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MOLECULAR DIAGNOSTIC AND BIOLOGICAL METHODS: AN ALTERNATIVE IN THE PREVENTION AND CONTROL OF BACTERIAL FISH DISEASES

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AUTORIZACIÓN DEL DIRECTOR DE LA TESIS

MOLECULAR DIAGNOSTIC AND BIOLOGICAL METHODS: AN ALTERNATIVE IN THE PREVENTION AND CONTROL OF BACTERIAL FISH DISEASES

Dª Maria Ysabel Santos Rodríguez

INFORMA:

Que la presente tesis, se corresponde con el trabajo realizado por Dª. Clara Fernández Álvarez, bajo mi dirección, y autorizo su presentación, considerando que reúne los requisitos exigidos en el Reglamento de Estudios de Doctorado de la USC, y que como director de esta no incurre en las causas de abstención establecidas en la Ley 40/2015.

De acuerdo con el artículo 41 del Reglamento de Estudios de Doctorado, declara también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de COMPENDIO DE PUBLICACIONES, en los que la participación del doctorando/a fue decisiva para su elaboración.

La utilización de estos artículos en esta memoria, está en conocimiento de los coautores, tanto doctores como no doctores. Además, estos últimos tienen conocimiento de que ninguno de los trabajos aquí reunidos podrá ser presentado en ninguna otra tesis doctoral.

En Santiago de Compostela a 12 de Diciembre de 2018.

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MOLECULAR DIAGNOSTIC AND BIOLOGICAL METHODS: AN ALTERNATIVE IN THE PREVENTION AND CONTROL OF BACTERIAL FISH DISEASES

D./Dña. Clara Fernández Álvarez

Presento mi tesis, siguiendo el procedimiento adecuado al Reglamento, y declaro que:

1) *La tesis abarca los resultados de la elaboración de mi trabajo.*
2) *En su caso, en la tesis se hace referencia a las colaboraciones que tuvo este trabajo.*
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El Trabajo presentado en esta Tesis Doctoral ha sido parcialmente financiado por dos proyectos dentro del Programa “Acelerador de Transferencia” concedidos por la Universidade de Santiago de Compostela y el Banco Santander.

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Aspectos éticos

Todos los ensayos de experimentación con animales han sido realizados en el Animalario experimental de la Facultad de Biología de la Universidade de Santiago de Compostela (REGA ES150780263301), en el marco del proyecto de experimentación animal con autorización número 15004/16/004. En los estudios con animales de experimentación incluidos en esta tesis se han tenido en cuenta las consideraciones legales y técnicas recogidas en el R.D. 53/2013 del 1 de febrero, BOE nº 34, del 8 de febrero, en el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos. Los ensayos de experimentación animal han sido realizados por la Profesora Ysabel Santos Rodríguez, directora de la tesis.

Conflicto de interés

El doctorando no declara ningún conflicto de interés

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A mi madre

A mis abuelos Angustias e Isidro

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Aún me parece mentira que después de tanto tiempo esta etapa haya llegado a su fin. Y la verdad, es que no puedo estar más feliz por ello. Por eso, quisiera expresar mi agradecimiento a todas esas personas que han contribuido en hacer posible este trabajo.

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List of publications derived from this PhD work

The research developed during the realization of this PhD has originated various articles that have been published in international journals and two Spanish patents, which are presented below:

Articles

Fernández-Álvarez, C., Gijón, D., Álvarez, M., Santos, Y. (2016) First isolation of *Aeromonas salmonicida* subspecies *salmonicida* from diseased sea bass, *Dicentrarchus labrax* (L.), cultured in Spain. (2016). *Aquaculture Reports*, 4, 36-41.

Fernández-Álvarez, C., F. González, S., Santos, Y. (2016) Development of a SYBR Green I real-time PCR assay for specific identification of the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida*. *Applied Microbiology and Biotechnology*, 100(24), 10585-10595.

Fernández-Álvarez, C., Torres-Corral, Y., Santos, Y. (2017) MALDI-TOF mass spectrometry for rapid differentiation of *Tenacibaculum* species pathogenic for fish. (2017). *Applied Microbiology and Biotechnology* 101(13), 5377-5390.

Fernández-Álvarez, C., Torres-Corral, Y., Santos, Y. (2018) Use of ribosomal proteins as biomarkers for identification of *Flavobacterium psychrophilum* by MALDI-TOF mass spectrometry. *Journal of Proteomics*, 170, 59-69.

Fernández-Álvarez, C., Torres-Corral, Y., Santos, Y. (2018) Comparison of serological and molecular typing methods for epidemiological investigation of *Tenacibaculum* species pathogenic for fish. *Applied microbiology and biotechnology* 102 (6), 2779 - 2789.

Fernández-Álvarez, C., F. González, S., Santos, Y. (2019) Quantitative PCR coupled with melting curve analysis for rapid detection and quantification of *Tenacibaculum maritimum* in fish and environmental samples. *Aquaculture* 498, 289-296.

Fernández-Álvarez, C., Santos, Y. (2018) First isolation and characterization of *Lacinutrix venerupis* isolated from *Trachurus trachurus*, an emerging pathogen for wild and cultured fish. *Research in Veterinary Science*. Sent for publication.

