higher for the virulent isolate. Although the bacterium was able to survive, the rates obtained always indicated a certain degree of bacterial inactivation inside phagocytes.

Survival of the non-virulent strain in contact with sole phagocytes was significantly lower compared with the virulent strain. The non-virulent strain also showed lower catalase activity. These results suggest that bacterial inactivation could be due to the accumulation of hydrogen peroxide, the precursor of hydroxyl radicals, after decomposition of superoxide radicals by bacterial SOD. This accumulation would not take place to such an extent in the virulent strain, as levels of catalase are higher. The important role of catalase in the protection against oxidative damage in *P. damselae* ssp. *piscicida* has been pointed out by Barnes *et al.* (1999a), who observed that the addition of exogenous catalase to the medium protected the bacteria from inactivation by photochemically generated superoxide anions.

Both virulent and non-virulent strains assayed by Barnes *et al.* (1999a) showed high susceptibility to cell-free generated superoxide radicals. In contrast, we have observed that a non-virulent strain, EPOY-8803-II, is significantly more susceptible to killing by sole phagocytes than a virulent strain ($Lg_{h41/01}$). Besides the lower catalase activity present in the non-virulent strain, the lack of a capsule in cells of EPOY-8803-II could contribute to the high inactivation rates observed. Thus, the capsule could protect bacterial cells from oxidative radicals or even prevent activation of phagocytes (Miller & Britigan 1997; Arijo *et al.* 1998).

The important role of iron in microbial infections has been pointed out by several authors (Miller & Britigan 1997; Weinberg 2000). The pathogen needs to obtain iron from the host, where this metal is linked to high-affinity proteins and iron availability is very low; also, a transition metal catalyst such as iron plays an important role in the generation of hydroxyl radicals *in vivo*. Indeed, at physiological pH, generation of hydroxyl radical from hydrogen peroxide and superoxide anions is of little biological importance unless a metal such as ferric iron is present (Haber–Weiss reaction) (Miller & Britigan 1997). *Photobacterium damselae* ssp. *piscicida* is more susceptible to killing by sole phagocytes when bacterial cells have been cultured under iron-depleted conditions. This could be due to the lower levels of catalase detected in both the virulent and avirulent cells, the lowest rates corresponding to strain EPOY-8803-II. Thus, although the presence of iron in environments where superoxide and hydrogen peroxide are generated, such as in phagocytes, may promote the generation of highly toxic hydroxyl radicals, it is also true that bacteria require iron for growth and replication and synthesize SOD and catalase to deal with the oxidizing anions. Thus, the ability to obtain iron from the host seems to be crucial for *P. damselae* ssp. *piscicida*. Indeed, it has been demonstrated that immune-activated macrophages modify intracellular distribution and dampen iron influx in order to diminish iron availability for invaders (Weinberg 2000).

Photobacterium damselae ssp. *piscicida* posses a high-affinity iron uptake system (Magariños *et al.* 1994; Naka, Hirono & Aoki 2005). However, despite its ability to obtain iron from high-affinity systems, several authors have reported that cells grown under iron-limited conditions have a reduced amount of capsular material covering the cells (Do Vale, Ellis & Silva 2001). These cells with reduced capsule would be more susceptible to phagocytosis and oxidative stress. Our results show that iron plays an important role in survival of *P. damselae* ssp. *piscicida* in contact with sole phagocytes; whether this is attributable to its contribution to capsular material or SOD and catalase synthesis by the bacterium needs to be investigated.

In conclusion, we have shown that *P. damselae* ssp. *piscicida* is able to survive in contact with sole phagocytes, survival rates being higher for a virulent strain. The increased levels of catalase activity detected in the virulent strain indicate a possible role for this enzyme in bacterial survival.

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ARTÍCULO 2.1.

ARTICLE 2.1.

Effect of dietary administration of *Porphyridium cruentum* on the respiratory burst activity of sole (*Solea senegalensis,* Kaup 1858) phagocytes

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Abstract

The stimulatory effect of the red microalga *Porphyridium cruentum* on respiratory burst activity of sole phagocytes was evaluated *in vitro* and *in vivo*. Sole phagocytes incubated *in vitro* with aqueous and ethanolic extracts (10, 5, 2 and 1 mg ml^{-1}) isolated from *P. cruentum* did not show increased superoxide anion production. By contrast, incubation of phagocytes with β-glucan from *Euglena gracilis* (10 mg ml^{-1}) increased respiratory burst activity. However, oral administration of a diet supplemented with lyophilized *P. cruentum* cells (10 g kg^{-1}) stimulated respiratory burst activity after 4 weeks feeding only in sole vaccinated with *Photobacterium damselae* subsp. *piscicida* bacterin. Results obtained are discussed in terms of the usefulness of the administration route of immunostimulant and synergistic effect with a vaccine.

Keywords: Immunostimulants; *Porphyridium cruentum;* Respiratory burst; Phagocytes; Sole (*Solea senegalensis*, Kaup 1858); Teleosts.

1. Introduction

Fish aquaculture is an expanding industry worldwide. Marine aquaculture in southern Europe has focused on species such as gilthead seabream (*Sparus aurata*, L.) and sea bass (*Dicentrarchus labrax*, L.). However, the diversification of the species farmed is required and Senegalese sole (*Solea senegalensis*, Kaup) is a species with a high economic value, which is farmed in Portugal and Spain [1]. Diseases of bacterial origin constitute the most significant cause of economic losses suffered in the aquaculture industry [2]. The most frequently isolated pathogen from outbreaks detected in farmed Senegalese sole is *Photobacterium damselae* subsp. *piscicida*, which is responsible for high mortalities in cultured fish [3]. Antibiotic treatment of bacterial diseases affecting farmed fish has been applied for many years. However, the occurrence of antibiotic resistance in pathogenic bacteria is limiting the usefulness of these substances [4-6] and therefore, several alternative strategies to the use of antimicrobials have been proposed, including the use of immunostimulants which represent a promising tool in aquaculture. Indeed, many authors have reported that the injection of immunostimulants, such as glucans, enhances the function of leucocytes and protection against pathogens [7-10]. Several authors have observed that *P. damselae* subsp. *piscicida* is highly susceptible to oxidative radicals generated during the macrophage respiratory burst [11, 12]. However, other authors have reported the presence of intact cells of this pathogen inside fish cells, suggesting the ability of the bacterium to survive as an intracellular pathogen [13-17]. For this reason, the stimulation of the respiratory burst activity of the phagocytes of Senegalese sole could facilitate a more effective destruction of *P. damselae* subsp. *piscicida*.

Marine organisms constitute a potential alternative source of substances for the prevention and treatment of infectious diseases [18]. In this connection, algae have been studied as dietary ingredients for fish nutrition [19] and as a source of bioactive compounds such as pharmaceutical [20, 21] and immunostimulant [22-25] agents.

Several authors [26] have suggested a possible stimulation of the metabolic and functional action of phagocytic system cells from Balb/c mice after intraperitoneal administration of polysaccharides isolated from cultures in the stationary phase of red

alga *Porphyridium cruentum*. For this reason, this alga has been selected in this work to evaluate its potential immunostimulant effect on farmed fish. However, most studies performed to examine the immunostimulant ability of algae have been carried out by *in vitro* incubation of immune cells with algal extracts, and information on the *in vivo* effects of whole algal cells is still scarce [27]. In addition, algal extracts are inoculated intraperitoneally in theses studies. This route of administration, although very effective, is also very laborious, time-consuming, stressful for fish and difficult to apply to fingerlings [28, 29]. Oral administration of immunostimulants is a non-stressful method with minimum economic cost and effort and enables mass administration regardless of the fish size [7], but studies addressing this route of administration are scarce and usually include only algal extracts instead of whole cells [22].

In this study the potential immunostimulant effect of aqueous and ethanolic extracts obtained from *P. cruentum* on the respiratory burst activity of Senegalese sole phagocytes has been determined. In addition, the potential stimulation of the respiratory burst activity of phagocytes isolated from fish fed with a commercial diet supplemented with *P. cruentum* cells has been studied. In this case, potential synergetic or antagonic effects resulting from the alga diet and vaccination against *P. damselae* subsp. *piscicida* have been evaluated.

Materials and Methods

2.1. Microorganisms

The virulent strain $Lg_{h41/01}$ of *Photobacterium damselae* subsp. *piscicida* isolated from diseased Senegalese sole [16] was selected to test the respiratory burst activity of sole phagocytes. The bacterial strain was cultured on tryptic soy agar (Oxoid) supplemented with 1.5% NaCl (TSAs) for 24 h at 22 ºC. Bacterial suspensions for respiratory burst assays were obtained from tubes containing tryptic soy broth (Oxoid) added with 1.5% NaCl (TSBs) inoculated with one colony from a TSAs plate and incubated at 22 ºC for 24 h. Then, the cultures were centrifuged at 6000 xg for 15 min at 4 ºC, and pellets were resuspended in L-15 medium at an optical density (600 nm) equal to 1(10^8 cells ml^{-1}).

2.2. Alga culture

The red microalga *Porphyridium cruentum* (S.F. Gray) Näegli was obtained from the collection at the Centro de Investigaciones Marinas de Cádiz, Cádiz, Spain. It was grown in *Porphyridium* medium [30] in batch culture at 25ºC, with a 12h photoperiod for 7 days. The algal biomass was centrifuged at 3000 xg, 15 min at 4ºC and the pellet was lyophilized.

2.3. Obtention of aqueous and ethanolic extracts

The preparation of water-soluble extract, aqueous extract, was carried out as follows: 10 g of lyophilized alga was resuspended in 100 ml of HBSS (Hank's Balanced Salt Solution) using a mortar and pestle. The extract was sonicated for 20 min and centrifuged at 3000 xg, 5 min. The supernatant was separated from the pellet and lyophilized and 10 mg of the lyophilized extract was resuspended in 1 ml of HBSS. Extraction of the non-soluble fraction of the alga, ethanolic extract, was carried out as described above, but instead of HBSS, ethanol was used. Dilutions from both extracts were prepared in HBSS to achieve concentrations of 10 mg ml^{-1} of lyophilized extract, 5 mg ml^{-1}, 2 mg ml^{-1} and 1 mg ml^{-1}.

Commercial β-1,3-glucan from *Euglena gracilis* (BioChemika Fluka, Sigma) was used as a positive control of stimulation of respiratory burst activity. β-1,3-glucan (10 mg) was dissolved following commercial instructions, and diluted in HBSS to achieve concentrations of 10 mg ml^{-1}, 5 mg ml^{-1}, 2 mg ml^{-1} and 1 mg ml^{-1}.

2.4. Fish and experimental design

Experiments to test the *in vitro* effect of *P. cruentum* on the respiratory burst activity of sole phagocytes were carried out. Sole of 200 g body weight, stocked in 250 l tanks with recirculating, aerated seawater at 20 ºC, 35‰ salinity, were used to isolate kidney phagocytes and determine the production of anion radicals in contact with aqueous and ethanolic extracts from *P. cruentum* and the commercial β-1,3-glucan.

Feeding assays were carried out with soles of 80 g mean body weight, which were randomly separated into six experimental groups, and stocked in six 250 l tanks (20 fish per tank) with similar culture conditions to those described above.

The diet assayed was prepared in the laboratory from the commercial pellet diet routinely used in fish farms (Skreeting, Trouw España, Nutreco, Burgos, Spain). Briefly, the commercial pellet diet was crushed and mixed with tap water before adding the lyophilized alga *Porphyridium cruentum* at the desired concentration (10 g kg^{-1}), and then made into pellets again. The re-made pellets were allowed to dry and stored at 4 ºC until use.

