UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

CIHEAM
Instituto Agronómico Mediterráneo de Zaragoza

MASTER OFICIAL EN CULTIVOS MARINOS

Las Palmas de Gran Canaria, España

2018-2019

ESTUDIO DEL EFECTO DE LA CEPA PROBIÓTICA *Vagococcus fluvialis* L21 SOBRE LA EXPRESIÓN DE GENES INVOLUCRADOS EN LA RESPUESTA INMUNE DE LA LUBINA (*Dicentrarchus labrax*,L)

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TESIS PRESENTADA Y PUBLICAMENTE DEFENDIDA PARA LA OBTENCIÓN DEL TÍTULO DE MASTER OFICIAL EN CULTIVOS MARINOS

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Trabajo realizado en Laboratorio de Ictiopatología de la Universidad de Las Palmas de Gran Canaria bajo la dirección del Dr. Félix Acosta Arbelo

Presentado como requisito parcial para la obtención del Título oficial de Máster Universitario en Cultivos Marinos otorgado por la Universidad de Las Palmas de Gran Canaria y del Diploma de Master of Science en Acuicultura otorgado por el Centro Internacional de Altos Estudios Agronómicos Mediterráneos (CIHEAM).

El tutor

Félix Acosta Arbelo

La Autora

Lorena Román

Índice General

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Lorena Román

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Abreviaturas empleadas

ADN	Ácido desoxirribonucleico
ADNc	Ácido desoxirribonucleico complementario
AF	Actividad fagocítica
APROMAR	Asociación Empresarial de Productores de Cultivos Marinos de España
ARN	Ácido ribonucleico
BHIA	Agar infusión cerebro corazón
BHIB	Caldo infusión cerebro corazón
CANEXMAR	Canarias Explotaciones Marinas
Casp-3	Caspasa-3
CCNs	Células citotóxicas no específicas
CGEs	Células granulares eosinofílicas
COX	Ciclooxigenasa
Ct	Valores de umbral de ciclo
CTAB	Cetiltrimetilamonio bromuro
DEPC	Dietilpirocarbonato
DHA	Ácido Docohexanoico
DMSO	Dimetil sulfóxido
ECPs	Productos extracelulares
EGHs	Eosinófilos granulares homogéneos
EPA	Ácido Eicosapentanoico
FAO	Organización de las Naciones Unidas para la Agricultura y la Alimentación
HBSS	Solución salina balanceada de Hanks

HUFA	Ácidos grasos poliinsaturados
ICCM	Instituto Canario de Ciencias Marinas
IFN	Interferón
IgM	Inmunoglobulinas M
IL	Interleuquina
LAB	Bacterias ácido-lácticas
LMR	Límite máximos de residuos
LPS	Lipopolisacáridos
MOI	Multiplicidad de infección
mRNA	Ácido ribonucleico mensajero
NBT	Nitroazul de tetrazoilo
NHI	Necrosis Hematopoyética Infecciosa
NK	Células Natural Killer
OIE	Oficina Internacional de Epizootias
OMS	Organización Mundial de la Salud
OMPs	Proteínas de membrana externa
ONGs	Organizaciones no gubernamentales
PAMPs	Patrones moleculares asociados a patógenos
PBS	Tampón fosfato salino
PKR	Proteína Kinasa R
PMA	Acetato de forbol miristato
POLY I:C	Ácido poliinosínico-policitidinico
PRS	Porcentaje relativo de supervivencia

qPCR	Reacción en cadena de la polimerasa semicuantitativa
RT-PCR	Reacción en cadena de la polimerasa a tiempo real
SOD	Superóxido dismutasa
TGF	Factor de crecimiento transformante
TMB	Tretrametilbenzidina hidroclorhídrica
TNF	Factor de necrosis tumoral
UE	Unión Europea
ufc	Unidad formadora de colonias
UV	Luz ultravioleta

RESUMEN

La acuicultura es una de las industrias de mayor crecimiento en el sector de la alimentación, aumentando en las últimas décadas el consumo de productos procedentes de la acuicultura. El rápido crecimiento de esta actividad, se ha visto afectado por la aparición de enfermedades en los peces, principalmente bacterianas, originando grandes mortalidades y pérdidas en el sector. Las restricciones en el uso de antibióticos y quimioterápicos así como la ineficacia de algunas vacunas, convierten a los probióticos en una alternativa eficaz, y a la vez respetuosa con el medio ambiente para la prevención y control de enfermedades en acuicultura.

Entre los múltiples mecanismos de acción de los probióticos, la modulación de las respuesta inmune es uno de los más estudiados. En el presente trabajo nos hemos planteado estudiar *in vitro* el efecto inmunomodulador de una cepa consideradas como probiótica para la acuicultura. Teniendo en cuenta que en estudios previos se evaluó el efecto inmunomodulador *in vitro* de la cepa *Vagococcus fluvilalis* L21 tanto viva como inactivada por calor y luz ultravioleta, en diferentes especies de interés acuícola para la acuicultura: Dorada (*Sparus aurata*), lubina (*Dicentrarchus labrax*), corvina (*Argyrosomus regius*) y bocinegro (*Pagrus pagrus*). En el presente trabajó se evaluó el efecto sobre la expresión de genes relacionados con la respuesta inmune como son (IL-1β, TNF-α, IL-6, IL-10, COX-2, Casp-3 y Mx), que tanto por la cepa *V. fluvialis* L21 viva como sus productos extracelulares (ECPs) ejercen sobre los leucocitos de lubina.

Los resultados sugieren que la cepa de *V. fluvialis* L21, tanto viva como inactivada, y sus ECPs estimulan la expresión de citoquinas proinflamatorias en la lubina,

siendo esta expresión mayor en los leucocitos que fueron estimulados con los ECPs de *V. fluvilalis* L21, por lo que se puede considerar tanto a *V. fluvialis* L21 como sus ECPs potentes inmunomoduladores para esta especie.

SUMMARY

Aquaculture is one of the faster growing industries in the food sector. In recent decades the consumption of aquaculture products has increased. The rapid growth of this activity has been affected by the occurrence of fish diseases, mainly bacterial, causing high mortality and losses in this sector. Restrictions on the use of antibiotics and chemotherapy and the ineffectiveness of some vaccines, makes probiotics an effective alternative, friendly with the environment, for the control of diseases in aquaculture.

Among multiple mechanisms of action of probiotics, the modulation of the immune response is one of the most studied. In this study, the *in vitro* immunomodulatory effect of a strain considered as probiotic for aquaculture has been developed. We initially studied the *in vitro* immunomodulatory effect of the strain *Vagococcus fluvilalis* L21, alive and inactivated by heat-shock and by ultraviolet light, in sea bass (*Dicentrarchus labrax*). Later, the effect on the immune related- genes expression such as (IL -1 , TNF- , IL-6 , IL-10, COX-2, Casp -3 and Mx of the strain *V. fluvialis* L21 and their extracellular products (ECPs) on sea bass leukocytes was also studied.

The results suggest that the strain *V. fluvialis* L 21 alive and inactivated, and their ECPs, stimulate proinflammatory cytokine expression in sea bass and this expression was higher in leukocytes were stimulated with ECPs of *V. fluvialis* L21, therefore, both being considered as potent immunomodulators for this specie.

1.- INTRODUCCIÓN

El incesante incremento de la población mundial junto con el aumento de la demanda de productos marinos y el descenso de la pesca extractiva hacen que para el año 2030 sean necesarias 37 millones de toneladas adicionales de pescado para mantener los niveles actuales de consumo (FAO, 2008).

Actualmente y según los últimos datos del informe sobre el estado actual de la pesca y la acuicultura (FAO, 2018) La acuicultura sigue creciendo más rápido que otros sectores principales de producción de alimentos, aunque ya no muestra las elevadas tasas de crecimiento anuales de las décadas de 1980 y 1990 (11,3% y 10,0%, excluidas las plantas acuáticas). El crecimiento anual medio descendió al 5,8% durante el período 2000-2016, aunque siguió registrándose un crecimiento de dos dígitos en un pequeño número de países individuales, especialmente en África entre 2006 y 2010. La producción acuícola mundial en 2016 fue de 80,0 millones de toneladas de pescado comestible y 30,1 millones de toneladas de plantas acuáticas, así como 37 900 toneladas de productos no alimentarios. Estos datos nos indican que es necesaria la intensificación de las producciones para poder responder a la gran demanda de productos de acuicultura.

La acuicultura intensiva supone un mayor número de peces por volumen de agua, que sumado, en muchas ocasiones, a las malas condiciones higiénico-sanitarias, y junto al estrés de los animales, favorece la penetración y desarrollo de agentes patógenos y, como consecuencia, la aparición de enfermedades.

Si bien la quimioterapia es quizás el método más rápido para tratar las enfermedades bacterianas, hoy en día hay un creciente reconocimiento de sus limitaciones en acuicultura debido a que, en algunos casos, más que proporcionar una solución, puede ocasionar efectos adversos en la salud del animal mediante la activación de la toxicidad, la resistencia, producción de residuos, etc, dando lugar a graves consecuencias ambientales, ya que los residuos de los antibióticos pueden permanecer durante mucho tiempo en el medio acuático, y por ello ocasionar problemas en la salud pública (FAO, 2018).

Debido a los nuevos requisitos legales de los gobiernos de numerosos países, a la demanda por parte de los consumidores de productos más seguros y saludables, así como a la preocupación por la conservación del medio ambiente, se está reforzando la necesidad de aplicar un enfoque más integrador en el sector de la producción acuícola. Por esta razón, la Unión Europea ha planteado serias restricciones sobre el uso de antibióticos en acuicultura, sugiriendo la necesidad de utilizar métodos alternativos sostenibles medioambientalmente para el control de las enfermedades infecciosas en las producciones acuícolas.

Sin duda, un diagnóstico rápido y preciso de las enfermedades, junto a unos estudios epidemiológicos adecuados, constituyen la clave para minimizar el impacto de las enfermedades en piscicultura. La prevención es una de las mejores herramientas de lucha, y ésta se puede realizar mediante el uso de vacunas, inmunoestimulantes, o mediante el uso de probióticos.

En el mercado existen vacunas comerciales para una gran variedad de enfermedades pero, en ocasiones, su aplicación no siempre es viable ni efectiva.

Por ello, hoy en día las investigaciones se centran en la búsqueda de métodos profilácticos alternativos, mediante la manipulación microbiana de las poblaciones en el medio ambiente de cultivo. En este sentido, el uso de probióticos ha despertado gran interés en las últimas décadas, debido a los enormes beneficios que juegan las bacterias no patógenas en la salud y bienestar de su hospedador, demostrando efectos positivos sobre el control de enfermedades, crecimiento, supervivencia, y sobre la producción general.

Hasta la fecha se han ofrecido muchas definiciones sobre los probióticos, pero una de las más completas y reconocidas es la propuesta por Verschuere y cols. (2000a), que definen a un probiótico como "*un suplemento vivo microbiano que tiene efecto beneficioso en el hospedador, modificando la flora asociada al mismo y la flora asociada al ambiente*". No obstante, la definición de probiótico está en continua evolución en un intento de adaptarse a los nuevos conocimientos que surgen de los trabajos de investigación con probióticos. En este sentido, se ha descrito que microorganismos inactivados, e incluso los componente celulares, pueden ejercer un efecto beneficioso para el hospedador (Ouwehand y Salminen, 1998; Isolauri y cols., 2002; Díaz-Rosales y cols., 2006; Sharifuzzaman y cols., 2011; Román y cols. 2012). Por tanto, a la definición de probiótico se le debería añadir, como así sugieren Díaz-Rosales y cols. (2006): "*microorganismos, no necesariamente vivos, que tienen un efecto beneficioso en el hospedador*".