Fernández-Álvarez, C., Santos, Y. (2018) Isolation and characterization of *Flavobacterium psychrophilum*-like bacteria from diseased rainbow trout and eggs (*Oncorhynchus mykiss*) in Spain. Diseases of Aquatic Organisms. Sent for publication.

Fernández-Álvarez, C., Santos, Y. (2018) Phenotypic and molecular identification of antimicrobial resistance in *Aeromonas salmonicida* subsp. *salmonicida* isolated in Spain. Aquaculture. Sent for publication.

Fernández-Álvarez C., A. R Sánchez-Arévalo, A. Martínez, Ysabel Santos. (2018) Dietary administration of a commercial nutraceutical: antimicrobial activity and effect in the resistance against *Flavobacterium psychrophilum* infections on rainbow trout. *Aquaculture*. In review.

Fernández-Álvarez C., A. R Sánchez-Arévalo, A. Martínez, Ysabel Santos. (2018) Screening of the antibacterial activity of chemical and plant-based products against *Aeromonas salmonicida* subsp. *salmonicida* and *Vibrio anguillarum. Journal of Fish Diseases*. Sent for publication.

Reviews

Fernández-Álvarez, C., Santos, Y. (2018) Identification and typing of fish pathogenic species of the genus *Tenacibaculum*. *Applied Microbiology and Biotechnology* 102 (23), 9973-9989.

Contents

Resumen

El crecimiento demográfico y la constante demanda de productos de mayor calidad por parte del consumidor favorecen el crecimiento del sector acuícola, situándolo como una de las industrias de mayor crecimiento económico a nivel mundial. Al igual que ocurre en otros sectores de producción animal, uno de los principales problemas de la industria de la acuicultura son las enfermedades infecciosas, que limitan el desarrollo socioeconómico en muchos países de todo el mundo. Estas pérdidas están relacionadas con la mortalidad de los animales, los costes de los tratamientos, el lento crecimiento de peces enfermos y las deformidades que acumulan los animales supervivientes que no los hacen aptos para su comercialización.

Las enfermedades infecciosas que afectan a los cultivos de peces pueden ser producidas por patógenos virales, parasitarios, fúngicos o bacterianos. Entre estas, las enfermedades bacterianas producidas por patógenos Gram-negativos son las más frecuentes. En concreto, la forunculosis típica (producida por *Aeromonas salmonicida* subsp. *salmonicida*), la tenacibaculosis (producida por *Tenacibaculum* spp.), la enfermedad bacteriana del agua fría (producida por *Flavobacterium psychrophilum*) y la vibriosis clásica (producida por *Vibrio anguillarum*) son algunas de las enfermedades que más impacto tienen en la acuicultura marina y continental de todo el mundo. Por ello, la presente investigación doctoral se centra en el estudio de diferentes aspectos relacionados con el diagnóstico, epidemiología, prevención y control de estas patologías.

La determinación de la causa de una patología clínica es necesaria para el establecimiento de un tratamiento eficaz. Tradicionalmente, el diagnóstico de enfermedades de peces se ha basado en la observación de los signos clínicos, el

aislamiento del patógeno a partir de los tejidos de los peces y su identificación mediante métodos fenotípicos o serológicos. Sin embargo, estos métodos son engorrosos y lentos y no siempre permiten la diferenciación entre bacterias filogenéticamente relacionadas. Por lo tanto, es evidente la necesidad de desarrollar métodos de diagnóstico rápidos, específicos y sensibles que permitan la detección e identificación de microorganismos a partir de tejidos de peces enfermos y portadores, así como de agua de los sistemas de cultivo. Estas herramientas facilitarán la aplicación de medidas adecuadas para la prevención y control de las enfermedades.

El estudio de la diversidad proteómica, genética y serológica intra- o inter-específica de patógenos de peces es importante para el diseño de estrategias de prevención (como el desarrollo de vacunas) y control así como para el estudio epidemiológico de las infecciones bacterianas.

Durante las últimas décadas se han utilizado de forma indiscriminada los antimicrobianos no solo como medida de tratamiento contra enfermedades bacterianas (uso terapéutico), sino también como una forma de prevención (uso profiláctico). Esto tiene repercusiones negativas sobre el medio ambiente y la salud humana; como la acumulación de residuos en el ambiente y en los tejidos de peces, la aparición y propagación de bacterias resistentes a los antimicrobianos y la transferencia de genes de resistencia a patógenos humanos y veterinarios. Esta problemática ha impulsado el diseño y desarrollo de nuevas estrategias de prevención y control de enfermedades que eviten el uso de antimicrobianos, como son el uso de inmunoestimulantes, probióticos, aceites vegetales esenciales, extractos de plantas o algas.

Por todo lo expuesto anteriormente, la presente tesis doctoral se ha centrado en resolver algunos de los principales problemas relacionados con las enfermedades producidas por patógenos bacterianos de los géneros

Tenacibaculum, Aeromonas, Flavobacterium. y *Vibrio.* Los objetivos específicos establecidos para la presente tesis fueron:

1. Determinación de la posición taxonómica y estudio de la virulencia de bacterias Gram-negativas que causan enfermedades en especies de peces cultivadas (Capítulo III).
2. Desarrollo de métodos moleculares altamente sensibles para la detección e identificación de patógenos de peces y su diferenciación de especies genéticamente relacionadas (Capítulo IV).
3. Evaluación de métodos proteómicos, serológicos y moleculares para la tipificación de bacterias patógenas de peces y la realización de estudios epidemiológicos (Capítulo V).
4. Caracterización fenotípica y molecular de la resistencia a antimicrobianos comúnmente utilizados en acuicultura en bacterias patógenas de peces (Capítulo VI).
5. Evaluación de la actividad antimicrobiana de compuestos naturales y químicos y su efecto sobre la resistencia de los peces frente a enfermedades bacterianas de gran impacto en el sector de la acuicultura (Capítulo VII).

