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Selección y evaluación de cepas probióticas para la prevención de la Lactococosis en la trucha arco iris (Oncorhynchus mykiss)

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SELECCIÓN Y EVALUACIÓN DE CEPAS PROBIÓTICAS PARA LA PREVENCIÓN DE LA LACTOCOCOSIS EN LA TRUCHA ARCO IRIS (ONCORHYNCHUS MYKISS)

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Selección y evaluación de cepas probióticas para la prevención de la Lactococosis en la trucha arco iris (*Oncorhynchus mykiss*)

Memoria presentada por **Tania Pérez Sánchez**
Para optar al grado de Doctor
julio 2011

Dr. IMANOL RUIZ ZARZUELA, Profesor Ayudante Doctor del Departamento de Patología Animal de la Facultad de Veterinaria de la Universidad de Zaragoza, y Dr. JOSÉ LUIS BALCÁZAR ROJAS, Investigador del Grupo de Microbiología del Institut Català de Recerca de l'Aigua, como Directores,

C E R T I F I C A N :

Que Dª. TANIA PÉREZ SÁNCHEZ ha realizado bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada "Selección y evaluación de cepas probióticas para la prevención de la Lactococosis en la trucha arco iris (*Oncorhynchus mykiss*)" que se ajusta con el Proyecto de Tesis presentado y cumple las condiciones exigidas para optar al Grado de Doctor por la Universidad de Zaragoza, por lo que autorizan su presentación como compendio de publicaciones y con la mención "Doctor Europeo" para que pueda ser juzgada por el Tribunal correspondiente.

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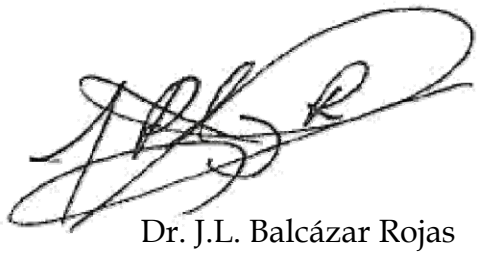

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Índice

I. Justificación 1

II. Introducción 3

I. Presentación de trabajos y justificación de unidad temática 3

II. Estado actual de la acuicultura 5

III. Lactococosis 7

IV. Probióticos 12

IV.1. Selección de cepas probióticas 14

IV.2. Mecanismos de acción de los probióticos 15

IV.2.1. Exclusión competitiva 15

IV.2.2. Optimización de la nutrición y la digestión 16

IV.2.3. Estimulación de la respuesta inmune 17

IV.3. Principales microorganismos probióticos utilizados en acuicultura 18

IV.3.1. Bacterias Gram positivas 19

IV.3.2. Bacterias Gram negativas 21

IV.3.2. Levaduras 22

III. Publicaciones **25**

I. Fish & Fisheries (en revisión) 25

II. Mucosal Immunology, 2010 49

III. Journal of Fish Diseases, 2011 57

IV. Fish & Shellfish Immunology, 2011 69

V. International Journal of Systematic and Evolutionary Microbiology, 2011 77

IV. Resumen general **95**

I. Resumen 95

I.1. Resumen 95

I.2. Summary 96

II. Objetivos de los trabajos presentados 98

III. Metodología 100

III.1. Animales 100

III.2. Instalaciones 100

III.3. Aislamiento e identificación de cepas probióticas con efecto inhibitorio frente a *Lactococcus garvieae* en la trucha arco iris 101

III.4. Caracterización de las cepas probióticas seleccionadas con efecto inhibitorio frente a *L. garvieae* 102

III.5. Evaluación de la supervivencia tras la administración de cepas probióticas para la prevención de la Lactococosis en la trucha arco iris 105
III.6. Evaluación *in vivo* de la capacidad de colonización de las cepas bacterianas con potencial probiótico 105
III.7. Evaluación in vivo de la capacidad protectora de las cepas probióticas seleccionadas frente a *L. garvieae* 106
III.8. Identificación de una nueva subespecie, *Lactococcus lactis* subsp. *tructae* subsp. *nov*. 107

V. Conclusiones **109**
I. Conclusiones 109
II. Conclusions 110

VI. Bibliografía **111**

VII. Apéndices **121**
I. Características de las revistas 121
II. Contribución del doctorando 123

I. Justificación

La presente tesis doctoral está constituida por un compendio de trabajos de investigación previamente publicados y/o en proceso de publicación en diversas revistas científicas de carácter internacional.

A continuación, se presentan las referencias bibliográficas de cada uno de los artículos mencionados:

1. Tania Pérez-Sánchez, José L. Balcázar, Ignacio de Blas, Imanol Ruiz-Zarzuela. Probiotics in aquaculture: a current assessment. ***Fish & Fisheries***. (en revisión).

2. Tania Pérez, José Luis Balcázar, Imanol Ruiz-Zarzuela, Nabil Halaihel, Daniel Vendrell, Ignacio de Blas, José Luis Múzquiz. Host-microbiota interactions within the fish intestinal ecosystem. ***Mucosal Immunology***. 2010; 3: 355-360.

3. Tania Pérez-Sánchez, José Luis Balcázar, Yaneisy García, Nabil Halaihel, Daniel Vendrell, Ignacio de Blas, Daniel Merrifield, Imanol Ruiz-Zarzuela. Identification and characterization of lactic acid bacteria isolated from rainbow trout *Oncorhynchus mykiss* (Walbaum) with inhibitory activity against *Lactococcus garvieae*. ***Journal of Fish Diseases***. 2011; 34: 499-507.

4. Tania Pérez-Sánchez, José Luis Balcázar, Daniel Merrifield, Oliana Carnevali, Giorgia Gioacchini, Ignacio de Blas, Imanol Ruiz-Zarzuela. Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection. ***Fish & Shellfish Immunology***. 2011; 31; 196-201.

5. Tania Pérez, José L. Balcázar, Álvaro Peix, Ángel Valverde, Encarna Velázquez, Ignacio de Blas, Imanol Ruiz-Zarzuela. *Lactococcus lactis* subsp. *tructae* subsp. *nov*. isolated from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). ***International Journal of Systematic and Evolutionary Microbiology***. 2010; doi:10.1099/ijs.0.023945-0.

II. Introducción

I. Presentación de los trabajos y justificación de su unidad temática

Durante los últimos años la acuicultura se ha convertido en una de las actividades productivas con una mayor expansión y capacidad de desarrollo en todo el mundo. Sin embargo, de forma paralela y como consecuencia del carácter intensivo de las instalaciones acuícolas, se ha observado un incremento exponencial tanto en el número de patologías como en la gravedad de las mismas, incidiendo en la mayor parte de los casos un uso indiscriminado de agentes terapéuticos como elección más eficaz para el control de las mismas, propiciando al mismo tiempo la aparición de una gran variedad de agentes patógenos y de resistencia antimicrobiana.

En la actualidad, dentro de las patologías de origen bacteriano destacan por su enorme repercusión económica y sanitaria, aquellos procesos sistémicos causados por cocos Gram positivos; especialmente debidos a *L. garvieae*, agente etiológico responsable de la Lactococosis, enfermedad de marcado carácter estacional que afecta principalmente a la producción de trucha arco iris. Uno de los aspectos más controvertidos de este proceso es el control y prevención del mismo, ya que en la mayor parte de las ocasiones se hace muy complejo, debido fundamentalmente a las limitaciones legislativas existentes en cuanto a la prescripción y empleo de sustancias quimioterápicas, así como a los altos índices de estrés provocado por una excesiva manipulación de los animales al tener que administrar los productos vacunales mediante inyección intraperitoneal (IP). Esto hace que la utilización de probióticos cobre cada vez mayor fuerza como una alternativa eficaz frente a los tratamientos más convencionales puesto que se ha observado que la administración de éstos de una forma regular es capaz de inhibir la proliferación de agentes patógenos mediante la activación de una serie de mecanismos en el organismo hospedador que favorecen los fenómenos de exclusión competitiva, un incremento de la respuesta inmunitaria así como la producción de sustancias antibacterianas.

En este contexto, el presente trabajo de investigación se ha enfocado inicialmente en la selección y caracterización de cepas bacterianas con potenciales propiedades probióticas para la prevención de la Lactococosis en la trucha arco iris.

Por todo ello, en el primer manuscrito hemos considerado necesario una evaluación del estado actual de desarrollo de los probióticos en acuicultura.

En el segundo manuscrito presentamos una profunda revisión bibliográfica sobre las interacciones más importantes que tienen lugar entre la microbiota intestinal de los peces y aquellos microorganismos con propiedades probióticas, incidiendo en los principales mecanismos de acción en el organismo hospedador.

Posteriormente, en el tercer manuscrito se recoge la selección de aquellas cepas bacterianas, capaces de producir sustancias inhibitorias frente a *L. garvieae,* agente patógeno en estudio. Así mismo, se ha llevado a cabo la caracterización de algunas de sus principales cualidades probióticas, como la velocidad de crecimiento, la hidrofobicidad y la tolerancia a bajas condiciones de pH y elevadas concentraciones de bilis.

En el cuarto manuscrito se ha estudiado la capacidad de colonización de las cepas probióticas previamente seleccionadas, así como la capacidad de incrementar los mecanismos de defensa del hospedador aumentando de esta forma la resistencia a la Lactococosis.

Finalmente, queremos destacar que tras el proceso de selección e identificación de las cepas candidatas se ha identificado una nueva subespecie perteneciente al género *Lactococcus*, a la que se ha denominado *Lactococcus lactis* subsp. *tructae* subsp. *nov*. (quinto manuscrito)..

II. Estado actual de la acuicultura

La acuicultura, se define como "*el cultivo de organismos acuáticos bajo condiciones controladas o semi-controladas*", en la que están implicadas una gran variedad de especies, gran parte de ellas destinadas al consumo humano (Stickney, 2009).

Según los últimos datos aportados por el informe sobre el "*Estado mundial de la acuicultura y la pesca en 2010*", la producción acuícola mundial, incluida la pesca de captura, alcanzó en el año 2009, 145 millones de toneladas, de las cuales alrededor del 38% del suministro total de pescado correspondió a la acuicultura; un incremento tres veces mayor que la producción mundial de carne contabilizando el sector avícola y vacuno juntos, en el mismo período. Algunos factores tan importantes como la necesidad de buscar soluciones a la sobreexplotación de los recursos naturales existentes, el estancamiento de la producción pesquera y/o la puesta a punto de la tecnología necesaria han incidido notablemente en este espectacular crecimiento (FAO, 2010).

Por otro lado, en Europa, la acuicultura constituye igualmente, una fuente cada vez más importante de pescado, destacando la producción de especies de un alto valor comercial (salmón, trucha, dorada, lubina, etc.) y los moluscos (APROMAR, 2007). Sin embargo, esta importancia no es la misma en todos los países de la Unión; en algunos como en España su relevancia económica y social supera ya a la de la pesca extractiva, aportando además el 25% de la producción total europea (JACUMAR, 2007).

Sin embargo, conforme se desarrollan nuevos métodos intensivos de producción y mejora el nivel de control sobre los procesos productivos se ha observado un incremento notable en el número y aparición de enfermedades infecciosas, constituyendo el impacto de estas patologías uno de los principales retos que debe resolver la acuicultura moderna, sobre todo por las graves pérdidas económicas que están ocasionando al sector.

Las alteraciones sobre las condiciones ambientales ocasionadas por las actividades acuícolas provocan situaciones que inducen cambios en la microbiota bacteriana y las interrelaciones con el resto de organismos presentes en el medio acuático. Estos desequilibrios pueden manifestarse finalmente como procesos patológicos de carácter multifactorial (McVicar, 1997). A la presentación de factores ambientales adversos y la presencia y/o naturaleza de algunos microorganismos patógenos debemos añadir determinadas características ligadas al propio hospedador cuya interacción con el agente patógeno y el medio puede desencadenar en una ruptura del

equilibrio ecológico existente entre estos factores y favorecer la presentación de diversas patologías (Hedrick y cols., 1998).

La mayor parte de las bacterias presentes en el medio acuático actúan como patógenos oportunistas, sin embargo en determinadas circunstancias pueden comportarse como agentes patógenos, generalmente en hospedadores ya dañados o debilitados. Para combatir este tipo de procesos, los peces son tratados frecuentemente con antimicrobianos, sin embargo, el uso continuado de estos compuestos ha propiciado un incremento en la aparición de bacterias resistentes por lo que estos tratamientos no resultan efectivos, y en muchos casos, aumentan los costes de producción.

El futuro de la acuicultura comprende por tanto el desarrollo de prácticas de cría sostenibles, mediante la reducción progresiva del uso de antibióticos y la búsqueda de nuevas y mejores herramientas para el control de los procesos patológicos, que conlleven además, una mejora en las producciones y un incremento en la resistencia de los animales.

III. Lactococosis

Dentro de las patologías de origen bacteriano que afectan a los peces, cabe destacar la Lactococosis, cuyo agente etiológico es *Lactococcus garvieae*, enfermedad de gran relevancia que afecta a numerosas especies de peces tanto dulceacuícolas (trucha arco iris, tilapia, etc.) como marinas (seriola, múgil, etc.) y que causa importantes pérdidas económicas, de hecho constituye una de las patologías que mayor mortalidad produce en la cría intensiva de trucha arco iris.

L. garvieae es una bacteria incluida dentro de la familia Streptococcaceae, género *Lactococcus* descrita por primera en Inglaterra a partir de un caso de mamitis en ganado vacuno (Collins y cols., 1983). También ha sido aislado, a partir de diferentes muestras procedentes del tracto urinario, sangre, y procesos neumónicos, en humanos, lo que ha sugerido por parte de algunos autores que pueda tratarse de un potencial agente zoonótico (Fefer y cols., 1998).

Las primeras infecciones causadas por *L. garvieae* se describieron en Japón en el año 1958 (Hoshina y cols., 1958), y desde entonces se ha ido extendiendo paulatinamente por el resto del mundo, causando importantes pérdidas en el sector acuícola, debido a la mortalidad inducida (superior al 50%), la disminución de la tasa de crecimiento y la imposibilidad de comercializar los peces afectados (Vendrell y cols., 2006). En España, los primeros brotes de Lactococosis datan de finales de los años 80 en piscifactorías de trucha arco iris (Palacios y cols., 1993).

Desde un punto de vista morfológico es un coco Gram positivo, inmóvil y se dispone generalmente formando parejas o cadenas cortas. En medio sólido las colonias son esféricas, de superficie lisa y coloración blanquecina. Puede crecer en un rango de temperaturas muy amplio, entre 4 y 45°C, siendo 37°C la temperatura óptima para el crecimiento de la bacteria (Vendrell y cols., 2006). Dicho crecimiento puede darse en medios con pH 9.6 y que contengan un 6.5% de NaCl, 40% de sales biliares y 0.1% de azul de metileno (Kusuda y cols., 1999). También se desarrolla en medios de cultivo Agar Infusión Cerebro Corazón (BHIA), Agar Tripticasa Soja (TSA) y Agar Sangre, entre otros. Según Cheng y Chen (1999), las condiciones óptimas de crecimiento para este patógeno son en medio Caldo Infusión Cerebro Corazón (BHIB), a pH 7-8 y a una temperatura de 25-30°C.

Los primeros signos clínicos de la enfermedad son anorexia, melanosis, letargia y natación errática. Externamente los peces enfermos presentan exoftalmia, que puede ser mono o bilateral, hemorragias periorbitales e intraoculares, en la base de las aletas, en la región perianal, en el opérculo y en la boca (Vendrell y cols., 2006). Debido a la extravasación de sangre que

provoca la bacteria, el endotelio vascular aparece lesionado, con la consiguiente aparición de hemorragias y petequias en la superficie de diversos órganos, principalmente en aquellos que están más irrigados, como la región perianal, bucal y aletas (Prieta y cols., 1993).

Se han identificado diferentes tipos de toxinas, tanto intra- como extracelulares, capaces de producir sintomatología clínica al inocularlo en un hospedador susceptible (Kimura y Kusuda, 1982; Kusuda y Hamaguchi, 1989).

Cuando se realiza la necropsia, se observa líquido ascítico, que puede ser purulento o hemorrágico, y los principales órganos afectados son bazo, hígado, cerebro, intestino, riñón y corazón (Pereira y cols., 2004; Austin y Austin, 1999; Afonso y cols., 2003).

Los principales factores del medio acuático que condicionan la presentación del proceso son la temperatura y la calidad del agua. La temperatura del agua tiene una gran importancia en el desarrollo del proceso ya que éste presenta una clara estacionalidad. Está asociado a temperaturas altas del agua; por encima de 18ºC se desencadenan brotes agudos con elevadas tasas de mortalidad, aunque no hay que olvidar que la enfermedad se ha descrito igualmente con temperaturas de 14-15ºC (Prieta y cols., 1993; Ghittino y Múzquiz, 1998). Por otra parte, la evolución del proceso está favorecida por una mala calidad del agua, lo que se asocia a deficiencias en las condiciones higiénico sanitarias de la explotación (Ghittino y Múzquiz, 1998). La virulencia de la bacteria se acentúa cuando la calidad del agua es pobre y deficiente en oxígeno (Fukuda y cols., 1997a; Fukuda y cols., 1997b). Así mismo, una excesiva concentración de amonio incrementa la mortalidad (Hurvitz y cols., 1997).

La trucha arco iris es la especie más susceptible y la que padece de forma más aguda y con mayores tasas de mortalidad esta enfermedad, afectando en la actualidad, de forma natural a animales de cualquier tamaño, desde juveniles a partir de 5 g hasta animales de más de 1 kg de peso (Chang y cols., 2002; Pereira y cols., 2004).

La transmisión del agente y la aparición de brotes de Lactococosis en explotaciones donde nunca antes se habían producido indican que el microorganismo ha llegado del exterior a través de diferentes fuentes de infección, vías de entrada y mecanismos de transmisión (Vendrell y cols., 2006). La introducción de nuevos lotes de animales, huevos o gametos en una piscifactoría es la vía de entrada más frecuente para el agente. Los portadores asintomáticos son la principal fuente de infección, ya que aunque no padecen la enfermedad son portadores de la bacteria en su microbiota intestinal y la eliminan por las heces infectando al resto de la población sana, y de esta forma mantienen latente la infección

desencadenando el proceso cuando las condiciones ambientales son óptimas para el agente (Ghittino y Múzquiz, 1998).

La transmisión de la enfermedad se produce principalmente por mecanismos horizontales, señalándose la vía feco-oral como la forma más frecuente entre peces sanos y enfermos o portadores asintomáticos que eliminan el agente a través de las heces, o del mucus (Múzquiz y cols., 1999; Afonso y cols., 2003). Así mismo, también hay que considerar como posible vía de transmisión el pienso puesto que el agente ha sido descrito como parte de la microbiota intestinal de algunas especies ícticas, utilizadas como materia prima en la elaboración de piensos, que pueden infectar a los peces si los tratamientos térmicos son insuficientes (Minami, 1979; Yasunaga, 1982).

Las medidas sanitarias son fundamentales para reducir la entrada de agentes patógenos a las instalaciones. Es importante un adecuado manejo de los animales y mantenimiento de las instalaciones. Disminuir al mínimo la manipulación de los peces, eliminar los peces muertos o enfermos, mantener densidades bajas de cultivo, una limpieza y desinfección periódica de los estanques y de todos los utensilios y aparejos de la piscifactoría, utilizando productos como el sulfato de cobre, formol, amonios cuaternarios, cloramina T, cloruro sódico, agua oxigenada o el permanganato potásico (de Kinkelin y cols., 1991; Romalde, 2004).

Así mismo, el control de la alimentación mediante análisis químicos y microbiológicos es de gran importancia ya que *L. garvieae* ha sido detectado en harinas de pescado, siendo viable incluso después de seis meses de congelación (Yasunaga y cols., 1982). El control de la calidad microbiológica y las características físico-químicas del agua constituye otro factor que se debe considerar para evitar la proliferación de la enfermedad.

Todos los peces o huevos que se introduzcan en las explotaciones deben estar libres del agente patógeno, para lo cual se deben exigir certificados sanitarios, además de llevar a cabo los correspondientes controles sanitarios y establecimiento de cuarentenas (De Kinkelin y cols., 1991; Romalde, 2004).

El tratamiento de la Lactococosis está basado en la utilización de numerosos antibióticos como la doxiciclina, amoxicilina y eritromicina (Munday, 1994; Treves-Brown, 2000).

Por otro lado, para el control de la enfermedad se han desarrollado algunas vacunas adyuvantadas mediante aceites minerales compuestos con distintos títulos de células bacterianas. Algunos autores como Ghittino (1999), observaron tras realizar distintas experiencias en condiciones de campo y/o de laboratorio que la inoculación IP con una dosis única de 0.1 mL, confería una protección completa a las 3 semanas post-vacunación

con las bacterianas y de 5 semanas con la utilización de vacunas oleosas, obteniendo una duración de inmunidad de 3-4 meses con las primeras, y de 4-5 con las segundas.

En la actualidad las estrategias de vacunación para la prevención de la Lactococosis no han variado mucho y consisten en vacunar a los peces, con un peso mínimo de 50 g, por vía IP con una temperatura del agua entre 12 y 14ºC (Ghittino y Múzquiz, 1998).

Sin embargo, debido fundamentalmente a las limitaciones actuales existentes en el uso de sustancias terapéuticas y la aparición de resistencias bacterianas, la Lactococosis resulta difícil de controlar. Estas restricciones han hecho que se busquen nuevas estrategias, que permitan mantener la producción y que aumente la resistencia a las patologías. En cuanto a las vacunas para el tratamiento de la Lactococosis, son eficaces cuando se realizan por inyección IP, pero esta vía de administración supone una serie de procedimientos perjudiciales para los peces derivados de una manipulación excesiva.

En este contexto, la selección de bacterias probióticas se convierte en una alternativa sumamente interesante para la prevención de procesos bacterianos en general y de la Lactococosis en la trucha en particular, a través de diferentes mecanismos de acción tales como la producción de sustancias inhibitorias, la competición por nutrientes esenciales o sitios de adhesión, o la modulación de la respuesta inmune (Balcázar y cols., 2006; Merrifield y cols., 2010).

Brunt y Austin (2005) observaron que tras la administración de *Aeromonas sobria*, aislada a partir de intestino de trucha arco iris, durante 14 días a una concentración de $5x10^7$ g^{-1} de pienso, una disminución de la mortalidad en el grupo tratado del 70% con respecto al grupo control. Los resultados obtenidos mostraron una estimulación de la respuesta innata, mediante el incremento de leucocitos y la actividad respiratoria y fagocítica.

Por otro lado, *Lactobacillus plantarum* CLFP 238 y *Leuconostoc mesenteroides* CLFP 196 fueron administradas durante 30 días a una concentración 10^7 UFC/g de pienso, observándose una reducción de la mortalidad producida por *L. garvieae* del 76% en el grupo control al 46% y 54%, respectivamente en los grupos tratados con las cepas probióticas señaladas.

Durante los últimos años se han desarrollado numerosos inmunoestimulantes para su uso en acuicultura, se trata de compuestos naturales que modulan el sistema inmune incrementando la resistencia del hospedador frente a las enfermedades. Hasta el momento, se han realizado algunos estudios con peptidoglucanos para el control de la Lactococosis

administrados por vía IP y oral, con la finalidad de inducir una protección de tipo innato frente a *L. garvieae*. Los resultados obtenidos demostraron una mayor protección en el grupo tratado con respecto al grupo control como consecuencia de una mayor actividad fagocítica y un incremento de la respuesta inmunitaria inespecífica (Itami y cols., 1996).

IV. Probióticos

En los últimos años la acuicultura se ha convertido en una importante actividad económica en todo el mundo. El incremento en la productividad, así como su intensificación, ha venido acompañado por la incidencia de una amplia variedad de agentes patógenos, especialmente de origen bacteriano.

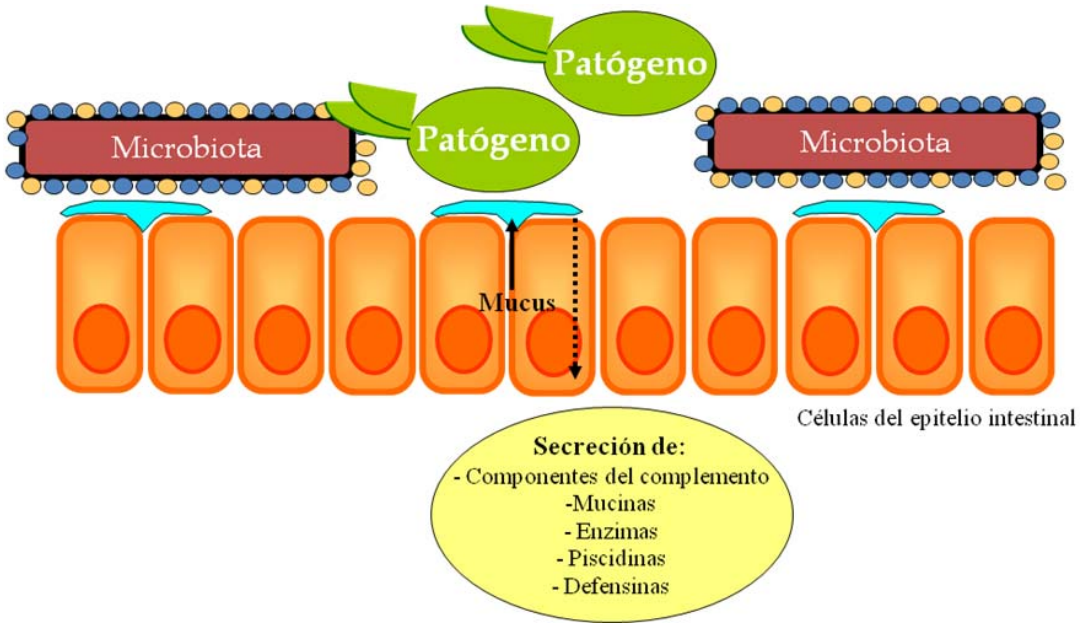
Como ya hemos señalado anteriormente, los antibióticos han sido utilizados en gran parte no sólo para el tratamiento de infecciones bacterianas, sino también, en muchas ocasiones, para la prevención de las mismas, lo que ha supuesto la aparición de fenómenos de transferencia de resistencias bacterianas a otras bacterias acuáticas generalmente de vida saprófita, así como a otros patógenos animales y de origen humano y, el acumulo de residuos en los productos derivados de la acuicultura (Sharifuzzaman y Austin, 2009a). Además, en muchos países, sobre todo en aquellos en vías de desarrollo, el tratamiento con este tipo de moléculas ha supuesto un coste económico muy elevado (Harikrishnan y cols., 2010), suscitando el desarrollo de nuevas estrategias de control y prevención de enfermedades basadas fundamentalmente en el uso de vacunas, inmunoestimulantes y/o probióticos (Gatesoupe, 1999; Balcázar y cols., 2006).

Se ha observado que los agentes terapéuticos son capaces de alterar la composición normal de la microbiota intestinal, cuyo papel como barrera de protección frente a la entrada de agentes patógenos es fundamental. Los probióticos son bacterias que forman parte de esta microbiota, y por tanto su presencia puede hacer que una alteración de la misma recupere su composición y estructura habitual.

La composición de la microbiota endógena en los animales acuáticos tiene una mayor influencia en el estado de salud del individuo que en los animales terrestres, por lo tanto, la manipulación de dicha microbiota mediante la administración de niveles adecuados de probióticos desempeña un papel importante para el mantenimiento del equilibrio y desarrollo normal (Hansen y Olafsen, 1999).