Entre los numerosos efectos beneficiosos que los probióticos ejercen sobre los hospedadores, la modulación del sistema inmune es uno de los mecanismos más estudiados.

1.1.-El medio ambiente como determinante de enfermedad en las especies acuícolas

El rápido desarrollo de la acuicultura y, consecuentemente, la intensificación en las producciones se ha traducido inevitablemente en un aumento de los problemas asociados a la producción animal intensiva. El mantenimiento de la salud y prevención de enfermedades con éxito y/o control no dependen de un solo factor sino que son la consecuencia de la integración de distintos factores así como una buena gestión de la relación que existe entre la calidad ambiental y la salud de los peces. (Plumb, 1999) (Figura I).

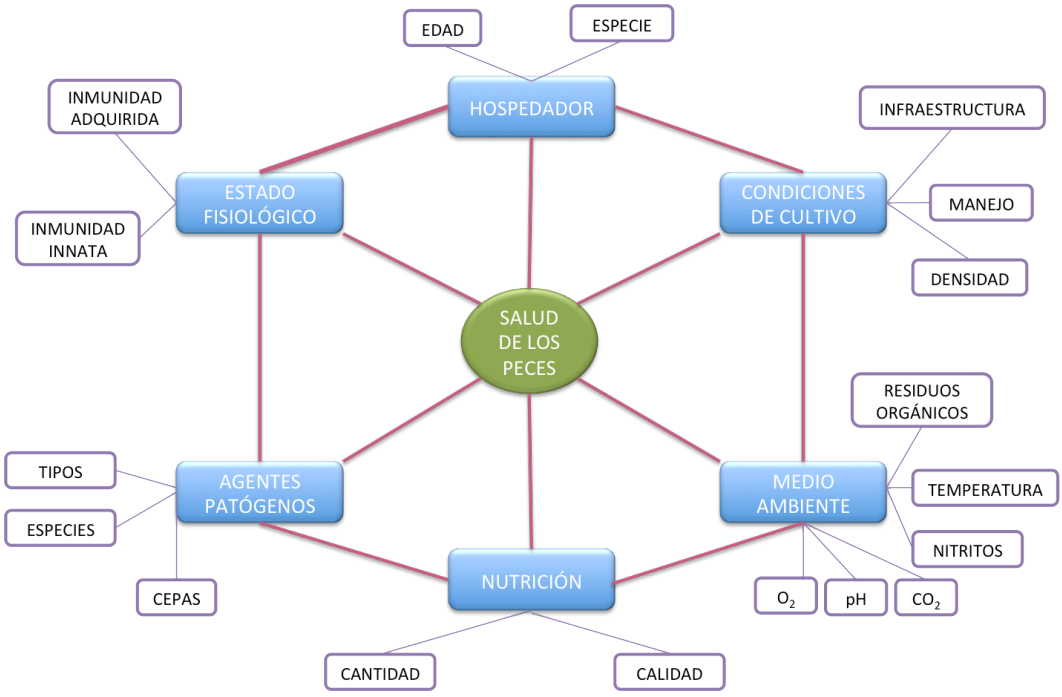

Figura I: Relación entre las condiciones ambientales, factores biológicos y prácticas de gestión acuícola y su influencia en la aparición de enfermedades infecciosas en los peces. Adaptado de Plumb (1999).

Bajo condiciones ambientales adecuadas, los peces se encuentran en buen estado de salud y sin signos clínicos ni lesiones, aunque pueden ser portadores

asintomáticos de agentes patógenos. Bajo condiciones ambientales estresantes, el hacinamiento, deficiencias nutricionales, prácticas de manejo inadecuado, condiciones higiénico-sanitarias inadecuadas, estos peces que en principio son portadores asintomáticos pueden desarrollar la enfermedad (Plumb, 1999) (Figura II). Por lo tanto, evitar el estrés mediante el mantenimiento de la calidad ambiental es esencial para mantener una población de peces sanos y libres de enfermedad.

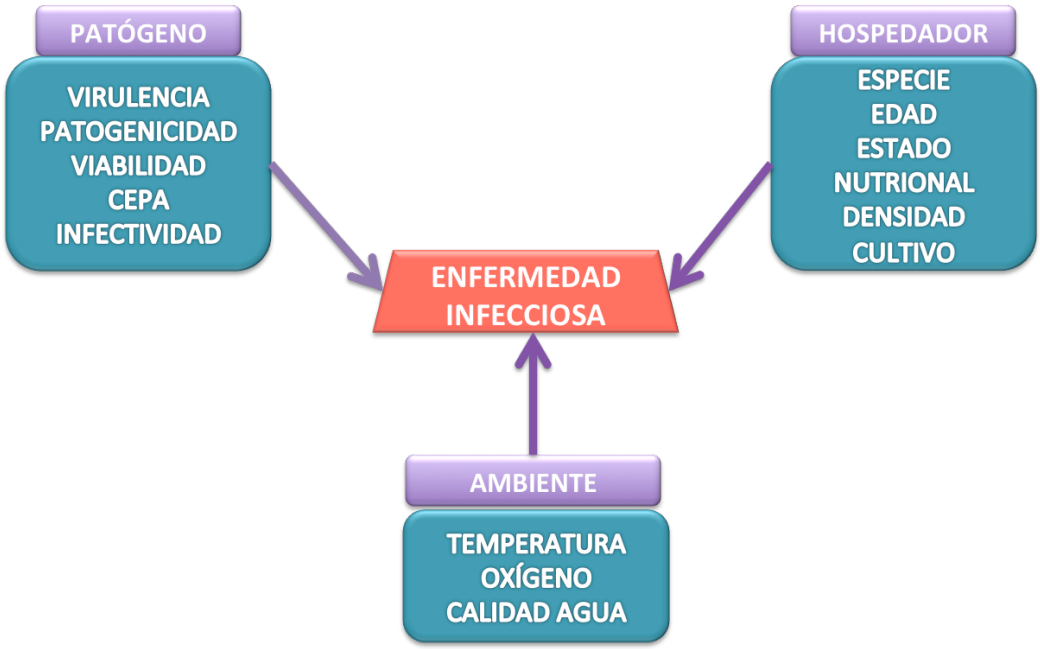

Figura II: Relación entre agente patógeno, hospedador y medio ambiente en el desarrollo de enfermedades infecciosas. Adaptado de Plumb (1999).

Son muchas las enfermedades que pueden afectar a las granjas acuícolas, convirtiéndose en problema que se traduce, sobre todo, en grandes pérdidas económicas si no son controladas adecuadamente. En la bibliografía se describen numerosas enfermedades producidas por bacterias, virus, parásitos. La mayoría de

estos microorganismos forman parte del medio marino, y sólo en determinadas circunstancias van a sobrepasar las barreras defensivas de los peces causando así la enfermedad.

1.2-El control de las enfermedades en acuicultura

La acuicultura es, sin duda, una de las industrias que más ha crecido en los últimos años en nuestro país, tanto respecto de la producción total obtenida como en cuanto al número de centros de crianza que existen. Esta expansión e intensificación de los cultivos, junto a unas condiciones medioambientales, muchas veces desfavorables, conlleva a la aparición de mayores riesgos sanitarios en las granjas, favoreciendo la colonización y desarrollo de agentes patógenos, y como consecuencia, la aparición de las enfermedades bacterianas (Kurath, 2008). Las enfermedades de los peces se han convertido en el principal obstáculo para el desarrollo óptimo de la acuicultura llegando a provocar grandes pérdidas económicas en el sector.

A diferencia de lo que ocurre en las granjas de animales terrestres, donde todos los parámetros ambientales están controlados, en el cultivo de peces las condiciones son altamente variables. Numerosos factores han contribuido a los problemas de salud a los que actualmente se enfrenta la acuicultura:

- Durante las tres últimas décadas, la acuicultura se ha expandido, intensificado y diversificado, basándose en gran medida en los movimientos de animales y sus productos, tales como reproductores, huevos y piensos. Estos movimientos están reconocidos como factores fundamentales en la introducción y

propagación de agentes patógenos y enfermedades en el sector de la acuicultura. Además, aumenta el periodo de permanencia de una instalación en un lugar determinado, lo que hace que el tiempo de residencia de los patógenos sea mayor y se "asienten".

- La intensificación de la acuicultura ha implicado un aumento de la carga de población, induciendo ambientes desfavorables para la salud de los peces, como una alta densidad de población, problemas de estrés, manipulación y perturbaciones físicas (Bullock y Mclaughlin, 1970; Tarazona y Muñoz, 1995). La superpoblación acarrea una deficiente calidad del agua debido a la disminución del nivel de oxígeno, a productos metabólicos y acumulación de excrementos, a un rápido crecimiento y transmisión de parásitos nocivos, hongos, bacterias y virus (Zhao, 2001).

Hay que tener presente que la producción de peces tiene que ser sostenible, lo cual significa que deben utilizarse medidas preventivas aceptables desde un punto de vista biológico y ambiental para mantener los problemas sanitarios en la acuicultura de manera sostenible.

1.3.-Probióticos

La mayoría de las definiciones de probiótico hacen referencia más a la especie humana y otros mamíferos. Durante muchos años las investigaciones se han centrado en estas especies, y cuándo se referían al término probiótico, normalmente lo hacían considerando bacterias ácido-lácticas, la mayoría pertenecientes a los géneros *Bifidobacterium, Lactobacillus, Streptococcus* (Verschuere y cols. 2000a).

En las especies acuáticas, a diferencia de los animales terrestres, la microbiota depende en gran medida del medio en el que viven, es decir, del flujo de agua que está en contacto con el tracto digestivo, con el que está en constante interacción, siendo este medio y la dieta los responsables de la microbiota. Moriarty (1999) sugiere que la definición de probiótico en acuicultura debe incluir la adición de una fuente viva de bacteria tanto a los tanques como a los estanques donde viven los peces. Sin embargo, hay que precisar, que la influencia del medio ambiente es mucho mayor en los animales acuáticos que en los terrestres. Además del tracto intestinal, las bacterias probióticas pueden ser activas en las branquias y en la piel, e incluso en el agua. Esto implica que muchos probióticos se obtienen del entorno el pez (Fuller, 1992). Una definición más completa, en la que se incluye la importancia de la microbiota ha sido propuesta: "*Un probiótico es un suplemento vivo microbiano que tiene efecto beneficioso en el hospedador modificando la flora asociada al mismo y la flora asociada al ambiente*" (Verschuere y cols., 2000a). Más tarde, Reid y cols. (2003) modificaron está definición incluyendo la frase "*cuando son administradas en cantidades adecuadas confieren un beneficio saludable para el hospedador*". Una definición más reciente es la ofrecida por Salminen y cols. (2005), que sugieren que los probióticos pueden ser parte de la microbiota gastrointestinal saludable y que su adición puede ayudar a devolver a la normalidad una microbiota alterada.

No obstante, la definición de probiótico está en continua evolución en un intento de adaptarse a los nuevos conocimientos que surgen de los trabajos de investigación con probióticos. En este sentido, se han descrito que microorganismos inactivados, e incluso los componente celulares, pueden ejercer

un efecto beneficioso para el hospedador (Ouwehand y Salminen, 1998; Isolauri y cols., 2002; Díaz-Rosales y cols., 2006; Sharifuzzaman y cols., 2010; Román y cols. 2012). Por tanto, a la definición de probiótico se le debería añadir, como así sugieren Díaz-Rosales y cols. (2006): "*microorganismos no necesariamente vivos, que tienen un efecto beneficioso en el hospedador*".