En el **Capítulo III**, se determinó la posición taxonómica y el potencial de virulencia de diferentes microorganismos Gram-negativos aislados a partir de peces enfermos cultivados de agua marina (lubina y jurel) y dulce (trucha); y a partir de huevas de trucha. Los aislados se caracterizaron utilizando métodos fenotípicos (test bioquímicos, sistemas API, análisis de los ácidos grasos), serológicos (ensayo Dot-Blot), genotípicos (ERIC y REP-PCR) y proteómicos (espectrometría de masas MALDI-TOF) y/o la secuenciación del gen rRNA 16S. Además, la potencial virulencia de las bacterias se evaluó mediante infección experimental de los peces.

Los microorganismos aislados a partir de lubina (*Dicentrarchus labrax*) (**artículo nº1**) fueron muy homogéneos a nivel fenotípico, serológico, molecular y proteómico y fueron muy similares a la cepa de referencia de *Aeromonas salmonicida* subesp. *salmonicida* utilizada con fines comparativos. Los aislados fueron susceptibles a todos los antimicrobianos testados excepto a oxitetraciclina. Además, las infecciones experimentales demostraron que las cepas no tenían especificidad de hospedador ya que fueron virulentas para rodaballo y trucha. Por lo tanto, el **artículo nº1** representó el primer aislamiento del patógeno *A. salmonicida* subesp. *salmonicida* a partir de lubina cultivada en España.

Por otro lado, las cepas aisladas a partir de jurel (*Trachurus trachurus*) (**artículo nº2)**, fueron identificadas como *Lacinutrix* spp. en base a los caracteres bioquímicos y al perfil de ácidos grasos. La posterior secuenciación parcial del gen 16S rRNA confirmó estos resultados, y permitió identificar las cepas como *Lacinutrix venerupis*. La secuencia presentó una mayor similitud con la secuencia del gen 16S rRNA de la cepa tipo de *L. venerupis* CECT 8573^T (99.1%), mientras que otras especies mostraron similitudes de 98% (*L. jangbogonensis*) y 97% (*L. algicola* o *L. mariniflava*). La caracterización molecular de los aislados mediante REP- y ERIC-PCR y la caracterización proteómica por MALDI-TOF-MS determinaron la existencia de heterogeneidad entre las cepas aisladas de jurel y la cepa tipo CECT 8573^T. Las cepas de *L. venerupis* no fueron virulentas para rodaballo (*Scophthalmus maximus*), lubina (*D. labrax*) o lenguado senegalés (*Solea senegalensis*) utilizando altas dosis bacterianas ($1x10^9$ UFC / ml). Por lo tanto, se necesitan más estudios para determinar la importancia de esta especie como patógeno para peces.

Por otro lado, las cepas similares a *Flavobacterium psychrophilum* aisladas a partir de truchas enfermas (*Oncorhynchus mykiss*) y huevas (**artículo nº3**) no pudieron ser identificadas utilizando medios fenotípicos de caracterización

(métodos bioquímicos y sistemas API). Las cepas mostraron características fenotípicas similares entre ellas, pero fueron completamente diferentes a las mostradas por *F. psychrophilum*, *Flavobacterium branchiophilum* o *Flavobacterium flevense*. Las técnicas ERIC y REP fueron efectivas para tipificar y diferenciar todos los aislados de *Flavobacterium* incluidos en el estudio. Sólo dos cepas de un total de nueve cepas analizadas pudieron ser identificadas de forma presuntiva como *Flavobacterium tructae* y *Flavobacterium collinsi* utilizando las técnicas ERIC y REP-PCR y la espectrometría de masas MALDI-TOF. Los ensayos de virulencia sugirieron que algunos de estos aislamientos podrían considerarse como una amenaza potencial para el cultivo de trucha arco iris. Sin embargo, se necesitan estudios más amplios basados en la secuenciación del gen 16S rRNA y el análisis de los ácidos grasos que permitan determinar la posición taxonómica estas bacterias y determinar el riesgo que suponen para el cultivo de trucha.

En el **Capítulo IV**, se desarrollaron técnicas de diagnóstico basadas en PCR específicas para la identificación y/o detección de los patógenos *A. salmonicida* subesp. *salmonicida* y *Tenacibaculum maritimum* en los tejidos de peces enfermos y/o en agua.