Las superficies mucosas del tracto gastrointestinal son el principal lugar en el que interactúan los agentes patógenos con la microbiota intestinal (Pérez y cols., 2010). Por tanto, la primera barrera de protección del hospedador es la capa de mucus que recubre el epitelio intestinal. Las células epiteliales ante la presencia de microorganismos patógenos son capaces de secretar sustancias antimicrobianas, como componentes del complemento, mucinas, enzimas, piscidinas y defensinas (Silphaduang y cols., 2006; Zou y cols., 2007) (Figura 1).

Figura 1. La microbiota intestinal constituye una barrera de protección frente a los agentes patógenos al crear un ambiente hostil para los mismos.



Los probióticos previenen las enfermedades bacterianas a través de una serie de mecanismos de acción, entre los que destacan, el desarrollo de un ambiente hostil para las bacterias patógenas, la producción de sustancias inhibitorias, la competición por nutrientes esenciales o sitios de adhesión, o la modulación de la respuesta inmune (Balcázar y cols., 2006; Merrifield y cols., 2010).

El término probiótico, que deriva del vocablo latín *pro* (a favor de) y del griego *bios* (vida), se utilizó por primera vez para denominar a las sustancias producidas por algunos microorganismos capaces de favorecer el crecimiento de otros microorganismos (Lilly y Stillwell, 1965).

De acuerdo a la Organización de Naciones Unidas y la Organización Mundial de la Salud, los probióticos se definen como microorganismos vivos que cuando se administran en las concentraciones adecuadas confieren un efecto beneficioso para el hospedador (FAO/WHO, 2001).

Los efectos beneficiosos de los probióticos en los peces han sido ampliamente demostrados por numerosos autores (Balcázar y cols., 2006; Gatesoupe, 2007 Kesacordi-Watson y cols., 2008; Merrifield y cols., 2010), por lo que en la actualidad son varios los países que los están incluyendo en sus protocolos de cultivo, con el objetivo de mejorar el rendimiento económico en las explotaciones.

Resulta importante señalar, que en acuicultura los efectos beneficiosos no se limitan al tracto gastrointestinal, sino que también pueden mejorar la

calidad del agua modificando la comunidad microbiana del agua y el sedimento (Verschuere y cols., 2000).

Actualmente la utilización de bacterias probióticas está cobrando una gran importancia en el campo de la acuicultura, no sólo porque se ha observado una mejora sustancial en los índices de crecimiento, sino también, una mayor resistencia a las enfermedades, lo que conlleva mejores resultados en la producción (Nayak, 2010).

IV.1. Selección de cepas probióticas

La selección de cepas probióticas está condicionada por varios criterios que hay que considerar, entre los que destacan: el origen y la seguridad de la cepa bacteriana; la producción de sustancias antimicrobianas; la capacidad para modular la respuesta inmune en el hospedador o la competición con los agentes patógenos por los sitios de adhesión.

Uno de los criterios más empleados para la selección de cepas probióticas es la capacidad de inhibir el crecimiento de bacterias patógenas, mediante la realización de pruebas *in vitro* en las que los productos extracelulares en medio líquido y/o sólido de las bacterias candidatas se enfrentan a los patógenos. Sin embargo, los resultados obtenidos durante el proceso de selección deben ser interpretados con precaución, ya que estudios previos han demostrado que las pruebas in vitro no pueden predecir el efecto que las cepas tendrán en el organismo del hospedador (Gram y cols., 2001).

La elección incorrecta de un microorganismo probiótico puede provocar efectos no deseados en el hospedador, por tanto, es fundamental conocer el origen de los mismos y es preferible utilizar aquellas cepas aisladas en el propio hospedador (Fjellheim y cols., 2010), puesto que además se ha demostrado que éstas poseen una mayor capacidad de competir con los microorganismos presentes en la microbiota normal (Sun y cols., 2010). Carnevali y cols. (2004) observaron una disminución significativa de la mortalidad de larvas y alevines de dorada (*Sparus aurata*), cuando se les administraba *Lactobacillus fructivorans*.

Vine y cols. (2006) propusieron que las cepas candidatas deberían de proceder de individuos sanos, preferentemente de la especie en la que se fueran a utilizar con posterioridad.

La capacidad de colonización del tracto gastrointestinal, caracterizada por su atracción a la superficie mucosa, seguida por su asociación en el mucus y/o adherencia a las células epiteliales, es otro factor importante a considerar en el proceso de selección de un probiótico. Balcázar y cols.

(2007), demostraron que tres cepas aisladas de salmónidos, *Lactococcus lactis* ssp. *lactis*, *Labtobacillus sakei* y *Leuconostoc mesenteroides*, tenían una gran capacidad de adherirse al mucus intestinal y sobrevivir en el mismo. Por otro lado, estudios llevados a cabo con *Pseudomonas chlororaphis* administrada en la perca (*Perca fluviatilis*) revelaron que dicha bacteria colonizaba el intestino de los peces de manera transitoria, por lo que si se quería usar como probiótico habría que administrarlo en intervalos regulares de tiempo (Gobeli y cols., 2009).

El tiempo de administración de las cepas probióticas es un factor muy variable, así Sharifuzzaman y Austin (2009), observaron que tras la administración de *Kocuria* SM1 entre 1 y 4 semanas en trucha arco iris infectadas experimentalmente con *Vibrio anguillarum*, conseguían reducir la mortalidad de manera significativa, especialmente a las 2 semanas post-tratamiento.

Por otro lado, dos cepas de *Bacillus* spp. aisladas del intestino de mero (*Epinephleus coioides*) disminuyeron significativamente el índice de conversión tras ser administradas durante 60 días (Sun y cols., 2010); sin embargo, la utilización de la cepa *Zooshikella* JE-34 en platija japonesa (*Paralichthys olivaceus*) durante una semana no produjo incremento alguno en la ganancia media diaria, pero sí tras su administración durante 4 semanas de tratamiento (Kim y cols., 2010).

Dichos estudios demuestran que la aportación de bacterias probióticas debe ser de forma continuada con el fin de que puedan estar presentes en el intestino, en la piel o en el agua de los estanques.

IV.2. Mecanismos de acción de los probióticos

La mayor parte de los estudios con probióticos en acuicultura han sugerido como principales mecanismos de acción: exclusión competitiva con bacterias patógenas, optimización de la nutrición y digestión por el suministro de nutrientes y enzimas esenciales en los animales y la estimulación de la respuesta inmune en el hospedador (Irianto y Austin, 2002a; Gómez y Balcázar, 2008).

IV.2.1. Exclusión competitiva

La exclusión competitiva en el tracto gastrointestinal, consiste en la prevención o reducción de la colonización de una bacteria patógena, por parte de las bacterias presentes en el tracto (Lara-Flores y Aguirre-Guzmán, 2009).

Una de las principales características que destacan en la exclusión competitiva, es la producción de sustancias inhibitorias. Este fenómeno fue observado por primera vez por De Giaxa (1889), al constatar el efecto inhibitorio producido por bacterias de origen marino sobre el crecimiento de *Vibrio* spp.

Posteriormente, Rosenfeld y Zobell (1947) estudiaron la capacidad de algunos microorganismos marinos como posibles sintetizadores de algunos antibióticos. Estos estudios hicieron que despertara el interés por la búsqueda de microorganismos capaces de producir compuestos inhibitorios, como los antibióticos, ácidos orgánicos, peróxido de hidrógeno, enzimas, sideróforos y bacteriocinas (Verschuere y cols., 2000; Balcázar y cols., 2006).

En el tracto gastrointestinal, la microbiota endógena constituye una barrera frente a la proliferación de agentes patógenos debido en gran parte a la producción de estas sustancias inhibitorias que pueden actuar de forma independiente o combinada. Se ha demostrado que existen algunas bacterias ácido lácticas (BAL), pertenecientes a los géneros *Lactobacillus* y *Carnobacterium*, que forman parte de la microbiota endógena normal de los peces y que son capaces de producir bacteriocinas que inhiben el crecimiento de ciertas bacterias patógenas (Ringø y Gatesoupe, 1998).

Por tanto, resulta fundamental el establecimiento de una microbiota para mantener el equilibrio ecológico entre los microorganismos beneficiosos y los microorganismos patógenos. Además, la presencia de una microbiota protectora tiene un efecto positivo sobre determinadas funciones inmunoreguladoras, por lo que es esencial el mantenimiento de la misma (Pérez y cols., 2010).

En acuicultura las interacciones que se establecen entre la microbiota y las bacterias patógenas no se limitan al tracto gastrointestinal, sino que también están presentes en las branquias, en la piel y en el propio medio acuático (Tinh y cols., 2008).

IV.2.2. Optimización de la nutrición y la digestión

Los probióticos desempeñan un papel beneficioso en el hospedador mediante la mejora del suministro de nutrientes y/o vitaminas (Ringø y Gatesoupe, 1998). Sakata (1990) describió la producción de ácidos grasos y vitaminas por algunos microorganismos pertenecientes a los géneros *Bacteroides* y *Clostridium*.

En algunas especies como la tilapia (*Oreochromis niloticus*) o la anguila japonesa (*Anguilla japonica*), se ha observado la implicación de los géneros

Aeromonas y *Pseudomonas* en la producción de amilasas (Sugita y cols., 1997). Así mismo, Ringø y cols. (1995), demostraron la contribución de los géneros *Agrobacterium*, *Pseudomonas*, *Brevibacterium*, *Microbacterium* y *Staphylococcus* en el proceso digestivo del salvelino del Ártico (*Salvelinus alpinus*).

Por otro lado, la administración de la levadura *Debaryomyces hansenii* incrementó la concentración de RNA mensajero (RNAm) de lipasa y amilasa, sugiriendo que el efecto de la misma podía deberse a la concentración de poliaminas secretadas en el lumen (Tovar-Ramírez y cols., 2004).

Los probióticos también pueden mejorar el proceso digestivo mediante el incremento de la actividad enzimática. Sin embargo, resultan necesarios un mayor número de estudios que demuestren este mecanismo de acción.

IV.2.3. Estimulación de la respuesta inmune

El sistema inmune de los peces puede ser estimulado por la presencia de cepas probióticas, ya que presentan compuestos en sus paredes celulares del tipo de los lipopolisacáridos, peptidoglucanos y β-glucanos, que mantienen en alerta los mecanismos de defensa del hospedador. De hecho, muchos inmunoestimulantes utilizados en peces y moluscos proceden de componentes de la pared celular, como el muramil dipéptido, los glucanos o los lipopolisacáridos (Anderson, 1992).

La modulación de la respuesta inmune por parte de los probióticos se ha traducido en una gran variedad de efectos, entre los que destacan: la producción de citoquinas pro-inflamatorias, la estimulación de las células NK el incremento en la producción de anticuerpos y/o la actividad de lisozima, la actividad complemento y/o fagocítica (Harikrishnan y cols., 2010). De hecho, las propiedades inmuno-estimuladoras de los probióticos han sido consideradas como el principal mecanismo de acción para proteger a los peces de las infecciones bacterianas (Sharifuzzaman y Austin, 2010).

Se ha constatado que la administración oral de *Clostridium butyricum* en trucha arco iris aumenta la resistencia de los peces a la Vibriosis como consecuencia de un incremento en la actividad fagocitaria de los leucocitos (Sakai y cols., 1995). De forma similar, Sun y cols. (2010) constataron un incremento en la actividad fagocítica tras administrar *Bacillus pumilus* y *Bacillus clansii* durante 60 días.

La fagocitosis es una forma de endocitosis, en la que partículas grandes, como los detritus o los propios microorganismos, son ingeridas en unas vesículas llamadas fagosomas. El papel fundamental de las células fagocíticas (monocitos, macrófagos y neutrófilos) es limitar la diseminación o el crecimiento de organismos infecciosos (Neumann y cols., 2001).

El incremento de la actividad lisozima es otro de los mecanismos de acción en los que se ha observado intervienen los organismos con propiedades probióticas. Un estudio realizado en la trucha arco iris demostró que la administración de *Aeromonas hydrophila* A3-51, *Vibrio fluvialis* A3-47S, *Carnobacterium* sp. BA211 y *Micrococcus luteus* A1-6 fueron capaces de incrementar la actividad de la lisozima (Irianto y Austin, 2002b). Por el contrario, Merrifield y cols. (2009) observaron que el incremento en la actividad lisozima era insignificante tras administrar *Bacillus subtilis* en esta misma especie.

Los probióticos también son capaces de estimular la producción de citoquinas pro- y anti-inflamatorias, mediadores de naturaleza proteica producidos por células inmunitarias que contribuyen al crecimiento y diferenciación celular de los mecanismos de defensa del hospedador (Nayak, 2010).

Entre las principales citoquinas pro-inflamatorias estudiadas en la trucha arco iris, se encuentran la interleuquina-1β1 (IL-1β), la interleuquina-8 (IL-8) y el factor de crecimiento β (TGF-β) (Secombes y cols., 2001; Gioacchini y cols., 2008; Mulder y cols., 2007), cuya principal función es evitar la colonización y diseminación de las bacterias patógenas (Kim y Austin, 2006). Por otro lado, la interleuquina-10 (IL-10) se encarga de regular la respuesta inflamatoria, minimizando el daño que puede provocar una respuesta excesiva (Raida y Buchmann, 2009).

En este sentido, Panigrahi y cols. (2007) observaron un aumento en la regulación de las citoquinas pro-inflamatorias como la IL-1β1 y TGF-β en trucha arco iris, tras la administración junto con el alimento de *Lactobacillus rhamnosus*, *Enterococcus faecium* y *Bacillus subtilis*. Así mismo, se constató un incremento de la expresión de la IL-8 en el intestino de ejemplares de trucha arco iris, previamente infectadas con *Aeromonas salmonicida* (Mulder y cols., 2007).

IV.3. Principales microorganismos probióticos utilizados en acuicultura

Actualmente, el uso de bacterias probióticas está presente tanto en la alimentación humana como animal observándose unos excelentes resultados (Balcázar, 2007); esto ha hecho que en los últimos 10 años haya despertado un gran interés en la utilización de este tipo de estrategias de control en acuicultura (Irianto y Austin, 2002; Balcázar y cols., 2006).

La primera vez que se utilizaron cepas probióticas en acuicultura fue en 1980 por Yasuda y Taga, quienes establecieron que ciertas bacterias podrían servir no solamente como fuente de alimento, sino también como agentes

de control biológico de las enfermedades y como activadores de la regeneración de nutrientes. Desde entonces, las investigaciones se han centrado en la búsqueda de este tipo de microorganismos, especialmente bacterias y levaduras.

En la Tabla 1 se recogen algunas de las bacterias probióticas utilizadas en acuicultura, así como su efecto en el hospedador.

IV.3.1. Bacterias Gram positivas

La mayor parte de las bacterias utilizadas como probióticos en la acuicultura son agentes Gram positivos, especialmente bacterias ácido-lácticas (BAL) pertenecientes a los géneros *Lactobacillus*, *Carnobacterium* y *Bacillus* (Balcázar y cols., 2006).

Las BAL constituyen un grupo de bacterias de morfología cocoide y/o bacilar, no esporuladas y de naturaleza inmóvil, capaces de producir ácido láctico como principal producto final tras la fermentación de los carbohidratos. Dichas bacterias han demostrado ser eficaces para el control de algunas enfermedades como la Edwardisellosis, Forunculosis y las Vibriosis (Harikrishnan y cols., 2010).

Gatesoupe (1991) demostró el efecto beneficioso de algunas bacterias del género *Lactobacillus* (*L. plantarum* y *L. helveticus*) en rodaballos (*Scophtalmus maximus*), tras observar un incremento significativo en los índices de crecimiento.

Jöborn y cols. (1997) determinaron que *Carnobacterium inhibens*, aislado del tracto gastrointestinal del salmón atlántico (*Salmo salar*), era capaz de producir sustancias inhibitorias in vitro frente a varios agentes patógenos. Además, en los experimentos in vivo se observó que se trataba de una cepa metabólicamente activa tanto en la mucosa intestinal como en las heces de los salmónidos.

La administración de *Pediococcus acidilactici* en tilapia roja (*O. niloticus*) supuso una tasa de supervivencia del 100%, mientras que en el grupo control fue del 88.33%. La razón de esta diferencia no pudo ser establecida ya que no se llevó a cabo ningún tipo de infección experimental y tampoco se detectaron síntomas patológicos.

Algunas BAL pertenecientes a los géneros *Lactobacillus* y *Enterococcus* utilizadas en la alimentación humana y animal, han sido consideradas para su uso en acuicultura. Así, *Enterococcus faecium* (utilizado como probiótico en humanos) y *Bacillus toyoi* (utilizado en animales), han sido aplicados satisfactoriamente en la anguila europea (*Anguilla anguilla*) para reducir la prevalencia de la Edwardsielosis (Chang y Liu, 2002).

Tabla 1. Probióticos utilizados en acuicultura y su efecto en el hospedador.

Especies de peces	Probióticos	Referencias
Epinephelus coioides	*Lactobacillus plantarum*	Son y cols. (2009)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Streptococcus* sp. o iridovirus.		
Oncorhynchus mykiss	*Vibrio fluvialis* A3-47S *Aeromonas hydrophila* A3-51 *Carnobacterium* sp. BA211 *Micrococcus luteus* A1-6	Irianto y Austin (2002)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Aeromonas salmonicida*.		
Oncorhynchus mykiss	*Lactobacillus rhamnosus* ATCC 53103	Nikoskelainen y cols. (2001, 2003)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Aeromonas salmonicida*.		
Oncorhynchus mykiss	*Aeromonas sobria* GC2	Brunt y Austin (2005)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Lactococcus garvieae* y *Streptococcus iniae.*		
Oncorhynchus mykiss	*Carnobacterium maltaromaticum* B26 *Carnobacterium divergens* B33	Kim y Austin (2006a, 2006b)
Expresión genética de citoquinas y mejora de la supervivencia tras una infección experimental con *Aeromonas salmonicida* y *Yersinia ruckeri*.		
Oncorhynchus mykiss,	*Lactobacillus sakei* CLFP 202 *Lactococcus lactis* CLFP 100 *Leuconostoc mesenteroides* CLFP 196	Balcázar y cols. (2007a, 2007b, 2009)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Aeromonas salmonicida*.		
Oncorhynchus mykiss	*Lactobacillus rhamnosus* ATCC 53103 *Bacillus subtilis* *Enterococcus faecium*	Panigrahi y cols. (2007)
Estimulación de la respuesta inmune y expresión genética de citoquinas.		
Oncorhynchus mykiss	*Bacillus subtilis* AB1	Newaj-Fyzul y cols. (2007)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Aeromonas* sp. ABE1.		
Oncorhynchus mykiss	*Lactobacillus plantarum* CLFP 238 *Leuconostoc mesenteroides* CLFP 196	Vendrell y cols. (2008)
Exclusión competitiva y mejora de la supervivencia tras una infección experimental con *Lactococcus garvieae.*		
Oncorhynchus mykiss	*Aeromonas sobria* GC2 *Brochothrix thermosphacta* BA211	Pieters y cols. (2008)
Estimulación de la respuesta inmune y mejora de la supervivencia tras una infección experimental con *Aeromonas bestiarum* y *Ichthyophthirius multifiliis.*		
Sparus aurata	*Lactobacillus delbriieckii* CECT 287 *Bacillus subtilis* CECT 35	Salinas y cols. (2008)
Estimulación de la respuesta inmune.		

Así mismo, se ha observado que la administración de *E. faecium* en el siluro (*Silurus glanis*), a una concentración de 2×10^8 UFC/g de alimento durante58 días, incrementaba el índice de masa corporal y reducía la incidencia de *Escherichia coli*, *Staphylococcus aureus* y *Clostridium* spp. con respecto al grupo control (Bogut y cols., 2000).

Por otro lado, la administración de *Lactobacillus rhamnosus* en trucha arco iris a una concentración entre 10^9 y 10^{12} UFC/g de alimento durante 51 días redujo la mortalidad tras un desafío experimental con *Aeromonas salmonicida* de 52.6% a 18.9% (109 UFC/g de alimento) y a 46.3% (10^{12} UFC/g de alimento) (Nikoskelainen y cols., 2001). Estos resultados pusieron de manifiesto que la concentración de probióticos no estaba directamente relacionada con el grado de protección en el hospedador

Algunos autores como Balcázar y cols. (2007b) y Vendrell y cols. (2008), observaron que *Leuconostoc mesenteroides* era capaz de conferir protección en truchas arco iris frente a una infección de *A. salmonicida* y *L. garvieae* La competición por los nutrientes y los sitios de adhesión podría ser el mecanismo de acción de esta cepa probiótica, puesto que los análisis moleculares realizados posteriormente demostraron su presencia en el intestino.

El género *Bacillus* se caracteriza por producir endosporas cuando las condiciones ambientales son adversas. La mayor parte de las especies pertenecientes a este género no son patógenas ni para el hombre ni para los animales, lo que facilita la utilización de los metabolitos que producen, entre los que se incluyen principalmente antibióticos y enzimas. Moriarty (1998) observó que la utilización de forma continuada durante 160 días de una cepa de *Bacillus* spp. como complemento alimenticio disminuía significativamente la incidencia de infecciones causadas por el agente *Vibrio* spp. en poblaciones de langostino tigre (*Penaeus monodon*); en comparación con el grupo control donde la mortalidad fue próxima al 100% de la población en un tiempo inferior a 80 días.

De forma similar, Balcázar y cols. (2007c), observaron que *Bacillus subtilis* reducía la mortalidad producida por una cepa patógena de *Vibrio parahaemolyticus* en camarón blanco (*Litopenaeus vannamei*), del 33% en el grupo control, al 19% en el grupo tratado.

IV.3.2. Bacterias Gram negativas

Los géneros más comunes de bacterias Gram negativas para su uso como potenciales probióticos en acuicultura son *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Shewanella* y *Vibrio* (Nayak, 2010). Se ha observado que el incremento en la tasa de supervivencia y en el crecimiento de las larvas de

camarón blanco en numerosas explotaciones ecuatorianas se atribuye a las propiedades probióticas de *Vibrio alginolyticus*. Garriques y Arevalo (1995) sugirieron que los efectos beneficiosos eran debidos a la acción de mecanismos de exclusión competitiva activados por la cepa patógena. Algunos autores como Austin y cols. (1995) demostraron el papel protector de *V. alginolyticus* en el salmón del Atlántico frente a infecciones causadas por *A. salmonicida*, *V. anguillarum* y *Vibrio ordalii*.

Gibson y cols. (1998) observaron que *Aeromonas media* reducía la proliferación de *Vibrio tubiashii* en larvas de ostras del Pacífico (*Crassostrea gigas*). Así mismo, Ruiz-Ponte y cols. (1999) vieron que *Roseobacter* sp. en co-cultivo con *V. anguillarum* presentaba un efecto inhibitorio frente a éste último, lo que incrementaba la supervivencia de larvas de pectínidos.

El efecto antagonista de *Pseudomonas* I-2 se ha demostrado al inhibir el crecimiento de algunas bacterias patógenas para los peneidos como *V. harveyi, V. fluvialis, V. parahaemolyticus, Vibrio vulnificus* y *Photobacterium damselae* por medio de inhibidores de bajo peso molecular (Chythanya y cols., 2002).

Así mismo, la administración por baño de *P. fluorescens* AH2 durante un período de 5 días redujo la mortalidad del 47 al 32% en truchas arco iris infectadas experimentalmente con *V. anguillarum* (Gram y cols., 1999).

Irianto y Austin (2002b) demostraron la efectividad de *Aeromonas hydrophila* y *Vibrio fluvialis* en el control de infecciones causadas por *A. salmonicida* en explotaciones de trucha arco iris. Por otro lado, *Pseudomonas* sp. MSB1 era capaz de inhibir el crecimiento *in vitro* de *Flavobacterium psychrophilum*, sugiriendo su papel como potencial probiótico en acuicultura, si bien sería necesario llevar a cabo estudios adicionales *in vivo* (Ström-Bestor y Wiklund, 2011).

IV.3.2. Levaduras

Las levaduras presentan la ventaja de no ser afectadas por los antibióticos, lo que ayuda a restablecer la microbiota tras un tratamiento con este tipo de moléculas terapéuticas. Además, algunos géneros de levaduras son capaces de sintetizar diferentes tipos de poliaminas (Tovar-Ramírez y cols., 2004), que presentan la habilidad de adherirse al mucus intestinal (Andlid y cols., 1998).

Tovar-Ramírez y cols. (2002), al administrar una dieta suplementada con *Saccharomyces cerevisae* y *Debaryomyces hansenii* en larvas de lubina (*Dicentrarchus labrax*), observaron un incremento en las tasas de supervivencia y en los índices de crecimiento en los animales tratados.

En carpas indias (*Catla catla*) se han evaluado las cualidades probióticas de diversas bacterias y levaduras, observando un incremento en la supervivencia y en el índice de masa corporal (Mohanty y cols., 1996).

La inclusión de *Phaffia rhodozyma* al 1% en la dieta de *L. vannamei* mostró un efecto positivo en el porcentaje de supervivencia y de crecimiento, a la vez que se incrementó la resistencia frente a la Vibriosis (Scholz y cols., 1999). Resultados similares fueron obtenidos con *Sacharomyces cerevisae* en la producción de tilapia (Lara-Flores, 2003).

Reyes-Becerril y cols. (2008) demostraron que una dieta suplementada con *Debaryomyces hansenii* estimulaba el sistema inmune de la cabrilla sardinera (*Mycteroperca rosacea*).

III. Publicaciones

I. Fish & Fisheries *(en revisión)*

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Probiotics in aquaculture: A current assessment

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Abstract

Because of environmental concerns, the use of chemotherapeutic agents has been restricted in many countries over recent years. This restriction has resulted in a demand for alternative strategies to improve aquaculture production and enhance disease resistance. Among these options, probiotics, live microorganisms that confer a health benefit to the host by providing both a nutritional benefit and protection against pathogens, represent a practical and useful approach, and their use may replace some of the inhibitory chemicals currently used in fish farms. Our article explores the current state of knowledge of the impacts of probiotics on aquaculture while placing particular emphasis on the criteria used for selection and evidence of their beneficial effects.

Keywords: Aquaculture; Microbiota; Pathogens; Probiotics

Introduction **xx**

Selection of probiotics **xx**

Mechanisms of action **xx**

Competitive exclusion: antagonistic compounds and adhesion xx

Source of nutrients and enzymatic contribution to digestion xx

Enhancement of the immune response xx

Probiotic strains studied in aquaculture **xx**

Gram-positive bacteria xx

Gram-negative bacteria xx

Yeasts xx

Concluding remarks **xx**

Introduction

Aquaculture has become an important economic activity in many countries. Although this activity has expanded, diversified and intensified, the emergence of a large variety of pathogens is considered to be the major limiting factor. Antibiotics have been used not only for the treatment of bacterial infections, but also for preventing them. However, antibiotic use in aquaculture may be detrimental to the environment and human health, and involve the development and transfer of resistance to other aquatic bacteria, animal and human pathogens, the accumulation of residual antibiotics in aquaculture products, and affect the microbial diversity (Sharifuzzaman and Austin, 2009a). Moreover, antibiotic treatment is cost-prohibitive to farmers in many undeveloped and developing countries (Harikrishnan et al., 2010). In this sense, several alternative strategies for the prevention and control of diseases have been proposed, such as the use of vaccines, immunostimulants and probiotics (Gatesoupe, 1999; Balcázar et al., 2006). Probiotics are usually members of the healthy microbiota associated with the host; therefore, they may provide an alternative way to reduce the use of antibiotics in aquaculture, because their addition can assist in returning a disturbed microbiota to its normal beneficial composition. Probiotics may prevent bacterial diseases through a variety of mechanisms, such as the creation of a hostile environment for pathogens by the production of inhibitory compounds, by competing for essential nutrients and adhesion sites, or by modulating the immune responses (Balcázar et al., 2006; Merrifield et al., 2010).