Por tanto, en el tracto digestivo de las especies acuáticas se han asilado cepas tanto gram positivas pertenecientes a los géneros *Carnobacerium, Enterococcus, Bacillus, Lactobacillus* (Irianto y Austin, 2002b) como bacterias gram negativas pertenecientes a los géneros *Vibrio* y *Pseudomonas* (Vine y cols. 2006) así como levaduras hongos (Burr y cols., 2005). Los probióticos más usados en acuicultura pertenecen a diferentes grupos, como las bacterias ácidos lácticas y los Géneros *Vibrio, Pseudomonas*, *Roseobacter.* Asimismo, aunque en menos interés los Géneros *Aeromonas, Alteromonas, Flavobacterium*, al igual que algas unicelulares y levaduras (Ringo y cols., 2010).

1.3.1-Criterios de selección de cepas probióticas

En acuicultura, la selección de cepas probióticas es un proceso bastante complejo ya que el hábitat microbiano está sometido a constantes alteraciones, y permite cambios en la composición estructural y funcional de las comunidades microbianas, debido a su interacción con el ambiente (Verschuere y cols., 2000b). Algunas investigaciones sugieren que los probióticos deben ser seleccionados del propio hospedador en los cuales van a ser usados, y de esta manera, minimizar los efectos provocados por las diferencias entre los ambientes en que se desarrollan los organismos (Duwat y cols., 2000), aunque existen numerosos trabajos donde se

ha demostrado el efecto beneficioso de bacterias aisladas de otros hospedadores sobre la salud de los peces (Gatesoupe, 2007; Liu y cols., 2012).

En general, para que una cepa sea considerada como cepa probiótica, debe cumplir una serie de características:

- **Antagonismo frente a patógenos:** Esta es una vía muy común para la primera selección de los posibles probióticos, donde cepas patógenas son expuestas *in vitro* a cepas probióticas o a sus productos extracelulares, siendo éste requisito fundamental para muchos autores, a la hora de escoger una cepa como probiótica (Fuller, 1989; Austin y cols., 1995; Chang y Liu, 2002; Irianto y Austin, 2002a).

- **Resistencia al pH y a la bilis:** La habilidad para sobrevivir el tránsito a través del tracto intestinal es una capacidad que presentan pocos microorganismos, debido a las variaciones en el pH que presenta dicha zona orgánica. Los microorganismos que se puedan utilizar como probióticos deberán resistir a las enzimas de la cavidad oral (lisozima, amilasa), el pH bajo del estómago, y también resistir a las concentraciones de sales biliares y jugos pancreáticos segregados en el intestino delgado (Duwat y cols., 2000). Es importante analizar inicialmente si la cepa candidata seleccionada tiene capacidad de llegar viva al intestino, mantenerse y proliferar de manera eficiente, una vez ha sido ingerida, y poder observar su efecto a largo plazo, o en su defecto ser adicionada regularmente en la dieta (Duwat y cols., 2000; Nikoskelainen y cols., 2001b), contribución enzimática para la digestión,

- **Inocuidad y efecto beneficioso para el hospedador:** Los microorganismos utilizados como probióticos deben ser no patógenos y no tóxicos, y por tanto, no producir efectos indeseables al administrarlos a los animales acuáticos (Irianto y Austin, 2002b). No sólo es necesario que protejan al hospedador frente a enfermedades, sino que ofrezcan efectos beneficiosos en la interacción con el mismo (Fuller, 1992; Irianto y Austin, 2002b).

- **Capaz de adherirse a células intestinales:** Para sobrevivir y competir en un ecosistema complejo como el intestino, es necesario que la cepa seleccionada pueda adherirse a la mucosa intestinal, siendo este criterio importante a la hora de evaluar una cepa como probiótica, ya que solo las cepas capaces de adherirse podrían llegar a colonizar el intestino, y a su vez formar parte de la primera barrera defensiva de los peces frente a los patógenos que utilizan este tracto como puerta de entrada. En caso contrario, la cepa se convertirá en un microorganismo transitorio, limitando sus posible efectos positivos (Verschuere y cols., 2000a; Vine y cols., 2006).

1.3.2- Mecanismos de acción de los probióticos

Durante las últimas décadas muchas son las publicaciones en la que se hace referencia a los mecanismos de acción de los probióticos (Balcázar y cols., 2006; Kesarcodi-Watson, 2008; Dimitroglou y cols., 2011). La capacidad de los probióticos para ejercer su acción depende fundamentalmente de la exactitud con la que alcancen el lugar específico donde deben actuar (Verschuere y cols., 2000a). Ejercer su acción protectora en el hospedador está relacionada con sus diferentes mecanismos de acción, que son evaluados tanto *in vitro* como *in vivo.* Entre los mecanismos de acción que han sido descritos para los probióticos podemos

destacar los siguientes: La producción de compuestos inhibidores (Naidu y cols., 1999), competencia por compuestos químicos o por energía disponible (Fredrickson y Stephanopoulos, 1981), competencia por los lugares de fijación (Tapia-Paniagua y cols., 2010), mejora de la calidad del agua (Antony y Philip, 2006), contribución enzimática para la digestión (Sáenz de Rodrigáñez y cols., 2009), fuente de macro y micronutrientes (Sugita y cols., 1998), efecto antiviral (Harikrishnan y cols., 2010, y uno de los mecanismos más importantes y en el que nos vamos a centrar en este trabajo es sobre el aumento de la respuesta immune (Villamil y cols., 2002; Balcázar y cols., 2007b; Salinas y cols., 2008; Son y cols., 2009; Chiu y cols., 2010). Figura III

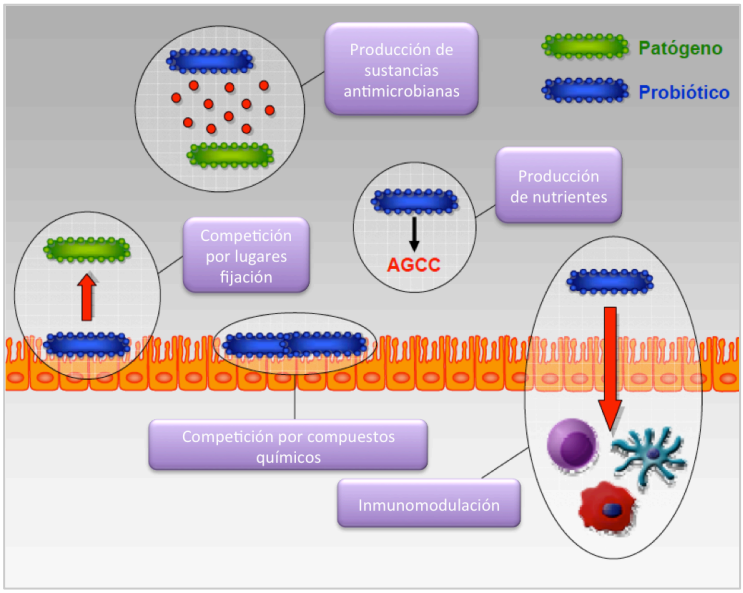

Figura III: Representación de algunos de los mecanismos de acción de los probióticos.

1.3.3-Los Probióticos y el sistema inmune de peces teleósteos

Entre los numerosos efectos beneficiosos de los probióticos, la modulación del sistema inmune es uno de los efectos más estudiados, sobre todo en humanos y

animales terrestres (Nayak, 2010). La mayoría de los estudios realizados con probióticos en peces estaba relacionado, hasta no hace mucho tiempo, con la capacidad protectora y la capacidad de promover el crecimiento (Nayak, 2010). En los últimos años, una gran cantidad de trabajos cercioran el efecto beneficioso de los probióticos tanto *in vivo* como *in vitro* sobre el sistema inmune de los peces teleósteos (Díaz- Rosales y cols., 2006; Balcázar y cols., 2007b; Brunt y cols., 2007; Panigrahi y cols., 2007; Kim y Austin, 2008; Balcázar y cols., 2009).

En la literatura se indica que los probióticos, tanto de manera individual como combinados, pueden mejorar tanto de forma sistémica como de forma localizada la inmunidad en los peces. A continuación se van a detallar algunos efectos de los probióticos sobre el sistema inmune de los peces así cómo los factores que pueden determinar dicha respuesta.

1.3.4-Los probióticos y la inmunidad innata

Los estudios realizados en humanos y animales con probióticos nos pueden proporcionar una idea de cómo influyen éstos sobre la inmunidad de los peces (Mishra y cols., 2008). Pueden estimular en los peces diferentes parámetros inmunes. Interactúan con células fagocíticas (monocitos y macrófagos), con leucocitos polimorfonucleares (neutrófilos), así como con células *natural killer* (NK) mejorando la respuesta innata inespecífica. Al igual que en los vertebrados superiores, los probióticos pueden aumentar el número de eritrocitos, granulocitos, macrófagos y linfocitos en diferentes especies acuícolas (Irianto y Austin, 2003; Kumar y cols., 2008). Del mismo modo, tanto *in vivo* como *in vitro*, estimulan activamente la proliferación de linfocitos B en los peces. El aumento de

las inmunoglobulinas producido por la suplementación con probióticos se ha demostrado en muchas especies animales, incluidos los peces (Panigrahi y cols., 2004; Nayak y cols., 2007; Al-Dohail y cols., 2009). Diferentes cepas bacterianas pertenecientes a las bacterias ácido-lácticas (LAB), ya sean viables o no, puede aumentar las inmunoglobulinas en los peces (Panigrahi y cols., 2005), incluso con sólo una semana de suplementación de las cepas con el alimento, como demostraron Nikoskelainen y cols. (2003), que observaron diferencias significativas en la cantidad de inmunoglobulinas de la truchas arcoiris (*Oncorhynchus mykis*) alimentadas con 2,8 x 10^8 ufc/g de alimento de *Lactobacillus rhamnosus.* Sin embargo, Balcázar y cols. (2007c) no observaron diferencias significativas en la cantidad de inmunoglobulinas de la trucha común (*Salmo trutta*), cuando se alimentaron durante dos semanas con una mezcla de 10^6 ufc/g de alimento de *Lactococcus lactis* subsp *lactis*, *Lactobacillus sakei* y *Leuconostoc mesenteroides*.

a) Actividad fagocítica:

La actividad fagocítica es la responsable de la activación temprana de la respuesta inflamatoria antes de la producción de anticuerpos, y juega un papel importante en las defensas frente a las bacterias. *Lactobacillus rhamnosus, Lactobaccilus lactis* y *Lactobacillus acidophilus* aumentan la actividad fagocítica en diversos animales (Rutherfurd-Markwick y Gill, 2004). Muchos de éstos probióticos se han utilizado en la práctica acuícola suplementados en el pienso, ya sea de forma viva o inactivada, observándose efectos beneficiosos sobre la actividad fagocítica (Irianto y Autin, 2003; Panigrahi y cols., 2004; Brunt y Austin,

2005; Brunt y cols., 2007; Pieters y cols., 2008). Pirarat y cols. (2006) observaron en la tilapia (*Oresochromis niloticus*) un aumento significativo en la actividad fagocítica de los leucocitos tras dos semanas de alimentación con *Lactobacillus rhamnosus.* Igualmente, Sakai y cols. (1995) encontraron un aumento en la actividad fagocítica de los leucocitos de riñón anterior en trucha arcoíris, tras la administración oral de *Clostridium butyricum*. Sin embargo, *Lactobacillus lactis* aumenta la actividad fagocítica de los leucocitos de riñón anterior en rodaballo (*Scophtalmus maximus*) (Villamil y cols., 2002).