Para *A. salmonicida* subesp. *salmonicida*, (**artículo nº 4**) se diseñó un protocolo basado en PCR en tiempo real empleando el fluoróforo SYBR Green I para la detección del producto de amplificación y el análisis de la temperatura de desnaturalización (Tm) para determinar la especificidad de la reacción. Para ello, se diseñaron un par de cebadores que amplifican un fragmento de la secuencia del gen *aopO* de *A. salmonicida* subesp. *salmonicida*, que codifica una proteína vinculada a la virulencia. Los ensayos de especificidad demostraron que el 100% de las cepas de *A. salmonicida* subesp. *salmonicida* testadas eran correctamente identificadas, generándose un producto de amplificación con una Tm de 80,75 ± 0,35ºC. El nivel de especificidad de la

técnica fue del 97,3%. El protocolo mostró una elevada sensibilidad utilizando muestras de cultivos bacterianos puros (1-2 células bacterianas por reacción) y de tejidos de peces infectados (6 a 60 células bacterianas por reacción de PCR). El ensayo fue altamente reproducible y permitió la detección específica de *A. salmonicida* subesp. *salmonicida* en tejidos de peces infectados de forma natural. Además, no se detectaron amplificaciones inespecíficas cuando se analizaron tejidos de peces sanos o de peces infectados con otras enfermedades.

Para la identificación específica de *T. maritimum* (**artículo nº 5)** se desarrolló un sistema de PCR basado en un par de cebadores que amplifican un fragmento de 164 pb de la secuencia del gen 16S rRNA. El sistema mostró unos niveles de especificidad del 100% para *T. maritimum*, ya que no se observó amplificación cuando se testaron otras especies de *Tenacibaculum* así como otros patógenos de peces no relacionados. La temperatura de fusión del producto de amplificación fue de 80,25 ± 0,35ºC. La sensibilidad de detección del protocolo fue de 4 x 10^{-10} ng/µl rRNA equivalente a 2,22 copias del gen. El ensayo permitió detectar y cuantificar el patógeno *T. maritimum* en diferentes tipos de muestras generadas en el laboratorio, incluyendo cultivos bacterianos puros y mixtos, muestras de riñón, sangre y moco de peces y agua de mar infectados experimentalmente con *T. maritimum* y en muestras de tejidos de rodaballo, lenguado y lubina afectados por tenacibaculosis. Este ensayo de PCR en tiempo real demostró ser una herramienta de diagnóstico rápida, sensible y fiable que puede ser utilizada en estudios epidemiológicos, para monitorizar la presencia de *T. maritimum* en sistemas de cultivo de peces, y evaluar la calidad de las aguas que entran y salen de las instalaciones acuícolas. Esto ayudará en la prevención de la aparición de brotes de tenacibaculosis.

En el **Capítulo V** **(artículos nº 6, 7, 8)** el objetivo fue la evaluación de técnicas proteómicas (espectrometría de masas MALDI-TOF), serológicas (aglutinación en portaobjetos y Dot-Blot) y/o moleculares (ERIC y REP-PCR)

para el tipado de patógenos bacterianos del género *Tenacibaculum* y *Flavobacterium* y para estudios epidemiológicos.

En el **artículo nº6** se evaluó un nuevo enfoque proteómico basado en la espectrometría de masas MALDI-TOF para la identificación y diferenciación de especies patógenas de peces del género *Tenacibaculum* (*T. maritimum, Tenacibaculum soleae, Tenacibaculum discolor, Tenacibaculum gallaicum, Tenacibaculum dicentrachi* and *Tenacibaculum ovolyticum*). Esta técnica permitió identificar ocho picos específicos de género en todas las cepas de *Tenacibaculum* analizadas. Por otro lado, se encontró al menos un pico específico de especie en *T. maritimum, T. soleae, T. dicentrarchi, T. litoreum* y *T. ovolyticum*. El dendrograma construido y en análisis de los componentes principales (PCA) permitió separar claramente las cepas de las especies *T. maritimum, T. soleae, T. dicentrarchi* y *T. ovolyticum* en diferentes grupos. Sin embargo, las especies de *T. discolor* y *T. gallaicum* fueron difíciles de distinguir basándose en sus perfiles proteómicos. En conclusión, la técnica MALDI-TOF permitió identificar las especies de *Tenacibaculum* analizadas mediante la detección de biomarcadores proteómicos específicos y los análisis de agrupamiento y de componentes principales. De forma similar, la metodología MALDI-TOF también permitió diferenciar la especie bacteriana *F. psychrophilum* de otras especies de *Flavobacterium* estrechamente relacionadas (*F. flevense, Flavobacterium succinicans, Flavobacterium columnare, F. branchiophilum y Flavobacterium johnsoniae*) mediante la definición de catorce biomarcadores específicos y mediante el análisis de agrupamiento **(artículo nº 8)**. Estos resultados demostraron que la espectrometría de masas MALDI-TOF representa una herramienta de utilidad para la identificación precisa del patógeno *F. psychrophilum* y de las especias patógenas para peces del género *Tenacibaculum* que podría integrarse como un método de diagnóstico rutinario en laboratorios de microbiología clínica.

Además, en el **artículo nº7** y **nº8** se evaluó el potencial de la espectrometría de masas MALDI-TOF para la identificación de biomarcadores específicos de los principales serotipos de las especies *T. marimum, T. soleae* y *T. discolor* y *F. psychrophilum*, así como biomarcadores asociados a las especies de peces o áreas geográficas de aislamiento En el **artículo nº7**, se identificaron picos específicos para los serotipos O1 y O2 del patógeno *T. soleae* y del serotipo O1 de *T. discolor*. Sin embargo, no se pudo identificar ningún pico específico de serotipo para la especie *T. maritimum*, ni ningún biomarcador asociado a la especie de pez hospedadora o área geográfica de aislamiento. El análisis de cepas de la especie *F. psychrophilum* permitió seleccionar conjuntos de biomarcadores específicos de los serotipos O1, O2a, O2b y O3. Además, se encontraron picos asociados a las especies de aislamiento (salmón o trucha). Estos resultados, indican que la técnica MALDI-TOF podría ser de utilidad tanto para estudios epidemiológicos como para la identificación de serotipos. Sin embargo, para confirmar estos resultados se necesita realizar más estudios incluyendo un mayor número de cepas aisladas de diferentes especies de peces de diferentes partes del mundo.