The commercial pellet Sanostim™ (Skreeting, Trouw España, Nutreco, Burgos, Spain), containing β-glucans, was used to test the response of sole phagocytes. Two groups of fish received daily one of the different diets assayed: diet consisting of non-supplemented commercial diet (control group); diet composed of the commercial diet containing immunostimulant Sanostim™; and finally, a commercial diet supplemented with lyophilized alga (1%). Fish were fed at a rate of 20 g dry diet kg^{-1} biomass (2 %) per day for 4 weeks. The biomass of the fish in each aquarium was measured before the experiment and daily ration, being adjusted accordingly. No mortality was observed during the experiment.

2.5. Immunization assay

Two weeks after beginning the feeding trial, fish from one tank per treatment were intraperitoneally inoculated with a bacterin of *P. damselae* subsp. *piscicida*. The formalin-killed aqueous vaccine was prepared with a virulent strain of *P. damselae* subsp. *piscicida* ($Lg_{h411/01}$) isolated from diseased sole [16] according to the following protocol. Briefly, bacteria were cultured on TSAs for 24 h and one colony was inoculated in tubes containing 5 ml of TSBs. After 18 h incubation at 22 ºC, an aliquot of the culture, 50 µl, was inoculated in flasks with 50 ml TSBs and incubated at 22ºC with continuous shaking. After 18 h incubation the culture achieved O.D. 600 of 1.2. The total bacterial number was counted, obtaining a bacterial concentration of 6 x 10^8 bacteria ml^{-1}. Then bacterial cells were killed by addition of formaldehyde to achieve 1% final concentration, and overnight incubation. Sterility tests were performed by

spreading an aliquot of the bacterin on TSA plates and incubation for 2 days at 22 ºC. The vaccine was administered by intraperitoneal injection (0.1 ml per fish). Control fish were injected with phosphate buffer saline (PBS, pH 7.2).

2.6. Isolation of head kidney phagocytes

Sole phagocytes were isolated from the kidney following the technique described by Secombes [31]. Briefly, the kidney was removed aseptically and pushed through a 100 μm nylon mesh with Leibovitz medium (L-15) containing 2% foetal calf serum (FCS, Sigma), 1% penicillin-streptomycin (Sigma), 0.1 % (5 mg ml^{-1}) gentamicine (Sigma) (P/S/G) and 10 U heparine ml^{-1}. This cell suspension was layered on a 30 to 51% Percoll (Amersham) gradient and centrifuged at 600 xg for 30 min. Then the bands separated at the interface were resuspended in L-15 medium supplemented with P/S/G. The viable cell concentration was determined after staining with trypan blue and microscope counting. Aliquots of 100 μl containing $1x10^7$ cells ml^{-1} in L-15 medium supplemented with P/S/G were added to 96-well microtitre plates. After 3 h incubation at 22 ºC, non-adherent cells were removed and medium was substituted by L-15 and P/S/G supplemented with 2% FCS. Monolayers were incubated overnight at 22 ºC.

2.7. Respiratory burst activity

The generation of intracellular superoxide radicals by sole phagocytes was determined by the reduction of nitro-blue tetrazolium (NBT) according to the technique described by Secombes [31] and Boesen et al. [32]. Phagocyte monolayers were washed with L-15 medium and HBSS (Hank´s Balanced Salt Solution) to remove any trace of the antibiotic.

In order to test the *in vitro* effects of algal extracts on the production of superoxide radicals by sole phagocytes, a volume of 20 μl of the extracts (aqueous, ethanolic or β-glucan) were added to the wells (15 wells from 5 fish) containing phagocyte monolayers prepared as above. Then, 100 μl of NBT dissolved at 1 mg ml^{-1} in HBSS was added to the wells and the phagocytes incubated at 22 ºC for 30 min. Wells containing phagocytes were infected with 20 μl of *P. damselae* subsp. *piscicida*

(10^8 bacterias ml^{-1}) and used to determine the response of the phagocytes to the fish pathogen. As a positive control phorbol myristate acetate (PMA, Sigma) (1 μg ml^{-1}) was used to stimulate the respiratory burst activity of non-infected phagocytes (data not shown). The specificity of the reaction was tested by adding superoxide dismutase (SOD) (300 I.U. per well) to some wells containing PMA-stimulated phagocytes (data not shown). After incubation, cells were fixed in 70% methanol and the reduced formazan within phagocytes was solubilised by adding 120 μl 2M KOH and 140 μl dimethyl sulfoxide (DMSO, Sigma). Finally, absorbance was read at 630 nm in a multiscan spectrophotometer (UV-1601 Spectrophotometer, Whitakker Bioproducts).

In the *P. cruentum* feeding experiments, the effect of oral administration of the alga or Sanostim™ was determined on phagocyte monolayers as described above, but algal extracts or bacteria were not added to the wells.

2.8. Statistical analysis

Results are expressed as the stimulation index (mean + standard error, SE), obtained by dividing each sample value by the mean control value. Values higher than 1 reflect an increase and lower than 1 a decrease in each parameter compared to the control. Data were statistically analysed by one-way analysis of variance (ANOVA) and Tukey's comparison of means using SPSS for Windows. Differences were considered statistically significant when $P< 0.05$.

3. Results

3.1. In vitro assays

Results have been expressed as the ratio of the absorbance at 630 nm of treated phagocytes to the absorbance of non-treated phagocytes. The treatment of phagocytes consisted of incubation with aqueous (Figure 1a) or ethanolic (Figure 1b) algal extracts or β-glucan (Figure 1c), in the presence or absence of *P. damselae* subsp. *piscicida*. Therefore the data represented in the Figures are the result of a correlation between phagocytes incubated with the algal extract or β-glucan, with or without bacteria, and phagocytes incubated without the potential immunostimulant (algal extract or β-glucan).

The results obtained show that extract from *P. cruentum* is not able to significantly increase the respiratory burst activity of sole phagocytes, both incubated with and without bacteria (Figures 1a and 1b). Only the commercial β-glucan (10 mg ml^{-1}) significantly enhanced (P<0.05) the respiratory burst activity compared to cells incubated both with or without *P. damselae* subsp. *piscicida* (Figure 1c). Incubation only with *P. damselae* subsp. *piscicida* cells did not induce superoxide anion production of sole phagocytes, the stimulation index obtained being 1.042 ± 0.069.

3.2. Feeding assays

A ratio between superoxide anion production (absorbance at 630 nm) of phagocytes from unvaccinated or vaccinated fish fed with Sanostim™ or with a diet supplemented with algal cells to absorbance values obtained in phagocytes from fish fed with a commercial pellet diet is represented in Figures 2a and 2b, non-immunized and immunized fish, respectively.

Significant differences were not detected between fish fed with Sanostim™ or algal supplemented diets and fish fed with non-supplemented diets for 2, 3 or 4 weeks (Figure 2a). However, significant (P<0.05) stimulation of the production of superoxide anions of phagocytes from fish fed with *P. cruentum* cells was observed only when fish were vaccinated with *P. damselae* subsp. *piscicida* bacterin and after 4 weeks of treatment (Figure 2b).

4. Discussion

No stimulation of the production of superoxide anions has been observed in sole phagocytes incubated with aqueous or ethanolic extracts isolated from *P. cruentum*. By contrast, immunostimulant effects on phagocytes of several fish species by different extracts from several algal species have been reported [23-25, 33, 34]. Previously, we have observed stimulation of sole phagocytes after *in vitro* contact with aqueous extracts from *Fucus distichus* [24]. Also, sole phagocytes increase the production of superoxide anion after 30 min contact with the higher concentration (10 mg ml^{-1}) of β-glucan from *Euglena gracilis* assayed but not with lower concentrations. These results

are in agreement with the data reported by Castro et al. [35], who observed increases in the respiratory burst activity of the head kidney phagocytes of turbot and gilthead seabream incubated with different concentrations of β-glucans obtained from fungi and yeasts. The fact that several authors found significant variations in the stimulatory capacities of algal extracts depending not only on their origin, but on the concentrations used and time of incubation [23] does not rule out a potential *in vitro* modulation of respiratory burst activity by *P. cruentum* extracts. Thus, higher concentrations and longer incubation times need to be assayed.

The results obtained in the *in vivo* assays showed that fish immunized with a bacterin against *P. damselae* subsp. *piscicida* and receiving the diet supplemented with the alga *P. cruentum* significantly increased the respiratory burst activity of their phagocytes after four weeks of the feeding treatment. This increase was higher than that obtained in the group of fish fed with the diet supplemented with Sanostim™ for four weeks. These stimulated phagocytes may be more efficient in the inactivation of intracellular pathogens such as *P. damselae* subsp. *piscicida* than other phagocytes which had not been in contact with the components of the alga assayed. However, experiments to test the bactericidal activity of phagocytes from sole specimens fed with this diet should be considered in order to confirm this hypothesis.

Evidence is increasing to support the essentiality of nutrients for maintaining a normal immune system in fish [22, 36]. These nutrients are proteins, essential fatty acids, polysaccharides, vitamins C and E, and some oligoelements such as Se and Zn [37, 38]. *P. cruentum* is a red microalga member of *Rodophyta,* of the order of *Porphyridiales,* with spherical cells that lack a rigid cell wall. This alga accumulates large amounts of lipids, specially arachidonic acid and noticeable amounts of eicosapentaenoic acid, these being substances which have been reported as immunostimulants [39, 40]. The available carbohydrates ranged from 40 to 57% [41]. Different carbohydrates have been reported previously as immunostimulant active on fish phagocytes [7, 23, 33, 40-48]. Morris et al. [26] suggested a potential immunostimulant activity of the polysaccharides isolated from cultures of *P. cruentum* on the metabolic and functional action of mice phagocytes. This alga also contains large amounts of carotenes [41] and several studies have reported that the carotenoids

increase the resistance to diseases [49] and they modulate some of the innate defense mechanisms in fish such as rainbow trout [22]. Other substances present in this alga are vitamins such as tocopherol, vitamins K and C, the latter having been reported as a very important immunostimulant [44, 50-52]. Due to the fact that this alga contains different immunostimulant substances, its use could generate a more general immune response as has been proposed for other microorganisms such as yeasts [8, 53].

Castro et al. [23] reported that when turbot phagocytes were pre-incubated with water–soluble extracts of *Chondrus crispus* and then incubated with PMA, the extracts had a priming effect on fish cells. Supplementation of the diet with whole cells of *P. cruentum* fed to the fish during a four-week period did not show a potential priming effect on the respiratory burst activity of sole phagocytes. This finding could indicate that this alga does not have the ability to induce the priming effect reported for *C. crispus* or that the oral administration of the alga did not supply the phagocytes with levels of immunostimulant capable of facilitating this priming effect.

Valente et al. [19] suggested that the inclusion of dry pellet of the red macroalga *Gracilaria bursa-pastoris* and the green macroalga *Ulva rigida*, up to 10% can be considered as very interesting ingredients in diets for sea bass juveniles, as no negative consequences on growth performance, nutrient utilization or body composition were observed. On the other hand, the inclusion of the red macroalga *Gracilaria cornea* should be limited to 5% of the diet.