b) Explosión respiratoria

La explosión respiratoria es un importante mecanismo de defensa innata de los peces. Los resultados obtenidos para la explosión respiratoria en los estudios realizados con probióticos en peces son a menudo contradictorios. Mientras que por un lado existen estudios en los que se demuestra que no hay efecto de los probióticos sobre la explosión respiratoria (Nayak y cols., 2007; Sharifuzzaman y Austin, 2009; Díaz-Rosales y cols., 2009), otros trabajos realizados, tanto *in vivo* como *in vitro*, han demostrado que los probióticos ejercen su acción sobre la explosión respiratoria de muchos animales acuáticos, incluidos los peces. *Bacillus subtilis* y ciertas cepas pertenecientes a las bacterias ácido-lácticas (LAB) pueden estimular la actividad de la explosión respiratoria en los peces (Ortuño y cols., 2002; Salinas y cols., 2005; Salinas y cols., 2006; Salinas y cols., 2009; Zhou y cols., 2010). Salinas y cols. (2006), observaron *in vitro* que 5 x 10^7 ufc/ml de *Lactobacillus delbrueckii* subps. *lactis* y *B. subtilis* inactivadas con calor eran

capaces de estimular al explosión respiratoria en los leucocitos de riñón anterior de la dorada (*Sparus aurata*).

c) Lisozima:

La lisozima es una de las enzimas más importantes de la respuesta innata inespecífica que ayuda al hospedador a combatir los agentes infecciosos (Rutherfurd-Markwick, 2004). Los probióticos, sólos o combinados, pueden aumentar la actividad lisozima del suero en los peces teleósteos, como *L. rhamnosus*, *Carnobacterium maltaromaticum*, *Carnobacterium divergens* en trucha arcoiris (Panigrahi y cols., 2004; Kim y Austin, 2006) o *L. lactis* subsp. *lactis*, *L. mesenteroides* y *L. sakei* en trucha común (Balcázar y cols., 2007a). A aparte de aumentar la lisozima del suero, también pueden mejorar el nivel de lisozima en el mucus de la piel de los peces (Taoka y cols., 2006; Song y cols., 2006).

d) Contenido en peroxidasa

La peroxidasa es una enzima que utiliza radicales oxidativos para producir ácido hipocloroso para matar patógenos. Se produce sobre todo durante la explosión respiratoria, y es liberada principalmente por los gránulos de los neutrófilos. Wang y cols. (2008) observaron que *Enterococcus faecium*, administrado a través del agua, aumenta el contenido en peroxidasa del suero en la tilapia del nilo (*Oreochromis niloticus*). Reyes-Becerril y cols. (2008), apreciaron diferencias significativas en el contenido en peroxidasa de doradas alimentadas durante 4 semanas con 10^6 ufc/g de alimento con la levadura *Debaryomyces hansenii*. Por el contrario, *L. delbrueckii* subsp. *lactis* y *B. subtilis* administrados

durante 3 semanas no aumentaron el contenido en peroxidasa de los leucocitos de riñón anterior de la dorada (*Sparus aurata*) (Salinas y cols. 2006).

e) Actividad del complemento

En los teleósteos, el sistema complemento desempeña un papel clave en la respuesta inmune, ya que ayuda a la quimiotaxis, opsonización, fagocitosis y a la degradación de los patógenos. Se ha demostrado que el sistema complemento puede ser estimulado tanto por cepas probióticas vivas (Panigrahi y cols., 2005; Salinas y cols., 2008) como inactivadas, como comprobaron Choi y Yoon (2008), tras las administración durante 4 semanas de cepas probióticas pertenecientes a la Familia *Vibrionaceae* (Pdp11 y 51M6).

f) Citoquinas

Las citoquinas son mediadores producidos por las células del sistema inmune y ayudan a contribuir al crecimiento y a la diferenciación celular, entre otras funciones (Peddie y cols., 2002). En la literatura se indica que numerosos probióticos pueden modular la producción de diferentes citoquinas proinflamatorias, tales como la interleuquina-1 (IL-1β), interleuquina-6 (IL-6), interleuquina-12 (IL-12), factor de necrosis tumoral-α (TNF-α), el interferón-γ (IFN-γ), así como citoquinas antiinflamatorias tales como la interleuquina-10, el factor de crecimiento transformante-β (TGF-β) en muchos animales (Christensen y cols., 2002; Peddie y cols., 2002; Niers y cols., 2005). Probióticos como *Bifidobacterium longum*, *L. acidophilus*, *L. lactis*, *Lactobacillus paracascei* pueden regular la expresión de citoquinas en diferentes hospedadores (Kato y cols., 1999;

Rutherfurd-Markwick, 2004). Los probióticos como *L. rhamnosus*, *E. faecium* y *B. subtilis* son capaces de regular la expresión que citoquinas como la IL-1β y el TGF-β, tanto en el bazo como en el riñón anterior de la trucha arcoíris (*Oncorhynchus mykis*) (Panigrahi y cols., 2007). Resultados similares obtuvieron Kim y Austin (2006) con una sobre estimulación de IL-1β, IL-8, TNF-α y TGF-β en los leucocitos de riñón anterior de trucha arcoíris que fueron alimentadas con *C. maltaromaticum* y *C. divergens*, sugiriendo que estas cepas posiblemente están involucradas en la respuesta antiinflamatoria. Por otro lado, Picchietti y cols. (2009) observaron inhibición en la expresión de la Ciclooxigenasa-2 (Cox-2), junto con la inhibición de la expresión de la IL-1β y el TGF-β en los leucocitos de larvas de lubina (*Dicentrarchus labrax*) alimentadas con *Lactobacillus delbrueckii*, suplementada a través de alimento vivo (artemia y rotíferos).

2.- OBJETIVOS

Por esta razón, y continuando con el estudio realizado por Sorroza y cols. (2012) y Sorroza y cols. (2013), la finalidad de este trabajo es estudiar el efecto inmunomodulador de la cepa identificada y caracterizada como probiótico *Vagococcus fluvilalis* L21 (Sorroza y cols., 2012), sobre el sistema inmune inespecífico de la lubina, para conocer con mayor exactitud el mecanismo por el cual esta cepa ejerce su acción sobre los hospedadores. Este trabajo está relacionado con el área de sanidad en acuicultura dentro del Máster de Cultivos Marinos. Por tanto, los objetivos que nos planteamos para este estudio son:

OBJETIVO GENERAL

Evaluar *in vitro* el efecto de las cepa probiótica *Vagococcus fluvialis* L21 sobre el sistema inmune inespecífico de la lubina (*Dicentrarchus labrax*).

OBJETIVOS ESPECÍFICOS

1- Estudiar el efecto de la cepa *Vagococcus fluvialis* L21, tanto viva como inactivada, sobre las dinámica de expresión de genes relacionados con la respuesta inmune inespecífica en los leucocitos de lubina (*Dicentrarchus labrax*)

2- Valorar el efecto de los productos extracelulares (ECPs) de la cepa *Vagococcus fluvialis* L21 sobre la dinámica de expresión de genes relacionados con la respuesta inmune inespecífica en los leucocitos de lubina (*Dicentrarchus labrax*)

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Artículo I:

Cytokine expression in head-kidney leucocytes of European sea bass (*Dicentrarchus labrax* L.) after incubation with the probiotic *Vagococcus fluvialis* L-21

Este capítulo contiene datos publicados en:

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4.1-Tabla de contenidos

ABSTRACT

The European sea bass (*Dicentrarchus labrax* L.), is one of the most extensively farmed marine fish in the Mediterranean sea. Under the high-density condition, common in aquaculture, the infectious diseases can cause significant economic losses. Probiotics are presented as an alternative to antibiotics for the control of aquaculture diseases. This study used real-time PCR to investigate *in vitro* the dynamic of expression of immune-related genes in sea bass after incubation with live and inactivated (heat and Uv-light) probiotic *Vagococcus fluvialis* L-21 at different times (T1, T12, T24, T48). The immune associated genes, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin 10 (IL-10), Tumor necrosis factor-α (TNF-α), ciclo-oxigenase-2 (COX-2), caspase-3 (Casp-3) and Mx were studied in head-kidney (HK) leucocytes of sea bass after incubation with the probiotic strain. Transcript of pro-inflammatory cytokines (IL-1β, TNF-α, and COX-2) was highly up-regulated after 1 h of incubation with the probiotic strain *V. fluvialis* L-21. We found statistically significant difference in pick of expression of TNF-α after 1 h of incubation with Uv-light inactivated probiotic strain. The COX-2 expression was highly up-regulated at all times studied, with the exception of 12 and 24 h post inbubation for the Uv-light inactivated bacteria. Transcript of IL-10 and Casp-3 showed the higher statistically significant differences of expression after 48 h post incubation with live bacteria. In the contrast, sea bass HK leucocytes expressed Mx at 12 and 48 h without statistically differences among treatments. Our results suggest that *V. fluvialis* L-21 is able to stimulate *in vitro* some immune-related

genes associated with the early inflammatory response. Future studies *in vivo* are necessary to clarify this process in sea bass.

1. Short Comunication

Sea bass (*Dicentrarchus labrax* L.) is the most intensively farmed marine fish in south Europe. The intensive culture practices render increase in the transmission of infectious diseases [1], and several strategies in disease control have been proposed. Among them, the administration of probiotics has appeared as a very promising biological control for aquaculture [2] and are considered as excellent tool to control fish diseases [3-8]. Up to day, several *in vivo* studies on the immune modulation of probiotics have been carried out [6-13]. However, *in vitro* studies that evaluate the immune-modulator effects of bacteria on the immune cells are particularly scarce [14-16]. Taking into account that in most of the studies, the results obtained *in vitro* show correspondence to those obtained *in vivo* [10,17], the *in vitro* assays are being developed to reliably identify the most interesting bacterial strains [18]. The mechanisms of action of probiotics are far from completely understood, although it is widely accepted that they can produce inhibitory compounds, compete against pathogens bacteria for nutrients and adhesion sites, improve the microbial balance, and modulate the physiology of the immune system [19]. Cytokines are protein mediators produced by immune cells that contribute to cell growth, differentiation and defence mechanisms of the host [20]. The literature indicates that probiotics can modulate effectively the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α) and gamma interferon (IFN-

γ), and anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) in many species [21-23]. The strain *Vagococcus fluvialis* L-21 is a Gram-positive coccus isolated from the intestine of gilthead sea bream (*Sparus aurata* L.) and sole (Solea solea L.), that according to *in vitro* and *in vivo* characteristics tested in our laboratory, make it suitable for use as potential fish probiotic strain [24]. The strain *V. fluvialis* L-21 showed *in vitro* inhibitory effect against different marine and continental fish pathogen, fish bile and pH resistance, adhesion and growth to intestinal mucus, protecting sea bass in a challenge against *V. anguillarum* with a relative percent of survival of 42.3% [24]. Román et al. 2012 [16] showed *in vitro* the immune modulatory effect of HK leucocytes in sea bream and sea bass with the probiotic strain *V. fluvialis* L-21 live and inactivated. The aim of this study was to evaluate the levels of immune-associated related genes (IL-1β, IL-6, IL-10, TNF-α, Casp-3, COX-2 and Mx) in HK leucocytes of sea bass after incubation with the probiotic strain *V. fluvialis* L- 21.

The strain *V. fluvialis* L-21, characterized as probiotic strain in fish by Sorroza et al. 2012 [24] was grown at 22 ºC in brain heart infusion broth (BHIB) (Pronadisa) supplemented with 1.5% NaCl, with continuous shaking for 24 h. All bacterial cultures were heat-inactivated for 2 h at 60 ºC and Uv-light inactivated for 2.5 h and stored at -80 ºC until required.

Sea bass with an average body weight of 100 g were provided by Instituto Canario de Ciencias Marinas (ICCM), Canary Island (Spain). Fish were maintained in 500 l stocking tanks and fed *ad libitum* with a commercial diet (Skretting).

HK leucocytes were isolated from a total of 35 sea bass under sterile conditions following the technique described by Secombes [25] with some modifications. To

study the expression of immune related genes, aliquots of 2.5 ml containing 1 x 10^7 cells ml^{-1} in L-15 medium supplemented with P/S/G were seed in 6-well plates (Nunc, Roskilde, Denmark). After 3 h of incubation at 22 °C, non-adherent cells were removed and medium was replaced by L-15 and P/S/G supplemented with 2% FCS. Finally, monolayers were incubated overnight at 22 °C.