Asimismo, en estos estudios (**artículo nº7**) se evaluó el potencial de métodos serológicos (aglutinación y Dot-Blot) y moleculares (REP-PCR y ERIC-PCR) para la tipificación de las especies *T. maritimum, T. soleae* y *T. discolor*. Los ensayos de aglutinación en portaobjetos y Dot-Blot demostraron la falta de relación inmunológica entre las diferentes especies de *Tenacibaculum* analizadas. Además, se confirmó la existencia de dos serotipos dentro de la especie T. *soleae* y al menos uno dentro de la especie *T. discolor*. Las técnicas moleculares REP-PCR y ERIC-PCR demostraron su potencial como herramientas de diagnóstico, ya que las especies *T. maritimum, T. soleae* y *T. discolor* mostraron perfiles específicos de especie. El análisis genotípico mostró variabilidad genética dentro de cada una de las especies de *Tenacibaculum*

analizadas. Sin embargo, esa variabilidad no se pudo relacionar con el serotipo, fuente de aislamiento o zona geográfica de las cepas analizadas en este estudio.

En el **Capítulo VI** (**artículo nº 9)** se realizó un estudio sobre la existencia de resistencia a antimicrobianos en cepas de *A. salmonicida* aisladas en España. Los resultados demostraron que el 66,6% de las cepas analizadas fueron resistentes a sulfamidas potenciadas; el 55,5% mostraron resistencia a oxitetraciclina; mientras que el 33,3% de las cepas fue resistente a cloranfenicol. La resistencia a florfenicol (7,4% de las cepas), enrofloxacina (3,7%) fue menos frecuente, mientras que ninguna de las cepas presentó resistencia a la flumequina. Además, el 66,6% de las cepas analizadas mostraron resistencia a múltiples antimicrobianos. La detección mediante PCR de genes de resistencia a antimicrobianos en las cepas de *A. salmonicida*, mostró que el gen *sul1* (que confiere resistencia frente a trimetoprim/sulfametoxazol) fue el más frecuente (66,6% de las cepas), seguido del gen *tet (A)* (que confiere resistencia a la oxitetraciclina) (55,5% de las cepas) y el gen *cat* (que confiere resistencia al cloranfenicol) (33,3% de las cepas). Por último, los genes *tet (E)* y *flor* solo estuvieron presentes en el 7,4% de las cepas. Este estudio proporciona una visión sobre la presencia de resistencia a antimicrobianos en cepas de *A. salmonicida* subsp. *salmonicida* aisladas en España. Los resultados obtenidos sugieren que este patógeno es un reservorio importante de genes de resistencia a antimicrobianos y debe ser monitoreado más frecuentemente.

En el **Capítulo VII** (**artículos nº10 y 11**) se evaluó la actividad antimicrobiana de nuevos compuestos naturales y químicos frente a los patógenos de peces *A. salmonicida*, *Vibrio anguillarum* y *F. psychrophilum*. En el **artículo nº10** se evaluó la actividad antibacteriana de dos productos disponibles comercialmente (Bronopol y Liptosa P203) frente a los patógenos de peces *A. salmonicida* subesp. *salmonicida* (*n*= 20 cepas) y *V. anguillarum*

(*n*=30 cepas). La concentración mínima inhibitoria (CMI) del nutraceútico Liptosa-P203; que se basa en ácidos grasos vegetales y aceites esenciales; fue de 292,96-585,92 ppm para *V. anguillarum* y *A. salmonicida* utilizando los sistemas de dilución en agar y en caldo. El agente químico Bronopol (agente bactericida) evaluado con fines comparativos mostró valores de CMI de 4,57 y 36,62 ppm para *V. anguillarum* y *A. salmonicida*. El producto Liptosa-P203 fue capaz de eliminar más del 99,99% de las células de *V. anguillarum* y *A. salmonicida* en sólo una hora a concentraciones 4x CMI (1171,84 ppm). El bronopol eliminó más del 99,99 % de las células de *V. anguillarum* en 2 horas y de *A. salmonicida* en 1 hora utilizando concentraciones de 4x CMI.