The term probiotic, meaning "for life", is derived from the Greek words "pro" and "bios". It was first used by Lilly and Stillwell in 1965 to describe "substances secreted by one microorganism which stimulates the growth of another" (Lilly and Stillwell, 1965) and was thus contrasted with the term antibiotic. The Joint Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO) have stated that probiotics are "live microorganisms, which when consumed in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). It is important to point out that probiotic effects in aquaculture are not limited to the intestinal tract, but also can improve the health of the host by controlling pathogens and improving water quality by modifying the microbial community composition of the water and sediment (Verschuere et al., 2000).

Selection of probiotics

In aquaculture, there are several criteria to be considered when choosing the appropriate probiotic strain. The characteristics to consider include: host origin, safety of the strain, production of antimicrobial substances, ability to modulate host immune response, or efficient competition with pathogens for intestinal mucosa adhesion sites. One of the most common ways to obtain a source of these bacteria is to perform *in vitro* antagonism tests, in which pathogens are exposed to the candidate probiotics or their extracellular products in liquid and/or solid medium. However, previous studies have demonstrated that the *in vitro* tests cannot be used to predict a possible *in vivo* effect and thus these observations should be interpreted with great caution (Gram et al., 2001). As a result, it is essential to know the origin, it is preferable to use strains isolated from the host (Fjellheim et al., 2010), safety and ability of the strain to survive to the transit through the gastrointestinal tract of the host (resistance to bile salts, low pH and enzymes).

Selection of probiotics is very critical because inappropriate microorganism can lead to undesirable effects in host. There is a general consensus that probiotics from autochthonous source have a greater chance of competing with resident microbes (Sun et al., 2010). For instance, Carnevali et al. (2004) recorded a significantly decreased larvae and fry mortality by using *Lactobacillus fructivorans*, isolated from gut of adult sea bream (*Sparus aurata,* Sparidae). Vine et al. (2006) proposed that candidate probiotics should be derived from healthy individuals, preferably of the target species.

According to Fuller (1989), a probiotic should not only be harmless but must also provide a beneficial effect to the host. Potential probiotics must therefore be safe, meaning neither invasive nor pathogenic. They must be amenable to industrial processes necessary for commercial production, must remain viable in the food product and during storage, and must be metabolically active to elicit an effect.

The ability to colonize the intestinal tract is clearly another important property of a probiotic. Balcázar et al. (2007a) demonstrated that *Lactococcus lactis* subsp. *lactis*, *Lactobacillus sakei* and *Leuconostoc mesenteroides*, isolated from the endogenous microbiota of salmonids, have a strong ability to adhere to and survive in the intestinal mucus, as high amounts of those probiotic strains were detected after one week of treatment. However, colonization experiments of juveniles and fingerlings of perch (*Perca fluviatilis*, Percidae) with *xylB*-labelled *Pseudomonas chlororaphis* revealed that

the bacterium only colonizes the fish transiently or perhaps remains in the intestine at levels that were undetected by *in vitro* bacterial culture. If *P. chlororaphis* were to be used as a probiotic, it would probably have to be administered to the host at regular intervals (Gobeli et al., 2009).

Studies with probiotics to date have involved the use of different feeding durations, for example 1-8 weeks feeding regimes, leading to improved disease resistance in farmed fish. Sharifuzzaman and Austin (2009a) when administered between one and four weeks, dietary supplementation of *Kocuria* SM1 led to a reduction in mortalities after challenge with *Vibrio anguillarum*. In particular, a two-week feeding regime led to the maximum reduction in mortalities. Two potential beneficial *Bacillus* strains isolated from the gut of fast growing groupers (*Epinephelus coioides,* Serranidae) were evaluated in a 60-day feeding trial and significant decrease of Feed Conversion Ratio (FCR) was observed in fish fed diets supplemented (Sun et al., 2010). Olive flounder (*Paralichthys olivaceus*, Paralichthyidae) fed three enriched diets with *Zooshikella* strain JE-34 did not significantly increase PGW on first week, but after fourth week all the enriched diets significantly increased the PGW (Kim et al., 2010). This study clearly showed that the probiotics containing feed must be given to fish continuosly to facilitate the retention of probiotic bacteria in the gut, skin and tank water and that the adhesion properties of probiotic bacteria may influence the speed of washout.

The selection and design of probiotics remains an important challenge and requires a solid foundation of basic information regarding the physiology and genetics of candidate strains, especially as it related to their intestinal roles, functional activities, and interaction with other resident microbiota (Klaenhammer and Kullen, 1999).

Mechanisms of action

Most studies in aquaculture suggest that most common mechanisms by which probiotics may offer a beneficial effect include (i) competitive exclusion of pathogenic bacteria, (ii) enhancement of host nutrition and enzymatic contribution to digestion, and (iii) stimulation of host immune response (Irianto and Austin, 2002a; Gómez and Balcázar, 2008).

Competitive exclusion: antagonistic compounds and adhesion

Competitive exclusion as it applies to the gastrointestinal tract is a phenomenon whereby an established microbiota prevents or reduces the colonization of a competing

bacterial challenge for the same location in the intestine (Lara-Flores and Aguirre-Guzmán, 2009). Microorganisms use a variety of mechanisms to compete for numerous resources, including nutrients, space (adhesion sites on epithelial surfaces), and oxygen.

One important mechanism of competition that also has important implications for pathogen control is the production of inhibitory compounds. This phenomenon was first noted in 1889 by De Giaxa, when marine bacteria were observed to inhibit *Vibrio* species (De Giaxa, 1889). In 1947, Rosenfeld and ZoBell investigated the bacteriostatic or bactericidal properties of seawater on nonmarine bacteria in culture and reported the presence of antibiotic activity in marine microorganisms (Rosenfeld & ZoBell, 1947). These studies stimulated a great interest in finding microorganisms that can synthesize inhibitory compounds.

However, antibiotic substances are not alone in producing inhibition. Antagonism may be mediated by other substances besides antibiotics, such as organic acids, hydrogen peroxide, lytic enzymes, iron-chelating compounds and bacteriocins (Verschuere et al., 2000; Balcázar et al., 2006).

In the gastrointestinal tract of healthy organisms, the indigenous microbiota constitutes a layer against the proliferation of pathogenic bacteria. It has been determined that some indigenous lactic acid bacteria, such as those belonging to the genus *Lactobacillus* and *Carnobacterium,* are able to produce bacteriocins that can inhibit the growth of some pathogenic bacteria (Ringø and Gatesoupe, 1998). Therefore, the establishment of a normal or protective microbiota is a key component in excluding potential invaders and maintaining health.

Colonization of intestinal mucosal surfaces with a normal microbiota has also a positive effect on immune regulatory functions, and disturbance of these functions by an imbalanced microbiota may contribute to the development of diseases. Significant attention has therefore been focused on the role of probiotics in the induction or restoration of a disturbed microbiota to its normal beneficial composition (Pérez et al., 2010).

The ability of a probiotic strain to colonize the gut and adhere to the mucus layer is considered a good criterion when preselecting probiotic candidates (Balcázar et al., 2006; Vine et al., 2006). Such a feature is important because a longer residence time in the intestinal tract could extend potential beneficial effects (Balcázar et al., 2007a).

However, in aquaculture systems, the interaction between the microbiota and the host is not limited to the intestinal tract. Probiotic bacteria can also be active on the gills or the skin of the host or in its surrounding environment (Tinh et al., 2008). It has been reported that bacterial strains associated with intestinal and skin mucus of adult marine turbot (*Scophtalmus maximus*, Scophthalmidae) and dab (*Limanda limanda*, Pleuronectidae) suppressed the growth of the fish pathogen *Vibrio anguillarum* (Olsson et al., 1992).

We have recently demonstrated that probiotic strains (*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris,* and *Lb. sakei*) reduced the adhesion of fish pathogens, such as *Aeromonas salmonicida*, *Carnobacterium piscicola,* and *Yersinia ruckeri* to intestinal mucus from rainbow trout (*Oncorhynchus mykiss*, Salmonidae) (Balcázar et al., 2007b). This sort of evidence suggests that is desirable to use adhering strains when designing probiotic supplements.

The administration of dead/ inactivated cells or the supernatant of probiotics does not necessarily reduce bacterial infections, indicating that the maximum benefits of probiotics are mediated in some cases by live bacterial cells (Sharifuzzaman and Austin, 2009b). Abbass et al. (2010) demonstrated the beneficial properties of subcellular components of *Aeromonas sobria* GC2 and *Bacillus subtilis* JB-1 when administered to rainbow trout, because of those probiotic strains conferred protection against a new biogroup of *Yersinia ruckeri*. It should be noted that the protection rate by extracellular components of bacteria can vary with the growth phase, nutrient level, pH, temperature and owing to differences of other unknown *in vivo* vs. *in vitro* growth conditions, and due to the quantity of inocula (Sharifuzzaman et al., 2010).

Source of nutrients and enzymatic contribution to digestion

Probiotics are also expected to have a direct growth promoting effect on the host either by a direct involvement in nutrient uptake, or by providing nutrients or vitamins (Ringø & Gatesoupe, 1998). Some members of the genera *Bacteroides* and *Clostridium* have been reported to contribute to host nutrition by supplying fatty acids and vitamins (Sakata, 1990). In some fish species, such as tilapia (*Oreochromis niloticus*, Cichlidae) or Japanese eel (*Anguilla japonica*, Anguillidae), members of the genera *Aeromonas* and *Pseudomonas* have been implicated in the production of amylase (Sugita et al., 1997). Moreover, members of the genera *Agrobacterium*, *Pseudomonas*, *Brevibacterium*, *Microbacterium*, and *Staphylococcus* have been also shown to

contribute to nutritional processes in Arctic charr (*Salvelinus alpines*, Salmonidae) (Ringø et al., 1995). A previous study has demonstrated that European sea bass (*Dicentrarchus labrax*, Moronidae) larvae fed live yeast (*Debaryomyces hansenii*) showed increased activity and concentrations of mRNA trypsin and lipase, suggesting that the dose-dependent effect of yeast on larval performance could be attributed to the amount of polyamines secreted by the yeast in the gut lumen of larvae (Tovar-Ramírez et al., 2004).

Probiotics can also influence the digestive processes by improving the microbial population and by enhancing the enzyme activity that contributes to more efficient digestion and food utilization. However, the significance of bacterially-produced enzymes to host health *in vivo* remains unclear and thus additional studies are needed to characterize enzyme contributions to the host.

Enhancement of the immune response

Probiotics can stimulate the host immune response since bacterial walls have components (lipopolysaccharides, peptidoglycan and β-glucans) that may cause a range of innate and adaptive host immune responses. Many immunostimulants have been tested on fish and shellfish and some of them originated from microbial cell walls, e.g, muramyl dipeptide, glucans, lipopolysaccharides (Anderson, 1992).

Modulating immune responses with probiotic bacteria have been shown to have several effects in a variety of fish, such as induction of proinflammatory cytokines, stimulating the activity of natural killer cells, increasing mucosal and systemic antibody production, activating phagocytic activity and increasing lysozyme and complement activity (Harikrishnan et al., 2010). Certainly, the immune-stimulating properties of probiotics have been recognized as the key mode of action protecting fish against bacterial infections (Sharifuzzaman and Austin, 2010).

Probiotic strains have been shown to modulate innate immune responses in their hosts and thereby facilitate the exclusion of potential pathogens. In rainbow trout, the oral administration of *Clostridium butyricum* has been shown to enhance the trout resistance to vibriosis by increasing the phagocytic activity of leucocytes (Sakai et al., 1995). Significant increases of phagocytic activity and phagocytic index were also observed in *Epinephelus coioides* fed *Bacillus pumilus* or *Bacillus clansii* containing diets for 60 days compared with those fed the control diet (Sun et al., 2010). Phagocytosis is a form

of endocytosis where large particles (i.e. cellular debris or microorganisms) are ingested into endocytic vesicles called phagosomes. The fundamental role of phagocytic cells (monocytes/macrophages and neutrophils) in host defence is to limit the initial dissemination and/or growth of infectious organisms (Neumann et al., 2001).

The augmentation of lysozyme activity is another mechanism of probiotic protection. Irianto & Austin (2002b) demonstrated that the administration of *Aeromonas hydrophila* A3-51, *Vibrio fluvialis* A3-47S, *Carnobacterium* sp. BA211 and *Micrococcus luteus* A1-6 was able to increase lysozyme activity. In contrast, Merrifield et al. (2009) demonstrated that the effect of *Bacillus subtilis* on serum lysozyme activity of rainbow trout was negligible in a 10-week feeding trail. This cationic enzyme is widely distributed in several fish tissues, including the serum, kidney, spleen and intestine. Although its exact physiological role is not yet understood, there is a general acceptance that lysozymes are involved in the defence against microorganisms. Lysozyme hydrolyses *N*-acetylmuramic acid and *N*-acetylglucosamine, which are both constituents of the peptidoglycan layer of bacterial cell walls (Gómez & Balcázar, 2008).

Sharifuzzaman and Austin (2009a) observed that rainbow trout fed *Kocuria* SM1 at concentrations of 10^8 cells g^{-1}, and then challenged intraperitoneally with *Vibrio anguillarum* at weekly intervals, enhanced cellular and humoral immune response, notably greater head kidney macrophage phagocytic and peroxidise activities, and higher serum lysozyme and total protein levels were recorded.

Lowest mortality rate of lymphocystis disease virus (LCDV) infected olive flounder (*Paralichthys olivaceus*) was seen in groups fed with Lactobacil individually or mixed with Sporolac supplemented diets, and significantly enhanced the immune parameters such as phagocytic activity superoxide anion production, complement activity and plasma lysozyme (Harikrishnan et al., 2010).

In addition, Nikoskelainen et al. (2003) showed that the administration of a lactic acid bacterium *Lactobacillus rhamnosus* stimulated the respiratory burst in rainbow trout. The administration of *L. rhamnosus* has also been involved in the stimulation of superoxide anion production from head kidney leukocytes in the same species (Panigrahi et al., 2005).

Probiotics have also been found able to modulate the production of pro- and anti-inflammatory cytokines, which are protein mediators produced by immune cells that contribute to cell growth, differentiation and defence mechanism of the host (Nayak, 2010). Probiotics such as *L. rhamnosus*, *Enterococcus faecium* and *Bacillus subtilis* were found to up regulate the pro-inflammatory cytokines like interleukin-1β1 and transforming growth factor β (TGF-β) in rainbow trout (Panigrahi et al., 2007).

Recent studies by our research group have shown a correlation between colonization with probiotic bacteria (*Lc. lactis* subsp. *lactis*, *Leuc. mesenteroides* and *Lb. sakei*) and host innate humoral responses, such as alternative complement pathway activity and lysozyme activity in brown trout (*Salmo trutta*, Salmonidae) (Balcázar et al., 2007a). We have also demonstrated that feeding rainbow trout with members of its intestinal microbiota resulted in a higher survival rate after challenge with *A. salmonicida*, a pathogen that initially infects the intestine and induces furunculosis in various wild and farmed fish species (Balcázar et al., 2007c).

Stimulation of the immune system by probiotic bacteria has also been reported in crustaceans, particularly shrimp. It is important to state that there are three types of circulating haemocytes in crustaceans: hyaline, semi-granular and large granular cells. Haemocytes are involved not only in phagocytosis but also in the production of melanin via the prophenoloxidase system, which is an important component of the cellular defence reaction (Johansson and Söderhäll, 1989). In this sense, the administration of *Bacillus* S11 stimulated phagocytic activity in tiger shrimp (*Penaeus monodon*, Penaeidae) (Rengpipat et al., 2000). Moreover, the administration of *Lactobacillus plantarum* stimulated phenoloxidase and superoxide dismutase activities, enhanced the efficiency of *Vibrio alginolyticus* clearance, and augmented peroxinectin mRNA transcription in white shrimp (*Litopenaeus vannamei*, Penaeidae) (Chiu et al., 2007).

Probiotic strains studied in aquaculture

One of the first studies on this subject was conducted by Yasuta and Taga (1980), who suggested that bacteria could act to control fish disease and activate nutrient regeneration in addition to serving as food. Since then, research effort has been dedicated to identifying beneficial microorganisms, especially Gram-positive and Gram-negative bacteria and yeasts.

Gram-positive bacteria

Most probiotics under consideration in aquaculture belong to the lactic acid bacteria (*Lactobacillus* and *Carnobacterium* species) and *Bacillus* (Balcázar et al., 2006). Lactic acid bacteria (LAB) are a group of Gram-positive rod- and coccus-shaped organisms that do not form spores, are non-motile and produce lactic acid as their major end product during the fermentation of carbohydrates. They are generally regarded as safe (GRAS) for use in food production and are widely used as probiotics.

The LAB probiotics have been shown to be effective against edwardisellosis, furunculosis and vibriosis (Harikrishnan et al., 2010). Gatesoupe (1994) demonstrated a significant increase in the mean weight and survival rate of turbot larvae that were fed rotifers enriched with lactic acid bacteria and these strains provided a significant protection against a pathogenic *Vibrio*. Jöborn et al. (1997) determined that *Carnobacterium inhibens*, which was isolated from the gastrointestinal tract of Atlantic salmon (*Salmo salar*, Salmonidae), produced inhibitory substances active against several fish pathogens *in vitro*. Furthermore *in vivo* results demonstrated that this strain is metabolically active in both the intestinal mucus and the faeces of salmonids and does not have a detrimental effect on the host.

Ferguson et al. (2010) observed that red tilapia (*Oreochromis niloticus,* Cichlidae) fed the diet supplemented with *Pediococcus acidilactici* showed a survival rate of 100%, whereas survival was 88.33% in those fed the control diet. The reason for this has not been elucidated as no pathogenic challenge was conducted, and no signs of disease were detected during the trial, but perhaps *Ped. acidilactici* may improve general vigour.

Some lactic acid strains that are generally used as human probiotics (e.g. lactobacilli and enterococci), have been considered for use in fish. Administration of *L. rhamnosus* to rainbow trout for 51 days reduced host mortality caused by *A. salmonicida* from 52.6% in the control to 18.9 and 46.3% in the 10^9 cells g^{-1} feed and the 10^{12} cells g^{-1} feed groups, respectively (Nikoskelainen et al., 2001). It is apparent that a high dose does not necessarily result in a greater amount of protection.

Panigrahi et al. (2010) assessed the effect of feeding viable and non viable forms of *L. rhamnosus* on blood profiles in rainbow trout. A significant elevation of plasma cholesterol and triglyceride and alkaline phosphatise activity level was found in the freeze-dried (FD) probiotic fed groups at 20 and 30 days postfeeding. This was

concomitant with the increased plasma protein and hematocrit values in FD group at 20 and 30 days. Likewise, the heat-killed probiotic fed group registered significantly high values of triglycerides, alkaline phosphatase activity, and plasma protein after 20 days of feeding. Alterations in the blood profiles could serve as supplementary information when examining the benefits of probiotics for fish.

In addition, survival rates of European eels (*Anguilla Anguilla*, Anguillidae) fed with *Enterococcus faecium* were significantly higher compared to controls following challenge with *Edwardsiella tarda* (Chang et al., 2002). This strain has also been useful in improving the growth of sheat fish (*Silurus glanis*, Siluridae). After feeding for 58 days on a dose equivalent to 2 x 10^8 bacteria g^{-1} of food, fish achieved better growth and also showed reduced incidence of *Escherichia coli*, *Staphylococcus aureus* and *Clostridium* spp. (Bogut et al., 2000).

We have also shown that the administration of *Leuc. mesenteroides* confers protection against *A. salmonicida* and *Lactococcus garvieae* in rainbow trout. Competition for nutrients and adhesion receptors could be the basis for this protection since microbiological and molecular analyses have revealed the presence of this probiotic strain in the fish intestine (Balcázar et al., 2007c; Vendrell et al., 2008).

The genus *Bacillus* constitutes a diverse group of rod-shaped, Gram-positive bacteria that are characterized by their ability to produce endospores in response to adverse environmental conditions. Most *Bacillus* species are not harmful to humans or animals and are commercially important as producers of a high and diverse amount of secondary metabolites, including antibiotics and enzymes. When a *Bacillus subtilis* strain isolated from the common snook (*Centropomus undecimalis*, Centropomidae) was inoculated into the rearing water, the results was an apparent elimination of *Vibrio* species from whole snook larvae (Kennedy et al., 1998). Moriarty (1998) also demonstrated that the application of several *Bacillus* spp. cultures to shrimp culture ponds permitted the continuous culture of the shrimp for over 160 days. Farms that did not use *Bacillus* spp. cultures experienced almost complete failure in all ponds in less than 80 days as a result of mortality caused by a luminescent *Vibrio* spp. pathogen. Similarly, Balcazar et al. (2007d) reported that *Bacillus subtilis* reduced the mortality of white shrimp infected with a pathogenic *Vibrio parahaemolyticus* strain. Controls inoculated with the pathogenic bacterium have a cumulative mortality of 33% at 14 days post-infection, whereas the mortality was 19% in the treated shrimp.

Gram-negative bacteria

The common Gram-negative probiotics that are used for aquaculture practices include *Aeromonas*, *Enterobacter*, *Pseudomonas*, *Shewanella* and *Vibrio* species (Nayak, 2010). The increased survival and growth of white shrimp post-larvae in Ecuadorian hatcheries has been attributed to the probiotic properties of *Vibrio alginolyticus*. The authors suggest that beneficial effect observed derives from the competitive exclusion of potential pathogenic bacteria in intensive larviculture systems (Garriques and Arevalo, 1995). Similar observations have been described by Balcazar et al. (2007d), who noted that administration of *V. alginolyticus* offer protection against *V. parahaemolyticus* in white shrimp.

Riquelme et al. (1996) showed a significantly improved rate of survival in scallop larvae (*Pecten maximus*, Pectinidae) treated with *Alteromonas haloplanktis* following challenge with *Vibrio anguillarum*. Gibson et al. (1998) also found that *Aeromonas media* reduced the proliferation of *Vibrio tubiashii* in Pacific oyster larvae (*Crassostrea gigas*, Ostreidae). Similarly, Ruiz-Ponte et al. (1999) found that *Roseobacter* sp. in co-culture with *V. anguillarum* displayed an inhibitory effect on *Vibrio*, enhancing the survival of larval scallop.

Gram et al. (1999) reported that *Pseudomonas fluorescens* reduced the mortality of rainbow trout infected with a pathogenic *V. anguillarum* strain. Controls inoculated with the pathogenic bacterium had a cumulative mortality of 47% after 7 days, whereas in the treated fish mortality was only 32%. Moreover, Irianto and Austin (2002b) reported that cultures of *Aeromonas hydrophila* and *Vibrio fluvialis* were effective at controlling *A. salmonicida* infections in rainbow trout. *Pseudomonas* sp. isolate MSB1 efficiently inhibited the growth of *Flavobacterium psychrophilum* in vitro, which suggested a potential use of MSB1 as a probiotic in rainbow trout aquaculture, especially in early life stages of the fish, but future *in vivo* experiments need to be carried out (Ström-Bestor and Wiklund, 2011).

Yeasts

Yeasts have the advantage that they are not affected by antibiotics, which may help to re-establish the normal microbiota after antibiotic treatment. They could also be an appropriate organism because some strains synthesize and secrete different polyamine molecules (Tovar-Ramírez et al. 2004), and they have strong adhesion to fish intestinal

mucus (Andlid et al., 1998). In fact, Tovar-Ramírez et al. (2002) investigated the secretion of digestive enzymes in sea bass larvae fed a compound diet supplemented with different strains of *Saccharomyces cerevisae* and *Debaryomyces hansenii*. The growth and survival of the larvae fed yeast-incorporated diet were higher than those of the larvae fed control diet.

The probiotic properties of both bacteria and yeasts have been evaluated in catla (*Catla catla*, Cyprinidae) and both have been found to increase fish survival and body weight (Mohanty et al., 1996). It has also been demonstrated that the addition of *Phaffia rhodozyma* to the diet confers protection against vibriosis in juvenile white shrimp (Scholz et al., 1999). Similar results have been observed with *Saccharomyces cerevisiae* in tilapia (Lara-Flores et al., 2003). Reyes-Becerril et al. (2008) demonstrated that diet supplemented with live yeast *Debaryomyces hansenii* stimulated the immune system of juvenile leopard grouper (*Mycteroperca rosacea*, Serranidae).

Concluding remarks

The growth of aquaculture in the last few years has resulted in the occurrence of diseases. While large amounts of antibiotics have traditionally been used to treat disease, their indiscriminate use has led to an increase in antibiotic resistance, a phenomenon that will have unpredictable long-term effects on public health. Chemotherapeutic agents can also alter the community composition of indigenous intestinal microbiota and it is well known that such assemblages play an important role in animal health. Probiotics are usually members of this microbiota, and their addition can assist a disturbed microbiota in returning to normal. Nowadays, probiotics are becoming an integral part of the aquaculture practices for improving growth and disease resistance and obtain high production (Nayak, 2010).

The use of probiotic bacteria as biological control agents should be considered as an alternative to the chemotherapeutic agents commonly used in fish farming for disease prevention. Furthermore, they can contribute to improved nutrition in aquaculture. These bacteria are emerging as significant food supplements in the field of prophylaxis because of their ability to counter fish infections.

The mechanisms by which probiotics act *in vivo*, which have been rarely investigated, merit further research. It is also crucial to investigate the interaction between probiotic bacteria and the host gastrointestinal microbiota. Such research will allow us to

establish efficient criteria for probiotic strain selection, which will lead to the use of effective and safe (harmless to the host) microorganisms in commercial aquaculture.

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II. Mucosal Immunology, 2010

Tania Pérez, José Luis Balcázar, Imanol Ruiz-Zarzuela, Nabil Halaihel, Daniel Vendrell, Ignacio de Blas, José Luis Múzquiz. Host-microbiota interactions within the fish intestinal ecosystem. ***Mucosal Immunology.*** 2010; 3: 355-60.

Host–microbiota interactions within the fish intestinal ecosystem

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Teleost fish are in direct contact with the aquatic environment, and are therefore in continual contact with a complex and dynamic microbiota, some of which may have implications for health. Mucosal surfaces represent the main sites in which environmental antigens and intestinal microbiota interact with the host. Thus, the gut-associated lymphoid tissues (GALT) must develop mechanisms to discriminate between pathogenic and commensal microorganisms. Colonization of intestinal mucosal surfaces with a normal microbiota has a positive effect on immune regulatory functions of the gut, and disturbance in these immune regulatory functions by an imbalanced microbiota may contribute to the development of diseases. Significant attention has therefore been recently focused on the role of probiotics in the induction or restoration of a disturbed microbiota to its normal beneficial composition. Given this, this article explores the fascinating relationship between the fish immune system and the bacteria that are present in its intestinal microbiota, focusing on the bacterial effect on the development of certain immune responses.