Live and inactivated bacteria were adjusted in PBS at 10^9 cfu ml^{-1} and then, 150 μl of each suspension were added to samples of 1.5 ml of HK Leucocytes after 1 h of incubation. Culture medium was used as a negative control. Once cells were incubated, were washed twice with PBS and finally maintained with L-15 medium supplemented with 2% FCS. Cells were collected at different times (1, 12, 24 y 48 h) after incubation. Each sample was repeated by triplicate. The expression of seven selected immune-relevant genes (Mx, IL-1β, IL-6, IL-10, COX- 2, TNF-α, Casp-3) were analysed by qRT-PCR using SYBR Green Supermix (Biorad). Specific PCR primers for IL-1β (forward, 5'-ATTACCCACCACCCACTGAC-3'; reverse, 5'-TCTCTTCCACTATGCTCTCCAG-3'), β-actin (forward, 5'-ATGTGGATCAGCAAGCAGG-3'; reverse, 5'-AGAAATGTGTGGTGTGGTCG-3') Mx (fordward, 5'-GGTCAAGGAGCAGATCAAACAG3'; reverse, 5'-CTCGCATCAGGTTAGGGAATC-3'), Casp-3, (fordward, 5'-ACGAAGCAGGTCAATCATCC-3'; reverse, 5'-GCAGTTTAAGGGTATCCAGAGC-3' and TNF-α (fordward, 5'-GCCAAGCAAACAGCAGGAC-3'; reverse, 5'-GCCAAGCAAACAGCAGGAC-3') were designed using the GeneRunner® software, while for IL-10 , IL-6 and COX-2 we used the sequence of primers described by Picchietti *et al*. 2009 [26] and Sepulcre *et al*. 2007 [27]. β-actin was also used as house-keeping gene. The relative gene expressions were determined according to the ΔΔCt method using IQ5 software

(Biorad). The ΔΔCt method is also known as the comparative Ct method where: $\Delta\Delta Ct = [\Delta]Ct_{sample} - [\Delta]Ct_{control}$.

Data were analysed by one-way analysis of variance (ANOVA) and Tukey's comparison of means. Values higher 1 in a parameter express an increase (up-regulation) while values lower 1 express a decrease (down-regulation) in a parameter. Differences were considered statistically significant when $P<0.05$.

The results obtained after incubation of the HK leucocytes with the live bacteria, heat-inactivated bacteria and Uv-light inactivated bacteria in each time are shown in Figure 1. We can observe how pro-inflammatory cytokines IL-1β and TNF-α are expressed after 1 h (T1) of incubation with the probiotic strain *V. fluvialis* L-21. TNF-α was detected with statistically significant differences between Uv-light inactivated bacteria and the other treatments at T1. The kinetic of gene expression of IL-6 was down-regulation in each treatment and in each time, with the exception of T1 for live bacteria and 24 h (T24) for heat-inactivated bacteria, where statistically differences were found with respect to other treatments in this time. At 48 h (T48) up-regulation were observed in IL-6 expression but without statistically differences. Expression of IL-10 was detected at T1 in each treatment, here we noticed a significant difference between the Uv-light inactivated bacteria and the others treatments. At T48 statistically significant pick of expression in IL-10 appears when live bacteria was used for treatment. HK leucocytes of sea bass expressed Mx at T12 and T48 after incubation with *V. fluvialis* L-21 without statistically differences among treatments in both times. At T48 a pick of expression of Casp-3 appears in live bacteria, showing up-regulation, and also

statistically significant differences with other bacteria treatments. COX-2 was the only gene up regulated in almost each time; here we observe that live bacteria produced the highest expression of COX-2 and this treatment also produces statistically differences among treatments in each time in sea bass HK.

In previous studies, Román et al. (2012) [16] reported that the strain of *V. fluvilalis* L-21, live or inactivated, stimulate *in vitro* HK leucocytes of sea bream and sea bass, obtaining in sea bass the best results of phagocytic activity, respiratory burst and peroxidase content. This result suggests that this strain could be an immune modulator for this specie. Although many studies have already described *in vitro* cytokines responses of various cell lines, such as mammalian macrophages, human intestinal cells (CaCo-2; HT-29) and human PBMC, to various bacteria including lactobacilli [28]. So far, only Kim and Austin (2006) [14] have studied cytokines response patterns against bacteria *in vitro* using fish cells. We report the first *in vitro* study of the effect of *Vagococcus fluvilalis* L-21 on some immune-related genes, testing pro-inflammatory cytokines like IL-1, IL-6, TNF-α and COX-2, generally induced at the early stage of the immune response [29,30]. After 1 h of incubation with live and inactivated bacteria, IL-1β and TNF-α gene expression were up regulated in HK leucocytes, suggesting that *V. fluvialis* induces an early inflammatory response in HK leucocytes of sea bass. These results are similar to those obtained *in vitro* by Kim and Austin (2006) [14] in HK of rainbow trout (*Oncorhynchus mykiss*) after co-culturing with live probiotics. The RNA expression of TNF-α in HK leucocytes after the incubation with Uv-light probiotic produces statistically significant difference compared with other treatments at the same time. These results have been compared with other studies, which had shown that

the supplementation with probiotic bacteria increases the expression of pro-inflammatory cytokines, including IL-1β and TNF-α [31]. The COX-2, which is considered as a central mediator during inflammation, was highly up-regulated after the incubation with live and inactivated *V. fluvialis* L-21. In contrast, Picchietti et al. (2009) [26] found transcripts significantly lower of COX-2, IL-10 and IL-1β in HK leucocytes of sea bass larvae fed with *Lactobacillus delbrueckii*. An over-expression of COX-2 is related *in vivo* with chronic diseases [32], but it has not been demonstrated *in vitro* so far. On the other hand, Sepulcre et al. 2007 [27] found that IL-6 is expressed *in vivo* in the first hours after the infection of sea bass with *Vibrio anguillarum*, while in this *in vitro* study we found that the pick of expression of IL-6 is after 48 h incubation. Our results are dissimilar to those found in rainbow trout by Chettri et al. 2001 [33], where regulation between IL-6 an IL-10 is observed. In our case, the expression of IL-10 showed the higher expression at 48 h after incubation with the live bacteria. Our hypothesis is that HK leucocytes incubated with live probiotic bacteria at 48 h express not only cytokines like IL-10 which are able to reduce the inflammation, but also Casp-3, which is responsible of closing the inflammatory response. Nagata, 1997 [34] considers that apoptosis plays a role not only during development and homeostasis, but also in the regulation of the host response during infection with bacteria, viruses and parasites. Some bacteria used as candidate probiotics have antiviral effect [5]. IFN is one of the most known mechanisms against virus and Mx proteins are the molecular effectors of this gene [35]. Acosta et al. 2004 [36] showed that a bacterin of *Listonella anguillarum* stimulated Mx in Atlantic salmon (*Salmo salar*, L). Bravo et al. 2011 [37] showed that LPS of *Vibrio alginolyticus* stimulated Mx response in

sea bream (*Sparus aurata*). This is the first report of Mx stimulation produced by Gram positive bacteria, and in spite no significantly differences were found among treatments, *V. fluvialis* L-21 do stimulate Mx expression suggesting that other components of the bacterial wall are involved in the immune stimulation.

This study demonstrate that leucocytes of sea bass responding differently depending on the viability of the probiotic strain of *V. fluvialis* L-21. The cytokines gene expression is generally higher in those leukocytes incubated with live bacteria and UV-light inactivated bacteria, in contrast with those incubated with heat-inactivated bacteria where observed less expression. Pro-inflammatory cytokines such as IL-1, TNF-α and COX-2 become up-regulated following exposure to probiotic strain suggesting that *V. fluvialis* L-21 induces an early inflammatory response in HK leucocytes. Despite the *in vitro* results suggest that *V. fluvialis* L-21 is an immune stimulant, more studies *in vivo* are necessary to better understand the interaction between this probiotic strain and the immune response in sea bass.

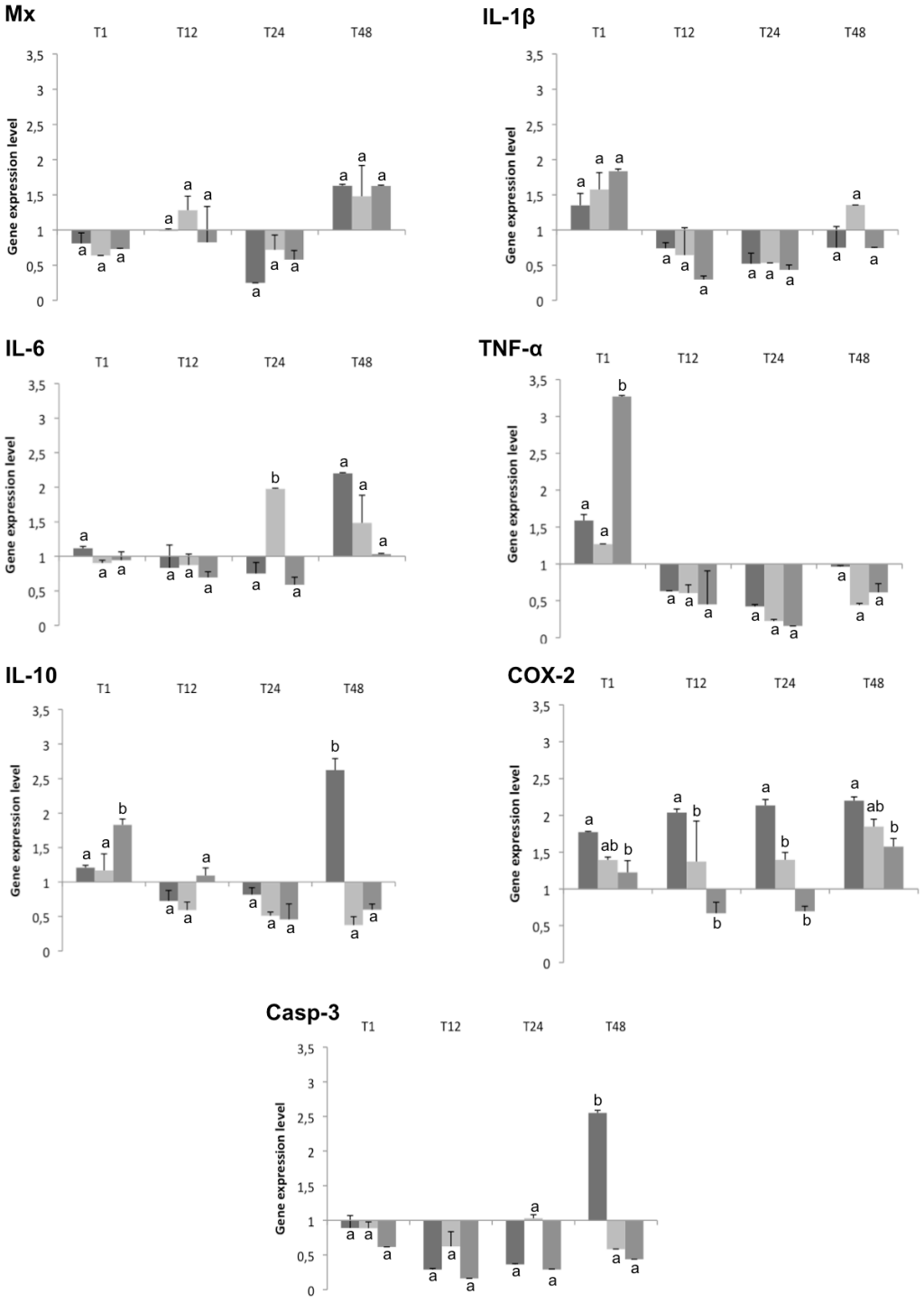

Fig. 1. Expression of immune-relevant genes determined by real-time PCR in head kidney leucocytes after incubation with 10^8 CFU ml^{-1} Live), heat) and Uv-light) inactivated bacteria compared to the control. Significant differences ($P<0.05$) between groups in each time is indicated by unlike letters on the bars (n=6).