Finalmente, en el **artículo nº11** el nutracéutico comercial Liptofry, fabricado también por Lipidos Toledo SA (Liptosa) mostró un mayor efecto inhibitorio frente a cepas de *F. psychrophilum* (CMI_{90} de 64 µg/ml); mientras que las cepas de *V. anguillarum* y *A. salmonicida* subesp. *salmonicida* fueron menos susceptibles (CMI_{90} de 1250 µg/ml y 3000 µg/ml, respectivamente). Para evaluar el efecto de las dietas suplementadas con Liptofry sobre la resistencia a la enfermedad bacteriana del agua fría, se alimentaron alevines de trucha arcoiris con dietas que contenían 0% (dieta de control) y 0,5% (dieta experimental) del nutracéutico durante 115 días y se infectaron experimentalmente con una cepa virulenta de *F. psychrophilum*. La mortalidad acumulada de los peces alimentados con la dieta suplementada con nutracéuticos fue significativamente menor ($p < 0,05$) que la de los peces alimentados con dietas control. Además, la administración de dietas suplementadas con Liptofry produjeron una sobreexpresión de los genes que codifican el factor de necrosis tumoral-α (TNF-α), el complemento (C5a) y el receptor del complemento (rC5a) en el bazo y la piel; mientras que el gen de inmunoglobulina M (IgM) sólo se sobre-expresó en el intestino. Nuestros resultados indicaron que el uso de dietas suplementadas

con el nutracéutico Liptofry confiere protección frente a enfermedades bacterianas producidas por *F. psychrophilum*.

La enfermedad es el resultado de una perturbación en el equilibrio entre el huésped, el patógeno y el medio ambiente. Por lo tanto, las estrategias para prevenir y controlar las enfermedades bacterianas en las piscifactorías deben considerar todos los aspectos implicados en la relación patógeno-huésped-ambiente para garantizar la protección de los peces a largo plazo. En este trabajo de doctorado se han estudiado diferentes aspectos del diagnóstico, la epidemiología y / o el control de las enfermedades producidas por los patógenos bacterianos *A. salmonicida*, *Tenacibaculum* sp., *Flavobacterium* sp. y *V. anguillarum*. Los resultados obtenidos mostraron que los métodos serológicos, moleculares y proteómicos son útiles para la identificación y tipificación de los patógenos de peces *A. salmonicida* subesp. *salmonicida*, *Tenacibaculum* sp. y *F. psychrophilum*. Además, las técnicas serológicas y proteómicas son herramientas útiles de tipificación de cepas y para estudios epidemiológicos. Las técnicas basadas en PCR en tiempo real desarrolladas en este trabajo permitieron la identificación de *A. salmonicida* y *T. maritimum* y su detección en tejidos de peces infectados y en agua de tanques de cultivo de peces. Todas estas metodologías permitirán monitorizar la presencia de estos patógenos bacterianos en los sistemas de producción de peces y evaluar la calidad de las aguas que entran y salen de las instalaciones acuícolas. Por último, aunque se encontró un notable número de cepas *A. salmonicida* subesp. *salmonicida* resistentes a alguno de los fármacos autorizados para uso en acuicultura, los nuevos antimicrobianos de origen vegetal evaluados en el presente estudio podrían representar una herramienta poderosa para la prevención y el control de la furunculosis y de otras enfermedades bacterianas.

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Abstract

Aquaculture is the fastest growing food-production industry because of the increasing demand for food fish consumption. However, as other food production sector, aquaculture is limited by infectious diseases. Economic impact is related with the high mortality rates that produce, the costs of treatments or the decreased growth rates of diseased and convalescent fish. Some of the most threatening bacterial diseases caused by Gram-stain-negative pathogens occurring in fish cultured in marine and continental farming worldwide include furunculosis, tenacibaculosis, flavobacteriosis and vibriosis. To reduce the impact of the fish diseases, it is necessary to apply prevention and control strategies. The development of rapid and sensitive diagnostic protocols for the detection of microorganism in diseased and carrier fish and farm waters is crucial for the establishment of effective control measures that reduce or eliminate the impact of the infectious diseases. On the other hand, typing of microbial pathogens, or identifying bacteria at the strain level is important for the establishment of effective prevention and control strategies, for the study of population dynamics and for epidemiological surveillance of bacterial infections.

To control bacterial fish diseases, feeding infected fish with formulated feed mixed with antibiotics is a general practice. The extensive use of antimicrobial compounds has led to the emergence and spread of antimicrobial-resistant bacteria in the environments and the transfer of resistance to human and veterinary pathogens, the accumulation of tissue residues and their implications for human health. For all these reasons, the use of antibiotics in aquaculture is no more a primary treatment option and new eco-friendly alternatives are being developed. These alternative measures include the use of improved husbandry/management practices, water disinfection biological control, vaccination, dietary supplements,

non-specific immunostimulants, probiotics, plant-based compounds (essential oils, plant extracts, algae and their metabolites) and new antimicrobial compounds.

This PhD work has focused on resolving the current concerns of bacterial fish diseases produced by *Tenacibaculum* sp., *Aeromonas* sp., *Flavobacterium* sp. and *Vibrio* sp. The general aims of the present work were:

1. The determination of the taxonomic position and study of the virulence of Gram-stain-negative bacteria causing diseases in different marine and freshwater farmed fish species (Chapter III).
2. Development of highly sensitive molecular methods for the detection and identification of fish pathogens and its differentiation from genetically closely related species (Chapter IV).
3. Evaluation of proteomic, serological and molecular methods for the typing of fish pathogenic bacteria and for epidemiological studies (Chapter V).
4. Phenotypic and molecular characterization of the resistance to antimicrobials commonly used in aquaculture of fish pathogenic bacteria (Chapter VI)
5. Evaluation of the antimicrobial activity of natural and chemical compounds and their effect on the resistance of fish against bacterial diseases of great impact in the aquaculture sector (Chapter VII).