The stimulation of sole phagocytes has been observed only when fish with *P. cruentum* were immunized with the *P. damselae* subsp. *piscicida* bacterin. Sakai [28] indicates that the combination of vaccination and immunostimulant administration may increase the potency of vaccines. Jeney and Anderson [54] reported that rainbow trout bathed in *A. salmonicida* O-antigen in combination with immunostimulants (levamisole, quaternary ammonium compound –QAC- and a polypeptide derived from fish products –ISK-) enhanced phagocytosis by leucocytes and antibody titers against *A. salmonicida*, and showed adjuvant effects with vaccination. Sakai et al. [55] reported that *R. salmoninarum*-vaccinated rainbow trout receiving EF203, the fermented product of chicken eggs, showed higher phagocytic activities and NBT responses in kidney leucocytes when compared to vaccinated fish without EF203 treatment or to

unvaccinated fish. However, the serum agglutinating antibody titers of vaccinated fish did not show a significant increase compared to control groups, and vaccinated fish treated with EF203 showed slightly increased survival in comparison with the other groups following *R. salmoninarum* challenge. The adjuvant effects of yeast glucan have also been demonstrated. Injection of the *A. salmonicida* vaccine and yeast glucan in Atlantic salmon enhanced antibody responses [56] and induced significantly increased protection against furunculosis over vaccines without yeast glucan [57]. The injection of yeast glucan alone did not afford protection. Baulny et al. [58] reported that oral administration of yeast glucan to turbot immersed in the *V. anguillarum* bacterin also increased protection compared to the bacterin alone, but similar to the results obtained in this work with β-glucan from *Euglena gracilis*, yeast glucan alone did not enhance protection against *V. anguillarum* infection.

P. cruentum has advantages in its culture, such as fast growth and low cost. This makes it easier to work with at known concentration, compared with using soluble substances such as vitamins, which exist as micronutrients in feed and are very sensitive to different factors (light, humidity, temperature). On the other hand *P. cruentum* could be a natural immunostimulant, which is biocompatible, biodegradable and safe for the environment and human health. In this way, these algae could be included in the groups of different whole microorganisms, alive or not, such as yeast [8, 53], fungi [59] and bacteria [60-62] which increase disease resistance in mammals and fish. In fish, as in other aquatic organisms, the whole microorganisms administered have been mainly bacterial species, such as probiotics [60-66], but algae can represent another promising alternative [18, 25], although studies about whole algae are still very scarce.

In conclusion, this study provides a description of the stimulation of the respiratory burst activity of phagocytes from sole vaccinated with a bacterin against *P. damselae* subsp. *piscicida* and fed with cells from *P. cruentum*.

Acknowledgements

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Figure legends

Figure 1. Respiratory burst activity of sole phagocytes incubated with aqueous **(a)** or ethanolic **(b)** extracts of *Porphyridium cruentum* (1, 2, 5, 10 mg ml^{-1}) or β-glucan **(c)** in absence (■) or presence (□) of *Photobacterium damselae* subsp. *piscicida* ($2x10^6$ cells per well). Results are expressed as stimulation index (mean ± SE; n=15) obtained by dividing each sample value by its mean control value (HBSS or *P. damselae* subsp. *piscicida* cells). The symbol * denotes statistically significant differences ($P<0.05$) compared to control wells.

Figure 2a. Relative superoxide anion production in sole phagocytes fed with commercial immunostimulant, Sanostim™, positive control (■) and with diet supplemented with *Porphyridium cruentum* (□). The samples were collected after 2, 3 and 4 weeks.

Figure 2b. Relative superoxide anion production in sole phagocytes fed with commercial immunostimulant, Sanostim™, positive control (■) and with diet supplemented with *Porphyridium cruentum* (□) and immunized with a bacterin against *P. damselae* subsp. *piscicida*. The samples were collected after 3 and 4 weeks, $P<0.05$ denotes a significantly higher response than that obtained with sole fed with normal pellet diet (negative control). The last column represents the ratio obtained from sole fed with normal diet and immunized and non-immunized sole (▩). The symbol * denotes statistically significant differences ($P<0.05$) with respect to the negative control group, sole fed with normal pellet diet.

Figure 1a

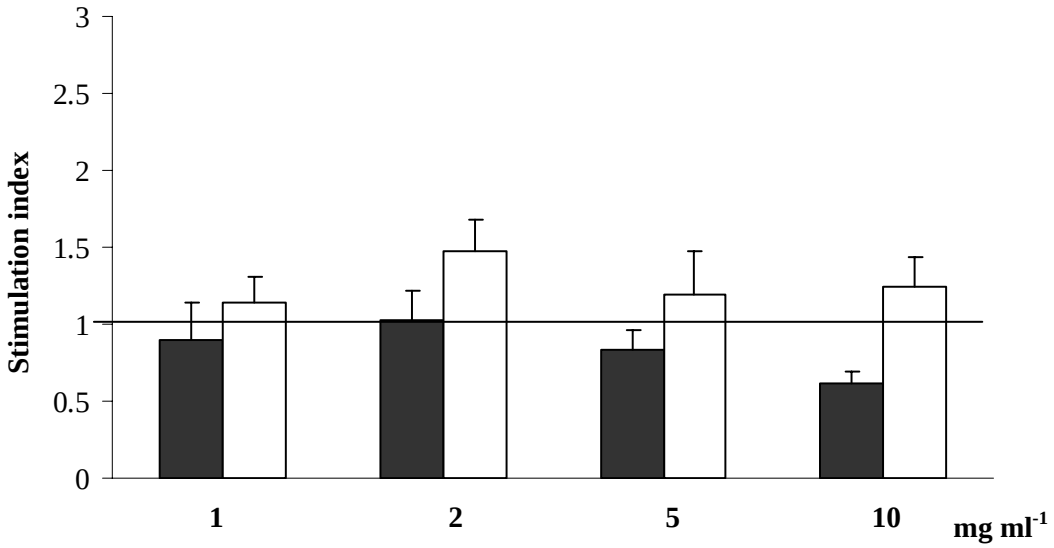


Figure 1b

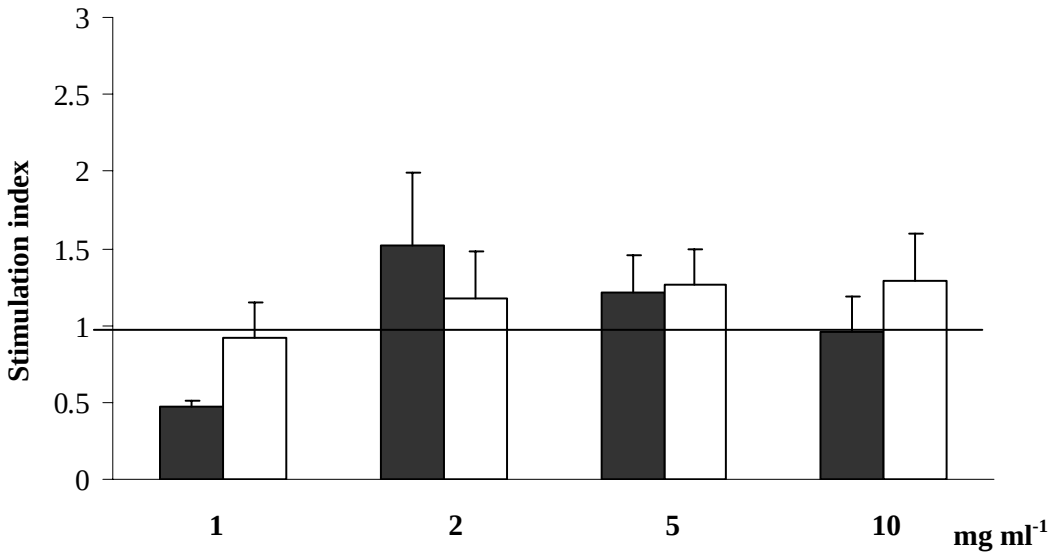


Figure 1c

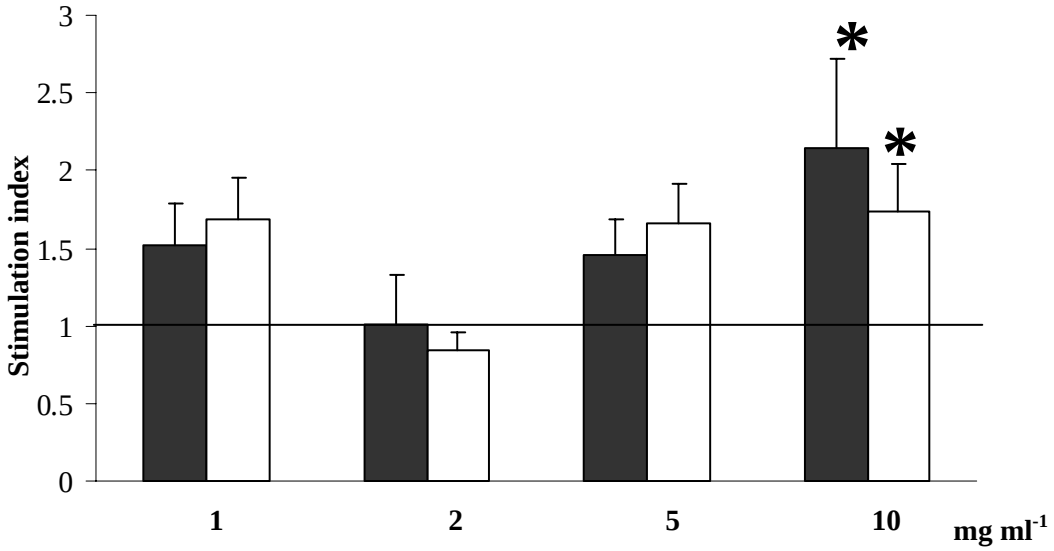


Figure 2a

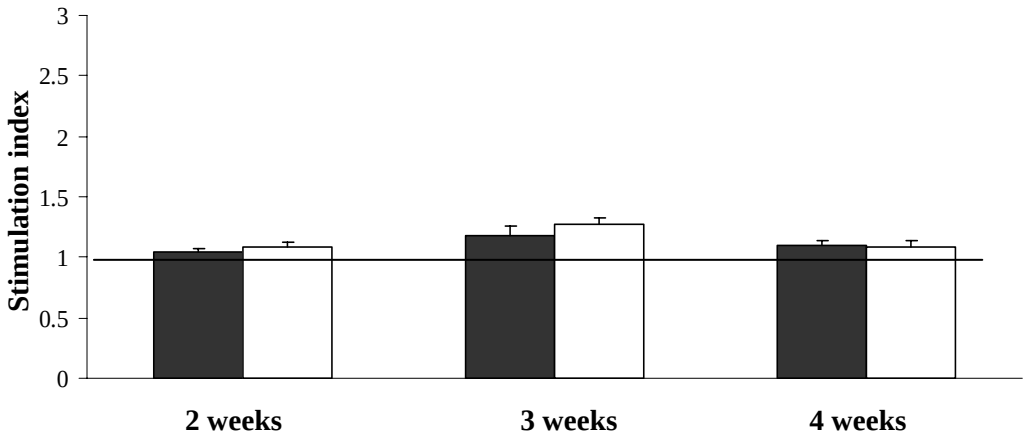
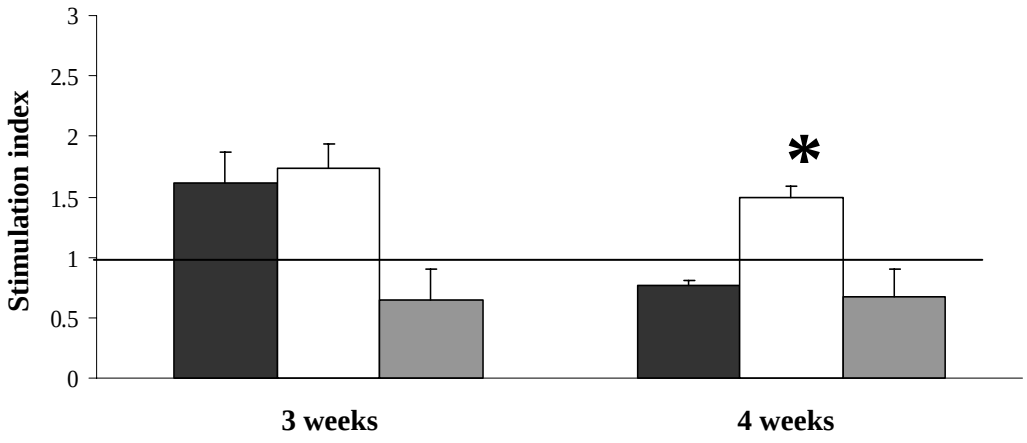


Figure 2b



ARTÍCULO 2.2.