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Short communication

Cytokine expression in head-kidney leucocytes of European sea bass (*Dicentrarchus labrax L.*) after incubation with the probiotic *Vagococcus fluvialis* L-21

L. Román, F. Real, D. Padilla, F. El Aamri, S. Déniz, V. Grasso, F. Acosta*

Instituto Universitario de Sanidad Animal, Universidad de Las Palmas de Gran Canaria, Trasmontaña s/n, 35413 Arucas, Spain

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ABSTRACT

The European sea bass (*Dicentrarchus labrax* L.) is one of the most extensively farmed marine fish in the Mediterranean sea. Under the high-density condition, common in aquaculture, the infectious diseases can cause significant economic losses. Probiotics are presented as an alternative to antibiotics for the control of aquaculture diseases. This study used real-time PCR to investigate *in vitro* the dynamic of expression of immune-related genes in sea bass after incubation with live and inactivated (heat and Uv-light) probiotic Vagoccus *fluvialis* L-21 at different times (T1, T12, T24, T48). The immune associated genes, interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin 10 (IL-10), TumourTumour necrosis factor-(TNF-), ciclo-oxigenase-2 (COX-2), caspase-3 (Casp-3) and Mx were studied in head-kidney (HK) leucocytes of sea bass after incubation with the probiotic strain. Transcript of proinflammatory cytokines (IL-1, TNF-, COX-2) was highly up-regulated after 1 h of incubation with the probiotic strain V. *fluvialis* L-21. We found statistically significant difference in pick of expression of TNF-, after 1 h of incubation with Uv-light inactivated probiotic strain. The COX-2 expression was highly up-regulated at all times studied, with the exception of 12 and 24 h post incubation for the Uv-light inactivated bacteria. Transcript of IL-10 and Casp-3 showed the higher statistically significant differences of expression after 48 h post incubation with live bacteria. In the contrast, sea bass HK leucocytes expressed Mx at 12 and 48 h without statistically differences among treatments. Our results suggest that V. *fluvialis* L-21 is able to stimulate *in vitro* some immune-related genes associated with the early inflammatory response. Future studies in vivo are necessary to clarify this process in sea bass.

Sea bass (*Dicentrarchus labrax* L.) is the most intensively farmed marine fish in south Europe. The intensive culture practices render increase in the transmission of infectious diseases [1], and several strategies in disease control have been proposed. Among them, the administration of probiotics has appeared as a very promising biological control for aquaculture [2] and is considered as excellent tool to control fish diseases [3–8]. Up to day, several in vivo studies on the immune modulation of probiotics have been carried out [6–13]. However, *in vitro* studies that evaluate the immune-modulator effects of bacteria on the immune cells are particularly scarce [14–16]. Taking into account that in most of the studies, the results obtained *in vitro* show correspondence to those obtained in vivo [10,17], the *in vitro* assays are being developed to reliably identify the most interesting bacterial strains [18]. The mechanisms of action of probiotics are far from completely understood, although it is widely accepted that they can produce inhibitory compounds, compete against pathogens bacteria for nutrients and adhesion sites, improve the microbial balance, and modulate the physiology of the immune system [19]. Cytokines are protein mediators produced by immune cells that contribute to cell growth, differentiation and defence mechanisms of the host [20]. The literature indicates that probiotics can modulate effectively the production of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α) and gamma interferon (IFN-γ), and anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF-β) in many species [21–23]. The strain *Vagococcus fluvialis* L-21 is a Gram-positive coccus isolated from the intestine of gilthead sea bream (*Sparus aurata* L.) and sole (*Solea solea* L.), that according to *in vitro* and in vivo characteristics tested in our laboratory, make it suitable for use as potential fish probiotic strain Ref. [24]. The strain V. *fluvialis* L-21 showed *in vitro* inhibitory effect against different

* Corresponding author. Tel.: +34 928 45 97 40; fax: +34 928451142.
E-mail address: facosta@dpat.ulpgc.es (F. Acosta).

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Artículo II:

The i*n vitro* immunomodultaroy effect of Extrecelullar products (ECPs) of *Vagococcus fluvialis* L21 on European Sea Bass (*Dicentrarchus labrax*) leucocytes

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5.1.-Tabla de contenidos

ABSTRACT

The immune associated genes, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor-α (TNF-α), ciclo-oxigenase-2 (COX-2), and Mx gene were studied by real-time PCR in head-kidney leucocytes of sea bass after incubation with the extracellular products (ECPs) of the probiotic strain *Vagococcus fluvialis* L21 and polyinosinic:polycytidylic acid (POLY I:C), at different times (T1.5, T6, T12, T24, T48 and T72). In general, we can observe how pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and COX-2 studied displayed a strong peak after stimulation with 1.5 h of ECPs of *V. fluvialis* L21, significant differences ($P < 0.05$) exist with other periods and with the POLY I: C at the same time. Similarly to the case of IL-10 also produced a statistically significant ($P < 0.05$) peak of expression on leukocytes that were stimulated with the ECPs of *V. fluvialis* L21. In the case of Mx gene expression, we note that in almost all sampling times there is an up-regulation of the Mx gene in leucocytes incubated with ECPs and POLY I:C compared to the control and Mx expression was higher in leucocytes that were stimulated with the ECPs of *V. fluvialis* for all times, except in T24. With these results we can consider that the ECPs of *V. fluvialis* L21 have a great power of stimulating the *in vitro* expression of immune-related genes and may even be useful as adjuvants for vaccine in aquaculture.

Keywords: ECPs; *Vagococcus fluvialis* L21; Fish Immune System; Leucocytes; European Sea bass.

1. Introduction

Infectious diseases are one of the most limiting factors for the successful development of aquaculture industry. Among other control strategies, probiotics are considered an alternative tool for disease control, which consequently can prevent the use of antibiotics [1-4]. Today, not only alive bacterial strains are used as probiotics, but also inactivated ones, which have beneficial effects on the host, both *in vitro* and *in vivo* [5-7]. In addition, recent researches shows that purified components of the bacterial strains may have beneficial effect on the health of the hosts. Abbass et al. [8] showed that the cell wall proteins, outer membrane proteins (OMP) and lipopolysaccharides (LPS) of the probiotic strains *Aeromonas sobria* GC2 and *Bacillus subtilis* JB-1 conferred protection against *Yersinia ruckeri* in salmonids. Moreover, the probiotic supplementation has begun to be used as an adjuvant to increase the immunogenicity of vaccines in humans and animals [9,10].

In order to better understand the mechanism of action of the *Vagococcus fluvialis* L21 strain, identified and characterized as probiotic strain with protective effect against vibriosis in sea bass by *Vibrio anguillaum* by Sorroza et al. [11], and considering that the this strain has a clear immunomodulatory activity *in vitro*, as well as the ability to induce cytokine production related to the immune response [7,12], the aim of this study was to determine, *in vitro*, the effect of extracellular products (ECPs) of *V. fluvialis* L21 on the dynamics of expression of genes (IL-1β, IL-6, IL-10, TNF-α, COX-2, Mx) involved in the immune response in sea bass leucocytes.

2. Materials and methods

2.1. Fish

Thirty-six sea bass with an average weight of 150 g were provides by the Canary Institute of Marine Sciences (ICCM) to study the dynamics of expression of cytokines related to the immune response. The fish were kept in tanks of 1000 l of capacity with open water circulation, constant aeration and natural photoperiod of 12 h. Fish were fed daily with a commercial diet Skretting (Burgos, Spain). After checking the sanitary status, fish were slaughtered by overdose of 2-phenoxyethanol (Panreac), and then moved to the laboratory of Infectious Diseases and Fish Pathology of the Institute of Animal Health and Food Safety at the University of Las Palmas de Gran Canaria facilities.

2.2. Isolation of head kidney leucocytes and incubation with ECPs and POLY I:C

Twelve sea bass were used for extracting head kidney leucocytes according to the methodology of Secombes [13]. Each extraction was performed in triplicate. Once extracted, the head kidney leucocytes viability was observed by Trypan Blue staining and the concentration was adjusted to 10^7 cells ml^{-1} with the Neubauer chamber. To study the expression of genes involved in the immune response at different time, 2.5 ml of a solution of 10^7 cells ml^{-1} was added and allowed to set in polystyrene plates of 6 wells. After 3 h of incubation at 22 °C non-adherent cells were removed and medium was replaced with L-15 medium supplemented with antibiotic and with 2% foetal bovine serum (FBS). Monolayers were incubated overnight at 22 ºC.

Afterwards, leucocytes were incubated with 1 ml of the ECPs solution of *V. fluvilalis* L21 that were extracted following the methodology described by Barbey et al. [14], with modifications. This ECPs solution was prepared at a concentration of 0.025 mg ml^{-1} in L15 medium and incubated with sea bass leucocytes for 1.5 h. As a positive control, leucocytes by triplicate were incubated with 100 μg ml^{-1}polyinosinic: polycytidylic acid (POLY I:C) and, as a negative control we used leukocytes by triplicate that were incubated with PBS. After 1.5 h -stimulation which coincides with the first sample point (T1.5)- both, the solution of ECPs and the POLY I:C, were removed from the wells and replaced with L-15 medium supplemented with FBS 2% and antibiotic.

2.3. Analysis of gene expression

Cells were collected at different times for RNA extraction and then RT-PCR was performed after 1.5 h of incubation (T1.5), 6 h (T6), 12 h (T12), 24 h (T24), 48 h (T48) and at 72 h (T72) post-incubation with each of the solutions described above. Total RNA was extracted from head-kidney (HK) leucocytes using Extraction Kit (QIAGEN®). The RNA was eluted in 50 μl RNase-free *d*H_2O and used for real-time quantitative PCR. RNA was reverse transcribed to cDNA using the iScript Reverse Transcription Reagent kit (Bio-Rad) as follows: 2 μl of total RNA (approx. 10 μg), 5 μl of buffer reaction 5 x, and 12 μl of RNase-free *d*H_2O, heated the mixture to 70 ºC for 10 min and chilled on ice. Then, were added 1 μl of enzyme reverse transcriptase (200 U ml^{-1}) and 5 μl of RNase-free *d*H_2O. This was subsequently incubated at 25 ºC for 10 min, 50 min at 42 ºC and 15 min at 70 ºC, ending the PCR protocol keeping the samples at 16 ºC.