INTRODUCTION

The structure and composition of the gut microbiota in fish is influenced by its surrounding environment, in which diverse microbial species compete with each other for space, nutrients, and available energy.[1,2] Because conventional characterization of microorganisms has depended on cultivation-based techniques, our understanding of the gut microbiota is restricted to those that can be cultured. However, modern molecular methods, such as broad-range sequencing of 16S ribosomal RNA from amplified nucleic acid, indicate evolutionary divergence that can be used to identify and classify microorganisms. The availability of sequence data has facilitated the development of molecular probes for fluorescence *in situ* hybridization and DNA microarrays that can identify and enumerate specific species.[3] Recent studies are now finding that host–microbiota interactions are essential to many aspects of normal physiology, ranging from metabolic activity to immune homeostasis.[4] With the availability of new tools to examine complex microbial communities and the growing appreciation for the importance of the indigenous microbiota, this review will focus on the role of the microbiota in fish health status and how the immune system adapts to the presence of microorganisms in the fish gastrointestinal tract.

HOST–MICROBIOTA COMMUNICATION IN THE GUT

Fish intestinal mucosal surfaces interface with a complex and dynamic community of microorganisms. Although the composition of intestinal microbiota depends on genetic, nutritional, and environmental factors, it is generally accepted that the fish gut harbors an estimated 10^7–10^8 bacteria per gram.[2,5] Members of the genera *Aeromonas*, *Alcaligenes*, *Alteromonas*, *Carnobacterium*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Pseudomonas*, and *Vibrio* constitute the predominant intestinal microbiota of a variety of marine fish species. In contrast to marine fish, the intestinal microbiota of freshwater fish species tend to be dominated by members of the genera *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Lactococcus*, and *Pseudomonas*, representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides*, *Clostridium*, and *Fusobacterium*.[2,5] In rare circumstances these microorganisms cause disease, either directly, by damaging or traversing epithelial layers, or indirectly, by inducing tissue-damaging inflammatory responses.[2] If microbial pathogens invade the host, innate and adaptive defense mechanisms are activated for preventing further spread of the infection. In healthy hosts, the intestinal immune system is hyporeactive, but it is capable to mount an extensive immune reaction against pathogenic bacteria.[6] However, in addition to responding to these sporadic confrontations with microbial pathogens, the immune system must also properly calibrate responses to frequent confrontations with non-pathogenic commensal and microorganisms from the external environment.[3] It is well documented that gut

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microbiota is essential for intestinal development, homeostasis, and protection against pathogenic challenges, to the point that some investigators have referred to it as an "extra organ" of the host.[7] The gut represents the natural interface between intestinal microbiota and the host; in fact, the mucosal surfaces of the gastrointestinal tract are the main sites in which environmental microorganisms and antigens interact with the host, through intensive cross-talks.[8] Thus, the first line of defense is provided by the mucus layer, which covers the epithelium and contains various protective and antimicrobial substances secreted by epithelial cells, including complement components, mucins, enzymes, piscidins, and defensins.[9,10]

With intestinal microbiota in continuous direct contact with the gut mucosa, the gut-associated lymphoid tissue (GALT) must develop mechanisms to distinguish between potentially pathogenic microorganisms and commensal microbiota, and to determine whether tolerance or an immune response should be induced. GALT contains important regulatory cells of the mucosal immune system such as lymphocytes, which are equipped to organize and mount rapid, selective, and potent immune responses against harmful foreign pathogens and phagocytes, which have a role in the sampling, presentation, and destruction of pathogens.[11] Commensal bacteria exert an effect as an important antigenic stimulus for the maturation of GALT implicated in the induction of local immune responses. Immunoglobulin A is the most abundantly produced immunoglobulin isotype at the surface of mucous membranes in mammals, which is capable of entering Peyer's patches across M cells and targeting dendritic cells that may direct bacteria in the form of immune complexes into the GALT to permit continuous immune stimulation under non-inflammatory conditions.[12] Intestinal bacteria are essential for the normal development of GALT. In the absence of luminal bacteria, B cells and T cells do not home to the lamina propria of the intestine, and immunoglobulin A is not secreted.[13]

GALT in teleost fish lacks specialized structures—such as Peyer's patches in mammals—but the intestinal mucosa contains lymphocytes, plasma cells, granulocytes, and macrophages in the epithelial cells or disseminated throughout the lamina propria.[14] In this sense, *Lactobacillus delbrueckii* ssp. *delbrueckii*, isolated from intestinal microbiota of adult sea bass (*Dicentrarchus labrax*), was administered to sea bass larvae and post-larvae to investigate the effects on development and differentiation of GALT, and it was observed that the number of T cells and acidophilic granulocytes in treated fish was significantly higher than in controls.[15]

Intestinal epithelial cells process the combined information from the luminal microbiota and the intestinal immune system. In consequence, the activation status and immune regulatory function is dependent on the kind of bacteria and immune-derived stimuli they receive. Intestinal epithelial cells are capable of sensing components of the microbiota through the expression of pattern recognition receptors.[6] One of the most representative members of pattern recognition receptors is the Toll-like receptor family; these receptors recognize bacterial lipopolysaccharides and other characteristic microbial molecules such as pathogen-associated molecular patterns.[16] Toll-like receptors help direct the immune response by activating signaling events that increase expression of soluble mediators, which recruit and regulate the immune and inflammatory cells that initiate or enhance immune responses (**Figure 1**).[17] These mediators can exert an effect locally to recruit other immune cells to the site of activation, or can exert an effect systemically to mediate a systemic immune response. Important soluble mediators

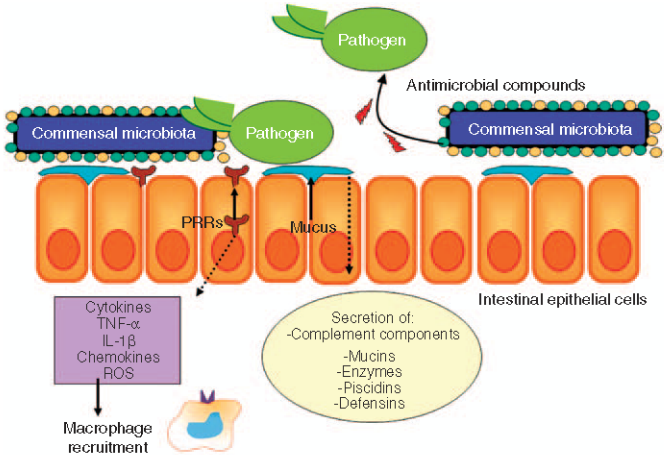

Figure 1 Mucosal immune system discriminates between pathogenic and commensal bacteria through pattern recognition receptors (PRRs), which mediate the detection of bacterial antigens and activate signaling cascades that regulate immune reactions (cytokines). Commensal bacteria (probiotics) can provide protection through the creation of a hostile environment for pathogenic bacteria by the production of inhibitory compounds, by competing for adhesion sites, or by modulating the immune response.

include the inflammatory cytokines, tumor necrosis factor-α, interleukin-1β (IL-1β), chemokines, and reactive oxygen species, including superoxide and nitric oxide, as well as the anti-inflammatory cytokine IL-10.[18–20] Activation by pathogen-associated molecular patterns also stimulates the expression of a distinctive subset of microbial defense mediators that are specific for the particular Toll-like receptor activated.[21] In fact, Picchietti *et al.*[15] quantified transcripts of immune-related genes (CD4, CD8-α, Cox-2, immunoglobulin A, IL-1β, IL-10, T cell receptor-β, and transforming growth factor-β) of the sea bass as markers of inflammation and cell-mediated immunity. Real-time PCR detected lower IL-1β transcripts, a typical pro-inflammatory cytokine, in probiotic group than in controls. Similarly, Cox-2, IL-10, and transforming growth factor-β transcripts were reduced in probiotic group, indicating a trend toward downregulation of inflammatory genes. These data differ from a study with rainbow trout (*Oncorhynchus mykiss*), in which upregulation of IL-1β_1 and transforming growth factor-β transcripts was observed in the spleen and head kidney after dietary administration of freeze-dried forms of *Lactobacillus rhamnosus* and *Enterococcus faecium*.[22]

In addition, to resolve the question of how the intestine calibrates its response against high lipopolysaccharide loads, Bates *et al.*[23] have recently shown that the zebrafish intestinal alkaline phosphatase is required to detoxify lipopolysaccharide and to prevent intestinal inflammation in response to the resident microbiota.

PROBIOTICS

Colonization of intestinal mucosal surfaces with a normal microbiota has a positive effect on immune regulatory functions, and disturbance of these functions by an imbalanced microbiota may contribute to the development of diseases. In fact, Rawls *et al.*[4] have shown that the establishment of the gut microbiota in zebrafish results in the induction of a complex pattern of gene expression. The genes expressed include those involved in stimulation of epithelial proliferation, promotion of nutrient metabolism, and innate immune responses. Significant attention has therefore been recently focused on the role of probiotics in the induction or restoration of a disturbed microbiota to its normal beneficial composition. Probiotics have been defined by the World Health Organization–Food and Agriculture Organization as "live microorganisms which when administered in adequate amounts, confer a health benefit on the host."[24]

The use of probiotic bacteria has been applied in human and animal nutrition with successful results.[25] In the past 10 years there has been a growing interest in fish farming to control diseases through alternative methods, such as probiotics.[26,27]

Recent evidence shows that some members of lactic acid bacteria, notably the genera *Lactococcus* and *Carnobacterium*, are important transient or permanent inhabitants in the gastrointestinal tract of fish.[28–30] They are believed to exert several beneficial effects there, such as nutritional contribution and protection against pathogens, either by the production of antimicrobial compounds or through competition for mucosal binding sites.[31] They are also vital to modulating interactions with the environment and the development and/or activation of beneficial immune responses associated with the mucous membranes that line the epithelial surfaces.[28,32]

A wide range of microalgae (*Tetraselmis*), yeasts (*Debaryomyces, Phaffia,* and *Saccharomyces*), and Gram-positive (*Bacillus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Micrococcus, Streptococcus,* and *Weissella*) and Gram-negative bacteria (*Aeromonas, Alteromonas, Phaeobacter, Pseudomonas,* and *Vibrio*) have been applied as probiotics to improve aquatic animal growth, survival, health, and disease prevention.[26,27] **Table 1** shows lactic acid bacteria and other microorganisms that are currently being used as probiotics, either singly or in combination. In this context, we have shown that feeding rainbow trout with members of its intestinal microbiota, such as *Lactobacillus sakei*, *Lactococcus lactis*, or *Leuconostoc mesenteroides*, for 2 weeks resulted in a higher survival rate after challenge with *Aeromonas salmonicida*. All three probiotic strains colonized the fish gastrointestinal tract and antagonized the populations of *A. salmonicida*, which initially infects the intestine and induces furunculosis in various wild and farmed fish species. In addition, there was a correlation between colonization with these probiotic strains and innate immune responses such as phagocytic activity and alternative complement pathway activity.[33]

Phagocytosis is responsible for early activation of the inflammatory response before antibody production, and is mediated by phagocytic cells, such as neutrophils, monocytes, and macrophages, in fish. The *in vivo* activation of phagocytic cells by immunomodulators may also lead to the secretion of a wide range of biologically active molecules, such as enzyme inhibitors, cationic peptides, and complement components, and to the production of reactive oxygen and nitrogen species that are involved in bactericidal activity.[34,35] Moreover, activation of the complement system initiates a cascade of biochemical reactions accompanied by the generation of biologically active mediators that result in antigen elimination through cell membrane lysis and activation of nonspecific mediators of inflammation.[36]

We have also shown that the administration of *L. mesenteroides* and *Lactobacillus plantarum* confers protection against *Lactococcus garvieae* in rainbow trout.[37] Competition for nutrients and adhesion receptors could be the basis for this protection, as microbiological and molecular analyses have revealed the presence of these probiotic strains in the fish intestine. In addition, it is well known that the gastrointestinal tract constitutes a preferred target for colonization by *L. garvieae*, and thus probiotic strains may create a hostile environment for the establishment of this pathogen.[38]

Probiotic microorganisms consist mostly of bacterial strains, although the use of other microorganisms such as yeasts has also been explored. Recently, Reyes-Becerril *et al.*[39] showed that administration of *Debaryomyces hansenii* resulted in an enhancement of the innate immune response in juvenile gilthead seabream (*Sparus aurata*). The mRNA transcript of Hep, immunoglobulin M, T cell receptor-β, nonspecific cytotoxic cell receptor protein 1, major histocompatibility complex class IIα,

Table 1 Probiotics used in fish and the effect on their host

Host species	Potential probiotic	Effect on host
Epinephelus coioides	*Lactobacillus plantarum* 7–40	Immune stimulation and improved survival after challenge with *Streptococcus* sp. or iridovirus.[48]
Oncorhynchus mykiss	*Vibrio fluvialis* A3-47S, *Aeromonas hydrophila* A3-51, *Carnobacterium* sp. BA211, *Micrococcus luteus* A1-6	Immune stimulation and improved survival after challenge with *Aeromonas salmonicida*.[49]
Oncorhynchus mykiss	*Lactobacillus rhamnosus* ATCC 53103	Immune stimulation and improved survival after challenge with *Aeromonas salmonicida*.[50,51]
Oncorhynchus mykiss	*Aeromonas sobria* GC2	Immune stimulation and improved survival after challenge with *Lactococcus garvieae* and *Streptococcus iniae*.[52]
Oncorhynchus mykiss	*Carnobacterium maltaromaticum* B26, *Carnobacterium divergens* B33	Expression of cytokine genes and improved survival after challenge with *Aeromonas salmonicida* and *Yersinia ruckeri*.[28,53]
Oncorhynchus mykiss, Salmo trutta	*Lactobacillus sakei* CLFP 202, *Lactococcus lactis* CLFP 100, *Leuconostoc mesenteroides* CLFP 196	Immune stimulation and improved survival after challenge with *Aeromonas salmonicida*.[32,33,54]
Oncorhynchus mykiss	*Lactobacillus rhamnosus* ATCC 53103, *Bacillus subtilis*, *Enterococcus faecium*	Immune stimulation and expression of cytokine genes.[22]
Oncorhynchus mykiss	*Bacillus subtilis* AB1	Immune stimulation and improved survival after challenge with *Aeromonas* sp. ABE1.[55]
Oncorhynchus mykiss	*Lactobacillus plantarum* CLFP 238, *Leuconostoc mesenteroides* CLFP 196	Competitive exclusion and improved survival after challenge with *Lactobacillus garvieae*.[37]
Oncorhynchus mykiss	*Aeromonas sobria* GC2, *Brochothrix thermosphacta* BA211	Immune stimulation and improved survival after challenge with *Aeromonas bestiarum* and *Ichthyophthirius multifiliis*.[56]
Sparus aurata	*Lactobacillus delbrueckii* CECT 287, *Bacillus subtilis* CECT 35	Immune stimulation[41]

colony stimulating factor 1 receptor, C3, tumor necrosis factor-α, and IL-1β genes were significantly higher in head kidney from fish fed the yeast-supplemented diet.

Multistrain and multispecies formulations, which are more than one strain of the same species or closely related species, have been proven to have synergistic beneficial effects on the host health, although the underlying mechanisms remain unclear. In higher vertebrates, there is conclusive evidence that adequately designed multistrain probiotic formulations possess health-promoting effects that are lacking in monospecies probiotic diets.[40] Some of the proposed mechanisms include greater survival, growth, viability, or adhesion to mucosal surfaces of one species in the presence of another species, the production of different enzymes or other proteins, the creation of a probiotic niche, and additive/synergistic effects of strain-specific properties.[40–43] Salinas *et al.*[41] studied the effect of either *Lactobacillus delbrüeckii* ssp. *lactis* or *Bacillus subtilis* or a combination of both strains on the innate immune response of gilthead seabream. They showed that increased phagocytic activity of the leukocytes from fish fed the monospecies diets disappeared at third week, whereas fish that received the multispecies diet maintained their greater leukocyte phagocytic ability. Moreover, cytotoxic activity, which had not varied during the first 2 weeks of feeding in any experimental group, was higher in the multispecies group at third week. Capkin and Altinok[44] also reported the effects of a multistrain probiotic formulation (*Enterobacter cloacae* and *Bacillus mojavensis*) on prevention of yersiniosis in rainbow trout. After feeding fish with multistrain formulation for 60 days, the fish survival rate increased to 99.2% after challenge with *Yersinia ruckeri* compared with controls that had 35% survival rate. The successful multistrain treatment may be the result of an optimal combination of strain-specific properties.

The effect of the indigenous microbiota is restricted to not only immune response but also to the structure, function, and metabolism of the digestive tract of fish. The gut microbiota and, by the same token, probiotics have a strong effect on priming immunophysiologic regulation in the intestine's mucosal barrier, which has opened up new angles in the science of nutrition.[8]

Physiological effects that have been suggested include serving as a source of nutrients, vitamins, and enzymes, and contributing to the microbial breakdown of indigestible components such as chitin, *p*-nitrophenyl-β-*N*-acetylglucosamine, cellulose, and collagen.[45] Studies in freshwater angelfish (*Pterophyllum scalare*), oscars (*Astronotus ocellatus*), and the marine southern flounder (*Paralichthys lethostigma*) suggest that the intestinal anaerobic bacteria can perform a role in the digestive process of the fish by providing a variety of enzymes such as carbohydrases, phosphatases, esterases, lipases, and peptidases that help in the absorption of nutrients.[46] According to Aly *et al.*[47] the administration of *B. subtilis* and *Lactobacillus acidophilus* increased the body-weight gain and this could be attributed to the improved digestive activity by enhancing the synthesis of vitamins, cofactors, and enzymatic activity, with a consequent improvement in the digestion, nutrient absorption, and weight gain.

CONCLUSIONS

The manipulation of the host microbiota may represent a new possibility in the prevention or management of pathological and physiological disorders. Probiotics are usually members of the normal indigenous microbiota, which has an important role in the health of animals, and their addition can assist in returning a disturbed microbiota to its normal beneficial composition. Better understanding of how bacteria are able to differentially modulate host cell function will result from the identification of bacterial cell constituents and corresponding host cell receptors that modulate downstream responses. It will be crucial to consider the role of microbial communities and provide new avenues to treat and prevent infections.

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DISCLOSURE

The authors declared no conflict of interest.

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Identification and characterization of lactic acid bacteria isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with inhibitory activity against *Lactococcus garvieae*

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Abstract

A study was conducted to evaluate the probiotic properties of endogenous rainbow trout microbiota against pathogenic *Lactococcus garvieae*. A total of 335 bacterial strains were isolated from rainbow trout and screened for antagonistic activity against *L. garvieae* using an agar spot assay. Antagonistic strains were grouped by PCR amplification of repetitive bacterial DNA elements (rep-PCR) and identified by 16S rRNA gene sequence analysis. The results revealed that the antagonistic strains belonged to the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc*. Further probiotic characteristics, such as specific growth rate, doubling time, resistance to biological barriers, antibiotic resistance, hydrophobicity and production of antimicrobial substances, were also studied. These strains were able to survive low pH and high bile concentrations, showed good adherence characteristics and a broad spectrum of antibiotic resistance. The antagonistic efficacy was maintained after sterile filtration and was sensitive to proteinase K, indicating that proteinaceous extracellular inhibitory compounds were at least partially responsible for pathogen antagonism. Based on these results, these strains should be further studied to explore their probiotic effects in challenge experiments *in vivo*. This study shows clear evidence that the indigenous trout-associated microbiota may provide a defensive barrier against *L. garvieae*.

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Keywords: aquaculture, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, probiotics.

Introduction

Lactococcus garvieae is a Gram-positive pathogen that causes haemorrhagic septicaemia and meningoencephalitis in several species of fish (Vendrell, Balcázar, Ruiz-Zarzuela, de Blas, Gironés & Múzquiz 2006) and mammals (Teixeira, Merquior, Vianni, Carvalho, Fracalanzza, Steigerwalt, Brenner & Facklam 1996). Moreover, this bacterium has also been isolated from humans, suggesting that *L. garvieae* could be classified as a potential zoonotic agent (Fefer, Ratzan, Sharp & Saiz 1998). *Lactococcus garvieae* outbreaks in aquaculture are treated with antibiotics; however, these are often ineffective, and their indiscriminate use has led to an increase in antibiotic resistance (Romalde & Toranzo 2002; Vendrell *et al.* 2006).

The last decade has seen a growing interest in the application of probiotics through the use of beneficial microorganisms to prevent pathogenic micro-

organisms and reduce the incidence of fish diseases (Irianto & Austin 2002; Balcázar, de Blas, Ruiz-Zarzuela, Cunningham, Vendrell & Múzquiz 2006; Kesarcodi-Watson, Kaspar, Lategan & Gibson 2008). Most probiotics proposed as biological control agents in aquaculture are lactic acid bacteria (LAB), such as *Lactococcus* spp., *Pediococcus* spp. or *Lactobacillus* spp., although other genera (e.g. *Vibrio*, *Bacillus* and *Pseudomonas*) and yeast have also been studied (Merrifield, Dimitroglou, Foey, Davies, Baker, Bøgwald, Castex & Ringø 2010; Pérez, Balcázar, Ruiz-Zarzuela, Halaihel, Vendrell, de Blas & Múzquiz 2010). Several mechanisms have been suggested for the inhibitory action of probiotics towards bacterial pathogens, including a decrease in localized pH, the production of antibacterial substances, competition for nutrients and adhesion sites, and stimulation of the host's immune responses (Balcázar *et al.* 2006; Merrifield *et al.* 2010).

When selecting a new microorganism for testing as an effective probiotic, a number of properties need to be considered. To colonize the gastrointestinal tract, potential probiotics should express high tolerance to acid and bile and have the ability to adhere to intestinal surfaces (Jöborn, Olsson, Westerdahl, Conway & Kjelleberg 1997; Nikoskelainen, Salminen, Bylund & Ouwehand 2001; Balcázar, Vendrell, de Blas, Ruiz-Zarzuela, Múzquiz & Gironés 2008). Because of the serious concerns about the emergence of antibiotic resistance, probiotic strains should also be carefully screened for antimicrobial susceptibility (Vizoso-Pinto, Franz, Schillinger & Holzapfel 2006).

Consequently, the aim of this study was to identify and characterize the properties of the endogenous microbiota of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against pathogenic *L. garvieae* by studying their antibacterial activity, pH and bile tolerances, antibiotic resistance and adherence characteristics to determine their potential use as probiotics.

Materials and methods

Isolation and initial screening

A total of 60 healthy rainbow tout weighing 35–40 g were collected from two fish farms (n = 30) in north-east Spain. The farms were participating in a health-improvement programme and thus underwent regular health monitoring. All fish were killed with tricaine methanesulphonate (MS-222; Syndel Laboratories) at a concentration of 150 mg L^{-1} of water for 15 min. The protocol (PI04/09) was approved by the Ethical Committee on Animal Experimentation of the Universidad de Zaragoza, Spain.

Mucus samples were collected as previously described (Balcázar, de Blas, Ruiz-Zarzuela, Vendrell, Gironés & Muzquiz 2007a; Balcázar, Vendrell, de Blas, Ruiz-Zarzuela, Gironés & Múzquiz 2007b). Briefly, the gill mucus was isolated after removing the gills, and cutaneous mucus was collected from the whole body by scraping the surfaces with a rubber spatula into 1 mL of phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.2). For intestinal mucus, the intestine was separated and mucus was collected and homogenized in 1 mL of PBS. Serial tenfold dilutions were then plated on tryptic soy agar (TSA; Scharlau) and de Man, Rogosa and Sharpe agar (MRS; Pronadisa) with incubation at 22 °C for 48 h. Colonies with different morphological characteristics from each sample were selected, subcultured in tryptic soy broth (TSB; Scharlau) or MRS broth (Pronadisa) and stored in sterile glycerol (15% v/v) at −80 °C.

To assess the growth inhibition of a virulent strain of *L. garvieae* CLFP LG 1, previously isolated during a natural lactococcosis outbreak in rainbow trout (Vendrell, Balcázar, Ruiz-Zarzuela, de Blas, Gironés & Múzquiz 2007), all strains (n = 335) were grown on TSA and MRS at 22 °C for 24–48 h. After incubation, a loop of each strain was spotted onto the surface of TSA or MRS agar previously inoculated with overnight cultures of the target strain (CLFP LG 1). Clear zones after overnight incubation at 22 °C indicated the presence of antibacterial substances.

Phenotypic characterization

All antagonistic strains (n = 11) were initially characterized by determining colony morphology, cell morphology, motility, Gram stain and the production of cytochrome oxidase and catalase. Further biochemical characteristics were determined using API 50 CH and API 20 Strep tests (bioMérieux), according to the manufacturer's instructions.

Rep-PCR genomic fingerprinting

Rep-PCR analysis was used to group the isolates. Genomic DNA of each isolate was extracted and purified following the method previously described

by Balcázar *et al.* (2007a). Amplification reactions were performed in a total volume of 25 μL containing 0.2 μL of *Taq* polymerase, 1.5 μL of $MgCl_2$, 1 μL of each deoxynucleoside triphosphate, 2 μL of 20 μM $(GTG)_5$ primer (5′-GTG GTG GTG GTG GTG-3′) (Versalovic, Schneider, De Brujin & Lupski 1994) and 5 μL of DNA template. The PCR temperature profile (MJ Mini Gradient Thermal Cycler; Bio-Rad Laboratories) consisted of an initial denaturation at 94 °C for 2 min followed by 40 cycles of 45 s at 93 °C, 1 min at 50 °C, 1 min at 72 °C and a final extension for 6 min at 72 °C. The genetic fingerprints were resolved in a 1.5% agarose gel in Tris–acetate-EDTA buffer.

Genotypic identification

Following DNA extraction, the 16S rRNA gene was amplified using primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) (Lane 1991) in a MJ Mini Gradient Thermal Cycler (Bio-Rad Laboratories). The PCRs were conducting using 0.4 μL of *Taq* polymerase, 1 μL of $MgCl_2$, 1.25 μL of each deoxynucleoside triphosphate, 1.25 μL of 1 μM of each primer and 5 μL of DNA template. The samples underwent an initial denaturation of 10 min at 95 °C, and then 30 cycles of 15 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by 10 min at 72 °C.

The PCR products were purified (Promega Biotech Iberica) and were directly sequenced on a MegaBACE 500 sequencer following the manufacturer's protocols (Amersham Biosciences). The sequences obtained were compared against the sequences available in the GenBank, EMBL and DDBJ databases obtained from the National Center for Biotechnology Information using the BLASTN (Altschul, Gish, Miller, Myers & Lipman 1990).

Growth

Following preculturing at 22 °C in MRS broth (pH 6.2) for 24 h, isolates were diluted and inoculated in MRS broth in triplicate with an initial concentration of 10^7 CFU mL^{-1} (OD600) and stirred gently to maintain a homogeneous bath culture. The optical density was recorded every 4 h for 24 h, and the readings of the profiles were averaged. Growth profiles of the three candidate probiotics, such as specific growth rate (μ) and doubling time (t_d), were determined as described by Vine, Leukes & Kaiser (2004).