ARTICLE 2.2.

Effect of the extracellular polysaccharidic fraction from the red microalga *Porphyridium cruentum* on the respiratory burst activity of sole (*Solea senegalensis*, Kaup 1858) phagocytes

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Abstract

The potential effect of extracellular polysaccharide fraction of the red microalga *Porphyridium cruentum* on respiratory burst activity of sole phagocytes was evaluated. *In vitro* assays were carried out and no immunostimulatory effects were obtained. In *in vivo* assays, fish were intraperitoneally injected with 500 μg of polysaccharide fraction and sampled after 24 h and 7 days. These fish were divided in two groups, immunized and not immunized with a bacterin against *Photobacterium damselae* subsp. *piscicida*. The results obtained from *in vivo* assays did not show increase on respiratory burst, on the contrary, the only significant differences were obtained from sole inoculated with polysaccharidic fraction or with bacterin, both of them showed a decrease on respiratory burst, 24 h post-inoculation.

Keywords: *Porphyridium cruentum; Photobacterium damselae* subsp. *piscicida*; Polysaccharides; Immunostimulants; Respiratory burst; Phagocytes; Sole (*Solea senegalensis*, Kaup 1858).

1. Introduction

Solea senegalensis (Kaup, 1858) is one of the new candidate species for aquaculture in the Portuguese and Spanish coasts [1]. One of the main limiting factors in its production is pseudotuberculosis, due to the bacterial pathogen *Photobacterium damselae* subsp. *piscicida* [2-4]. There are several means of protection against this microorganism, including antibiotherapy, although the use of antibiotics leads to the appearance of resistant strains [5]. Vaccination is another option which has been demonstrated effective protection against pseudotuberculosis in sole [4].

Since immunostimulants mainly facilitate the function of phagocytic cells and increase their bactericidal activities, it may be possible that immunostimulants administration to fish increase the respiratory burst activity of the phagocytes and enhance the protection against pseudotuberculosis. Several authors have reported that *P. damselae* subsp. *piscicida* has the ability to survive as an intracellular pathogen [6-10]. The search for new immunostimulant agents is mainly due to the great development of fish farming and the stress situations and diseases, which arise from intensive culture [11].

Several polysaccharides, such as chitin, chitosan and β-glucans obtained from crustaceans and yeasts have been used in fish and shellfish as immunostimulants [11-18]. Due to their content in polysaccharides, algae could be an alternative source to obtain polysaccharides easily. Polysaccharides from *Spirulina platensis* and *Chlorella* have been demonstrated their immunomodulating capacity in mammalian models [19-21]. However, few studies have been focused on the immunostimulant properties of algal polysaccharides in farmed fish [22, 23, 24]. The red microalga *Porphyridium cruentum* excrete a sulphurized polysaccharide, commercially used in the industry as thickener, stabilizer and emulsifier for its gelling properties [25-28]. This polysaccharide is an acidic heteropolymer composed of xylose, glucose, galactose and sulphate esters [29]. In a previous study, Morris *et al.* [30] suggested a possible stimulation of the metabolic and functional action of phagocytic cells of Balb/c mice after an intraperitoneal administration of polysaccharides isolated from cultures of *P. cruentum* in stationary phase.

The aim of this work is to evaluate the immunomodulatory capacity of the extracellular polysaccharidic fraction isolated from *P. cruentum* on respiratory burst activity of sole phagocytes against *P. damselae* subsp. *piscicida*.

2. Materials and Methods

2.1. Microorganisms

The virulent strain of *Photobacterium damselae* subsp. *piscicida* $Lg_{h41/01}$ isolated from diseased sole [17] was selected to test the respiratory burst activity of phagocytes from Senegalese sole treated with and without polysaccharidic fraction of *P. cruentum*. The bacterial strain was cultured on tryptic soy agar (Oxoid) supplemented with 1.5% NaCl (TSAs) for 24 h at 22 ºC. Bacterial suspensions were obtained from one colony of the previous culture on TSAs, inoculated in culture in tryptic soy broth (Oxoid) added with 1.5% NaCl (TSBs) and incubated at 22ºC for 24 h. Then, the cultures were centrifuged at 2000 xg for 20 min at 4ºC, and pellets resuspended in L-15 medium at an optical density (600 nm) equal to 1(10^8 cells ml^{-1}).

2.2. Isolation of the extracellular polysaccharidic fraction from Porphyridium cruentum

The red microalga *P. cruentum* (S.F. Gray) Nägeli obtained from the collection of Centro de Investigaciones Marinas de Cádiz, Cádiz, Spain was grown in *Porphyridium* medium [31] in batch culture at 25 ºC, with 12 h photoperiod for 7 days. Then, the algal culture was centrifuged at 10000 xg for 10 min.

The polysaccharidic fraction was obtained by selective precipitation of the exocellular polysaccharides from the culture supernatant with N-cetylpyridinium bromide (Cetavlon) 2% (p/v), following the method described by Morris et al. [30]. The pellet was redissolved with 4 M NaCl, and the polysaccharide was flocculated again with ethanol (96%), centrifuged (10000 xg, 10 min), dialyzed against 2M NaCl and finally lyophilized. Dilutions from the lyophilised extract were prepared in HBSS to achieve concentrations of 10 mg of lyophilised per ml, 5 mg ml^{-1}, 2 mg ml^{-1} and 1 mg ml^{-1}.

On the other hand, β-1,3-glucan from *Euglena gracilis* (BioChemika Fluka, Sigma) was used as a positive control. Ten mg of this compound were dissolved according to the manufacturer instructions, and diluted in HBSS to achieve concentrations of 10 mg ml^{-1}, 5 mg ml^{-1}, 2 mg ml^{-1} and 1 mg ml^{-1}.

2.3. Inoculation with the polysaccharidic fraction and immunization assay

Specimens of Senegalese sole of 50 g mean weight were randomly separated into groups of 20 fish each, and stocked into six 2500 l tanks with recirculating, aerated seawater at 22 ºC, 35‰ salinity and fed daily with a commercial pellet diet (Skreeting, Skreeting, Trouw España, Nutreco, Burgos, Spain).

Two groups of 20 fish were intraperitoneally injected with a dose of 500 µg of the extracellular polysaccharidic fraction from *P. cruentum* per fish. Phagocytes from two groups of sole were sampled at 2 and 8 days from algal extract administration. One group of them was intraperitoneally inoculated with bacterin of *P. damselae* subsp. *piscicida*, 24 h after the administration by intraperitoneal injection of the polyssacharide. The formalin-killed aqueous vaccine was prepared according to the following description. Briefly, the selected strain of *P. damselae* subsp. *piscicida* isolated from diseased sole in Spain was cultured on TSAs, and one colony was transferred to one tube containing 5 ml of TSBs for 18 h at 22 ºC. After incubation at 22 ºC for 18 h, an aliquot of the culture, 50 µl, was inoculated in a flask containing 50 ml TSBs and incubated at 22 ºC for 18 h with continuous shaking. When the culture achieved O.D.600 of 1.2, corresponding to 6 x 10^8 bacteria ml^{-1}, the cells were killed by addition of formaldehyde (1% final concentration) and incubated overnight. Sterility trials were performed by spreading an aliquot of the vaccine preparation on TSAs plates and incubating for 2 days at 22 ºC. The vaccine was administered by intraperitoneal injection (0.1 ml per fish). The other two groups of fish were used as the controls and phosphate buffer saline (PBS, pH 7.2) was inoculated instead of the algal polysaccharide. One of these groups was vaccinated with a bacterin against *P. damselae* subsp. *piscicida* as described above.

2.4. Isolation of head kidney phagocytes

Five specimens of soles of 200 g mean weight were sacrificed by an overdose of clave oil and head kidney phagocytes to test respiratory burst activity were isolated following the technique described by Secombes [32]. Briefly, the kidney was removed aseptically and pushed through a 100 μm nylon mesh with Leibovitz medium (L-15) containing 2% foetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin (Sigma), 0.1% (5 mg ml^{-1}) gentamicine sulfate (Sigma) (P/S/G) and 10 U heparine ml^{-1}. This cell suspension was layered on a 30 to 51% Percoll (Amersham) gradient and centrifuged at 600 xg for 30 min, without brake. Then, the bands separated at the interface were collected, centrifuged for 15 min at 500 xg and resuspended in L-15 medium supplemented with P/S/G. The viable cell concentration was determined after staining with trypan blue and microscope counting. Aliquots of 100 μl containing $1x10^7$ cells ml^{-1} in L-15 medium supplemented with P/S/G were added to 96-well microtitre plates. After 3 h incubation at 22ºC, non-adherent cells were removed and medium was substituted by L-15 and P/S/G supplemented with 2%FBS. Monolayers were incubated overnight at 22ºC, before use.

2.5. Respiratory burst activity

The generation of intracellular superoxide radicals by sole phagocytes, in response to *in vitro* contact with algal polysaccharide, was determined by the reduction of nitro-blue tetrazolium (NBT) according to the technique described by Secombes [32] and Boesen et al. [33]. Volumes of 20 μl containing 1, 2, 5 and 10 mg ml^{-1} of the lyophilized polysaccharidic fraction from *P. cruentum* were added to the wells containing phagocyte monolayers obtained as described above. The response of sole phagocytes to β-glucan from *Euglena gracilis* was also evaluated and 20 μl of serial dilutions containing 1, 2, 5 and 10 mg ml^{-1} to 12 wells with sole phagocyte monolayers. Response of sole phagocytes to the infection with *P. damselae* subsp. *piscicida* was determined after inoculation of algal polysaccharide or β-glucan treated monolayers with 20μl of bacterial suspensions containing 10^8 bacteria ml^{-1}. Phorbol myristate acetate (PMA, Sigma) (1 μg ml^{-1}) was used as a positive control to stimulate the respiratory burst activity of sole phagocytes (data not shown). The specifity of the

reaction was tested by adding superoxide dismutase (SOD) (300 I.U. per well) to some wells containing PMA-stimulated phagocytes (data not shown).