Gene name	Primers sequence		Annealing Tª	Accession number
IL-1β	Forward	5'-ATTACCCACCACCCACTGAC-3'	57.7ºC	AJ269472
	Reverse	5'-TCTCTTCCACTATGCTCTCCAG-3'		
β-actin	Forward	5'-ATGTGGATCAGCAAGCAGG-3'	57.7ºC	AJ537421
	Reverse	5'-AGAAATGTGTGGTGTGGTCG-3'		
Mx	Forward	5'-GGTCAAGGAGCAGATCAAACAG3'	57.7ºC	AM228974
	Reverse	5'-CTCGCATCAGGTTAGGGAATC-3'		
TNF-α	Forward	5'-GCCAAGCAAACAGCAGGAC-3'	60ºC	DQ200910
	Reverse	5'-ACAGCGGATATGGACGGTG-3'		
IL-6	Forward	5'-ACTTCCAAAACATGCCCTGA-3'	59.3ºC	AM490062 [15]
	Reverse	5'-CCGCTGGTCAGTCTAAGGAG-3'		
COX-2	Forward	5'-AGCACTTCACCCACCAGTTC-3'	59.3ºC	AJ630649 [15]
	Reverse	5'-AAGCTTGCCATCCTTGAAGA-3'		
IL-10	Forward	5'-ACCCCGTTCGCTTGCCA-3'	59.3ºC	AM268529 [16]
	Reverse	5'-CATCTGGTGACATCACTC-3'		

Table 1. Primers for real time PCR analysis

The expression of seven selected immune-relevant genes (Mx, IL-1β, IL-6, IL-10, COX- 2, TNF-α) was analysed by real-time PCR using a SYBR Green Supermix (Biorad). Specific PCR primers of IL-1β, β-actin, Mx and TNF-α, were designed using the GeneRunner® software. For the amplification of the other genes we used the sequence of primers described by Sepulcre et al. [15] and Picchietti et al. [16]. β-actin was use as house-keeping gene. Primers and annealing temperature are given in Table 1.

cDNA pooled from the sea bass HK leucocytes were used in each PCR reaction. The real-time analysis consisted of 1 cycle of 95 °C for 5 min, 40 cycles of 95 °C for 15 s and annealing temperature for 30 s, 1 cycle of 95 °C for 1 min, 1 cycle of 70 °C for 1 min, and a melting curve of 81 cycles (from 55 °C to 95 °C) for 30 s. Reactions were performed in triplicate for each template cDNA that was replaced with water in all blank control reactions.

The relative gene expressions were determined according to the ΔΔCt method using IQ5 software (Bio-Rad). The ΔΔCt method is also known as the comparative Ct method where:

$$\Delta\Delta Ct = [\Delta]Ct_{sample} - [\Delta]Ct_{control};$$

here ΔCt_{sample} is the Ct value for any sample normalized to the endogenous housekeeping gene and $\Delta Ct_{control}$ is the Ct value for the control sample also normalized to the endogenous housekeeping gene. The IQ5 software allows automatic normalization of the Ct values to the housekeeping gene (β-actin). The Ct values are also automatically compared between samples.

2.4. Statistical analysis.

Statistical analyses were carried out using the statistical software SPSS program version 17 (SPSS, Inc, Chicago, IL, USA). Data were analysed by two-way analysis of variance (ANOVA) and Tukey's comparison of means. Values higher 1 in a parameter express an increase (up-regulation) while values lower 1 express a decrease (down-regulation) in a parameter. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Immune-related gene expression

Fig. 1 show the immune-related gene expression in sea bass leucocytes incubated with the ECPs of *V. fluvialis* L21 and the POLY I: C. We can observe how pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and COX-2 studied displayed a strong peak after stimulation with 1.5 h of ECPs of *V. fluvialis* L21, significant

differences (P<0.05) exist with other periods and with the POLY I: C at the same time. The peak expression markedly diminished in the case of COX-2 or more is maintained prolonged as is the case of IL-1β and IL- 6. In the case of TNF-α after 6 hours of incubation the expression levels border on the baseline. For the expression of COX -2 in each of the sampling times are significant differences between the two products used to stimulate the leukocytes, whereas in the other genes studied, only appears in the first hours after stimulation and for IL-6 high as 24 hours post- inoculation. In the case of IL-10, after stimulation for 1.5 h with the ECPs of *V. fluvialis* there was a statistically significant (P<0.05) peak of expression. From T6 onwards there were no significant differences among treatments except at T24 (p<0.05). Similarly as in previous cases, the expression of IL-10 was higher in leucocytes that were stimulated with the ECPs of *V. fluvialis* L21. In general, we note that in almost all sampling times there is an up-regulation of the Mx gene in leucocyte incubated with ECPs and POLY I:C compared to the control. Down-regulation was observed in leucocytes incubated with POLY I:C at the times T1.5; T6 and T72. Mx expression was higher in leucocytes that were stimulated with the ECPs of *V. fluvialis* L21 for all times, except in T24. In this time a statistically significant (P<0.05) peak of expression was observed in leucocytes we incubated with POLY I:C.

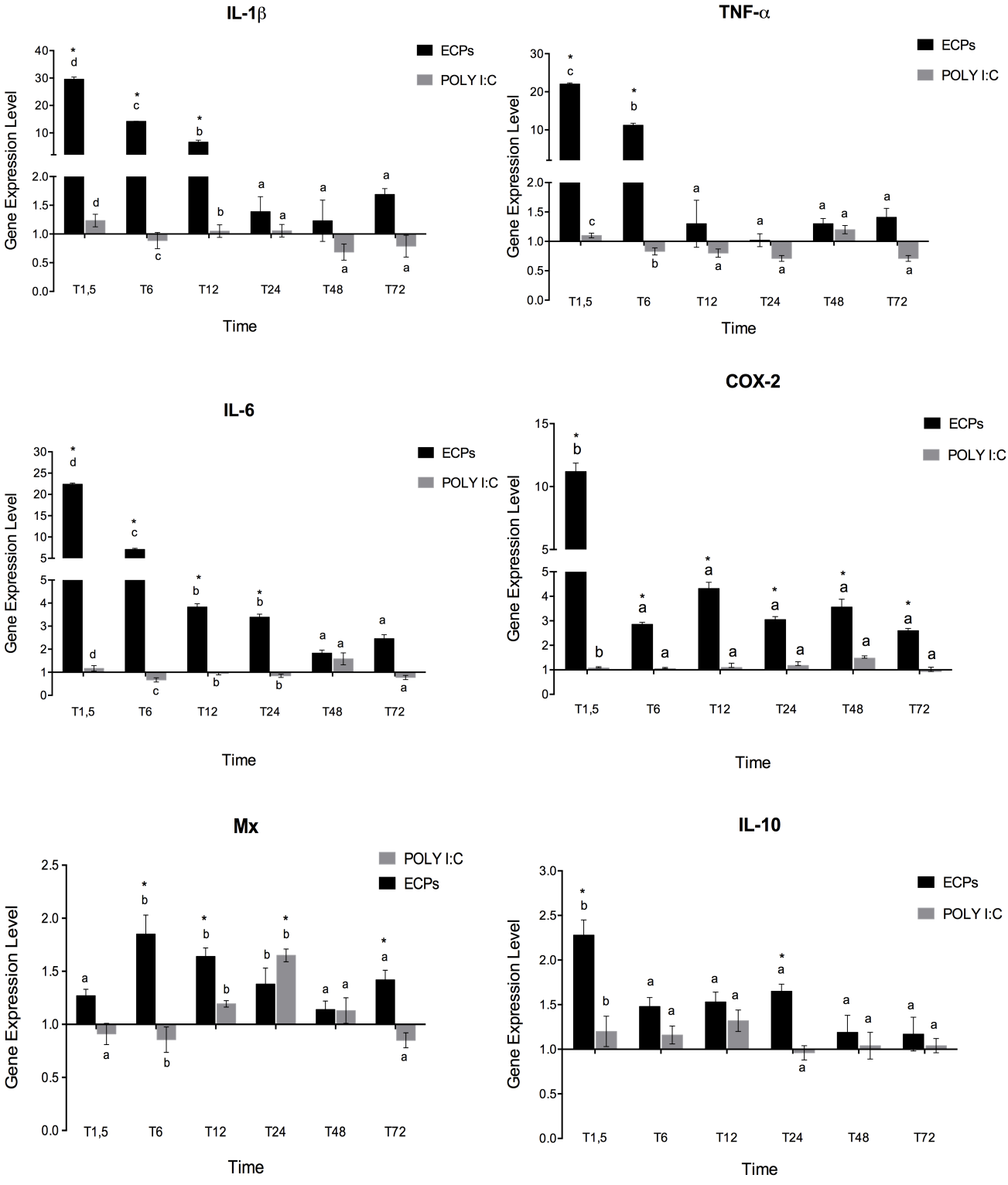

Fig. 1. Expression of immune-relevant genes determined by real-time PCR in head kidney leucocytes after incubation with ECPs of *Vagococcus fluvialis* L21 and POLY I:C compared to the control. Significant differences (P<0.05) between groups in each time is indicated by unlike letters on the bars (n=6). (a: time; (*): Products)

4. Discussion

Although the use of probiotic strains is an essential tool in aquaculture practice, sometimes there are certain risks caused by the use of alive probiotic strains since their administration may interfere with the ecosystems [5,17]. On the other hand, there is some concern in the scientific community about the possible development of virulence by some fish probiotic strains, due to the possible horizontal transfer of resistance plasmids [18]. Although so far there has been no notification, some bacterial genera used as probiotics in fish can be pathogenic for humans and for fish as for example, some strains belonging to the Genus *Aeromonas* [19] and *Vibrio* [20]. Species of the gender *Vagococcus* have been isolated from various sources such as sewage, faeces of domestic animals such as pigs, chickens, aquatic mammals such as seals, porpoises and otters, as well as from human clinical specimens [21,22]. In our case the strain of *V. fluvialis* L21 was isolated from flounder intestine and it is well documented its protective effect against an experimental infection with *Vibrio anguillarum* on sea bass with a relative survival rate of 42.3 % [23]. It is believed that some species of the genus Vagococcus as *Vagococcus salmoninarum* can cause sporadic fish deaths. Sorroza et al. [23] demonstrated the safety of *V. fluvialis* L21 and due to both its *in vitro* and *in vivo* characteristics demonstrated in our laboratory, it may be considered an appropriate probiotic strain to be used for marine aquaculture.

In an attempt to expand the current understanding of the mechanism of action of this strain, in this study we aimed to evaluate the *in vitro* immunomodulatory effect of the ECPs of the probiotic strain *V. fluvialis* L21 on the expression of genes

related to immune response. Cytokines such as IL-1β, IL-6, TNF-α, and the COX-2, generally produced in the first stage of the immune response, were examined [24-25]. We also include the study of IL-10 and Mx gene.

It is known that the cellular components can activate the immune system of many animals, including fish [26]. This is the case of lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, in small doses, can have immunomodulatory properties and induce the activation of T and B lymphocytes, stimulate complement activity and increase some cytokine expression such as IL-6, IL-1β and TNF-α [27-29], and also stimulate the production of Mx gene [30,31]. Furthermore, the outer membrane proteins (OMPs) of Gram-negative bacteria can stimulate the immune response of fish, both at humoral and cellular level. This was demonstrated by Boesen et al. [32] with the antigen of *V. anguillarum* serotype 01, which induced the immune response in rainbow trout (*Oncorhynchus mykiss*) or increased immune response after inoculation of *Photobacterium damselae* subsp. *piscicid*a antigens in sea bream [33].

It is demonstrated that certain bacterial ECPs exert an immunomodulatory effect on hosts [34, 35]. Although most of the work done to study the protective effect of both proteins in the cell wall, total protein or extracellular products of certain bacterial strains were performed *in vivo*, in this work we obtained *in vitro* a potent immunomodulatory effect of the ECPs of *V. fluvialis* L21 on sea bass head-kidney leucocytes. Even if we don't know the exact composition of the ECPs of *V. fluvialis* L21, we can assert that they have a higher capacity to stimulate the nonspecific immune response of leucocytes of sea bass. In general, we observed

that the ECPs of *V. fluvialis* L21 induce a strong pro-inflammatory response, at very high levels of expression, especially for IL-1β, TNF-α, IL-6 and COX-2 during the early hours post -incubation with ECPs, this dynamic expression was very similar among IL-1β, TNF-α and IL-6. These results differ somewhat to those obtained *in vivo* by Lund et al. [36] with the ECPs of *Aeromonas salmonicida* subsp. *salmonicida* in Atlantic salmon where no protection was obtained against furunculosis. However, our data are similar to those obtained by Evenberg et al. [34] in carp (*Cyprinus carpio*) vaccinated against *A. salmonicida* with ECPs, where protective effect was found.