Hydrophobicity

The ability of the organisms to adhere to hydrocarbons (as a measure of their hydrophobicity) was utilized as an indicator of their ability to adhere to intestinal epithelial cells (after Pan, Li & Liu 2006). The assay was conducted as described by Vinderola & Reinheimer (2003) with some modifications. The LAB strains were harvested in the stationary phase by centrifugation at 5000 ***g*** for 5 min at 5 °C, washed twice in 50 mM K_2HPO_4 (pH 6.5) buffer and finally resuspended in the same buffer. These cell suspensions were adjusted to 1.0 (OD560) with the buffer, and 3 mL of bacterial suspension was put in contact with 600 μL of toluene (Lab-Scan) and vortexed for 2 min. The two phases were allowed to separate for 1 h at 37 °C. The aqueous phase was carefully removed and optical density measured. The decrease in the absorbance of the aqueous phase was taken as a measure of the cell surface hydrophobicity (H%), which was calculated with the formula $H\% = [(OD_0 - OD)/OD_0] \times 100$, where OD_0 and OD are the absorbance before and after the extraction with toluene, respectively.

pH and bile tolerances

Tolerance to different pH conditions was determined using LAB cultures grown to stationary phase (18 h) in MRS broth and adjusted to 10^8 CFU mL^{-1} (OD between 0.6 and 0.7). These bacterial suspensions were harvested by centrifugation (2500 ***g***, 10 min, 4 °C) and washed once in PBS. Bacterial pellets were resuspended in PBS and used for *in vitro* acid tolerance studies. Five hundred microlitres of the bacterial suspension was added to 4.5 mL of sterile PBS and adjusted to a series of pH values (1.0, 2.0 and 3.0) with HCl (Prasad, Gill, Smart & Gopal 1998). The suspensions were incubated at 22 °C, and after 0, 1, 2 and 3 h, viable counts were determined by plate counting on duplicate MRS agar (24 h at 22 °C).

Bile used in these studies was desiccated ox-bile (Oxoid). The procedure of Klaenhammer & Kleeman (1981) was used to determine the tolerance of the strains to bile (at final concentrations of 0%, 0.2%, 0.4%, 0.6%, 0.8% and 1.0% w/v) on MRS agar in duplicate.

Sensitivity to antibiotics

Antibiotic susceptibilities were assessed by the disc diffusion test in Mueller-Hinton agar. The antibiotic sensitivity discs included amoxicillin/clavulanic acid (30 μg), ampicillin (10 μg), chloramphenicol (30 μg), chlortetracycline (30 μg), clindamycin (2 μg), doxycycline (30 μg), enrofloxacin (5 μg), erythromycin (15 μg), florfenicol (30 μg), flumequine (30 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), nitrofurantoin (300 μg), oxolinic acid (2 μg), penicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), tylosin (150 μg) and vancomycin (30 μg). Agar plates were incubated at 22 °C for 48 h. The diameters of the growth inhibition halos were measured and the antibiograms interpreted in agreement with the National Committee for Clinical Laboratory Standards recommendations.

Partial characterization of inhibitory substances

Antimicrobial activity was determined as described by Nikoskelainen *et al.* (2001). Briefly, the supernatant from cultures of LAB in 50 mL MRS broth for 48 h at 22 °C was prepared by centrifugation at 2000 ***g*** for 10 min and sterilized by passage through a 0.45-μm Millipore membrane (Millipore). After sterilization, 25 mL of each cell-free culture supernatant was neutralized (pH 6.8) with 5 N NaOH to prevent the inhibitory effect of lactic acid. The other 25 mL was assessed at the *in situ* pH. *Lactococcus garvieae* was grown in MRS broth overnight at 22 °C. The cells were harvested by centrifugation (2000 ***g***), washed twice with PBS and resuspended in 5 mL of PBS. The bacterial suspensions were spread on MRS plates in triplicate, and 6-mm wells were made in each agar plate with a sterile Pasteur pipette and were filled with either 50 μL of neutralized supernatant or 50 μL of untreated supernatant and then air-dried for 10 min. In a further two wells, neutralized MRS and pH 6.0 MRS were added as negative controls. After incubation of *L. garvieae* for 24 h at 22 °C, the clearing zone was determined.

The sensitivity of cell-free culture supernatants to proteinase K (GE Healthcare) at a final concentration of 1.0 mg mL^{-1} was also tested in buffers recommended by the supplier. Samples, with and without proteinase K, were incubated at 37 °C for 2 h, and residual activity was determined by the previously described well plate assay.

Statistical analysis

Data were assessed by one-way analysis of variance (ANOVA) (after checking normal distribution with the Kolmogorov–Smirnov test), with Duncan's *post hoc* test. All statistics were performed using SPSS for Windows version 15.0 (SPSS).

Results

Isolation and identification of the strains

Eight of 198 isolated strains from the intestinal mucosa exhibited inhibitory activity against *L. garvieae* CLFP LG 1, but only two from the 105 gill isolates and one from the 32 cutaneous mucus isolates exhibited inhibitory activity. Of the inhibitory bacteria, six strains showed strong inhibition, causing a clear zone > 20 mm in the agar spot assay (Table 1).

Rep-PCR analysis demonstrated that these strains produced different genetic fingerprints, which could be grouped into three groups (data not shown). Subsequently, this was confirmed by partial 16S rRNA gene sequence analysis, which revealed that the strains showed highest nucleotide alignment identities to one of the three LAB strains: *Lactobacillus plantarum* subsp. *plantarum* (n = 5), *Lactococcus lactis* subsp. *cremoris* (n = 5) and *Leuconostoc mesenteroides* subsp. *mesenteroides* (n = 1) (Table 1). However, the results of the phenotypic characterization differed from the genetic identification for six strains (Table 1). As only three different species were identified as displaying antagonism, the subsequent probiotic assays were conducted with one representative of each species (CLFP 3, CLFP 25 and CLFP 68).

Growth, pH, hydrophobicity, pH and bile tolerances and sensitivity to antibiotics

The resulting growth curves (data not shown) led to the determination of specific growth rate and doubling time (Table 2). *Lactococcus lactis* showed slower growth rates than *Lb. plantarum* and *Leuc. mesenteroides*, whereas *Leuc. mesenteroides* exhibited greater growth rate and doubling time compared to the other two bacteria (Fig. 1). Similarly, the pH of the medium after 24 h was significantly lower ($P < 0.05$) for *Lb. plantarum* and *Leuc. mesenteroides* than for *L. lactis* (Fig. 2).

The hydrophobicity percentages (H%), an indication of adherence ability, were 24.99% for

Table 1 Antagonistic activity and identification of lactic acid bacteria strains isolated from rainbow trout against *L. garvieae*

Isolate code	Accession number[a]	Source	Pathogen inhibition[b]	Phenotypic identification (% similarity)[c]	Genetic identification (% similarity)
CLFP 3	FR670524	Mucus	++	*Lactobacillus paracasei* subsp. *paracasei* (99.4)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 6	FR670525	Gills	++	*Lactobacillus plantarum* (98.7)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 9	FR670526	Gills	+	*Leuconostoc* spp. (94.8)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 18	FR670527	Intestine	+	*Leuconostoc* spp. (99.1)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 23	FR670528	Intestine	+	*Leuconostoc* spp. (94.8)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 24	FR670529	Intestine	+	*Lactococcus lactis lactis* (85.7)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 25	FR670530	Intestine	++	*Lactococcus lactis lactis* (85.7)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 30	FR670531	Intestine	++	*Leuconostoc* spp. (94.8)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 31	FR670532	Intestine	++	*Lactobacillus pentosus* (98.7)	*Lactococcus lactis* subsp. *cremoris* (99.9)
CLFP 52	FR670533	Intestine	+	*Lactobacillus plantarum* (98.7)	*Lactobacillus plantarum* subsp. *plantarum* (99.8)
CLFP 68	FR670534	Intestine	++	*Leuconostoc mesenteroides* (89.3)	*Leuconostoc mesenteroides* subsp. *mesenteroides* (99.5)

[a]Genbank partial sequence.
[b]+, clear zone of 15 mm or more; ++, clear zone of 20 mm or more.
[c]API 50 CH and API 20 Strep (bioMérieux).

Table 2 Growth rate and doubling time of the selected lactic acid bacteria strains

Growth parameters	Bacterial strains		
	Lactobacillus plantarum	*Lactococcus lactis*	*Leuconostoc mesenteroides*
μ^a	0.35	0.31	0.57
t_g^b	0.86	0.97	0.52

[a]The growth rate (μ) was calculated as the slope of the polynomials for the exponential phase of the dispersion curves obtained from the kinetic growth of each strain evaluated, using the method of adjustment.
CLFP 3: $y = 0.350x + 6.454$; $r^2 = 0.999$
CLFP 25: $y = 0.308x + 6.934$; $r^2 = 0.998$
CLFP 68: $y = 0.571x + 6.158$; $r^2 = 0.999$
where y: Log CFU mL^{-1} and x: time.
[b]The doubling time (t_g) was calculated as: $t_g = \ln 2/\mu$.

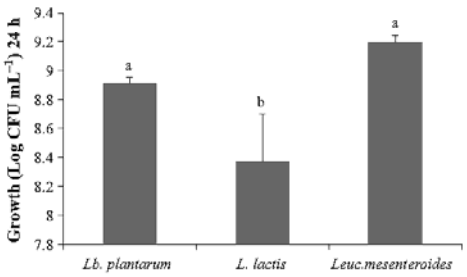

Figure 1 Growth of lactic acid bacteria strains after 24 h. Different letters indicate significant differences ($P < 0.05$).

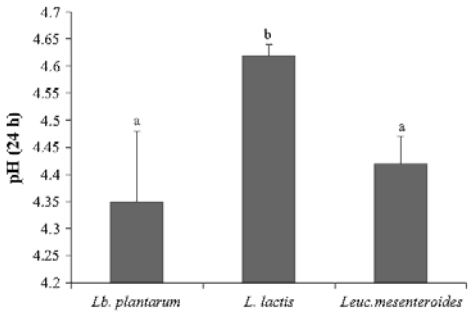

Figure 2 pH of Man, Rogosa and Sharpe broth cultures of lactic acid bacteria after 24-h incubation. Different letters indicate significant differences ($P < 0.05$).

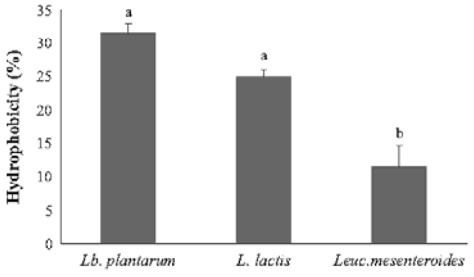

Figure 3 Hydrophobicity percentages of lactic acid bacteria strains. Different letters indicate significant differences ($P < 0.05$).

L. lactis, 31.59% for *Lb. plantarum* and 11.42% for *Leuc. mesenteroides* (Fig. 3). The hydrophobicity of *Leuc. mesenteroides* was significantly lower ($P < 0.05$) than for *Lb. plantarum* and *L. lactis*.

In addition, all LAB strains showed relatively high resistance to bile and low pH. No significant differences ($P > 0.05$) were observed between bile concentration sensitivity for the potential probiotics

tested. These strains were resistant to 1.0% bile and maintained the original population level through all tested concentrations. There were some indications that *Lb. plantarum* was less resistant to higher levels of bile (> 0.6%) than *L. lactis* and *Leuc. mesenteroides* (Table 3); however, there were no significant differences ($P > 0.05$) at 1.0% bile.

The three LAB strains remained viable after a 3-h exposure to pH values from 2.0 to 3.0, but none could tolerate exposure to pH 1.0 (Table 4). Significant differences ($P < 0.05$) of survival were observed between the LAB strains; *L. lactis* showed higher levels than *Lb. plantarum* and *Leuc. mesenteroides* at pH 2.0, whereas *Leuc. mesenteroides* showed higher levels than the other two bacteria at pH 7.4.

Antibiotic susceptibilities of *L. lactis*, *Lb. plantarum* and *Leuc. mesenteroides* are shown in Table 5. The three LAB strains were resistant to the majority of antibiotics tested. Some variations of susceptibility between strains were observed, but all were resistant to ampicillin, clindamycin, enrofloxacin, flumequine, kanamycin, nalidixic acid, oxolinic acid, penicillin, tetracycline, trimethoprim/sulfamethoxazole and vancomycin; all showed intermediate susceptibility to amoxicillin/clavulanic acid, and all were susceptible to gentamicin.

Table 3 Tolerance of lactic acid bacteria strains to different bile concentrations

	Log CFU mL^{-1} (SD)[1]		
Bile (%)	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
0.0	7.42 (0.01)	7.54 (0.01)	7.52 (0.08)
0.2	7.33 (0.02)	7.51 (0.08)	7.41 (0.08)
0.4	7.36 (0.03)	7.33 (0.18)	7.52 (0.01)
0.6	7.45 (0.01)^{a}	7.62 (0.04)^{b}	7.57 (0.04)^{b}
0.8	7.31 (0.08)^{a}	7.57 (0.02)^{b}	7.50 (0.04)^{b}
1.0	7.42 (0.04)	7.57 (0.08)	7.59 (0.01)

Different superscripts denote significant differences between the different strains at the respective bile concentration (i.e. between columns). There were no significant differences between the values at different concentrations for the respective probiotics (i.e. within columns).
[1]Data are presented as mean (standard deviations).

Table 4 Tolerance of LAB strains to different pH conditions for 3 h at 22 °C

	Log CFU mL^{-1} (SD)[1]		
pH	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
1.0	ND	ND	ND
2.0	3.82 (0.03)^{Aa}	4.24 (0.01)^{Ab}	3.80 (0.01)^{Aa}
3.0	6.51 (0.04)^{Ba}	6.48 (0.03)^{Ba}	6.51 (0.02)^{Ba}
7.4	6.46 (0.04)^{Ba}	6.55 (0.06)^{Ba}	6.66 (0.04)^{Bb}

ND, not detected; LAB, lactic acid bacteria.
Lowercase superscripts denote a significant difference between the LAB strains at the respective pH level (i.e. differences between columns).
Capital superscripts denote a significant difference between values for the respective strains at different pH concentrations (i.e. differences within columns).
[1]Data are presented as mean (standard deviations).

Partial characterization of inhibitory substances

After incubation of *L. garvieae* on MRS plates, measurable clearing zones were detected around the wells filled with both untreated and neutralized extracellular supernatants from the respective LAB strains. Additionally, the antimicrobial substances exhibited by the three LAB strains were found to be sensitive to proteinase K.

Discussion

There is no clear evidence that probiotic strains isolated from the host perform better than strains from a different habitat (Merrifield *et al.* 2010), but logically, a good initial site to screen for beneficial bacteria is the host's own microbiota (Fjellheim, Klinkenberg, Skjermo, Aasen & Vadstein 2010). In the present study, we obtained a pool of bacterial isolates from rainbow trout and characterized those with inhibitory activity against *L. garvieae*. Our study demonstrated that 3.28% (11) of the screened bacteria ($n = 335$) were antagonistic towards *L. garvieae*, which supports other studies that have also reinforced the view that antagonism between endogenous gut microorganisms of fish and bacterial pathogens occurs in nature and the establishment of a normal or protective microbiota might constitute a key component of defensive barrier function (Gómez & Balcázar 2008; Cain & Swan 2010). However, it should be noted that no isolates cultured on TSA displayed antagonism, and thus, it is recommended that future screening studies focus on using MRS agar to isolate antagonistic isolates. Several LAB isolated from fish and aquatic animals display antagonistic activity against fish pathogenic agents (Jöborn *et al.* 1997; Ringø, Sepploa, Berg, Olsen, Schilinger & Holzapfel 2002; Kim & Austin 2008; Rengpipat, Rueangruklikhit & Piyatiratitivorakul 2008; Ringø, Løvmo, Kristiansen, Bakken, Salinas, Myklebust, Olsen & Mayhew 2010); however, to our knowledge, this is the first report demonstrating a clear and consistent antagonism of

Table 5 Antibiotic susceptibilities of lactic acid bacteria strains

Antibiotic	*Lactobacillus plantarum*	*Lactococcus lactis*	*Leuconostoc mesenteroides*
Amoxicillin/clavulanic acid (bioMérieux)	I	I	I
Ampicillin (bioMérieux)	R	R	R
Chloramphenicol (Bio-Rad)	S	S	S
Chlortetracycline (Mast Diagnostics)	R	R	I
Clindamycin (Bio-Rad)	R	R	R
Doxycycline (bioMérieux)	S	I	I
Enrofloxacin (Bio-Rad)	R	R	R
Erythromycin (BBL Sensi-Disc)	S	R	S
Florfenicol (BBL Sensi-Disc)	R	I	I
Flumequine (Bio-Rad)	R	R	R
Gentamicin (Bio-Rad)	S	S	S
Kanamycin (BBL Sensi-Disc)	R	R	R
Nalidixic acid (BBL Sensi-Disc)	R	R	R
Nitrofurantoin (Bio-Rad)	I	R	R
Oxolinic acid (BBL Sensi-Disc)	R	R	R
Penicillin (bioMérieux)	R	R	R
Streptomycin (BBL Sensi-Disc)	S	R	R
Tetracycline (bioMérieux)	R	R	R
Trimethoprim/Sulfamethoxazole (Bio-Rad)	R	R	R
Tylosin (Neo-Sensitabs)	S	R	R
Vancomycin (bioMérieux)	R	R	R

R, resistant; I, intermediate; S, susceptible.

indigenous rainbow trout microbiota against *L. garvieae*, mediated, in part at least, by extracellular antimicrobial peptides.

The ability to adhere to intestinal epithelial cells is thought to be an important property of potential probiotic strains. Pan *et al.* (2006) assessed the ability of LAB strains to adhere to intestinal epithelial cells and concluded that the higher hydrophobic strains displayed stronger adhesive capability. *Lactobacillus plantarum* and *L. lactis* displayed significantly better hydrophobicity values than *Leuc. mesenteroides*. Thus, it is likely that the ability of *Leuc. mesenteroides* to adhere to intestinal epithelial cells is less than that of the other two LAB strains. Tolerance to bile is also important for the probiotic strains to grow and survive in the fish intestine (Nikoskelainen *et al.* 2001; Chabrillón, Arijo, Díaz-Rosales, Balebona & Moriñigo 2006; Fjellheim *et al.* 2010; Lazado, Caipang, Rajan, Brinchmann & Kiron 2010). However, there is still no consensus about the precise concentration to which the selected strain should be tolerant (Balcázar *et al.* 2008). In the present study, the three LAB strains tested showed little or no decrease in viable cell numbers after 3-h incubation at pH 3.0 and at a 1.0% bile. However, there were some indications that *Lb. plantarum* might be less resistant to higher bile acid concentrations than the other two strains. Potential probiotic strains with high hydrophobicity that are less sensitive to acid and bile may be more likely to survive passage through the gastrointestinal tract and potentially colonize the intestinal surfaces of the fish (Nikoskelainen *et al.* 2001; Zhou, Pan, Wang & Li 2007).

In the present study, the three LAB strains showed a broad spectrum of antibiotic resistance. It should be emphasized that antimicrobial compounds are still applied in aquaculture in addition to feed or by immersion (Cabello 2006). Thus, chemotherapy may disturb the homeostasis of gut microecology and physiology, which could cause fish to be vulnerable to infections (Kim & Austin 2008). In this respect, antibiotic-resistant probiotics may be advantageous in the case of administration of antibiotics to fish and in the establishment of the beneficial microorganisms in the intestine for prolonged periods (Kim & Austin 2008). However, in future, it should be ensured that such resistance cannot be transferred via plasmids.

The partial characterization of inhibitory substances suggested that the inhibitory activity was not caused only by organic acid production, as has been observed elsewhere (Brunt & Austin 2005), but also by proteinaceous compounds, which could be bacteriocins or bacteriocin-like inhibitory substances (BLIS). Bacteriocins are ribosomally synthesized, extracellularly released, bioactive peptides or peptide complexes that have a strong inhibitory

effect against other bacterial species (Dawn & Falkiner 1997; Riley & Wertz 2002) and are known to be produced by a wide range of LAB species, including those from the genera isolated in the present study (Klaenhammer 1993; Nes, Diep, Havarstein, Brurberg, Eijsink & Holo 1996).

Lactic acid bacteria form part of the normal microbiota of the gastrointestinal tract of both hatchery-cultured and wild-caught rainbow trout (González, Encinas, García-López & Otero 2000; Heikkinen, Vielma, Kemiläinen, Tiirola, Eskelinen, Kiuru, Navia-Paldanius & von Wright 2006; Balcázar *et al.* 2007a,b) as well as being found in other marine and freshwater fish species (Bucio, Hartemink, Schrama, Werreth & Rombouts 2006; Itoi, Abe, Washio, Ikuno, Kanomata & Sugita 2008). Thus, these antagonistic LAB may be beneficial for the control of intestinal microbiota by competition with pathogen species, as it has been suggested that the gastrointestinal tract is a possible port of entry for the bacterial fish pathogen *L. garvieae* (Vendrell *et al.* 2006) as well as a target for protective treatments, such as feeds containing probiotic bacteria (Brunt & Austin 2005; Vendrell, Balcázar, de Blas, Ruiz-Zarzuela, Gironés & Múzquiz 2008).

Given the good pH and bile tolerances, the ability to suppress pathogen growth under *in vitro* conditions as well as the positive indications towards epithelial adherence, *L. lactis*, *Lb. plantarum* and *Leuc. mesenteroides* should be further studied in challenge experiments *in vivo* for use as potential probiotics for the control of lactococcosis and may provide an alternative to the current use of antimicrobial compounds.

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Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection

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ABSTRACT

The aim of the present study was to investigate the effect of lactic acid bacteria (LAB) on the control of lactococcosis as well as to assess the impact of probiotics on the expression of immune-related genes in the head kidney and intestine of rainbow trout (*Oncorhynchus mykiss*). *Lactobacillus plantarum*, *Lactococcus lactis* and *Leuconostoc mesenteroides*, were administered orally at 10^6 CFU g^{-1} feed to fish for 36 days. Twenty-one days after the start of the feeding period, fish were challenged with *Lactococcus garvieae*. Only the fish fed the diet containing *Lb. plantarum* showed significantly ($P < 0.05$) improved protection against *L. garvieae* compared to the control. Subsequently, real-time PCR was employed to determine the mRNA levels of IL-1β, IL-8, IL-10 and TNF-α in the head kidney, and IL-8, Tlr5 and IgT in the intestine of the control and *Lb. plantarum* groups. IL-1β, IL-10 and TNF-α gene expression were significantly up-regulated by *Lb. plantarum*. Moreover, the mRNA levels of IL-10, IL-8 and IgT were significantly higher in the Lb. plantarum group after *L. garvieae* infection, suggesting that *Lb. plantarum* can stimulate the immune response of rainbow trout.

PCR-DGGE revealed no detectable levels of the probiotics or the pathogen present on the distal intestinal mucosa. These findings demonstrate that direct probiotic–host interactions with the intestine are not always necessary to induce host stimulatory responses which ultimately enhance disease resistance. Furthermore, as *L. garvieae* did not colonise the intestinal tract, and therefore likely did not infect via this route, the antagonistic properties of the probiotic candidate towards *L. garvieae* were likely of little influence in mediating the improved disease resistance which could be attributed to the elevated immunological response.

1. Introduction

Lactococcus garvieae is the causal agent of lactococcosis, a disease that causes severe economic losses in farmed marine and freshwater fish species, particularly during the summer months, given its association with high water temperatures [1]. The disease is characterised by a haemorrhagic septicaemia and meningoencephalitis in several species of marine and freshwater fish [1,2] and mammals [3]. Moreover, *L. garvieae* has also been isolated from humans in several cases, suggesting that this bacterium could be catalogued as a potential zoonotic agent [4].

In fish farming, bacterial disease outbreaks are typically treated with antibiotics; however, they are often ineffective and their indiscriminate and prophylactic use has led to an increase in antibiotic resistances [1]. Commercial vaccines are available for some pathogens [5], but vaccination cannot prevent disease development in immunologically immature fish. In the case of lactococcosis, immunity after vaccination gives a good level of protection, but it only lasts for a short period of time [6].

Over the last decade there has been a growing interest in the use of beneficial microorganisms to prevent or control pathogenic microorganisms as an alternative to traditional disease control treatments [7,8]. Probiotics have been defined as a viable microbial feed supplements which beneficially influence the health of the host [9], and they offer potential alternatives by providing benefits to the host primarily via the direct or indirect modulation of the gut microbiota. Suggested modes of action resulting from increased

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favourable bacteria in the gastrointestinal tract include the production of inhibitory compounds, competition with potential pathogens, inhibition of virulence gene expression, enhancing the immune response, improved gastric morphology and aiding digestive function [10–12].

Remarkable progress has been achieved in isolating and characterising cytokine genes from fish in recent years [13]. In particular, pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) and the anti-inflammatory cytokine IL-10 [13–15], these stimulatory and inhibitory molecules are commonly used immune-regulatory genes studied in rainbow trout (*Oncorhynchus mykiss*). These cytokines are thought to contribute to defence mechanisms of the host in response to bacterial colonization or invasion [16]. In contrast, the main function of IL-10 seems to be regulation of the inflammatory response, thereby minimizing damage to the host induced by an excessive response [17]. IgT, an immunoglobulin specialized in mucosal immunity which acts like a mucosal antibody [18] and toll-like receptor 5 (Tlr5) is involved in host survival before adaptive immunity [19].

Therefore, the aim of the present study was to determine the protective effects of three lactic acid bacteria against pathogenic *L. garvieae* and to assess the impact of probiotics on the expression of immune-related genes in the head kidney and intestine in rainbow trout.

2. Materials and methods

2.1. Bacterial strains

Lactobacillus plantarum subsp. *plantarum* CLFP 3, *Lactococcus lactis* subsp. *cremoris* CLFP 25 and *Leuconostoc mesenteroides* CLFP 68, isolated from rainbow trout and identified by 16S rRNA gene sequencing, were selected as potential probiotics [20]. These strains were chosen from a pool of 335 isolates obtained from distal intestinal mucosa, cutaneous mucus and gills of healthy rainbow trout, because of their positive *in vitro* characteristics, which include resistance to pH and bile, positive adhesion characteristics and antagonism against *L. garvieae* [20]. These strains were grown aerobically in the de Man, Rogosa and Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 22 °C. Stock cultures stored at −80 °C were prepared from overnight cultures to which 15% (vol/vol) glycerol (Scharlab, Barcelona, Spain) was added just prior to freezing.

2.2. Preparation of the feed

The three selected strains were grown in MRS broth in a shaking incubator at 22 °C overnight. After incubation, the cells were harvested by centrifugation (2000 × *g*), washed twice with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride [pH 7.2]), and re-suspended in the same buffer. The absorbance at 600 nm was adjusted to 0.25 ± 0.05 which corresponded to 10^7–10^8 CFU mL^{-1}. Dilution plating was used to verify the relationship between absorbance at 600 nm and CFU mL^{-1}.

Commercial feed (Skretting, Burgos, Spain; 42% protein and 24% lipid) was used as the basal diet for the supplementation with the three selected strains. In order to reach a final concentration 10^6 CFU g^{-1} feed, after [21,22], bacterial suspensions were slowly applied into the feed, mixing part by part in a drum mixer. The probiotic concentration in each feed was determined by plate counting on MRS agar.

2.3. Fish and experimental conditions

Rainbow trout were obtained from a commercial fish farm in the Autonomous Community of Aragon, Spain. The fish were fed a standard commercial feed at a rate of 1.5% of the biomass per day. The fish had not been vaccinated nor exposed to fish diseases and were deemed pathogen free by standard microbiological techniques and by a previously described PCR technique for the detection of *L. garvieae* [23], *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri* [24]. The fish were acclimated for 2 weeks to laboratory conditions in tanks before the start of the trial. After the acclimation period, the average weight of the fish was 26 g and the fish were divided into five 1000 L tanks (one for each treatment, control group and cohabitation fish), each containing 45 fish. All the fish were maintained in re-circulating aerated freshwater at 15 ± 1 °C with a 25% water change everyday and a 12 h dark/12 h light photoperiod. During 36 days of probiotic supplementation, the water temperature was increased progressively from 15 ± 1 °C to 19 ± 1 °C to induce the experimental infection.