The effect of inoculation of polysaccharidic fraction to unvaccinated and vaccinated soles against *P. damselae* subsp. *piscicida* on the production of superoxide anions by sole phagocytes was also determined. In this case, phagocytes from control fish, fish inoculated with algal polysaccharide, and fish inoculated first with the algal polysaccharide and with the bacterin were isolated and monolayers prepared. In all the cases, NBT (100 µl) dissolved at 1 mg ml^{-1} in HBSS was added to the wells and phagocytes incubated at 22 ºC for 30 min. After incubation, cells were fixed in 70% methanol and the reduced formazan within phagocytes was solubilised by adding 120 µl 2M KOH and 140 µl dimethyl sulfoxide (DMSO, Sigma). Finally, absorbance was read at 630 nm in a multiscan spectrophotometer (UV-1601 Spectrophotometer, Whitakker Bioproducts).

2.6. Statistical analysis

Results are expressed as phagocyte stimulation index, calculated by dividing each sample value by the mean control value. Values above 1 reflect an increase and under 1 a decrease in each parameter. Data were statistically analysed by one-way analysis of variance (ANOVA) and Tukey's comparison of means using SPSS for Windows. Differences were considered statistically significant when $P< 0.05$.

3. Results

Results obtained *in vitro* after incubation of phagocytes with algal extracts are shown in Figure 1a. The data are expressed as a phagocytic stimulation index obtained by dividing superoxide anion produced by phagocytes incubated with algal polysaccharide or with algal polysaccharide plus bacteria by the values of phagocytes incubated without the algal polysaccharidic fraction. Data obtained after incubation with β-glucan instead of algal polysaccharide are expressed in Figure 1b. Results obtained show that only at the commercial β-glucan significantly enhanced the respiratory burst activity of sole phagocytes at the highest concentration (10 mg ml^{-1}), compared to non

treated and algal extract treated phagocytes. Thus, the polysaccharidic fraction from *P. cruentum* did not show ability to increase the respiratory burst activity of sole phagocytes, neither alone or in the presence of *P. damselae* subsp. *piscicida*, being stimulation index obtained 1.042 ± 0.069.

Superoxide anion production of phagocytes isolated from sole inoculated with *P. cruentum* polysaccharide and *P. damselae* subsp. *piscicida* bacterin are shown in Figure 2. Inoculation of the polysaccharide or the bacterin alone resulted in a significant decrease ($P<0.05$) of respiratory burst activity of phagocytes in the 24 h within the inoculation. However, this decrease was not observed when the polysaccharide and the bacterin were jointly inoculated on 7 days post inoculation.

4. Discussion

The results obtained show that *in vitro* contact of sole phagocytes with different concentrations of the polysaccharidic fraction of *P. cruentum* does not stimulate the respiratory burst activity of phagocytes. Other authors have demonstrated immunostimulatory effect on respiratory burst activity of phagocytes isolated from several fish species incubated with seaweed extracts [23, 34, 35]. Castro et al. [23, 24] suggested that stimulation on the respiratory burst activity of turbot phagocytes in the presence of water-soluble extracts of seaweed species such as *Ulva rigida* and *Chrondus crispus* was associated with polysaccharides. Nevertheless, the modulatory ability of the respiratory burst activity of fish phagocytes reported by Castro et al. [23] varied greatly for different species of red algae. Composition of the polysaccharidic fraction isolated from *P. cruentum* has not been determined. Thus, results obtained in this study could be due either to the absence of polysaccharides capable to stimulate sole phagocytes or to the presence in low concentrations of active substances in the extracts assayed. On the other hand, polysaccharides such as glucans derived from yeasts have been reported as important stimulants of respiratory burst activity in farmed fish [22, 36-38]. In this way, in this study a significant increase of the respiratory burst activity of sole phagocytes incubated with 10 mg ml^{-1} of a commercial β-glucan from *Euglena gracilis* has also been detected.

Intraperitoneal inoculation of the polysaccharidic fraction of *P. cruentum* to sole specimens did not induce an increase of the respiratory burst activity. On the contrary, the respiratory burst activity decreased in fish inoculated with polysaccharide fraction or with bacterin within 24 h post-inoculation. This decrease in respiratory burst activity may be due to a immunosupression caused by stress after handling [39, 40] as it is not observed after 7 days of the inoculation.

In short, the results obtained *in vivo* indicate that the polysaccharidic fraction from *P. cruentum* does not enhance the respiratory burst activity compared to non inoculated fish.

Acknowledgements

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Figure legends

Figure 1. Respiratory burst activity of sole phagocytes incubated with polysaccharidic fraction from *Porphyridium cruentum* **(a)** or β-glucan **(b)** (1, 2, 5, 10 mg ml^{-1}) in absence (■) or presence (□) of *Photobacterium damselae* subsp. *piscicida* ($2x10^6$ cells per well). Results are expressed as stimulation index (mean ± SE; n=15) obtained by dividing each sample value by its mean control value (HBSS or *P. damselae* subsp. *piscicida* cells). Symbol * denotes statistically significant differences ($P<0.05$) compared to control wells.

Figure 2. Relative superoxide anion production of sole phagocytes after inoculation of 500 mg of polysaccharidic fraction and measured after 24 h (P-24) or 7 days (P-7), or a bacterin (B-24 and B-7) or a combination of both, polysaccharidic fraction and bacterin (PB-24 and PB-7). Data are means (±SD) of ten wells of cells from three fish. Symbol * denotes statistically significant differences ($P<0.05$) respect to the control group, soles inoculated with PBS.

Figure 1a

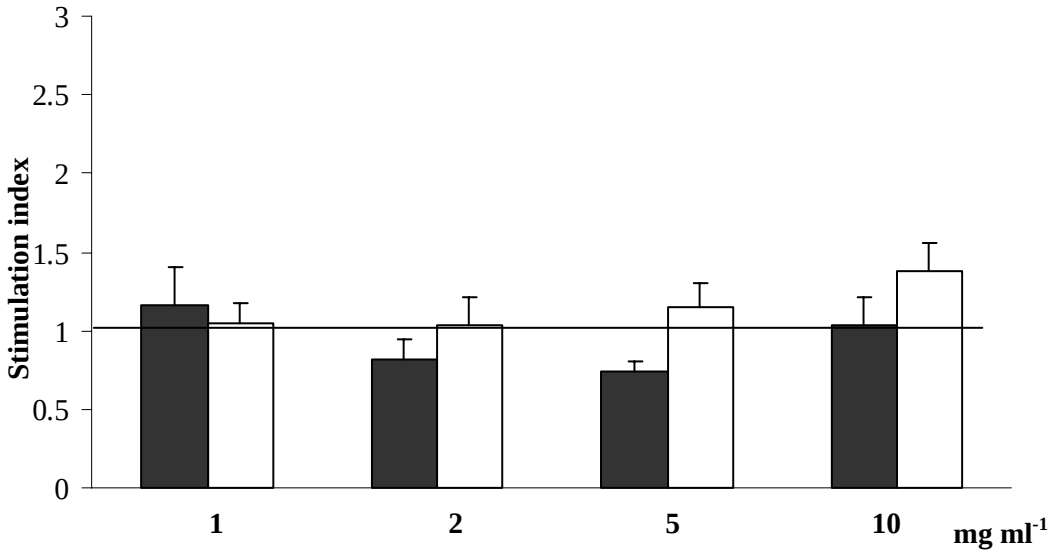


Figure 1b

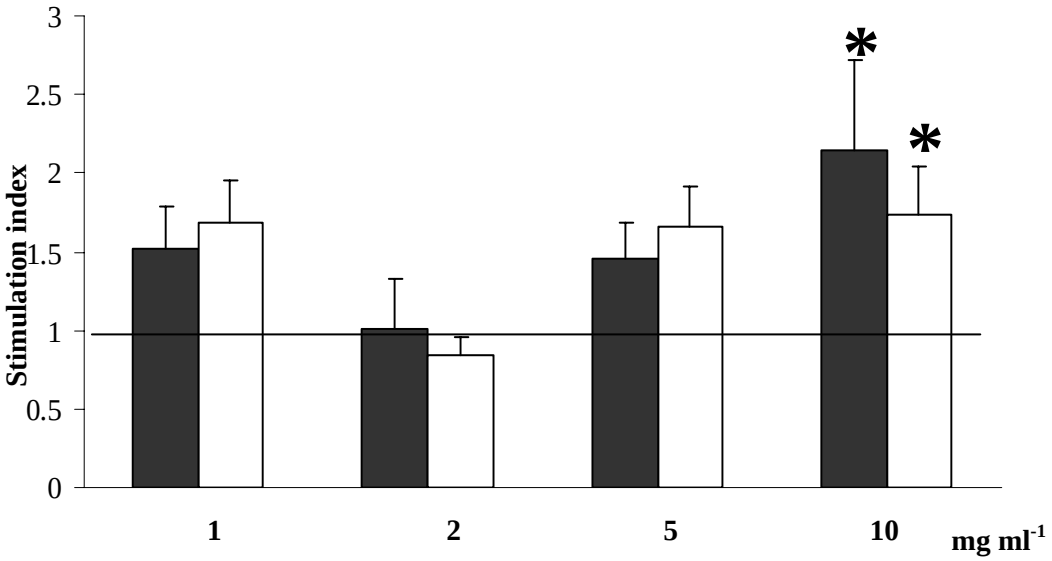
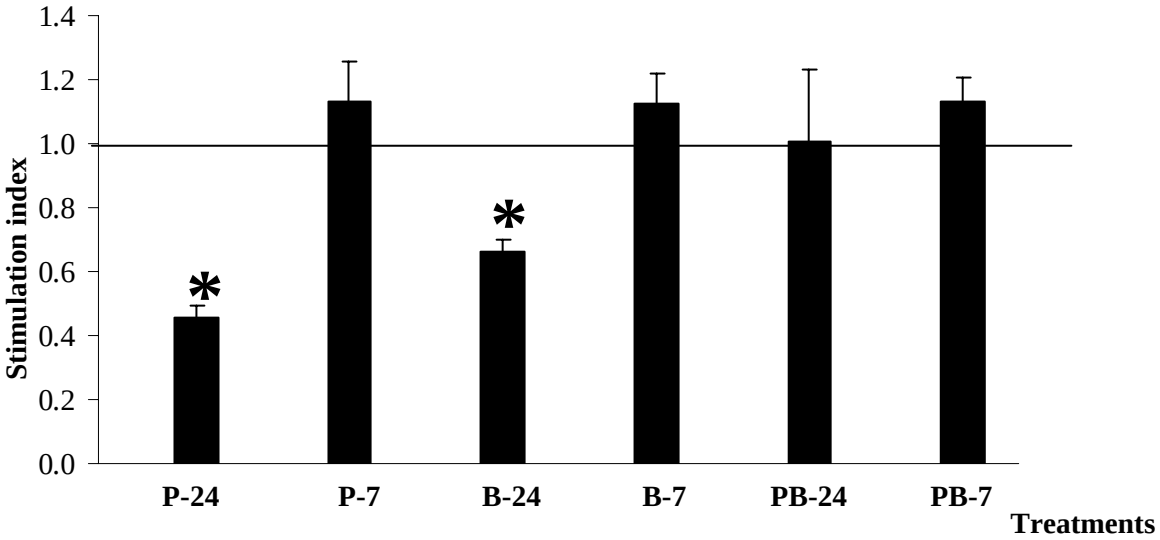


Figure 2


1.4
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1.0
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Stimulation index
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B-7
PB-24
PB-7
Treatments

ARTÍCULO 2.3.

ARTICLE 2.3.