In mammals the effects of regulatory cytokines exert on pro-inflammatory cytokines are well described [37]. Our results are similar to those found by Chettri et al. [38], in rainbow trout and Román et al. [12] in sea bass, where regulation between IL-6 and IL-10 were observed. All of these cytokines, except IL 10, are believed to contribute to the host defense mechanisms in response to the colonization and invasion. In fact, one of the main functions of IL-10 appears to be related to the regulation of the inflammatory response, to prevent this response is excessive [39]. In our case, the IL-10 showed higher levels of expression at 1.5 hours after incubation with the ECPs of *Vagococcus fluvialis* coinciding with the highest values of proinflammatory cytokines in the same time. Our data support the proposal by Raida and Buchmann [39] hypothesis.

Although it has been shown that LPS from different bacteria are capable of stimulating the Mx gene among other genes, the ECPs of *V. fluvialis* L21 stimulated Mx gene expression at 6 hours post-incubation in the head kidney leucocytes of sea

bass, producing a higher peak than the POLY I:C. Thus, we can consider that some component of the ECPs of *V. fluvialis* L21 is capable of activating the IFN system of sea bass leucocytes.

Furthermore, leucocytes that were stimulated with POLY I:C hardly expressed genes related to immune response. These results are in contrast with those obtained by Chettri et al. [38], who studied the response of leucocytes from rainbow trout that were stimulated with different compounds that refer to molecular pattern associated pathogens (PAMPs). In this study Chettri et al. [38] demonstrated that the rainbow trout respond differently to PAMPs, suggesting that these different routes activated cascade, which results in the activation of different immune effector cells. In our case, although this study was not performed in leucocytes of sea bass, we can consider that in our case the leucocytes of this species respond differently depending on the viability of the probiotic strain [12] with *V. fluvialis* L21 and the ECPs of *V. fluvialis* L21, reinforcing the hypothesis by Chettri et al. [38].

In numerous works the beneficial effects obtained after inoculation with different ECPs of Gram-positive and Gram-negative bacteria have been shown, so that many researchers emphasize to introduce these products as adjuvants for vaccines [40-42].

5. Conclusion

Overall, taking into account previous results obtained with strain *V. fluvialis* L21 in which it was demonstrated the protective effect of this strain in sea bass against experimental infection with *V. anguillarum* as well as stimulation of non-

specific cellular immune response and the effect on gene expression, we can consider that the ECPs of *V. fluvialis* L21 have a great power of stimulating *in vitro* the expression of immune-related genes and may even be useful as adjuvants for vaccine in aquaculture.

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Full length article

The *in vitro* immunomodulatory effect of extracellular products (ECPs) of *Vagococcus fluvialis* L21 on European sea bass (*Dicentrarchus labrax*) leucocytes

L. Román [a], F. Acosta [a], D. Padilla [a, *], F. El Aamri [a], J. Bravo [a], B. Vega [a], E. Rodriguez [b], J. Vega [a], S. Déniz [a], F. Real [a]

[a] Instituto Universitario de Sanidad Animal, Universidad de Las Palmas de Gran Canaria, Trasmontaña s/n, 35413 Arucas, Spain
[b] Facultad de Veterinaria, Universidad de Las Palmas de Gran Canaria, Trasmontaña s/n, 35413 Arucas, Spain

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ABSTRACT

The immune associated genes, interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin 10 (IL-10), tumor necrosis factor-α (TNF-α), ciclo-oxigenase-2 (COX-2), and Mx gene were studied by real-time PCR in head-kidney leucocytes of sea bass after incubation with the extracellular products (ECPs) of the probiotic strain *Vagococcus fluvialis* L21 and polyinosinic:polycytidylic acid (POLY I:C), at different times (T1.5, T6, T12, T24, T48 and T72). In general, we can observe how pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and COX-2 studied displayed a strong peak after stimulation with 1.5 h of ECPs of *V. fluvialis* L21, significant differences ($P < 0.05$) exist with other periods and with the POLY I: C at the same time. Similarly to the case of IL-10 also produced a statistically significant ($P < 0.05$) peak of expression on leukocytes that were stimulated with the ECPs of *V. fluvialis* L21. In the case of Mx gene expression, we note that in almost all sampling times there is an up-regulation of the Mx gene in leucocytes incubated with ECPs and POLY I:C compared to the control and Mx expression was higher in leucocytes that were stimulated with the ECPs of *V. fluvialis* for all times, except in T24. With these results we can consider that the ECPs of *V. fluvialis* L21 have a great power of stimulating the *in vitro* expression of immune-related genes and may even be useful as adjuvants for vaccine in aquaculture.

1. Introduction

Infectious diseases are one of the most limiting factors for the successful development of aquaculture industry. Among other control strategies, probiotics are considered an alternative tool for disease control, which consequently can prevent the use of antibiotics [1–4]. Today, not only alive bacterial strains are used as probiotics, but also inactivated ones, which have beneficial effects on the host, both *in vitro* and *in vivo* [5–7]. In addition, recent researches shows that purified components of the bacterial strains may have beneficial effect on the health of the hosts. Abbass et al. [8] showed that the cell wall proteins, outer membrane proteins (OMP) and lipopolysaccharides (LPS) of the probiotic strains *Aeromonas sobria* GC2 and *Bacillus subtilis* JB-1 conferred protection against *Yersinia ruckeri* in salmonids. Moreover, the probiotic supplementation has begun to be used as an adjuvant to increase the immunogenicity of vaccines in humans and animals [9,10].

In order to better understand the mechanism of action of the *Vagococcus fluvialis* L21 strain, identified and characterized as probiotic strain with protective effect against vibriosis in sea bass by *Vibrio anguillaum* by Sorroza et al. [11], and considering that the this strain has a clear immunomodulatory activity *in vitro*, as well as the ability to induce cytokine production related to the immune response [7,12], the aim of this study was to determine, *in vitro*, the effect of extracellular products (ECPs) of *V. fluvialis* L21 on the dynamics of expression of genes (IL-1β, IL-6, IL-10, TNF-α, COX-2, Mx) involved in the immune response in sea bass leucocytes.

2. Materials and methods

2.1. Fish

Thirty-six sea bass with an average weight of 150 g were provides by the Canary Institute of Marine Sciences (ICCM) to study the

* Corresponding author. Tel.: +34 928 45 97 40.
E-mail address: daniel.padilla@ulpgc.es (D. Padilla).

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D. FERNANDO REAL VALCÁRCEL CATEDRÁTICO DE UNIVERSIDAD Y COAUTOR DE LOS ARTÍCULOS:

-The *in vitro* immunomodulatory effect of extracellular products (ECPS) of *Vagococcus fluvialis* L21 on European sea bass (*dicentrarchus labrax*) leucocytes.

-Cytokine expression in head-kidney leuocyes of European sea bass (Dicentrarchus labrax L.) after incubation with the probiotic *Vagococcus fluvialis* L-21

DECLARA E INFORMA:

Que Dña. Lorena Román Fuentes , Licenciada en Veterinaria, ha realizado la parte fundamental del trabajo recogido en dichos artículos autorizando así su presentación en este trabajo y me comprometo a que no se utilicen en otras Tesis de Máster con el mismo fin.

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Fdo. Fernando Real Valcárcel

D. FÉLIX ACOSTA ARBELO PROFESOR DE UNIVERSIDAD Y COAUTOR DE LOS ARTÍCULOS:

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6.-CONCLUSIONES

PRIMERA.- Los ECPs de *Vagococcus fluvialis* L21 inducen una respuesta en los leucocitos de lubina de mayor intensidad y duración, que cuando se utiliza dicha cepa tanto viva como inactivada.

SEGUNDA.- Los ECPs de *Vagococcus fluvialis* L21 inducen una fuerte respuesta pro-inflamatoria en los leucocitos de lubina, presentando un índice de estimulación muy superior al POLY I:C, lo cual sugiere su utilización en la protección antivírica en esta especie.

TERCERA.- Se ha demostrado por primera vez que *Vagococcus fluvialis* L21 utilizada tanto viva como inactivada, han demostrado su efecto inmunomodulador *in vitro* en especies relevantes para la acuicultura marina.

7. ANEXOS

ANEXO I

Medios de cultivo y soluciones empleadas

Medios de cultivo:

- **Caldo infusión Cerebro Corazón (BHIB)**

  Composición (por litro):

Infusión de cerebro de ternera	7,5 g
Infusión de corazón de res	10 g
Dextrosa	2 g
Peptona de gelatina	10 g
Fosfato disódico (Na2HPO4)	2,5 g
Cloruro sódico (NaCl)	5 g

pH final: 7,4 ± 0,2 a 25ºC

Preparación:
Suspender 37 g en 1 l de agua destilada, calentar y agitar hasta disolución completa. Esterilizar a 121°C durante 15-20 minutos. Dejar enfriar hasta 45°C y conservar en la nevera.

- **Agar infusión Cerebro Corazón (BHIA)**

  Composición (por litro):

Infusión de cerebro de ternera	7,5 g
Infusión de corazón de res	10 g

Dextrosa	2 g
Peptona de gelatina	10 g
Fosfato disódico (Na2HPO4)	2,5 g
Cloruro sódico (NaCl)	5 g
Agar	15g

pH final: 7,4 ± 0,2 a 25ºC

Preparación:

Suspender 52 g en 1 l de agua destilada, calentar y agitar hasta disolución completa. Esterilizar a 121°C durante 15-20 minutos. Agitar el medio antes de servir. Dejar enfriar hasta 45°C y distribuir en placas de petri estériles con 20 ml en cada una.

Soluciones:

- **Tampón Fosfato Salino**

  Composición (por litro)

Dihidrogenofosfato potásico (KH2PO4) (1,4 mM)	0,24 g
Cloruro potásico (KCl) (2,7 mM)	0,2 g
Fosfato disódico (Na2HPO4) (0,01 M)	1,44 g
Cloruro sódico (NaCl) (0,137 M)	8 g

Preparación:

Mezclar todos los ingredientes en 1 l de agua destilada, agitar bien hasta que estén todos disueltos. Esterilizar a 121°C durante 30 minutos.

- **Agua DEPC**

  Composisicón (por 100ml)

Dietilpirocarbonato (C6H10O5)	0,1 ml

Preparación:
Mezclar el ingrediente en 100 ml de agua destilada, agitar toda la noche a temperatura ambiente y en oscuridad. Esterilizar a 121°C durante 30 minutos.

ANEXO II

Dirección de laboratorios y casas comerciales

Beckman	Beckman Coulter España. Costa Rica 72, 6G. Madrid
Biogen	Biogen, Científica. Castelar 15, 28028 Madrid
Biorad	Bio-rad Laboratories, S.A., Alcobendas, Madrid, España.
Chiesi	Chiesi España S.A. Torre Realis, Plaça D'Europa 41-43. Planta 10, 08908, Barcelona
Eppendorf	Eppendorf, Ibérica, S.L.U. Avda Tenerife, 2 Edificio 1, Planta 3 29703 San Sebastián de los Reyes,Madrid
Gibco	Gibco, Gaithersburg, MD, USA
Lonza	Lonza. Principe Vergara 55, 5ºB, 28006 Madrid
Millipore	Millipore Iberia S.A.U. Avd Burgos, 114, 28050 Madrid
Panreac Química, S.A.	Panreac Quimica S.A.U., Polígono Pla de la Bruguera, Castellar del Vallès, Barcelona, España.
Pronadisa	Laboratorios Conda, Torrejón de Ardoz, Madrid, España.

Sigma	Sigma-Aldrich Química, S.A., Tres Cantos, Madrid,
Skretting	Skretting España S.A., Cojóbar, Burgos, España.
Thermo	Thermo ScientificRoskilde, Dinamarca.
VWR	VWR International Eurolab S.L. C/ De la Tecnolocgía, 5-17 Llinars Park 08450 Llinars del Vallés, Barcelona