The first two groups were fed unsupplemented feed during the entire trial period. The first group served as the control, while the second group served to keep fish that were used for experimental infection (cohabitation method). The third, fourth and fifth groups were fed diet containing different viable lactic acid bacteria during the entire trial period. The third group was fed a diet supplemented with 10^6 CFU g^{-1} *Leu. mesenteroides*, the fourth one supplemented with 10^6 CFU g^{-1} *L lactis* subsp *cremoris* and the last group a diet supplemented with 10^6 CFU g^{-1} *Lb. plantarum*. Fish in all groups were fed at 1.5% of biomass over two feeding periods daily.

2.4. Experimental infection

After 21 days of probiotic feeding, the experimental infection was carried out by the cohabitation method. *L. garvieae* CLFP LG1, previously isolated during a natural outbreak in rainbow trout, was grown for 24 h at 22 °C in Tryptic Soy Agar (TSA; Scharlab, Barcelona, Spain). After incubation, the cells were harvested by centrifugation (2000 × g), washed twice with PBS, and re-suspended in the same buffer. The absorbance at 600 nm was adjusted to 0.125 ± 0.005 in order to standardize the number of bacteria (1×10^7 CFU mL^{-1}). The bacterial suspension was diluted to a density of 1×10^4 CFU mL^{-1}, and 0.1 mL of this suspension was injected intraperitoneally into cohabitation fish. The fish were anaesthetised with tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd Vancouver, Canada) before injection. All cohabitants (8 fish for each group) were marked by clipping the adipose fin after injection, and were placed into the appropriate tank with the experimental fish. Dead fish were collected and cumulative mortality was recorded and the probiotic efficacy was calculated on the last day of the trial by relative percent survival [25]. *L. garvieae* was isolated from tissue samples of freshly dead fish on Columbia Sheep Blood Agar (BioMérieux, Marcy l'Etoile, France) at 22 °C for 48 h, and its identity was verified by a previously described PCR method [23].

2.5. Sample collection

At the end of the 21-day probiotic feeding trial, 5 fish per treatment were sacrificed by immersion in a tank containing MS-222 at a final concentration of 150 mg L^{-1} for 15 min, according to the instructions given by the Zaragoza University Ethics Committee for Animal Experimentation.

Intestine and head-kidney samples were then collected from each treatment. Subsequently, the same number of samples ($n = 5$)

were taken at the end of the experimental infection. All samples were stored separately at −80 °C until use.

2.6. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE)

PCR-DGGE was employed to assess the potential colonization and population of the probiotics and pathogen on the distal intestinal mucosa in order to ascertain if this was an infection route for *L. garvieae* and whether the probiotic was able to colonise the mucosal surfaces. DNA was extracted from 10 (5 prior to infection with *L. garvieae* and 5 at the end of the challenge trial) intestinal samples per treatment (*Lb. plantarum*, *L. lactis* and *Leu. mesenteroides*) and the control group using QIAamp® Stool Mini Kit (Qiagen) prior to PCR amplification of V3 region of the bacterial 16S rRNA gene as previously described [26]. DGGE was performed using a Dcode Universal Mutation Detection System (Bio-Rad). Twenty μL of standardized PCR products were run on an 8% acrylamide gel with a denaturing gradient of 40–60% (where 100% denaturant is 7 M urea and 40% formamide). The gel was run at 65 V for 17 h at 60 °C in 1 ×TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA).

Visualization of the DGGE bands was achieved by SYBR Green staining (Molecular Probes, Eugene, OR, USA). Gels were scanned in a Bio-Rad universal hood II (Bio-Rad) and optimised for analyses by enhancing contrast and greyscale.

2.7. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, with some modifications. Briefly, 40 mg of tissue were homogenized in 1 mL of TRIzol and 200 μL of chloroform was added. After mixing, samples were centrifuged at 10,000 × *g* for 15 min. The upper aqueous phase was transferred in a tube containing an equal volume of isopropanol. Mixtures were thoroughly vortexed and centrifuged at 12,000 × *g* for 10 min. Supernatants were discarded and the precipitated RNA pellets were washed using 1 mL of 75% ethanol. Total RNA dissolved in diethylpyrocarbonate (DEPC) water was treated with DNAse (TURBO DNA-*free*™, Ambion) following the manufacturer's instructions, to remove the contaminating genomic DNA. RNA concentration and purity were measured spectrophotometrically (NanoDrop Technologies, Wilmigton, USA) and stored at −80 °C until use. A total amount of 1 μg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad). PCRs were performed with the SYBR green method in a iQ5 iCycler thermal cycler (Bio-Rad). Duplicate PCR reactions were carried out for each sample analyzed. The reactions were set on a 96-well plate by mixing, for each sample, 1 μL of diluted (1/20) cDNA, 5 μL of 2× concentrated iQ ™ SYBR Green Supermix (Bio-rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM forward primer and 0.3 μM of reverse primer. Table 1 presents the primer sequences used. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20s at 95 °C, 20s at 60 °C and 20s at 72 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak.

β-actin and 60S were used as house keeping genes in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative controls and no primer-dimer formations were observed in the control templates. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad). Modification of gene expression is represented with respect to the control sampled at the same time of the treatment.

2.8. Statistical analysis

Survival curves were calculated using the Kaplan–Meier method and compared by the log-rank test. One-way ANOVA and Tukey's multiple comparison test were used to determine the significant variation ($P < 0.05$) in the immune response between the control and experimental group. All statistics were performed using SPSS for Windows version 15.0 (SPSS, Chicago, USA).

3. Results

3.1. Disease challenge

To investigate whether the probiotics were able to protect rainbow trout against lactococcosis infection, fish were infected with *L. garvieae* by the cohabitation method. Fish fed diets containing probiotics showed a cumulative mortality ranging from 12.5% (*Lb. plantarum*) to 32.5% (*Leu. mesenteroides*), whereas mortality was 32.5% in fish not treated with the probiotics (Fig. 1). Statistical analysis demonstrated that fish fed the diet containing *Lb. plantarum* at 36 days had significantly ($P < 0.05$) lower mortality than fish fed diets containing *L. lactis*, *Leu. mesenteroides* and the control. Relative survival value was thus 61.5% in fish fed the diet containing *Lb. plantarum* compared with the control group. External examination and bacteriological analysis of fish that died during the study revealed the presence of *L. garvieae* in all cases.

3.2. PCR-DGGE analysis

PCR-DGGE analysis revealed that the intestinal mucosa of all fish sampled was devoid of *L. garvieae*. Additionally, no detectable probiotic populations were present on the intestinal mucosal tissues in the respective groups.

3.3. Relative mRNA expression of immune-related genes

As *Lb. plantarum* was the only strain to confer protection against *L. garvieae* infection in rainbow trout, this treatment was selected to investigate the expression of immune-related genes in the head

Table 1
Primers used for detection of target genes.

Gene	GenBank accession nos	Product size	Forward primer	Reverse primer
IL-1β	AJ223954	91	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCCTTG
IL-10	AB118099	70	CGACTTTAAATCTCCCATCGAC	GCATTGGACGATCTCTTTCTTC
TNF-α	AJ277604	75	GGGGACAAACTGTGGACTGA	GAAGTTCTTGCCCTGCTCTG
IL-8	AJ279069	69	AGAATGTCAGCCAGCCTTGT	TCTCAGACTCATCCCCTCAGT
IgT	AY870265	72	AGCACCAGGGTGAAACCA	GCGGTGGGTTCAGAGTCA
Tlr5	AB091105	89	GGCATCAGCCTGTTGAATTT	ATGAAGAGCGAGAGCCTCAG
β–actin	AJ438158	167	ACAGACTGTACCCATCCCAAAC	AAAAAGCGCCAAAATAACAGAA
60s	NM001165047	147	AGCCACCAGTATGCTAACCAGT	TGTGATTGCACATTGACAAAAA

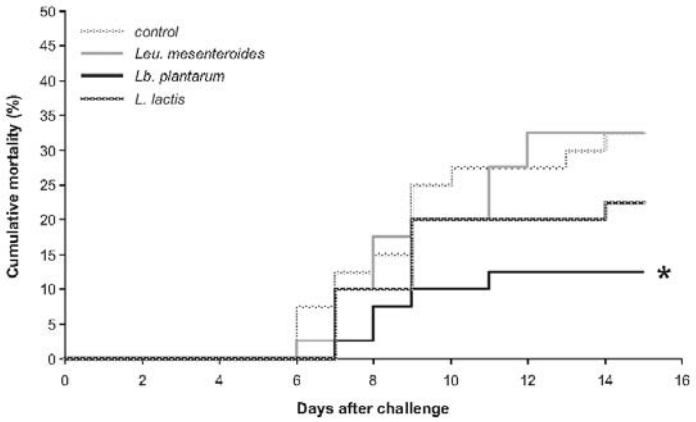

Fig. 1. Mortality curves of rainbow trout challenged with *L. garvieae* and treated with probiotics. Mortality between groups was compared using the Kaplan–Meier method. The asterisk indicates significant difference from the control ($P < 0.05$).

kidney (IL-1β, IL-10 and TNF-α) and the intestine (IL-8, IgT and Tlr5).

In the head kidney (Fig. 2), IL-1β, IL-10 and TNF-α gene expression in fish fed *Lb. plantarum* were significantly ($P < 0.001$) up-regulated prior to infection with *L. garvieae* compared to the control group. After the cohabitation, IL-1β and TNF-α gene expression were up regulated in the control group compared to pre-infection levels. In contrast however, IL-1β and TNF-α levels in the probiotic group were significantly lower than pre-infection levels with IL-1β expression significantly lower ($P < 0.001$) compared with the control group. However, IL-10 gene expression in the probiotic group was significantly ($P < 0.001$) higher after infection than in the control group.

In the intestine (Fig. 3), IL-8 gene expression was significantly ($P = 0.002$) up-regulated in the probiotic group prior to infection. After the infection, Tlr5 anf IgT levels were up-regulated in the intestine of fish from the control group whereas only IL-8 and IgT were up-regulated in the probiotic group; Tlr5 mRNA levels remained unaffected in the probiotic group post infection. After infection, IgT and IL-8 gene expression were significantly higher ($P < 0.001$) in the probiotic group than in the control group. In contrast, Tlr5 gene expression in the probiotic group was significantly ($P < 0.001$) lower after infection than in the control group.

4. Discussion

To date the beneficial effects of probiotic administration against *L. garvieae* infection in rainbow trout have been demonstrated with dietary *Aeromonas sobria* GC2 [27], *Leu. mesenteroides* CLFP 196 and *Lb. plantarum* CLFP 238 [22]. The present study confirmed the benefits of *Lb. plantarum* against *L. garvieae* infection as a significant reduction of cumulative mortality. In contrast, *L. lactis* and *Leu. mesenteroides* were ineffective at reducing *L. garvieae* induced mortalities.

To evaluate whether probiotic treatment had an effect on the expression of immune-related genes, four cytokines (IL-1β, IL-8, IL-10 and TNF-α), Tlr5 and IgT were examined using real-time PCR. In the present study, IL-1β, IL-10 and TNF-α gene expression were significantly up-regulated in the head kidney of fish fed dietary *Lb. plantarum* CLFP 3 compared to the control group. These findings

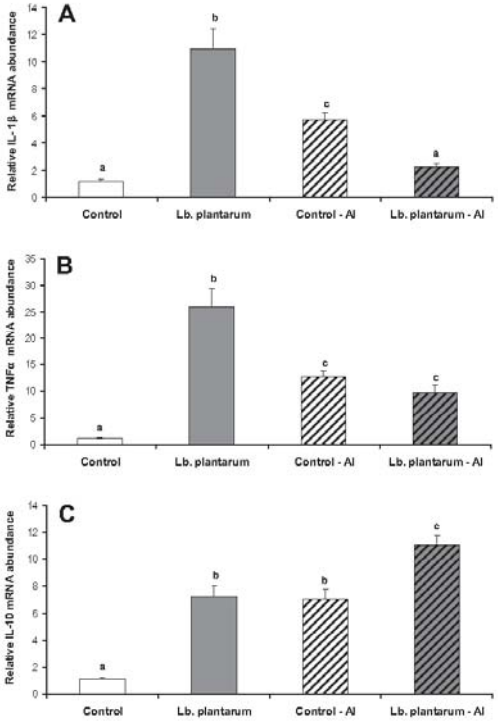

Fig. 2. IL-1β (A), TNF-α (B) and IL-10 (C) mRNA quantification in the kidney of control and fish fed diet containing *Lb. plantarum* CLFP 3 before and after *L. garvieae* infection (-AI). Values with different superscript letters are significantly different ($P < 0.05$).

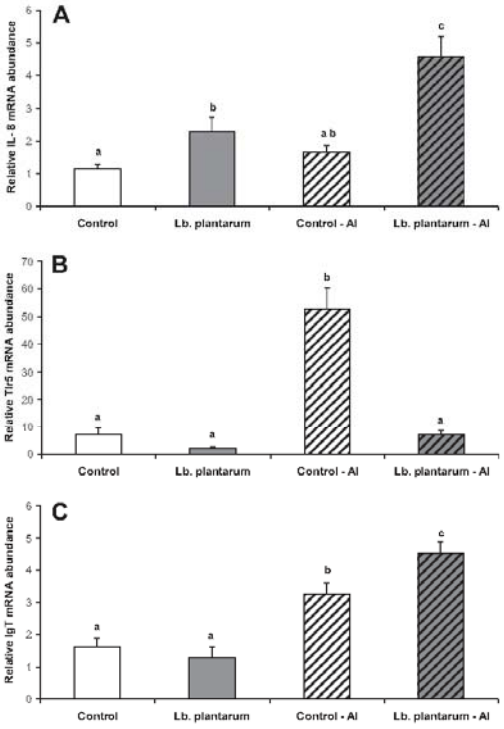

Fig. 3. IL-8 (A), Tlr5 (B) and IgT (C) mRNA quantification in the intestine of control and fish fed diet containing *Lb. plantarum* CLFP 3 before and after *L. garvieae* infection (-AI). Values with different superscript letters are significantly different ($P < 0.05$).

are in agreement with previous studies that have shown that the supplementation of probiotic bacteria increases the expression of pro-inflammatory cytokines, including IL-1β and TNF-α in rainbow trout [16,28]. The present study reports for the first time the up-regulation of rainbow trout IL-10, which is considered to have regulatory roles in immune responses [29], gene expression in response to probiotic feeding. Interestingly, the probiotic group displayed higher IL-10 gene expression after *L. garvieae* infection, both compared to the control group and compared to the pre-infection probiotic levels. This supports the findings from mice studies which show that probiotic strains displaying potential to induce higher levels of IL-10 offer good protection against *in vivo* diseases [30].

It is considered that resident mucosal immune cells in the intestine of fish may modulate the local immune site by secreting various cytokines and immune-regulatory substances. The intestinal epithelium may therefore play a key role in the initiation and regulation of mucosal immunity to bacteria by interacting with immune cells of the gut-associated lymphoid tissue, lamina propria lymphocytes and intraepithelial lymphocytes [31]. Previous studies have shown that the expressions of IL-8, as well as other cytokines, are elevated in the proximal intestine of rainbow trout when infected by *A. salmonicida* [15]. Similarly, *L. garvieae* infection in the present study induced a higher IL-8 mRNA abundance compared to pre-infection levels, but the differences were not significant. Compared to the control levels however, IL-8 gene expression was significantly up-regulated in the intestine of fish fed dietary *Lb. plantarum* both prior and after *L. garvieae* infection. *L. garvieae* infection in the present study also induced elevated IgT mRNA abundance levels in both the control and *Lb. plantarum* fed fish. In contrast to the effect on IL-8 in the present study, probiotic feeding had no effect on intestinal IgT mRNA levels in the absence of the bacterial challenge; however, post challenge with *L. garvieae*, IgT gene expression was significantly higher in the probiotic group than in the control group. Our results also demonstrated that Tlr5 gene expression in fish fed dietary *Lb. plantarum* CLFP 3 was not induced in the intestine, neither before nor after infection with *L. garvieae*. However, Tlr5 gene expression in the control group was significantly higher after infection. Although *L. garvieae* is not a flagellated bacterium, and Tlr5 is involved in the recognition of flagellin, previous studies have suggested that some pathogen-derived factors other than flagellin may contribute to its activation [32,33].

PCR-DGGE analysis revealed no detectable probiotic populations present on the intestinal mucosal tissues in the respective groups. Previous studies however have shown that the probiotic species used in the present study can populate the gastrointestinal tract of trout fed supplemented diets [20,21]. The present study indicates that these populations may be primarily luminal (allochthonous) populations which do not establish a resident population present on the epithelium itself. Despite this however, the immune-regulatory genes in the intestine were significantly affected by the application of dietary *Lb. plantarum* CLFP 3. These findings indicate that the direct association of the probiotic-epithelium is not always a prerequisite to induce localised effects which ultimately induce systemic immune responses. Therefore, probiotic candidates which do not show positive selection attributes (i.e. adhesion to epithelial cells, adhesion to mucus and growth within mucus, pathogen antagonism etc) in preliminary *in vitro* assays should not necessarily be dismissed as candidate strains. Alternative methods, such as the assessment of relevant immune-regulatory gene expression of intestinal mucosal cells and lymphocytes after exposure to probiotic cell wall components and extracellular products, could offer a viable *in vitro* method to be used in preliminary selection criteria.

Previous studies have indicated that the gastrointestinal tract can be a port of entry for *L. garvieae* [1]. The present study however revealed no detectable *L. garvieae* levels present from the intestinal mucosal samples which indicates that this was not the primary infection route for *L. garvieae* during the cohabitation challenge. These findings, along with the gene expression analyses, indicate that host immunological responses were responsible for mediating elevated diseases resistance of the *Lb. plantarum* fed fish in the present study, not probiotic antagonism and competition as has previously been observed with these strains *in vitro* [19].

5. Conclusions

Fish fed diet containing *Lb. plantarum* had significantly lower mortality than fish fed diets containing *L. lactis*, *Leu. mesenteroides* and the control. PCR-DGGE analysis revealed no detectable populations present on the intestinal mucosal tissues in the respective groups. In the head kidney, IL-1β, IL-10 and TNF-α gene expression were significantly up-regulated by *Lb. plantarum*. Moreover, IL-10 gene expression was also significantly higher in the *Lb. plantarum* group after *L. garvieae* infection. In the intestine, IL-8 was up-regulated in the probiotic group prior and after infection, but only after the infection was IgT significantly elevated. In contrast, Tlr5 gene expression in the probiotic group was lower after infection than in the control group. These findings indicate that host

immunological responses were responsible for mediating elevated diseases resistance of the *Lb. plantarum* fed fish.

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Lactococcus lactis subsp. _tructae_ subsp. nov. isolated from the intestinal mucus of brown trout (_Salmo trutta_) and rainbow trout (_Oncorhynchus mykiss_)

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Short title: _Lactococcus lactis_ subsp. _tructae_ subsp. nov.

Contents List Category: Gram positive of low G+C content

Accession number of 16S rRNA gene for strain L105^T: EU770697

Summary

The species *Lactococcus lactis* currently includes three subspecies isolated from milk sources (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*) and from the leafhopper *Hordnia circellata* (*L. lactis* subsp. *hordniae*). In this study, we isolated three strains from the intestinal mucus of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) closely related to this species. The 16S rRNA gene of the type strain L105^T showed 99.4% similarity with respect to the type strains of the subspecies *lactis* NCDO 604^T and *hordniae* NCDO 2181^T and 99.9% with respect to the type strain of *Lactococcus lactis* subsp. *cremoris* NCDO 607^T. The analysis of two housekeeping genes, *rpoB* and *recA*, confirmed the closeness of the trout strains with respect to the subspecies *cremoris* with 99.3% and 99.7% identities, respectively. However they can be differentiated on the basis of several phenotypic characteristics, as occurs in the case of the other two subspecies *lactis* and *hordniae*, which are also closely related on the basis of the 16S rRNA *rpoB* and *recA* genes. Therefore, we propose that the strains isolated in this study represent a new subspecies, *Lactococcus lactis* subsp. *tructae* subsp. nov. The type strain is L105^T (LMG 24662^T, DSM 21502^T).

The genus *Lactococcus* currently contains six species isolated from different vegetal, animal and food sources with *L. piscium* as the only species hitherto isolated from salmonid fish (Williams *et al.*, 1990). Within the species *L. lactis*, three subspecies are currently accepted (Scheleifer *et al.*, 1985), *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *hordniae*. The first two subspecies were isolated from milk sources and the last one was isolated from the leafhopper *Hordnia circellata* (Latorre-Guzmán *et al.*, 1977). Recently, strains from *L. lactis* subsp. *lactis* have been also isolated from intestinal tracts of freshwater fish (Itoi *et al.*, 2008 and 2009).

In this work, we isolated three strains from two different salmonid species, brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). The phylogenetic analysis based on the 16S rRNA, *rpoB* and *recA* genes placed these strains within the species *Lactococcus lactis* but the phenotypic characteristics were different to those of the three subspecies currently recognized in this species. Thus, we propose L105^T as the type strain of a new subspecies within *Lactococcus lactis* from which the name *Lactococcus lactis* subsp. *tructae* subsp. nov. is proposed.

The strains were isolated from intestinal mucus of salmonids in Man Rogosa and Sharpe agar (MRS, Pronadisa, Madrid, Spain) with incubation at 22°C for 24 h. The strain L105^T was isolated from brown trout and the strains L101 and I3 from rainbow trout. Colonies were white-cream coloured, opaque, round and convex.

Gram behaviour of cells was ascertained by staining (Doetsch, 1981), and they were Gram positive cocci, non sporulated and non motile as occurs in the remaining subspecies of *Lactococcus lactis*.

Amplification and sequencing of 16S rRNA gene was performed as described by Rivas *et al.* (2007). The sequences obtained were compared with those from GenBank using the BLASTN program (Alschul *et al.*, 1990) and aligned using the Clustal W software (Thompson *et al.*, 1997). The distances were calculated according to Kimura´s two-parameter model (Kimura, 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA 4.0 package (Tamura *et al.*, 2001) was used for all analyses.

The 16S rRNA gene sequences of the three strains isolated in this study were identical and thus only that of the type strain was analysed and deposited in Genbank. The comparison of the complete (1541 bp) 16S rDNA sequence with those held in the GenBank database indicated that L105^T belong to the species *Lactococcus lactis*, showing 99.4% similarity with respect to the type strains of the subspecies *lactis* NCDO 604^T and *hordniae* NCDO 2181^T and 99.9% with respect to the type strain of *Lactococcus lactis*

subsp. *cremoris* NCDO 607^T. In agreement with these results strain L105^T clustered with *Lactococcus lactis* subsp. *cremoris* NCDO 607^T (figure 1). In this analysis two strains isolated from fish by Itoi *et al.* (2009) were included showing that they clustered with *L. lactis* subspecies *lactis* and *hordniae* (figure S1 is available at IJSEM on line).

The REP-PCR pattern analysis using primer (GTG)$_5$ has been shown to be a good tool for *L. lactis* subspecies differentiation (Rademaker *et al.*, 2007; Svec & Sedlacek, 2008). Therefore we analysed these patterns according to the methodology described by Marilley *et al.* (2004) in our strains and in reference strains from each subspecies of *L. lactis* (see figure S2 is available at IJSEM online). The results showed that the strains belonging to the same subspecies displayed the same REP-PCR pattern that was clearly different among the subspecies of *L. lactis*. Therefore our results confirmed that REP-PCR fingerprinting is a rapid and reliable technique for the differentiation of subspecies within *L. lactis*. In order to analyse the genetic diversity of the three strains isolated in this study, we performed a RAPD analysis using the primer M13 (5'- GAG GGT GGC GGT TCT –3'), according to Rivas *et al.* (2006). The results showed that the patterns of the strains L105^T (lane 5), L101 (lane 4) and I3 (lane 5) were different (figure S3). These patterns were also different to those presented by the type strains of the three subspecies from *L. lactis* (lanes 1 to 3). The results of the RAPD analysis showed that the strains isolated in this study are genotypically diverse in spite of their identity in 16S rRNA gene.

The usefulness of housekeeping genes in bacterial taxonomy and phylogeny has been reported for several bacterial groups (Maiden, 2006) including Gram positive cocci of genus *Streptococcus*, a genus phylogenetically close to *Lactococcus* (Glazunova *et al.*, 2009; Pombert *et al.*, 2009). In this study we obtained partial sequences of two housekeeping genes, *rpoB* (about 460 nucleotides) and *recA* (about 330 nucleotides), in the type strains of the species of genus *Lactococcus* and in the strains isolated in this study. Gene amplification and sequencing were performed using the primers rpoBLac1F (5'-TAC GGK AAA CAC CGTA-3'), rpoBLac1R (5'-TCA ARC CAW GCT CCA CGG-3'), recALac1F (5'-GCA GCC TTT ATC GAT GCTG-3') and recA1R (5'-GCA CGA CCA CCA GG-3') designed on the basis of conserved regions of these genes in strains *L. lactis* subsp. *lactis* KF147 (accession number CP001834) and subsp. *cremoris* SK11 (accession number CP000425). The PCR conditions were as follows: pre-heating at 95 °C for 9 min; 35 cycles of denaturing at 95 °C for 1 min; annealing at 52°C (for *recA* gene) or at 47.5°C (for *rpoB* gene) for 1 min and extension at 72 °C for 1 min, and a final extension at 72°C for 7 min. The results of the phylogenetic analysis of *rpoB* and *recA*

gene are shown in figures 2 and 3, respectively. Since this is the first study of the *rpoB* and *recA* genes in the species of genus *Lactococcus*, there are no previous data about the identity levels of these genes among strains from the same species and among different species. Nevertheless, the results found in the genus *Streptococcus*, belonging to the same family than *Lactococcus*, showed that two close species, *S. salivarius* and *S. vestibularis*, presented about 97% and 91% identities in *rpoB* and *recA* genes, respectively (Drancourt *et al.*, 2004; Glazunova *et al.*, 2009; Pombert *et al.*, 2009). The results obtained in the genus *Lactococcus* showed lower identity values (up to 30% dissimilarity) in both genes among different species (figures 2 and 3). These results showed the high usefulness of the *rpoB* and *recA* genes for species differentiation within genus *Lactococcus*. Moreover these results showed that the type strains of subspecies *cremoris* and *lactis* from *L. lactis* showed identity values in *rpoB* and *recA* genes (97.2 and 91.8%, respectively) which are in the limit for species differentiation in the case of the genus *Streptococcus*. The type strain of the subspecies *hordniae* is closer to the subspecies *lactis* with about 99.8% identity in both genes (which represents 1 different nucleotide in both cases in the fragment analysed) than to the subspecies *cremoris* with about 97.3% and 92% identities in *rpoB* and *recA* genes, respectively. Concerning the new proposed subspecies *tructae* has an internal identity of 100% in these two genes and, as occurs in the case of the 16S rRNA gene, formed a cluster with the subspecies *cremoris* after the phylogenetic analysis of *rpoB* and *recA* genes (figures 2 and 3). The identity values with respect to this subspecies were 99.3% identity in the case of *rpoB* gene (3 different nucleotides in the fragment analysed) and 99.7% identity in the case of *recA* gene (1 different nucleotide in the fragment analysed). These data support the definition of the new subspecies *tructae* within *L. lactis*.