In **Chapter III,** the taxonomic position and the potential virulence of Gram-stain-negative pathogens isolated from marine (sea bass and mackerel) and freshwater (rainbow trout) farmed fish with clinical signs of disease was evaluated. The strains were characterized using different phenotypic (biochemical tests, API systems, chemotaxonomic analysis), serological (Dot-Blot assay), genotypic (ERIC and REP-PCR) and proteomic (MALDI-TOF-MS) methods and/or sequencing of the 16S rRNA gene. The potential virulence of the microorganisms was also evaluated by experimental infection of fish. The microorganisms isolated from sea bass (**article nº1**) were very homogeneous at phenotypical, serological, molecular

and proteomic levels and were very similar to the reference strains of *Aeromonas salmonicida* subsp. *salmonicida* used for comparative purposes. The strains showed lack of host specificity since they were also virulent for turbot and trout. Overall, **article nº1** represents the first isolation of the fish pathogen *A. salmonicida* subsp. *salmonicida* from farmed sea bass in Spain. On the other hand, strains isolated from *Trachurus trachurus* (**article nº2)** were identified as *Lacinutrix* spp. after biochemical and fatty acids analysis, while the partial sequencing of the 16S rRNA gene allowed to identify the strains as *Lacinutrix venerupis* (similarity levels of 99.1% with the type strains *L. venerupis* CECT 8573^{T}). However, the strains were not virulent for turbot, Senegalese sole or sea bass. Thus, further studies will be necessary to determine the importance of this species as fish pathogens. The *Flavobacterium psychrophilum*-like strains isolated from rainbow trout and trout eggs (**article nº3**) could not be identified using phenotypic characterization. Only two strains could be presumptively identified using the ERIC, REP and MALDI-TOF profiles as *Flavobacterium tructae* and *Flavobacterium collinsi*. Virulence studies suggested that some of these isolates could be considered as a potential threaten for rainbow trout farming. However, further studies basing on the sequencing of the 16S rRNA gene and the analysis of the fatty acids will allow classifying these bacteria and determining the risks for trout culture.

In **Chapter IV**, molecular diagnostic techniques based in PCR were designed for the specific detection of *Tenacibaculum maritimum* and *A. salmonicida* subsp. *salmonicida* in fish tissues and/or water. For *A. salmonicida* subsp. *salmonicida* (**article nº 4**), specific primers were designed on the basis of the sequence of the *AopO* gene, which is located in the low-copy-number *pAsa5* plasmid, which encodes for a serine/threonine protein kinase of the type III secretion system linked to virulence. This protocol showed specificity levels of 97.3 % for *A. salmonicida* subsp. *salmonicida*. The assay showed a high sensitivity when using pure cultures (1-2 cells per PCR reaction) and tissue samples of infected fish (6-60 cells per PCR reaction). For *T. maritimum* (**article nº 5)** a pair of primers that amplify a region of

the 16S rRNA gene were designed. The protocol showed 100% specificity for *T. maritimum*, since the other bacteria tested did not show any amplification. The assay allowed the specific detection and quantification of the bacterium in lethal and non-lethal fish samples and seawater samples. Thus, this protocol could be used for epidemiological studies, monitoring the presence of *T. maritimum* in fish production systems, and to evaluate the quality of waters entering and leaving the aquaculture facilities, which may help to prevent the occurrence of tenacibaculosis outbreaks.

In **Chapter V** proteomic (MALDI-TOF-MS), serological (slide agglutination and Dot-Blot) and/or molecular (ERIC and REP-PCR) methods were evaluated for the typing and epidemiological study of the fish pathogens *Tenacibaculum* spp. and *F. psychrophilum*. In **article nº 6**, MALDI-TOF analysis of *Tenacibaculum* species (*T. maritimum*, *Tenacibaculum soleae*, *Tenacibaculum discolor*, *Tenacibaculum gallaicum, Tenacibaculum dicentrachi* and *Tenacibaculum ovolyticum*) allowed to differentiate all the species analysed through the detection of species-specific biomarkers, cluster and principal components analysis (PCA). Moreover, in **article nº 7** MALDI-TOF-MS allowed to identify serotype-specific mass peaks for the serotypes O1 and O2 of *T. soleae* and the serotype O1 of *T. discolor* but did not allow to identify biomarkers associated to the host species or geographical region of isolation. Serological analysis (**article nº 7)** of the *Tenacibaculum* (*T. maritimum*, *T. soleae* and *T. discolor*) strains demonstrated the lack of immunological relationship among the species analysed. Genotyping study (**article nº 7**) of the species *T. maritimum*, *T. soleae* and *T. discolor* by ERIC and REP-PCR revealed specific molecular profiles for each bacterial species. Genomic variability was found within each species analysed, however, these variations could not be related with the serotype, source or geographical area of the strains analysed. On the other hand, in **article nº 8** MALDI-TOF analysis allowed to differentiate *F. psychrophilum* strains from other species (*Flavobacterium flevense*, *Flavobacterium succinicans*, *Flavobacterium columnare*, *Flavobacterium*

branchiophilum and *Flavobacterium johnsoniae*) by defining species identifying biomarkers and by hierarchical cluster analysis. MALDI-TOF analysis also allowed to detect four mass peaks patterns associated with the main pathogenic *F. psychrophilum* serotypes: serotype O1, serotype O2a; serotype O2b and serotype O3. A positive relationships was detected between the proteomic profiles and the source of isolation of the *F. psychrophilum* strains. All these results suggest that protein biomarkers detected by MALDI-TOF could be used for identification of bacterial fish pathogens belonging to *Tenacibaculum* spp. and *Flavobacterium* spp. and for the detection of different serotypes. Further studies are necessary, including a higher number of strains belonging to different serotypes and isolated from different parts of the world from different fish species.