Effect of dietary administration of probiotics on respiratory burst activity of phagocytes and intestinal microbiota of Senegalese sole (*Solea senegalensis*, Kaup 1858)

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Abstract

The effects of the dietary administration of two bacterial probiotic strains from the *Alteromonadaceae* family, Pdp11 and Pdp13, on Senegalese sole intestinal microbiota were studied. The fish were fed four different diets: control (non-supplemented), or diets supplemented with alginate alone, or as a carrier for Pdp11 (10^9 cfu g^{-1}) or Pdp13 (10^9 cfu g^{-1}) for sixty days. The effect of the different dietary treatments was assessed by measuring respiratory burst activity, protection against experimental infection with *Photobacterium damselae* subsp. *piscicida* and analysis of intestinal microbiota by 16S ribosomal RNA gene-targeted PCR-DGGE (denaturing gradient gel electrophoresis). We also evaluated, to which extend and how this technique could be effectively applied to examine bacterial diversity and dynamics of the sole (*Solea senegalensis*) intestinal microbiota, as influenced by the diet. Bacterial ribotypic diversity was determined using DGGE analysis of the V6 to V8 and V3 regions of 16S rRNA genes using two different sets of primers for PCR amplification. To our knowledge, this is the first report related to the effect of probiotics in the intestinal microbiota of sole, by cultivation-independent molecular ecological approaches.

Keywords: Probiotics; *Alteromonadaceae*; Intestinal microbiota; DGGE; Sole (*Solea* senegalensis, Kaup 1858); Teleosts.

1. Introduction

Senegalese sole culture (*Solea senegalensis*, Kaup 1858) is nowadays a very promising industry in the Mediterranean countries [1]. One of the most serious problems concerning sole production is the existence of infectious diseases, pseudotuberculosis caused by *Photobacterium damselae* subsp. *piscicida*, being the main limiting factor [2].

In fish farms, control of this bacterial pathogen is achieved by the administration of chemotherapeutic agents. However, over the last decade, drug-resistant strains carrying a transferable R-plasmid have evolved, making treatment with antimicrobial chemotherapeutics less successful [3]. Therefore, immunoprophylaxis has become the best way to prevent pseudotuberculosis [4].

Throughout the last 20 years, there has been a variety of studies analyzing the effectiveness of immunization in preventing pseudotuberculosis [5]. A divalent vaccine against *V. harveyi* and *P. damselae* subsp. *piscicida* for sole has been reported [6]. This, however, only provides protection for a short period. Therefore, searching for new prophylactic methods, such as immunostimulants or probiotics seems a very promising alternative.

Probiotics have been defined as live microbial preparations that improve the health and well-being of the host [7-10]. The research on probiotics for aquatic animals is increasing with the demand for environment-friendly aquaculture. Most of these studies have been related to challenge trials and suppression of pathogen growth by probiotic bacteria [11-21]. Recent works have been focused on the immunological enhancement of fish defence mechanisms by probiotic bacteria [22-27] and the effects that dietary administration of probiotics could exert on the intestinal microbiota of the host fish [28].

As immunomodulators, probiotics may enhance phagocytic activity and increase the production of reactive oxygen species by macrophages from species such as gilthead seabream [27, 29] and rainbow trout (*Onchorynchus mykiss*) [22]. The means of invasion and survival of *P. damselae* subsp. *piscicida* inside the host are still unknown, and while authors have reported the presence of intact bacteria inside fish cells,

suggesting the ability of the bacterium to survive as an intracellular pathogen [30-33], others have observed that this pathogen is highly susceptible to oxidative radicals generated during the macrophage respiratory burst [34, 35]. For this reason, the stimulation of the respiratory burst activity of Senegalese sole phagocytes could facilitate a more effective destruction of *P. damselae* subsp. *piscicida*.

Hence, the first objective of the present study was to evaluate the possible immunostimulatory effect of dietary administration of two probiotics [36-38] on respiratory burst activity of sole phagocytes. In addition, possible changes in the intestinal microbiota were evaluated by determining the genetic diversity of gut microbiota by DGGE (denaturing gradient gel electrophoresis) [39]. Several authors have reported on the composition of intestinal microbiota of different farmed fish [40-44], although none of them have studied sole microbiota. On the other hand, the possible effect of probiotics on fish intestinal microbiota is still largely unknown [28]. Thus, this is the first report on sole gut microbiota and the possible changes in response to probiotic treatment.

2. Materials and Methods

2.1. Microorganisms

Two bacteria strains isolated from gilthead seabream skin, Pdp11 and Pdp13, belonging to *Alteromonadaceae* family, *Shewanella* genus, were selected for this study because their *in vitro* characteristics suggested they could be considered as potential fish probiotics [36-38].

These strains were grown in tubes containing 5 ml of trypticase soy broth (Oxoid Ltd., Basingstoke, UK) supplemented with 1.5 % NaCl (TSBs) for 18 h at 22 ºC, with continuous shaking. Appropriate dilutions of the culture were spread onto plates of trypticase soy agar (Oxoid) supplemented with 1.5 % NaCl (TSAs). The number of culturable bacteria was determined by plate counting on TSAs. Bacteria were recovered from the plates, lyophilized and the concentration of lyophilized bacteria was adjusted per gram of pellet diet.

2.2. Fish and experimental design

Senegalese sole specimens (*Solea senegalensis*, Kaup, 1838) of 15-30 g weight, were kept in four 300 l seawater tanks (75 fish per tank), 36 ‰ salinity, at 22 ºC and fed with a commercial pellet diet (Gemma 0.5, Skreeting, Trouw España, Nutreco, Burgos, Spain). Specimens were sacrificed by overdose of clave oil.

Experimental diets were prepared in the laboratory from the commercial pellet diet. The diet preparation was carried out with alginate (0.5% kg^{-1}) and 50 mM calcium chloride (0.4% kg^{-1}) to facilitate the lyophilized bacteria incorporation to the pellet. Alginate and Ca Cl_2 were sprayed into the feed slowly, mixing with the required amount of lyophilized bacteria (10^9 cfu g^{-1}).

Thus, fish in each tank received one of four different diets: a commercial diet supplemented with Pdp11 (10^9 cfu g^{-1}) (diet A); the same diet supplemented with Pdp13 (10^9 cfu g^{-1}) (diet B); a diet consisting of the commercial diet (control without alginate) (diet C); and, finally, the fourth group of fish received a diet supplemented with alginate and calcium chloride (control with alginate) (diet D). Fish were fed at a rate of 15 g dry diet Kg^{-1} biomass (1.5 %) per day for 60 days. The biomass of the fish in each aquarium was measured at the beginning of the experiment and daily ratio was adjusted accordingly. No mortality was observed during the experiment.

Sampling was carried out at the end of the feeding trial, day 60, for the challenge test and study of intestinal microbiota. Samples for measurement of the respiratory burst activity were taken at the middle of the trial and at the end, at day 30 and 60, respectively.

2.3. Isolation of head kidney phagocytes

The influence of the treatment on respiratory burst activity was tested in phagocytes isolated from the kidney of soles following the technique described by Secombes [45]. Briefly, the kidney was removed aseptically and pushed through a 100 µm nylon mesh with Leibovitz medium (L-15) containing 2% foetal calf serum (FCS, Sigma), 1% penicillin-streptomycin (Sigma), 0.1 % (5 mg ml^{-1}) gentamicine (Sigma) (P/S/G) and 10 U heparine ml^{-1}. This cell suspension was layered on a 30 to 51% Percoll (Amersham) gradient and centrifuged at 600 × g for 30 min. Then, the bands

separated at the interface were collected, centrifuged for 15 min at 500 × g and resuspended in L-15 medium supplemented with P/S/G. The viable cell concentration was determined after staining with trypan blue and microscope counting. Aliquots of 100 µl containing $1x10^7$ cells ml^{-1} in L-15 medium supplemented with P/S/G were added to 96-well microtitre plates. After 3 h incubation at 22 ºC, non-adherent cells were removed and medium was substituted by L-15 and P/S/G supplemented with 2%FCS. Monolayers were incubated overnight at 22 ºC.

2.4. Respiratory burst activity

Fish of the four experimental groups, fed with diets A, B, C or D, were assayed for respiratory burst activity.

The generation of intracellular superoxide radicals by sole phagocytes was determined by the reduction of nitro-blue tetrazolium (NBT) according to the technique described by Secombes [45] and Boesen *et al.* [46]. Phagocyte monolayers were washed with L-15 medium and HBSS (Hank´s Balanced Salt Solution) to remove any trace of antibiotic. Then, NBT (100 µl) dissolved at 1 mg ml^{-1} in HBSS was added to the wells and the phagocytes incubated at 22 ºC for 30 min. Wells containing phagocytes were infected with *P. damselae* subsp. *piscicida* (10^8 bacterias ml^{-1}) and used to determine the response of the phagocytes to the fish pathogen. As a positive control phorbol myristate acetate (PMA, Sigma) (1 µg ml^{-1}), an activating agent of the respiratory burst, was used to stimulate the respiratory burst of non-infected phagocytes. The specificity of the reaction was tested by adding superoxide dismutase (SOD) (300 I.U. per well) to some wells containing PMA-stimulated phagocytes (data not shown).

After incubation, cells were fixed in 70% methanol and reduced formazan within phagocytes was solubilised by adding 120 µl 2M KOH and 140 µl dimethyl sulfoxide (DMSO, Sigma). Finally, absorbance was read at 630 nm in a multiscan spectrophotometer (UV-1601 Spectrophotometer, Whitakker Bioproducts).

2.5. Challenge with Photobacterium damselae *subsp.* piscicida

Groups of 10 fish (15-30 g weight) were challenged at the end of the feeding trial, after 60 days of beginning. For the challenge, three groups of fish were assayed, fed with diets A, B and C.

Soles were intraperitoneally injected with a dose of *P. damselae* subsp. *piscicida* ($Lg_{h41/01}$) of 5 x 10^4 cfu g^{-1} from a bacterial suspension in PBS (phosphate buffer saline) adjusted to 5 x 10^8 cfu ml^{-1}. As control, the same number of fish was inoculated with 0.1 ml PBS. Inoculated fish were followed daily for 10 days, and all mortalities were recorded, considering only those that could be linked to the bacterial challenge by re-isolation in pure culture from internal organs of dead fish.

2.6. Analysis of the sole intestinal microbiota

2.6.1. Sample collection

Three groups of fish were analyzed for intestinal microbiota composition, i.e. fish fed with diets A, B and C.

Per treatment group, three specimens of Senegalese sole were sacrificed at the end of the feeding trial (60 days), and whole intestines were collected and stored at -80 ºC until further analysis.

2.6.2. DNA isolation from intestinal content and probiotic cultures

The gut contents were homogenized in 4 ml PBS (phosphate buffer saline) pH 7.2, and a 1 ml aliquot was centrifuged at 1000 × g for 5 min. The supernatant was pre-treated by enzymatic digestion with 40 µl proteinase K (20 mg/ml) and 50 µl dodecyl sulphate (SDS) 10% and incubated at 65 ºC for 30 min. Subsequently, DNA extraction from the suspension was performed with the Fast DNA Spin kit for soil (Qbiogene, Inc., Carlsbad, CA) according to manufacturer's instructions. Agarose gel (1.5% [wt/vol]) electrophoresis in the presence of ethidium bromide was used to check visually for DNA quality and yield.