The analysis of G+C content was peformed by the Identification Service of DSMZ. For base composition analysis, DNA was extracted by and purified according to Cashion *et al.* (1977). The DNA was hydrolyzed with P1 nuclease and the nucleotides dephosphorylized with bovine alkaline phosphatase (Mesbah *et al.*, 1989). The mol % G+C content of DNA was determined by HPLC according to Mesbah *et al.* (1989) using a Shimadzu HPLC system (Shimadzu Corp., Japan). The DNA G+C content of strain L105^T was 36.0 mol%. The DNA-DNA hybridization analyses were performed according to the method of Ezaki *et al.* (1989), following the recommendations of Willems *et al.* (2001). DNA relatedness values for strain L105^T versus *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* and *L. lactis* subsp. *hordniae* were 62%, 90% and 60%, respectively. These values are in agreement with those of 16S rRNA and housekeeping gene sequences

showing that our strains are more closely related to the subspecies *cremoris* than to *lactis* and *hordniae*.

The cellular fatty acids were analysed by the Identification Service of DSMZ according to the instructions of the Microbial Identification System (MIDI; Microbial ID). In agreement with the 16S rRNA gene sequences, the fatty acid profile of our strain was similar to that of *L. lactis* according to the data recorded in the MIDI database. The main fatty acids found in strain L105^T were $C_{16:0}$ and $C_{19:0}$ cyclo ω8c comprising 37.6% and 26.6%, respectively. Other fatty acids detected in the strain L105^T were $C_{14:0}$ (13.1%), $C_{18:1\ \omega7c}$ (14.4%), $C_{18:0}$ (1.0%), $C_{20:2\ \omega6,9c}$ (1.0%), 11 methyl $C_{18:1\ \omega7c}$ (1.5%) and summed in feature 3: $C_{16:1\ \omega7c}$/$C_{15:0\ iso\ 2OH}$ (4.3%). This profile is closest to that of *L. lactis* subsp. *cremoris* than to those of subspecies *lactis* and *hordniae* although there were slight differences in the amounts of various fatty acids (see Table S1).

The acid production from carbohydrates were tested using the API 20STREP and API 50CH systems according to the manufacturer's instructions. The results were read as indicated by Schleifer *et al.* (1985). The growth in presence of 4% NaCl was checked using TSA as basal medium (Difco, Becton Dickinson, BBL). For testing the antibiotic resistance the following antibiotics were used: ampicillin (2 μg), erythromycin (2 μg), ciprofloxacin (5 μg), penicillin (10 IU), polymyxin (300 IU), cloxacillin (1 μg), oxytetracycline (30 μg), gentamycin (10 μg), cefuroxime (30 μg), or neomycin (5 μg), (Becton Dikinson, BBL). The disc diffusion method on Sheep Blood Agar prepared plates (Scharlau Microbiology) was used. The type strains of the three subspecies of *L. lactis* and the reference strains included in REP-PCR and RAPD analysis from each subspecies were included in the phenotypic study. Several differences were found among the strains isolated in this study with respect to the subspecies *lactis, cremoris* and *hordniae* (Table 1). The complete results of antibiotic resistance are in supplementary Table S2. Biocoding for the API 20 STREP tests resulted in 7203551 for strains L105^T and I3 and 7203551 for strain L101. Both codes match with *Lactococcus lactis* subsp. *lactis* with 39% and 87.8% identity, respectively, in APILAB database. The results of the phenotypic characterization showed that, in spite of the complete identity of the 16S rRNA, *rpoB* and *recA* genes of strains from the subspecies *tructae*, they were phenotypically diverse and variations were observed in the acid production from several carbon sources and in the natural antibiotic resistance. These strains differed in several characteristics from the remaining subspecies of *L. lactis* as can be seen in Table 1.

Our results confirmed the phenotypic differences between the subspecies *hordniae* and *lactis*, that were closely related on the basis of the genes analysed in this study, as was already pointed out by Schleifer *et al.* (1985) which proposed that the phenotypic characteristics are enough to differentiate among subspecies of *L. lactis*. In this way, the new subspecies *tructae* presented many phenotypic differences with respect to *L. lactis* subsp. *cremoris* that allow their differentiation in spite of the phylogenetic closeness of these two subspecies. The remaining subspecies can be clearly differentiated on the basis of the 16S rRNA and housekeeping genes and all subspecies of *L. lactis* can be differentiated by REP-PCR fingerprinting. All these results support the definition of a new subspecies for which the name *Lactococcus lactis* subsp. *tructae* subsp. nov. is proposed.

Description of *Lactococcus lactis* subsp. *tructae*

Description of *Lactococcus lactis* subsp. *tructae* (truc'ta.e L. gen. n. tructae, of a trout fish).

Characteristics additional to those reported in the original description of the species *Lactococcus lactis* (Schleifer *et al.*, 1985) that allow the differentiation from the remaining subspecies of this species are given below.

Arginine dehydrolase production positive after 24 h incubation. Growth in presence of 4% NaCl positive. Assimilation of gluconate is positive. Acid is produced from maltose, lactose, ribose, mannitol, sucrose and amygdalin. Acid is not produced from D-xylose. The production of acid from melibiose and raffinose is variable. Sensitive to cefuroxime and resistant to erythromycin and polymyxin B. Resistance variable to tetracyclin. The G+C content of strain L105^T was 36.0 mol%. The type strain L105^T (LMG 24662^T, DSM 21502^T) was isolated from the intestinal mucus of brown trout (*Salmo trutta*).

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Table 1. Differential characteristics for *Lactococcus lactis* subsp. *tructae* subsp. nov. from the remaining taxa of genus *Lactococcus*. 1, *L. lactis* subsp. *tructae* subsp. nov. L105^{T}; 2, *L. lactis* subsp. *tructae* subsp. nov. I3; 3, *L. lactis* subsp. *tructae* subsp. nov. L101; 4, data are from *L. lactis* subsp. *lactis* DSM 20481^{T} and LMG 7930; 5, data are from *L. lactis* subsp. *hordniae* DSM 20450^{T} and LMG 9462; 6, data are from *L. lactis* subsp. *cremoris* DSM 20069^{T} and LMG 8505; 7, *L. chungangensis* CAU 28^{T}; 8, *L. garvieae* KCTC 3772^{T}; 9, *L. pisicium* DSM 6634^{T}; 10, *L. plantarum* DSM 20686^{T}; 11, *L. raffinolactis* DSM 20443^{T}. *Results are from this study and agree with those of Teuber (2009) for taxa 4 to 6 and 8 to 11, Schleifer *et al.* (1985) for taxa 4, 5, 6, 8, 10 and 11 and Cho *et al.* (2008) for species 7. ¶Results are from Cho *et al.* (2008). ‡Results were positive for strain KCCM 40699^{T} (Cho *et al.*, 2008). #Results are from Wilson *et al.* (1990), Teuber (2009). □Results are from Schleifer *et al.* (1985), Teuber (2009). §After 24h incubation in API 20STREP. ¥After 24h incubation in API 50CH. *f*Data from antibiotic resistance are from this study. +: positive, -: negative, v: variable, w: weak, nd: no data, S: sensitive, R: resistant, WS: weakly sensitive.

Characteristic	**1***	**2***	**3***	**4***	**5***	**6***	**7¶**	**8□**	**9#**	**10□**	**11□**
Growth in 4% NaCl	+	+	+	+	-	-	-	+	-	+	-
Production of:											
Arginine dehydrolase§	+	+	+	+	+	-	-	+	-	-	-
Acid from:											
Ribose§,¥	+	+	+	+	-	-	nd	+	-	-	v
D-xylose¥	-	-	-	+	-	-‡	-	-	+	-	v
Mannitol§,¥	+	+	+	v	-	-	+	-	+	+	-
Lactose§,¥	+	+	+	v	-	+	-	+	+	-	+
Maltose¥	+	+	+	+	-	-	+	+	+	+	+
Melibiose¥	+	+	-	-	-	-	-	-	+	-	+
Sucrose¥	+	+	+	v	+	-	+	-	+	+	+
Raffinose§,¥	+	+	-	-	-	-	-	-	+	-	+
Amygdalin§,¥	+	+	+	w	-	-	+	+	+	+	-
Assimilation of:											
Gluconate¥	+	+	+	-	-	-	nd	nd	nd	-	-
Resistance/Sensibility to*f*:											
Cefuroxime	S	S	S	R	S	S	S	S	S	S	S
Tetracyclin	S	S	R	S	V	WS	S	S	S	S	S
Erythromycin	R	R	R	S	S	S	R	R	S	S	S
Polymyxin B	R	R	R	R	S	S	WS	R	R	R	WS

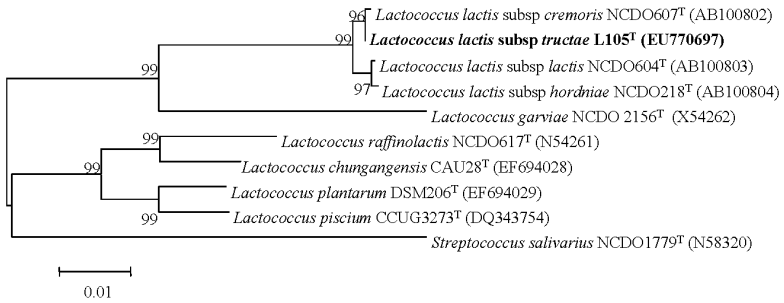

Figure 1. Neighbour-joining tree based on 16S rRNA gene sequences of *Lactococcus lactis* subsp *tructae* strains and other related taxa. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitutions per 100 nt.

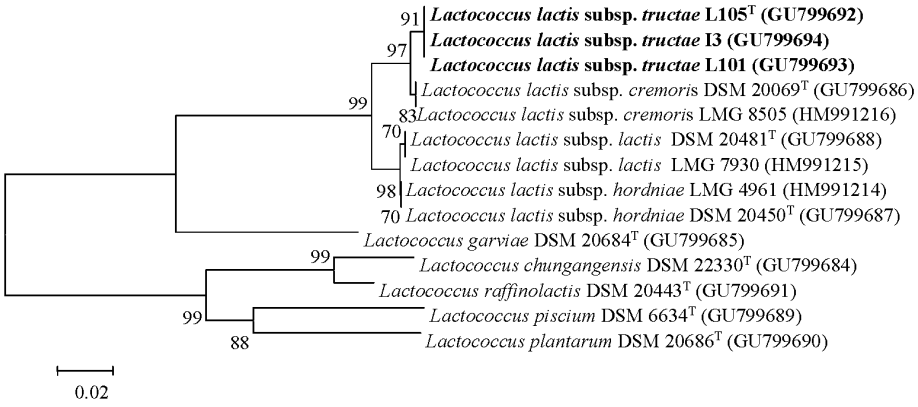

Figure 2. Neighbour-joining tree based on partial *rpoB* gene sequences of *Lactococcus lactis* subsp *tructae* strains and other related taxa. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.

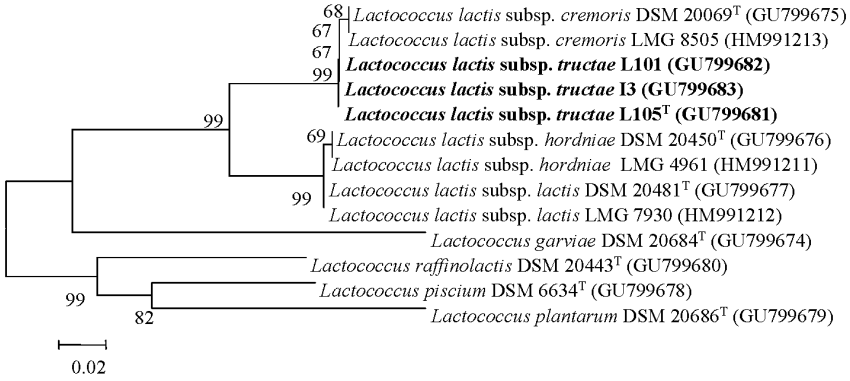

Figure 3. Neighbour-joining tree based on partial *recA* gene sequences of *Lactococcus lactis* subsp *tructae* strains and other related taxa. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 nt substitutions per 100 nt.

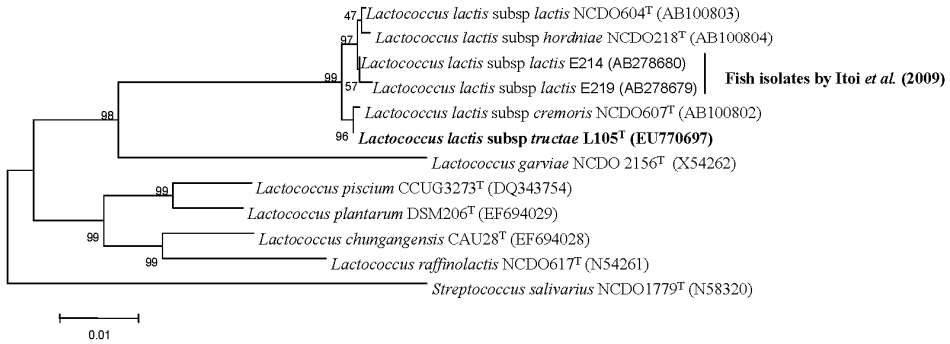

Figure S1. Neighbour-joining tree based on 16S rRNA gene sequences of *Lactococcus lactis* subsp *tructae* strains and other related taxa. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 1 nt substitutions per 100 nt.

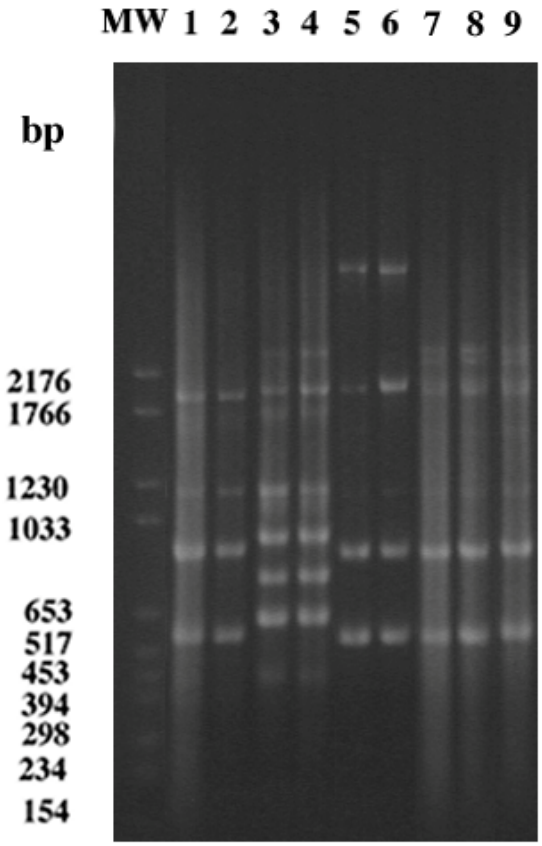

Figure S2. REP-PCR patterns of *L. lactis* subsp. *hordniae* DSM 20450^T (lane 1), *L. lactis* subsp. *hordniae* LMG 9461 (lane 2), *L. lactis* subsp. *lactis* DSM 20481^T (lane 3), *L. lactis* subsp. *lactis* LMG 7930 (lane 4), *L. lactis* subsp. *cremoris* DSM 20069^T (lane 5), *L. lactis* subsp. *cremoris* LMG 8505 (lane 6) , *L. lactis* subsp. *tructae* L105^T (lane 7) *L. lactis* subsp. *tructae* L101 (lane 8), and *L. lactis* subsp. *tructae* I3 (lane 9).

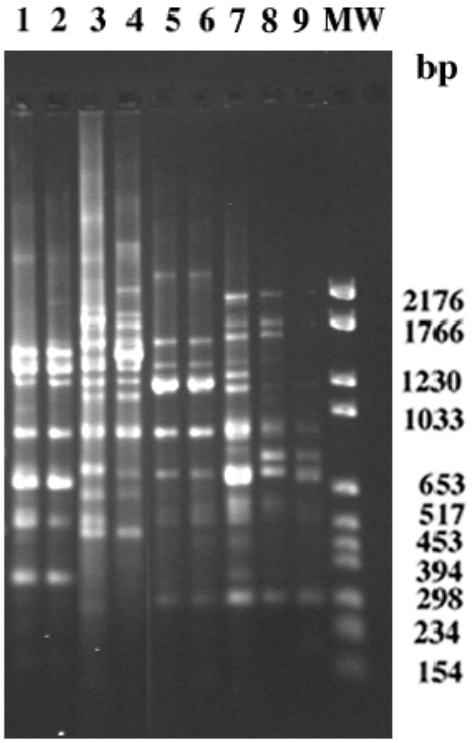

Figure S3. RAPD patterns of *L. lactis* subsp. *hordniae* DSM 20450^T (lane 1), *L. lactis* subsp. *hordniae* LMG 9461 (lane 2), *L. lactis* subsp. *lactis* DSM 20481^T (lane 3), *L. lactis* subsp. *lactis* LMG 7930 (lane 4), *L. lactis* subsp. *cremoris* DSM 20069^T (lane 5), *L. lactis* subsp. *cremoris* LMG 8505 (lane 6) , *L. lactis* subsp. *tructae* L105^T (lane 7) *L. lactis* subsp. *tructae* L101 (lane 8), and *L. lactis* subsp. *tructae* I3 (lane 9).

Table S1. Cellular fatty acid composition of the type strains from the subspecies of *Lactococcus lactis*. 1, *L. lactis* subsp. *tructae* subsp. nov. L105^T; 2, *L. lactis* subsp. *lactis* DSM 20481^T; 3, *L. lactis* subsp. *cremoris* DSM 20069^T; 4, *L. lactis* subsp. *hordniae* DSM 20450^T. Data are from this study and Cho *et al.*, 2008. Summed feature 3: $C_{16:1}\omega7c$/iso-$C_{15:0}$ 2-OH.

Fatty acid	1	2	3	4
$C_{14:0}$	13.07	8.9	10.0	3.4
$C_{15:0}$	-	-	0.5	0.3
$C_{16:0}$	37.6	45.7	40.3	32.6
$C_{18:0}$	0.98	2.7	0.7	1.3
Cyclo-$C_{17:0}$	0.55	-	0.7	-
$C_{18:1}\omega7c$	14.39	22.9	9.9	60.5
$C_{20:2}\omega6,9c$	1.04	-	1.5	-
Cyclo- $C_{19:0}\omega8c$	26.57	12.5	31.5	-
11-methyl $C_{18:1}\omega7c$	1.45	0.5	1.9	-
Summed feature 3	4.36	6.9	3.1	1.9

Table S2. Results from this study for the natural resistance to antibiotics of species from genus *Lactococcus*. 1, *L. lactis* subsp. *tructae* subsp. nov. L105^T; 2, *L. lactis* subsp. *tructae* subsp. nov. I3; 3, *L. lactis* subsp. *tructae* subsp. nov. L101; 4, *L. lactis* subsp. *lactis* DSM 20481^T and LMG 7930; 5, *L. lactis* subsp. *hordniae* DSM 20450^T and LMG 9462; 6, *L. lactis* subsp. *cremoris* DSM 20069^T and LMG 8505; 7, *L. chungangensis* CAU 28^T; 8, *L. garvieae* KCTC 3772^T; 9, *L. pisicium* DSM 6634^T; 10, *L. plantarum* DSM 20686^T; 11, *L. raffinolactis* DSM 20443^T. S: sensitive, R: resistant, WS: weakly sensitive, V: variable.

Characteristic	1*	2*	3*	4*	5*	6*	7¶	8□	9#	10□	11□
Resistance/Sensibility to[f]:											
Gentamycin	S	WS	S	S	S	V	R	R	R	R	R
Tetracyclin	S	S	R	S	V	S	S	S	S	S	S
Erythromycin	R	R	R	S	S	S	R	R	S	S	S
Cefuroxime	S	S	S	R	S	S	S	S	S	S	S
Cyprofloxacin	WS	WS	R	V	V	V	WS	S	S	S	R
Penicillin	S	S	S	S	S	S	S	S	S	S	S
Ampicillin	S	S	S	S	S	S	S	S	S	S	S
Polymyxin B	R	R	R	R	V	S	WS	R	R	R	WS
Cloxacillin	R	R	R	V	R	R	R	R	R	R	WS
Neomycin	S	S	S	S	S	WS	R	WS	R	R	WS

IV. Resumen general

I. Resumen

I.1. Resumen

Durante los últimos años la acuicultura se ha convertido en una importante actividad económica en todo el mundo. El incremento en la productividad derivada de esta actividad, así como su intensificación ha venido acompañado por diversos impactos ecológicos, entre los cuales destaca la aparición de una gran variedad de agentes patógenos y de resistencia antimicrobiana. Dichos impactos son debidos en parte al uso indiscriminado de agentes quimioterápicos, que ha sido la vía más común para controlar hasta el momento las principales enfermedades de origen bacteriano.

Se han propuesto numerosas estrategias para el control y prevención de estas enfermedades, entre ellas el uso de vacunas, inmunoestimulantes y probióticos. El uso de probióticos, los cuales controlan los patógenos a través de una serie de mecanismos, constituye una alternativa al tratamiento antibiótico, lo que permite que puedan ser utilizados para prevenir enfermedades bacterianas en acuicultura. El efecto beneficioso de los probióticos viene mediado mediante la competición por receptores específicos en la superficie mucosa, producción de compuestos inhibitorios, competición por componentes nutricionales o por un incremento de la respuesta inmune tanto innata como adaptativa.

Lactococcus garvieae es el agente etiológico de la Lactococosis, enfermedad de gran relevancia que afecta a numerosas especies de peces tanto de agua dulce como salada y que causa importantes pérdidas económicas, principalmente cuando la temperatura del agua supera los 16ºC.

Esta tesis incluye en primer lugar una visión general de la relación que se establece entre las bacterias presentes en la microbiota intestinal y el sistema inmune de los peces, centrándonos en los efectos de determinadas bacterias en el desencadenamiento de la respuesta inmune.

A continuación, hemos seleccionado aquellas cepas microbianas aisladas de trucha arco iris, que presenten exclusión competitiva frente al agente etiológico responsable de la Lactococosis de los peces y analizado algunas de sus principales características probióticas.

Posteriormente, con las bacterias seleccionadas se llevaron a cabo experimentos *in vivo* para determinar la capacidad de colonización, así como la capacidad de incrementar los mecanismos de defensa del hospedador aumentando de esta forma la resistencia a dicho proceso.

Finalmente, tras el proceso de selección e identificación de cepas candidatas, hemos identificado una nueva subespecie perteneciente al género *Lactococcus*, denominada *Lactococcus lactis* subsp. *tructae* subsp. *nov*.

I.2. Summary

In the last few years, aquaculture has become an important economic activity in the world. The increase in the productivity of this activity and its intensification has been accompanied by ecological impacts, including emergence of a large variety of pathogens and the appearance of antimicrobial resistance among pathogenic bacteria. These impacts are in part due to the indiscriminate use of chemotherapeutic agents, which have been the most common way to control diseases.

Several alternative strategies for the prevention and control of diseases have been proposed, such as the use of vaccines, immunostimulants and probiotics. The use of probiotics, which control pathogens through a variety of mechanisms, is increasingly viewed as an alternative to antibiotic treatment; therefore they can be used to prevent bacterial diseases in aquaculture. The beneficial effects of probiotics may be mediated by competition for specific pathogen receptor sites on the mucosal surface, production of inhibitory compounds, competition for nutritional substrates, or by enhancement of the host innate and adaptive immune response.

Lactococcus garvieae is the etiological agent of lactococcosis, a relevant disease which affects many fish species and causes important economic losses both in marine and freshwater aquaculture when water temperature increases over 16ºC.

Firstly, this thesis includes a general overview of the relationship between the fish immune system and the bacteria that are present in its intestinal microbiota, focusing on the bacterial effect on the development of certain immune responses.

Next, we have selected potential probiotic bacteria isolated from rainbow trout with competitive exclusion against the etiological agent of lactococcosis in fish and analyzed some of the main probiotic characteristics.

Afterwards, the bacteria selected were studied in challenge experiments *in vivo* for the control of lactococcosis. The immunological responses, as well as the colonization in the gut were studied to assess the impact of probiotic bacteria.

Finally, after the identification and characterization of the candidate strains, a new subspecies of the genera *Lactococcus*, *Lactococcus lactis* subsp. *tructae* subsp. *nov*, was identified.

II. Objetivos de los trabajos presentados

Probiotics in aquaculture: a current assessment. Tania Pérez-Sánchez, José Luis Balcázar, Ignacio de Blas, Imanol Ruiz-Zarzuela. *Fish & Fisheries*. (en revisión).

El objetivo del presente trabajo fue realizar una revisión bibliográfica sobre la situación actual de los probióticos en el campo de la acuicultura, con el fin de conocer los criterios que intervienen en el proceso de selección de este tipo de bacterias, sus principales mecanismos de acción y, finalmente los diferentes microorganismos investigados.

Se escribió este artículo con el fin de actualizar la información disponible, y especialmente para disponer de un trabajo en el que se recogieran los diferentes aspectos mencionados con posterioridad.

Host-microbiota interactions within the fish intestinal ecosystem. Tania Pérez, José Luis Balcázar, Imanol Ruiz-Zarzuela, Nabil Halaihel, Daniel Vendrell, Ignacio de Blas, José Luis Múzquiz. *Mucosal Immunology*. 2010; 3: 355-60.

El objetivo del presente artículo fue realizar una revisión bibliográfica de la relación que se establece entre las bacterias presentes en la microbiota intestinal y la respuesta inmune de los peces.

Se optó por la preparación de este trabajo ya que no se disponía de una fuente suficientemente amplia de información que abarcara todos los aspectos, especialmente los relacionados con las interacciones entre las bacterias con efectos beneficiosos para el hospedador y el desarrollo de determinados mecanismos inmunitarios

Identification and characterization of lactic acid bacteria isolated from rainbow trout Oncorhynchus mykiss (Walbaum) with inhibitory activity against Lactococcus garvieae. Tania Pérez-Sánchez, José Luis Balcázar, Yaneisy García, Nabil Halaihel, Daniel Vendrell, Ignacio de Blas, Daniel Merrifield, Imanol Ruiz-Zarzuela. *Journal of Fish Diseases*. 2011; 34: 499-507.

Se establecieron como objetivos principales del presente trabajo, el aislamiento, caracterización e identificación fenotípica y genotípica de las cepas bacterianas con efecto inhibitorio frente a *L. garvieae*.

Para ello, a partir muestras procedentes del tracto digestivo, mucus cutáneo y branquias de trucha arco iris, se valoró el efecto inhibitorio de las bacterias aisladas frente al agente patógeno en estudio. Posteriormente, una vez seleccionadas las cepas candidatas, se caracterizaron algunas de sus propiedades probióticas (tolerancia a

diferentes concentraciones de bilis y pH, adherencia al mucus, velocidad de crecimiento y producción de sustancias inhibitorias).

Expression of immune-related genes in rainbow trout (Oncorhynchus mykiss) induced by probiotic bacteria during Lactococcus garvieae infection. Tania Pérez-Sánchez, José Luis Balcázar, Daniel Merrifield, Oliana Carnevali, Giorgia Gioacchini, Ignacio de Blas, Imanol Ruiz-Zarzuela. *Fish & Shellfish Immunology*. 2011; 31; 196-201.

Los objetivos del estudio fueron la evaluación in vivo de la inocuidad y la capacidad de colonización de las cepas bacterianas con potencial probiótico, así como de la capacidad protectora de las mismas frente a *L. garvieae*.

Para ello, las bacterias seleccionadas fueron administradas por vía oral a través de la alimentación con el fin de valorar su eficacia, como método de control y prevención de la Lactococosis, mediante una infección experimental con *L. garvieae*.