Pure cultures of probiotic strains were grown until exponential phase in TSBs, and then centrifuged at 2500 × g for 15 min. Pellets were washed with PBS and centrifuged again. DNA was extracted from the resulting pellet resuspended in 500 µl PBS with the Fast DNA Spin kit (Qbiogene, Inc., Carlsbad, CA).

2.6.3. PCR amplification

Two different sets of primers were tested in this study to select those that yielded the best results to compare DGGE patterns based on their resolution and observed diversity (Table 1). Primer set Bact-0968-GC-F / Bact-1401-R was used to amplify the V6 to V8 regions of the 16S rRNA gene [47, 48] and PRBA-338-GC-F and PRUN-518-R amplified the V3 hypervariable region [44]. PCR was performed using the *Taq* DNA polymerase kit from Life Technologies (Gaithersburg, Md). PCR mixtures (50 µl) contained 0.5 µl *Taq* polymerase (1.25 U), 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 3 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 5 pmol of the primers, 1 µl of DNA template, and UV-sterilized water. The samples were amplified in a T1 thermocycler (Whatman Biometra, Göttingen, Germany), and the cycling conditions for each pair of primers are listed in Table 1. Aliquots (5 µl) were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels containing ethidium bromide to check for product size and quantity.

2.6.4. DGGE analysis

The amplicons obtained from the intestinal lumen-extracted DNA and the probiotic strains were separated by DGGE according to the specifications of Muyzer *et al.* [39] using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA). Electrophoresis was performed in an 8% polyacrylamide gel (37.5:1 acrylamide-bisacrylamide; dimensions, 200 by 200 by 1 mm) using a 30 to 55% denaturing gradient for separation of PCR products. The gels contained a 30 to 55% gradient of urea and formamide increasing in the direction of the electrophoresis. A 100% denaturing solution contained 7 M urea and 40% (vol/vol) deionized formamide. PCR samples were applied to gels in aliquots of 13 µl per lane. The gels were electrophoresed for 16 h at 85 V in 0.5 X TAE (20 mM Tris acetate [pH 7.4], 10 mM sodium acetate, 0.5 mM Na_2-EDTA) [50] buffer at a constant temperature of 60 ºC and subsequently stained with $AgNO_3$ [51].

Gel image processing and comparative analysis of banding patterns was done with Bionumerics version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarity between DGGE profiles was determined by calculating similarity indices of

the densitometric curves of the profiles compared using the Pearson product-moment correlation [52, 53].

Clustering of DGGE patterns was achieved by construction of dendrograms using UPGMA (Unweighted Pair Groups Method using Arithmetic Averages).

3. Results

3.1. Respiratory burst activity

Respiratory burst activity of phagocytes isolated from assayed soles was expressed as stimulation index, which was calculated as the ratio between the absorbance obtained from phagocytes from fish fed with the different experimental diets and absorbance from fish fed with the control diet, without any supplementation.

The respiratory burst activity of phagocytes was measured at days 30 and 60. Only the phagocytes from sole specimens fed with the diet supplemented with probiotic strain Pdp11 showed a significant increase ($P<0.05$) of respiratory burst activity after 60 days of feeding regardless of the phagocyte incubation with HBSS (Figure 1a) or with *P. damselae* subsp. *piscicida* (Figure 1b).

On the contrary, Pdp13 addition did not lead to a significant enhancement of respiratory burst activity.

3.2. Challenge trial

Mortality in the groups of Senegalese sole challenged with *P. damselae* subsp. *piscicida* $Lg_{h41/01}$ was observed from the second day post-challenge (Fig. 2). The cumulative percentage of mortality observed in the groups fed with commercial diet and Pdp11 supplemented diet was 100% three days after the challenge. Only when the fish were fed with the Pdp13 supplemented diet, a 20% survival was achieved. Hence, the mortality in fish receiving the diet supplemented with Pdp13 strain was lower (80%) than that in fish fed with the diet supplemented with Pdp11 strain and those receiving the normal diet (100%). All the mortalities in the experimental groups were caused by *P. damselae* subsp. *piscicida* $Lg_{h41/01}$, as demonstrated by culture from the kidney and liver (data not shown).

3.3. Intestinal microbiota analysis

In order to compare DGGE patterns of the intestinal microbiota of soles receiving the different diets assayed, two sets of primers, targeting different variable regions of the 16S rRNA gene, were used to select the optimal set with respect to diversity and resolution. DGGE profiles were compared using Pearson's similarity coefficient analysis, a test that considers quantitative values of a densitometric curve, representing bands and their intensities.

Profiles were compared within (Table 2) and between experimental groups (Table 3). The results obtained show that the V6-V8 targeting primers Bact-0968-GC-F and Bact-1401-R detected higher similarity in DGGE patterns among fish from the same group (intragroup) in two of the three groups assayed, control group, without any supplementation, and fish fed with Pdp11 strain (Table 2). On the other hand, the same set of primers (Bact-0968-GC-F and Bact-1401-R) detected lower percentages and more pronounced differences with respect to intergroup similarities (Table 3). There is only one statistically significant difference ($P<0.05$) comparing the similarity percentage obtained between fish from the control group and fish fed with Pdp13 strain (41.54±2.88) and the results obtained comparing control group with fish fed with Pdp11 (57.08±2.91) or fish fed with Pdp11 strain respect to fish fed with Pdp13 strain (65.08±1.87).

In addition, similarity values of profiles obtained with Bact-0968-GC-F and Bact-1401-R were used to construct a dendrogram by UPGMA (Unweighted Pair Groups Method using Arithmetic Averages) (Fig. 3). This analysis confirmed above observations, i.e. higher similarity of profiles within as compared to between experimental groups. Also, control samples clustered separately from the two probiotic treatment group, reflecting the lower inter-group similarities (Table 3).

4. Discussion

Most of the bacteria, for which a possible immunostimulant effect in fish was studied, belong to the group of lactic acid bacteria [54], while *Vibrio* and *Pseudomonas* are the most common genera in marine fish [55]. Therefore these genera could be the

most efficient probiotics for aquaculture, as a consequence of the specificity of aquatic microbiota [8]. Hence, we assessed the probiotic potential of bacteria of the genus *Shewanella*, naturally present on seabream skin [36]. Moreover, the selection of the probiotic Pdp11 was based on its adhesion to intestinal mucus, its resistance to intestinal mucus and bile [37, 38] and its ability to increase several immunological parameters in gilthead seabream [27, 29].

Despite of their affiliation within the same genus, *Shewanella*, and same origin of both microorganisms assayed in this study, their effect on the respiratory burst activity was completely different, as only Pdp11 strain produced statistically significant increase in superoxide anion production by sole kidney phagocytes. This could suggest that the immunostimulation of the respiratory burst of sole phagocytes is strain-dependent rather than a general characteristic of a bacterial species. This strain-dependence has been described for other characteristics such as the mucus adhesion of certain isolates of lactic acid bacteria [56] and fish isolates [37, 38]. In a previous work it was reported that Pdp11 strain did not induce an increase on the respiratory burst activity of phagocytes from gilthead seabream when the microorganism was administered heat-inactivated [27]. These results are in agreement with Panigrahi *et al.* [24], who indicated that probiotic viability could probably influence the induced immune responses. It was found that viable lactic acid bacteria were more efficient in enhancing certain aspects of immune response in rainbow trout (*Oncorhynchus mykiss*) compared to non-viable heat-killed cells, although the respiratory burst activity in this case, was not affected.

Although several authors reported the induction of respiratory burst activity by probiotics [23, 57-59]. The fact that the potential probiotic strain Pdp13 did not increase superoxide anion production does not rule out that the possibility to induce a significant change in other immunological parameters. This is supported by the fact that we observed increased survival after the challenge with the pathogenic bacteria *P. damselae* subsp. *piscicida* in fish fed with diet supplemented with Pdp13 strain, compared to control fish (fed with normal diet) and fish fed with the diet supplemented with Pdp11. Although the degree of protection achieved with Pdp13 was not very high (i.e. 20% survival), the reduced and retarded mortality might provide additional time that could be useful to prevent the infection by using other different treatments.

Several authors have reported a stimulatory effect of different probiotics on different immunological parameters, such as phagocytic [22, 24, 26, 27, 58], complement [24, 27], lysozyme [22, 24] or cytotoxic [26, 27] activities. Moreover it has been described that probiotic treatment resulted in an increase of immunoglobulin levels in serum [23, 59]. Hence, Pdp13 strain can be considered as probiotic although further studies should be carried out, evaluating additional immunological aspects.

On the other hand, the probiotic bacteria have to be administered at an optimal dose that may depend on the size and species of experimental fish and used probiotic strain [23]. This might explain different effects of both probiotic strains observed in this study when applied to sole, and differences of Pdp11 obtained for sole (this study) and gilthead seabream [27].

Besides the effect of the probiotics on the immune response of sole, the other objective of this work was to assess changes in bacterial community structure of the sole intestine, as influenced by dietary treatment with probiotics. The development of molecular methods for studying microbial communities has recently also resulted in numerous works focusing on fish microbiota [20, 40-44, 60]. Nevertheless, no study has been reported evaluating possible shifts in fish intestinal microbiota in response to probiotics administration. In the present study, PCR-DGGE has been applied to the analysis of fragments derived from different hypervariable regions of 16S rRNA genes. The set of primers selected are specific for all bacteria (V6-V8 region), and were previously used by several authors for the analysis of dietary effects on intestinal microbiota [47-49]. The results obtained in this study showed very simple patterns. Muyzer *et al.* [39] described that communities with a few dominant species will produce simpler patterns and that less abundant species may not be adequately represented in the community pattern. It could be possible that some of these bands may not represent individual species, but rather groups which have the same relative G+C content within the 16S V6-V8 region and have comigrated [61, 62]. These limitations may account in part for the decreased band number in the present study and may also have influenced the apparent diversity and similarity values [63]. The use of universal primers (e.g. those targeting the V3 or V6-V8 region of the 16S rRNA gene) allows any bacterial community to be analyzed, although in case of an ecosystem with a relatively high

bacterial diversity only the dominant microbiota (i.e. populations with relative abundances of > 1%) will be visualized on the DGGE gels [64].

Similar to the situation described by several authors in homeotherms [64, 65], each individual fish showed a unique DGGE pattern. As expected, the fish intestinal microbiota fluctuates over time and is more dependent on the environment than homeothers [8].

In our study, the only significant change in the intestinal microbiota could be observed in intergroup comparisons after comparing fish from the control group with fish fed with strain Pdp13. This might partly be explained by the fact that closely related populations were already installed in the tissue, as corresponding bands were also observed in control groups. This is in accordance with the idea that the microbiota of fish intestine consists of bacteria that are also present in the surrounding water, but which are able to persist and multiply in the environment provided by intestinal tract [66, 67]. Therefore, the gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, *Vibrio* being one of the most abundant genera in marine fish [55]. The probiotics were isolated from the skin of another farmed fish, gilthead seabream. It can be expected that similar microbiota colonize both hosts. Further studies using direct sequencing of excised bands and 16S rRNA gene clone library analysis need to be carried out to confirm that these bands correspond to populations identical to Pdp11 or Pdp13 strains.

Although probiotics did not produce dramatic changes in the intestinal microbiota detected by DGGE analysis, this does not imply that they do not exert any effect. According to Ouwehand *et al.* [68], it is not necessary that a probiotic induces changes in intestinal microbiota or so-called colonization to exert an effect locally or during transient passage through the gastrointestinal system. In fact, the variation of the microbiota in fish is substantial and fluctuates strongly on a daily basis [17, 20, 24]. Therefore, stabilisation of the protective intestinal microbiota may be a means of improving the natural resistance to potential pathogens.