Lactococcus lactis subsp. tructae subsp. nov isolated from the intestinal mucus of brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss). Tania Pérez, José L. Balcázar, Álvaro Peix, Ángel Valverde, Encarna Velázquez, Ignacio de Blas, Imanol Ruiz-Zarzuela. *International Journal of Systematic and Evolutionary Microbiology*. 2010; doi:10.1099/ijs.0.023945-0.

Tras el proceso de selección e identificación de las cepas candidatas, se obtuvo una bacteria, aislada a partir del mucus intestinal de trucha arco iris, que fue analizada junto con otras dos bacterias aisladas del mucus intestinal de trucha común.

Se llevaron a cabo pruebas bioquímicas y análisis filogéneticos que permitieron identificar una nueva subespecie perteneciente al género *Lactococcus*, denominada *Lactococcus lactis* subsp. *tructae* subsp. *nov*.

III. Metodología

III.1. Animales

Para la realización de todos los experimentos se utilizaron ejemplares de trucha arco iris (*Oncorhynchus mykiss*), como especie piscícola de referencia, puesto que se ha demostrado que presenta una gran sensiblidad a la enfermedad. En el primer experimento en el que se seleccionaron las diferentes cepas con efecto probiótico, se utilizaron animales procedentes de dos piscifactorías industriales situadas en las provincias de Huesca y Zaragoza, ambas pertenecientes a la Asociación de Defensa Sanitaria Acuícola de Aragón, en la cual se realizan periódicamente numerosos controles sanitarios, lo que nos aseguraba que dichas explotaciones eran libres a la enfermedad. En el segundo experimento, en el que se evaluó la capacidad de colonizar el tracto gastrointestinal y la respuesta inmune, los peces procedían de la piscifactoría industrial situada en la provincia de Zaragoza.

No obstante, una vez que los animales llegaron a las instalaciones del Laboratorio de Ictiopatología de la Facultad de Veterinaria de la Universidad de Zaragoza, se realizaron numerosos controles microbiológicos y moleculares (PCR), previos al inicio de cada uno de los experimentos, con el fin de descartar la presencia del agente patógeno *L. garvieae* y asegurar que no eran portadores del agente patógeno.

III.2. Instalaciones

Las diferentes experiencias se llevaron a cabo en la piscifactoría experimental que posee el Laboratorio de Ictiopatología de la Facultad de Veterinaria de la Universidad de Zaragoza. Ésta dispone de cinco tanques de 1000 L de capacidad cada uno, equipados con equipos de refrigeración del agua y oxigenación forzada. Así mismo, el suministro de agua provenía de la red municipal que era declorada y descalcificada antes de ser almacenada en dos depósitos de 1000 L de capacidad. Antes de llegar a los estanques, el agua pasaba a través de un filtro ultravioleta con el fin de disminuir la carga microbiana que pudiera interferir en las pruebas experimentales. Una vez en los tanques, el agua era sometida a un proceso de recirculación que la devolvía al tanque en forma de cascada para favorecer la oxigenación. La renovación de agua era diaria (25%) en cada uno de los tanques; así mismo, se realizaba una limpieza generalmente a

primera hora de la mañana retirándose las heces de los peces mediante aspiración.

Por otra parte, las técnicas analíticas y diagnósticas se realizaron en el laboratorio de la Unidad de Enfermedades Infecciosas y Epidemiología, y en el Laboratorio de Ictiopatología de la Facultad de Veterinaria de la Universidad de Zaragoza, los cuales disponen de todos los equipos necesarios para la realización de las técnicas microbiológicas y moleculares utilizadas en el presente trabajo. En cuanto a los análisis mediante Gel de Electroforesis con Gradientes Desnaturalizantes (DGGE), para determinar la composición, diversidad y dinámica de las muestras de tejido tomadas previamente a partir de trucha arco iris, tras la administración oral de cepas probióticas, así como tras la infección experimental con *L. garvieae*, se llevaron a cabo en la Unidad de Nutrición y Acuicultura de la Universidad de Plymouth (Reino Unido).

III.3. Aislamiento e identificación de cepas probióticas con efecto inhibitorio frente a *L. garvieae* en trucha arco iris.

Para el aislamiento de cepas bacterianas se utilizaron medios de cultivo sólidos, agar Tripticasa Soja (TSA; Scharlau) y agar De Man Rogosa Sharpe (MRS; Pronadisa), así como caldo MRS (Pronadisa) a partir de siembras realizadas de la porción final del intestino, branquias y mucus cutáneo de truchas arco iris (n=60). De forma paralela se realizaron siembras a partir de los órganos internos, hígado, bazo y riñón anterior, con el fin de confirmar el estado sanitario de la población.

En la selección de cepas con propiedades antibacterianas, se utilizó una cepa de referencia de *L. garvieae*, cepa CLFP LG 1, previamente aislada a partir de un brote natural de Lactococosis desencadenado en una explotación intensiva de trucha arco iris. Ésta fue sembrada en placas de TSA y MRS, utilizando 100 µL de la suspensión bacteriana a una concentración de 10^7 unidades formadoras de colonias por mililitro (UFC/mL). Posteriormente, se depositaron pequeñas cantidades de cultivo fresco procedentes de aquellas cepas bacterianas que se pretendían valorar. La temperatura de incubación fue de 22ºC durante 24-48 h. La presencia de áreas de inhibición en el crecimiento de *L. garvieae*, indicó la existencia de antagonismo bacteriano.

La identificación fenotípica se llevó a cabo mediante pruebas de crecimiento, tinción específica y microscopía, así como pruebas bioquímicas de identificación entre las que se incluyeron sistemas de identificación rápida API 20E, API 20NE, API 50CH y API 20Strep (bioMérieux).

Previamente a la identificación genética, se llevó a cabo una rep-PCR, con el objetivo de agrupar las diferentes cepas aisladas. Esta técnica está basada en la amplificación de elementos palindrómicos extragénicos repetitivos dentro del genoma bacteriano. Para ello, el ácido desoxirribonucleico (DNA) fue extraído mediante el método previamente descrito por Balcázar y cols. (2007d). El cebador seleccionado fue el GTG5 (5´-GTG GTG GTG GTG GTG-3´) (Versalovic y cols. 1994), y las reacciones de la PCR se realizaron en un termociclador MJ Mini Gradient (Bio-Rad). Para la visualización de las bandas se utilizó un gel de agarosa al 1.5%.

Para la secuenciación de las cepas probióticas candidatas, se utilizaron cebadores basados en las regiones conservadas del gen 16S RNA ribosomal (RNAr) para amplificar aproximadamente un producto de 900 pares de bases (pb). Los cebadores utilizados fueron 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') y 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') (Lane, 1991). Todas las amplificaciones se realizaron en un termociclador MJ Mini Gradient (Bio-Rad). Los productos de la PCR se purificaron utilizando un kit comercial (Promega), siguiendo las instrucciones del fabricante. Los productos se secuenciaron directamente en un secuenciador MegaBACE ET Terminators (Amersham Biosciences). Las secuencias obtenidas fueron editadas y alineadas utilizando el programa informático Clustal W (Thompson y cols., 1994) y comparadas con secuencias depositadas en la base de datos GenBank utilizando el programa BLAST (www.ncbi.nlm.nih.gov).

III.4. Caracterización de las cepas probióticas seleccionadas con efecto inhibitorio frente a *L. garvieae*

Para el estudio de la velocidad de crecimiento de las cepas probióticas seleccionadas, se preparó una suspensión de cada una de ella en caldo MRS (pH 6.20 ± 0.2) con una densidad óptica (DO) a 600 nm de 0.125, equivalente a 10^7 UFC/mL a partir de un cultivo de 18-24 h, incubado a 22ºC en un agitador orbital (Heidolph, Inkubator 1000 y Unimax 1010) con control de temperatura a 120 rpm para favorecer la homogenización y dispersión del cultivo. Se inocularon 5 mL del cultivo en erlenmeyers con 45 mL de caldo MRS (1/10, v/v). Cada matraz se consideró una unidad experimental y se utilizó un diseño completamente aleatorizado con tres repeticiones por cepa.

La dinámica de crecimiento de cada cepa se monitoreó durante 24 h (0, 4, 8, 12, 16 y 24 h) para determinar la velocidad específica de crecimiento y el tiempo de duplicación. De cada erlenmeyer se tomó 1 mL, se diluyó de forma seriada y se sembró en placas de agar MRS, que se incubaron

posteriormente a 22ºC durante 24-48 h para determinar las UFC/mL. El diluyente utilizado fue solución salina al 0.85 % (p/v).

La velocidad específica de crecimiento (μ) se calculó mediante la ecuación descrita por Prescott y cols. (2003):

$$\mu = \frac{\log N_t - \log N_0}{t_t - t_0}$$

donde N_t: UFC/mL en un tiempo t (t_t) y N_0 las UFC/mL en un tiempo anterior (t_0), correspondiente a la fase exponencial del cultivo. A partir de este valor se calculó el tiempo medio de duplicación (td): td=log 2/μ.

La hidrofobicidad (H%) se determinó como la habilidad de los microorganismos de adherirse a los hidrocarburos, según la metodología descrita por Vinderola y Reinheimer (2003), con algunas modificaciones. Los cultivos bacterianos en fase estacionaria se centrifugaron a 5000 g durante 5 min. Posteriormente, se lavaron dos veces con PBS (pH 6.5) y se resuspendieron en la misma solución tamponada. La densidad óptica (DO) a 560 nm de la suspensión se ajustó con PBS (pH 6.5) a un valor de 1.0 (A_0). De esta solución se tomaron 3 mL, a los que se añadieron 600 μL del tolueno (Lab-Scan) y se mezclaron durante 2 min. Después de 1 h de incubación a 37ºC, para facilitar la separación de las fases, se retiró la fase acuosa y se determinó nuevamente su DO a 560 nm (A_1). La prueba se realizó por triplicado y el porcentaje de hidrofobicidad se calculó mediante la fórmula:

$$H\% = \frac{A_0 - A_1}{A_0} \cdot 100$$

La tolerancia a pH ácidos y a las sales biliares se determinó a partir de cultivos en caldo MRS en fase estacionaria, cuyas DO a 600 nm se ajustaron a 0.6. Para el estudio de la tolerancia a pH ácidos se utilizó el procedimiento descrito por Prasad y cols. (1998). Los cultivos bacterianos en fase estacionaria se concentraron mediante centrifugación a 2500 g durante 10 min; posteriormente, se lavaron con PBS y se resuspendieron 500 μL en 4.5 mL de la misma solución tamponada, previamente ajustada con HCl a valores de pH de 1.0, 2.0 y 3.0, respectivamente. Las suspensiones se incubaron durante 0, 1, 2 y 3 h, y finalmente se determinó el número de colonias en agar MRS.

Para determinar la tolerancia a las sales biliares se utilizó el procedimiento de Klaenhammer y Kleeman (1981). Los cultivos se sembraron por duplicado en agar MRS con 0%,0.2%, 0.4%, 0.6%, 0.8% y 1.0% de sales biliares (Ox-Gall, Oxoid) y se incubaron durante 24 h a 22ºC.

Para confirmar la síntesis de posibles sustancias antibacterianas, todas las cepas que presentaron inhibición in vitro frente a *L. garvieae*, fueron cultivadas en 50 mL de caldo MRS durante 48 h a 22ºC. Tras este periodo de incubación, las cepas fueron sedimentadas por centrifugación y los sobrenadantes fueron esterilizados mediante filtros con un tamaño de poro de de 0.45 µm (Millipore). Después de la esterilización, la mitad del sobrenadante (25 mL) fue neutralizado con NaOH 5N hasta alcanzar un pH de 6.8 (Nikoskelainen y cols., 2001). La cepa patógena de *L. garvieae* se cultivó en caldo MRS durante 24 h a 22ºC, posteriormente fue centrifugada y lavada dos veces con tampón fosfato salino (PBS; pH 7.2) y resuspendida finalmente en la misma solución tamponada. Dicha suspensión bacteriana (100 µL; 10^7 UFC/mL) fue sembrada en placas de MRS por triplicado, sobre las que se habían realizado previamente cuatro pocillos de 6 mm de diámetro con una pipeta Pasteur estéril, para depositar en dos de ellos 50 µL de sobrenadante neutralizado (pH 6.8) y sin neutralizar. En los dos pocillos restantes, se agregó caldo MRS neutralizado y sin neutralizar para determinar la posible actividad inhibitoria del medio. Tras un periodo de incubación de 24 h a 22ºC, se evaluó la presencia de áreas de inhibición en el crecimiento de *L. garvieae*, indicando la producción de sustancias antibacterianas (Nikoskelainen y cols., 2001).

La sensibilidad a diversos antibióticos se analizó mediante la técnica de difusión en agar Mueller-Hinton. Los antibióticos elegidos fueron amoxicilina/ácido clavulánico (30 µg, bioMérieux), ampicilina (10 µg, bioMérieux), cloranfenicol (30 µg, Bio-Rad), clortetraciclina (30 µg, Mast-Diagnostics), clindamicina (2 µg, Bio-Rad), doxiciclina (30 µg, bioMérieux), enrofloxacina (5 µg, Bio-Rad), eritromicina (15 µg, BBL), florfenicol (30 µg, BBL), flumequine (30 µg, Bio-Rad), gentamicina (10 µg, Bio-Rad), kanamicina (30 µg, BBL), ácido nalidíxico (30 µg, BBL), nitrofurantoína (300 µg, Bio-Rad), ácido oxolínico (2 µg), penicilina (10 µg, bioMérieux), estreptomicina (10 µg, BBL), tetraciclina (30 µg, bioMérieux), trimetoprim/sulfametoxazol (1.25/23.75 µg, Bio-Rad), tilosina (150 µg, Neo-Sensitabs) y vancomicina (30 µg, bioMérieux). A partir de cultivos de 18-24 h en caldo MRS de las cepas seleccionadas, se diluyeron con caldo hasta obtener una turbidez de 0.5 en la escala de McFarland (bioMérieux, Francia) y se sembraron en placas de MRS. El periodo de incubación fue de 48 h a 22ºC, tras el cual se midieron los halos de inhibición y se interpretaron los resultados obtenidos siguiendo las recomendaciones establecidas por el Instituto de Estándares Clínicos y de Laboratorio (CLSI).

Tras comprobar una distribución normal con el test de Kolmogorov–Smirnov, los datos se analizaron mediante un ANOVA de una vía y posteriormente con la prueba de Duncan.

III.5. Evaluación de la supervivencia tras la administración de cepas probióticas para la prevención de la Lactococosis en la trucha arco iris

Se prepararon cinco grupos experimentales constituidos por 45 animales cada uno, tres de los cuales fueron alimentados diariamente con una dieta suplementada con cada una de las cepas bacterianas seleccionadas (tras 24 h de cultivo, una suspensión bacteriana en PBS fue mezclada con el pienso hasta alcanzar una concentración de 1×10^6 UFC/g pienso) durante 36 días. Mientras que los otros dos grupos fueron alimentados con el mismo pienso pero sin tratar.

Durante este tiempo la temperatura del agua se fue incrementando progresivamente hasta alcanzar los 19 ± 1ºC. Tras 21 días de tratamiento con las cepas probióticas, se procedió a realizar la infección experimental mediante la técnica de cohabitación con truchas (procedentes de uno de los grupos sin tratar), a las cuales se les inoculó 0.1 mL de suspensión bacteriana (*L. garvieae*) a una concentración de 1.0×10^4 UFC/ml, por vía IP, que representaban el 20% de la población. La infección se llevó a cabo en los tres grupos tratados y en un cuarto que sirvió como testigo de la actividad del agente. A continuación, los peces se mantuvieron en observación 15 días más, durante los cuales fue registrada la mortalidad en todos los grupos así como determinada la presencia del agente patógeno en todas las bajas mediante métodos microbiológicos y moleculares.

III.6. Evaluación *in vivo* de la capacidad de colonización de las cepas bacterianas con potencial probiótico

El análisis mediante DGGE es una técnica molecular mediante la cual se separan fragmentos de DNA, producto de una amplificación por PCR, que tienen una misma longitud pero difieren en la secuencia. Las regiones más conservadas se utilizan para el diseño de los cebadores, mientras que las variables para establecer diferencias entre las secuencias y poder así analizarlas filogenéticamente. Esta técnica se utilizó para determinar el grado de colonización de las cepas probióticas seleccionadas, así como para establecer la ruta de infección de *L. garvieae*.

Para la recogida de las muestras, se seleccionaron al azar 5 peces de cada uno de los tratamientos con probióticos, así como del grupo control. Inmediatamente después de la eutanasia se tomaron muestras del intestino distal, las cuales se congelaron a -80ºC hasta su posterior utilización. Utilizamos el kit de extracción QIAamp Stool Mini Kit (Qiagen) para

obtener un DNA de gran pureza, aislado del resto de componentes celulares y cualquier otra sustancia que pueda inhibir la PCR posterior. Los cebadores utilizados se diseñaron según las regiones específicas de los organismos de interés, en el caso de las bacterias, se utiliza generalmente la región V3 del RNAr. El DGGE para V3 se realizó en un gel con un 8% de acrilamida y entre un 40 y un 60% de agente desnaturalizante, que estuvo corriendo durante 16 h a 60 Voltios (V). Tras una tinción con SYBR Green, la visualización de las bandas se realizó con un transiluminador acoplado a un sistema de captación de imágnes (Bio-Rad Universal Hood II).

III.7. Evaluación *in vivo* de la capacidad protectora de las cepas probióticas seleccionadas frente a *L. garvieae*

A partir de las muestras de intestino y riñón anterior se procedió a la extracción del RNA, para la cual se utilizó el método TRIZOL con algunas modificaciones, descrito previamente por Zhang y cols. (2009). Para ello, se diseñaron cebadores específicos, mediante el programa informático Primer-BLAST (National Center for Biotechnology Information), que nos permitieron amplificar las siguientes citoquinas IL-1β, TNF-α, IL-10 en riñón anterior e IL-8, Tlr5, IgT en intestino. En la Tabla 2 se especifican las secuencias de los cebadores utilizados.

Tabla 2. Cebadores utilizados para detectar los genes estudiados.

Gen	Nº acceso GenBank	Tamaño (pb)	Sentido	Cebador
IL-1β	AJ223954	91	5′ - 3′	ACATTGCCAACCTCATCATCG
			3′ – 5′	TTGAGCAGGTCCTTGTCCTTG
IL-10	AB118099	70	5′ - 3′	CGACTTTAAATCTCCCATCGAC
			3′ – 5′	GCATTGGACGATCTCTTTCTTC
TNF-α	AJ277604	75	5′ - 3′	GGGGACAAACTGTGGACTGA
			3′ – 5′	GAAGTTCTTGCCCTGCTCTG
IL-8	AJ279069	69	5′ - 3′	AGAATGTCAGCCAGCCTTGT
			3′ – 5′	TCTCAGACTCATCCCCTCAGT
IgT	AY870265	72	5′ - 3′	AGCACCAGGGTGAAACCA
			3′ – 5′	GCGGTGGGTTCAGAGTCA
Tlr5	AB091105	89	5′ - 3′	GGCATCAGCCTGTTGAATTT
			3′ – 5′	ATGAAGAGCGAGAGCCTCAG
β actina	AJ438158	167	5′ - 3′	ACAGACTGTACCCATCCCAAAC
			3′ – 5′	AAAAAGCGCCAAAATAACAGAA
60s	NM001165047	147	5′ - 3′	AGCCACCAGTATGCTAACCAGT
			3′ – 5′	TGTGATTGCACATTGACAAAAA

Para la síntesis del DNA complementario (DNAc) se utilizó un kit iScript DNAc Synthesis kit (Bio-Rad), tras la cual se realizaron las PCR por duplicados para cada una de la muestras mediante el método de SYBR Green, utilizando un termociclador iQ5 iCycler (Bio-Rad). El programa informático iQ5 optical system (Bio-Rad) fue utilizado para calcular los niveles de expresión de las citoquinas estudiadas en comparación a la expresión de la β-actina y el gen 60S, utilizados como controles.

Los resultados obtenidos se analizaron estadísticamente mediante el método de Kaplan-Meier y el test log-rank. Para determinar diferencias significativas entre la respuesta inmune de los grupos tratados y el control se utilizó el test ANOVA de una vía y la prueba de Tukey.

III.8. Identificación de una nueva subespecie, *Lactococcus lactis* subsp. *tructae* subsp. *nov*.

Las tres cepas bacterianas aisladas a partir de muestras de mucus intestinal procedente de ejemplares de trucha arco iris y de trucha común, fueron sembradas en MRS e incubadas posteriormente a 22ºC durante 24 h. Una vez crecidas se determinó la morfología y movilidad de las colonias.

La amplificación y secuenciación del gen 16S RNAr, se realizó según la metodología descrita por Rivas y cols. (2007). Las secuencias obtenidas se compararon con las del GenBank, utilizando el programa informático BLAST (Alschul y cols., 1990) y alineadas con el programa informático Clustal W (Thompson y cols., 1994). Las distancias se calcularon según el modelo de Kimura (1980) y los árboles filogéneticos fueron construidos según la metodología previamente descrita por Saitou y Nei (1987). Para llevar a cabo todos los análisis se utilizó el programa MEGA 4.0 (Tamura y cols., 2007).

Con el objetivo de determinar el patrón de bandas de las cepas aisladas, se realizó una rep-PCR, utilizando el cebador GTG5 (5´-GTG GTG GTG GTG GTG-3´) y siguiendo la metodología propuesta por Marilley y cols. (2004).

Para determinar la diversidad genética de las cepas aisladas se analizaron mediante una amplificación al azar de las regiones polimórficas del DNA (RAPD), con el cebador M13 (5'-GAG GGT GGC GGT TCT-3'), de acuerdo con el método de Rivas y cols. (2006).

Para el análisis filogenético de distintas especies bacterianas, entre las que se incluyen cocos Gram positivos del género *Streptococcus* (Maiden, 2006), se ha demostrado la utilidad de los genes constitutivos. En este estudio se analizaron dos genes rpoB y recA. Para la amplificación y posterior

secuenciación se utilizaron los siguientes cebadores; rpoBLac1F (5'-TAC GGK AAA CAC CGTA-3'), rpoBLac1R (5'-TCA ARC CAW GCT CCA CGG-3'), recALac1F (5'-GCA GCC TTT ATC GAT GCTG-3') y recA1R (5'-GCA CGA CCA CCA GG-3'), diseñados a partir de las regiones conservadas de las especies *L. lactis* subsp. *lactis* KF147 (número de acceso CP001834) y *L. lactis* subsp. *cremoris* SK11 (número de acceso CP000425).

El análisis del contenido en Guanina-Citosina (G+C) se realizó en el Servicio de Identificación de la Colección Alemana de Microorganismos y Cultivos Celulares (DSMZ), para lo cual el DNA fue extraído y purificado según la metodología descrita por Cashion y cols. (1997). Posteriormente, el DNA fue hidrolizado con la nucleasa P1 y los nucleótidos desfosforalizados con la fosfatasa alcalina bovina (Mesbah y cols., 1989). El porcentaje de G+C se determinó mediante cromatografía líquida de alta resolución (HPLC) según Mesbah y cols. (1989).

Los análisis de hibridación del DNA se realizaron de acuerdo al método de Ezaki y cols. (1989), siguiendo las recomendaciones de Willens y cols. (2001).

Los ácidos grasos fueron analizados en el Servicio de Identificación de DSMZ, siguiendo las instrucciones del Sistema de Identificación Microbiológica (MIDI).

Para determinar la producción de ácido de los carbohidratos, se utilizaron los sistemas de identificación rápida API 20Strep y API 50CH (bioMérieux). Los resultados se interpretaron según las indicaciones de Schleifer y cols. (1985).

También se determinó el crecimiento en presencia de un 4% de NaCl en agar TSA. La sensibilidad a diversos antibióticos se analizó mediante la técnica de difusión en agar sangre (Sharlau). Los antibióticos elegidos fueron ampicilina (2 µg), eritromicina (2 µg), ciprofloxacina (5 µg), penicilina (10 UI), polimixina (300 UI), cloxacilina (1 µg), oxitetraciclina (30 µg), gentamicina (10 µg), cefuroxime (30 µg) y neomicina (5 µg), (BBL).

V. Conclusiones

I. Conclusiones

Bajo nuestras condiciones de estudio, se han obtenido las siguientes conclusiones:

PRIMERA: *Lactobacillus plantarum* subsp. *plantarum* ha demostrado ser la cepa más adecuada para su aplicación oral como probiótico frente a la Lactococosis de la trucha.

SEGUNDA: La respuesta inmune desencadenada mediante la liberación de citoquinas en el grupo tratado con *Lactobacillus plantarum* subsp. *plantarum*, ha permitido un mayor grado de protección frente a *Lactococcus garvieae*.

TERCERA: Las interacciones que se establecen en el intestino entre las bacterias probióticas y la microbiota endógena, no constituye un requisito necesario para desencadenar un efecto protector frente a la enfermedad.

CUARTA: La manipulación de la microbiota normal del hospedador mediante la administración de cepas probióticas podría constituir una vía para la prevención de trastornos patológicos en los peces.

II. Conclusions

Based on the study conditions, the results from the experiments carried out in this study and its discussion, the following conclusions have been obtained:

FIRST: *Lactobacillus plantarum* subsp. *plantarum* has demonstrated to be the most appropriate strain for use as oral probiotic against Lactococcosis in rainbow trout.

SECOND: The immunological responses in the group treated with *Lactobacillus plantarum* subsp. *plantarum* were responsible for mediating elevated disease resistance against *Lactococcus garvieae*.

THIRD: Direct probiotic-microbiota interactions with the intestine are not always necessary to induce a protector effect against the disease.

FOURTH: The manipulation of the host microbiota through the administration of probiotic bacteria may represent a new possibility in the prevention of pathological disorders.

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VII. Apéndices

I. Características de las revistas

En el presente apéndice se indican el factor de impacto (JIF) y las áreas temáticas correspondientes a las revistas donde se han publicado los trabajos incluidos en la presente tesis doctoral.

Todos los valores se han obtenido del Journal Citation Reports® disponible en ISI Web of Knowledge.

En cada una de las áreas temáticas señaladas se indica entre paréntesis la posición de la revista indicada sobre el total de revistas incluidas en el área de estudio.

Revista	Fish & Fisheries		
JIF	6.434	**Año**	2010
Áreas temáticas	Fisheries (1/46)		

Revista	Mucosal Immunology		
JIF	6.817	**Año**	2010
Áreas temáticas	Immunology (15/134)		

Revista	Journal of Fish Diseases		
JIF	1.603	**Año**	2010
Áreas temáticas	Fisheries (13/46) Marine & Freshwater Biology (37/92) Veterinary Sciences (27/145)		

Revista	Fish & Shellfish Immunology
JIF	3.044 **Año** 2010
Áreas temáticas	Fisheries (5/46) Immunology (58/134) Marine & Freshwater Biology (8/92) Veterinary Sciences (4/145)

Revista	International J. of Systematic & Evolutionary Microbiology
JIF	1.930 **Año** 2010
Áreas temáticas	Microbiology (68/107)

II. Contribución del doctorando

El doctorando es el primer autor de todos los trabajos presentados en esta tesis, lo que justifica plenamente su contribución.

Además, debemos indicar que todos los coautores son doctores, con la excepción de Dª Yaneisy García Hernández, quien renuncia expresamente a presentar el trabajos del que es coautora como parte de otra tesis doctoral, en función de lo cual firma el presente documento.

Dª Yaneisy García Hernández