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Figures legends

Table 1. Primers and PCR conditions used in this study.

Figure 1a. Respiratory burst activity of kidney phagocytes from sole specimens fed diets containing alginate alone, or in combination with 10^9 cfu g^{-1} Pdp11 or 10^9 cfu g^{-1} Pdp13 for 30 days (■) or 60 days (□). Results are expressed as stimulation index obtained by dividing each sample value by the mean control value. Symbol * denotes statistically significant differences ($P<0.05$) with respect to the control group.

Figure 1b. Respiratory burst activity of kidney phagocytes from sole specimens fed diets containing alginate alone, or in combination with 10^9 cfu g^{-1} Pdp11 or 10^9 cfu g^{-1} Pdp13 for 30 days (■) or 60 days (□), and incubated with *Photobacterium damselae* subsp. *piscicida* (10^8 bacteria ml^{-1}). Results are expressed as stimulation index obtained by dividing each sample value by the mean control value. Symbol * denotes statistically significant differences ($P<0.05$) with respect to the control group.

Figure 2. Cumulative percentage of mortality of fish after challenge with $5x10^7$ ufc ml^{-1} ($5x10^4$ ufc g^{-1}) of *P. damselae* subsp. *piscicida*.

Table 2. Intragroup comparison using Pearson's coefficient.

Table 3. Intergroup comparison using Pearson's coefficient.

Figure 3. UPGMA dendrogram of Pearson correlations between 16S rRNA gene-targeted DGGE fingerprints of bacterial communities in sole intestinal samples.

Table 1

Primer	Sequence (5'-3')	PCR	Reference
Bact-0968-GC-F	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	94 ºC for 2 min and 35 cycles of 95 ºC for 30 s, 56 ºC for 40 s, 72 ºC for 1 min, and 72 ºC for 5 min (final extension)	[47-49]
Bact-1401-R	CGG TGT GTA CAA GAC CC		
PRBA-338-GC-F	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG	92 ºC for 2 min and 30 cycles of 92 ºC for 1 min, 55 ºC for 30 s, 72 ºC for 1 min, and 72 ºC for 6 min (final extension)	[44]
PRUN-518-R	ATT ACC GCG GCT GCT GG		

*Primer with a 40-bp GC clamp at the 5'end.

Figure 1a

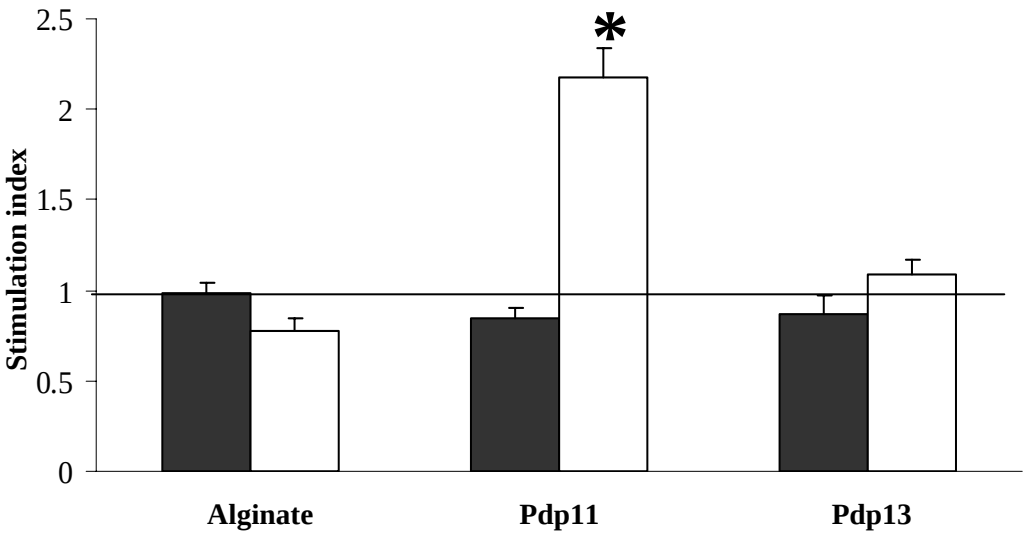


Figure 1b

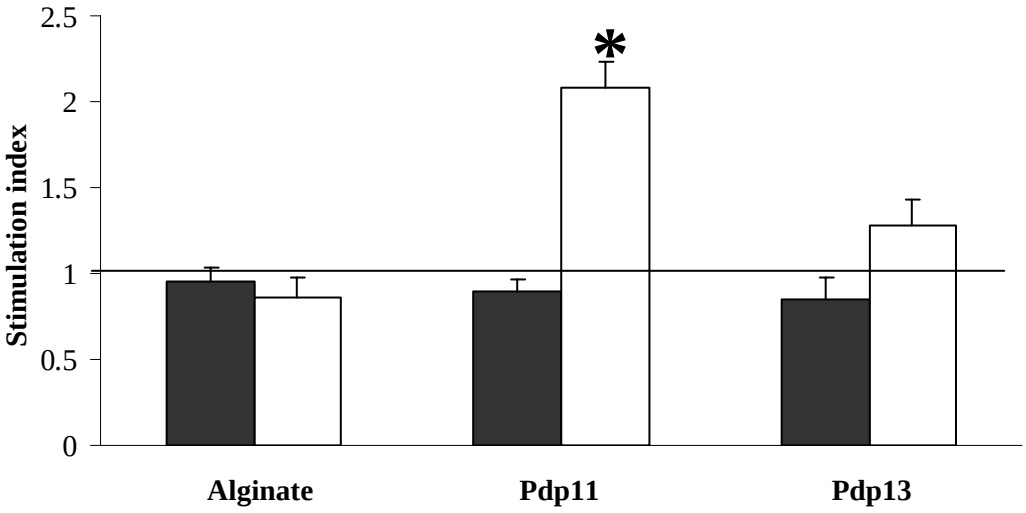


Figure 2

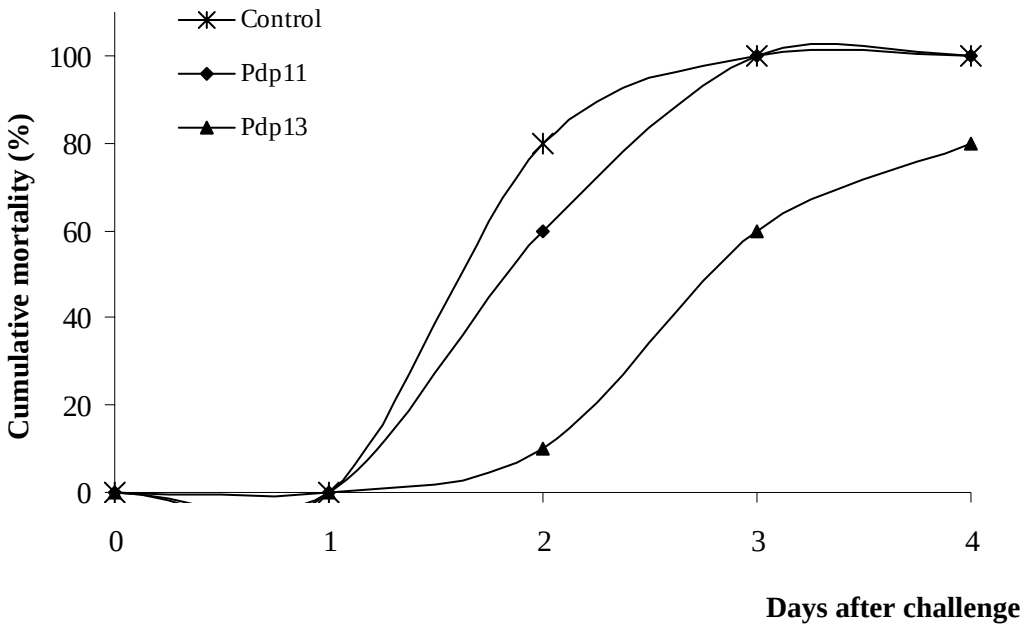

Control
Pdp11
Pdp13
Cumulative mortality (%)
100
80
60
40
20
0
0
1
2
3
4
Days after challenge

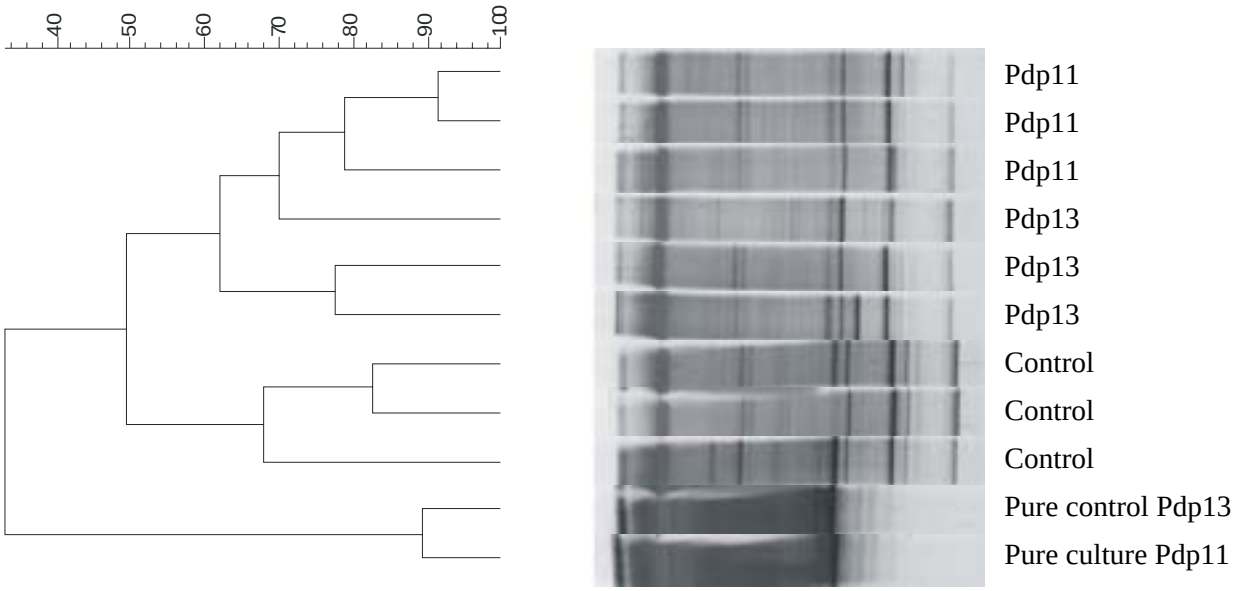
Table 2

Primer	Control	Pdp11	Pdp13
Bact-0968-GC-F & Bact-1401-R	72.85±7	83.01±4.22	65.81±4.92
PRBA-338-GC-F & PRUN-518-R	71.4±4.67	71.33±7.19	73.11±5

Table 3

Primer	Bact-0968-GC-F & Bact-1401-R	PRBA-338-GC-F & PRUN-518-R
Control-Pdp11	57.08±2.91	68.95±4.52
Control-Pdp13	41.54±2.88	70.21±3.82
Pdp11-Pdp13	65.08±1.87	79.78±4.18

Figure 3


40
50
60
70
80
90
100
Pdp11
Pdp11
Pdp11
Pdp13
Pdp13
Pdp13
Control
Control
Control
Pure control Pdp13
Pure culture Pdp11

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