In the **Chapter VI (article nº 9)** an assessment of the occurrence of resistance to antimicrobials in *A. salmonicida* isolated in Spain is presented. Resistance to trimethoprim/ sulfamethoxazole (66.6% of the strains), oxytetracycline (55.5% of the strains) and chloramphenicol (33.3% of the strains) was determined according to either disk diffusion and/or broth dilution assays. Resistance to florfenicol (7.4% of the strains) and enrofloxacin (3.7% of the strains) was less common, while resistance to flumequine was not present in the strains analysed. Molecular identification by PCR of antimicrobial resistance showed that the *sul1* gene; which encodes resistance to sulphonamides; was the most prevalent (66.6% of the isolates), followed by the *tet(A)* (55.5% of the strains) and *cat* (33.3% of the strains) genes; that encode for resistance to oxytetracycline and chloramphenicol, respectively. Lastly, the *tet(E)* and *flor* genes were only present in the 7.4% of the strains. This study provides an update on the antimicrobial resistance on *A. salmonicida* subsp. *salmonicida* isolated in Spain. All the results obtained suggest that this fish pathogen is an important reservoir of drug resistance genes and should be monitored more extensively.

In **Chapter VII** the antimicrobial activity of different commercial natural or chemical compounds was comparatively evaluated against the fish pathogens *A. salmonicida*, *Vibrio anguillarum* and/or *F. psychrophilum*. In **article nº 10** the nutraceutical Liptosa P-203 (Lípidos Toledo S.A., Liptosa), which is based on vegetable fatty acids and essential oils showed MIC values of 292.96-585.92 ppm for *V. anguillarum* and *A. salmonicida* using both the agar and broth dilution methods. The bactericidal chemical agent Bronopol, used for comparative purposes, showed MIC values ranging from 4.57 to 9.15 ppm for *V. anguillarum* and from 9.15-36.62 ppm for *A. salmonicida* using the agar and broth dilution methods. Time-killing assays showed that Liptosa-P203 was able to kill the 99.99% of cells of *V. anguillarum and A. salmonicida* in only 1h at concentrations of 4x MIC (1171.84 ppm). Bronopol killed >99.99% of *V. anguillarum* cells within 2h using at concentrations of 4x MIC (18.31 ppm) and >99.99% of *A. salmonicida* cells within 1h at concentrations of 4x MIC (156.24 ppm). In **article nº 11** the nutraceutical Liptofry (Lípidos Toledo S.A., Liptosa) showed the highest inhibitory effect against the bacterial strains of *F. psychrophilum* (MIC_{90} of 64 µg/ml); while *V. anguillarum* and *A. salmonicida* subps. *salmonicida* were less sensitive (MIC_{90} values of 1250 µg/ml and 3000 µg/ml, respectively). Moreover, rainbow trout fed diets containing 0.5 % (experimental diet) of the nutraceutical Liptofry showed lower mortality rates comparing to fish fed control diets after experimental infection with a virulent strain of *F. psychrophilum*. Interestingly, fish fed diets supplemented with Liptofry showed an over-expression of the genes encoding tumour necrosis factor-α (TNF-α), complement (C5a) and complement receptor (rC5a) in spleen compared to control fish, while immunoglobulin M (IgM) gene expression was only increased in intestine from fish fed nutraceutical-additives. These results demonstrated that the administration of nutraceutical-supplemented diets stimulates immune parameters of fish. This immunity stimulation could explain the higher levels of survival observed in fish fed Liptofry after infection with the virulent strain of *F. psychrophilum*.

Overall, disease is the result of a disturbance in the balance between host, pathogen and environment. Thus, strategies to prevent and control bacterial diseases in fish farms should consider all the different aspects implied in the pathogen-host-environment relationship to ensure a long-term protection of fish. In this PhD work different aspects of the diagnosis, epidemiology and/or control of the diseases produced by the fish pathogens *A. salmonicida*, *Tenacibaculum* sp., *Flavobacterium* sp., and *V. anguillarum* have been studied. Results showed that serological, molecular and proteomic approaches were useful for the identification and typing of the fish pathogens *A. salmonicida* subsp. *salmonicida*, *Tenacibaculum* sp. and *F. psychrophilum*. Moreover, MALDI-TOF and serological techniques are useful strain-typing tools for epidemiological studies. The techniques based on real time PCR developed in this work allowed identification of *A. salmonicida* and *T. maritimum* and their detection in tissues of infected fish and in water of fish holding tanks. All these methologies will allow monitoring the presence of bacterial pathogens in fish production systems, and to evaluate the quality of waters entering and leaving the aquaculture facilities. Although a high prevalence of antimicrobial resistance was found among *A. salmonicida* subsp. *salmonicida* strains isolated in Spain, the new plant-based antimicrobials evaluated in the present study could represent a powerful tool for the prevention and control of furunculosis and other bacterial diseases without selecting resistant strains.

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CHAPTER I. General Introduction

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1.1. Main pathologies in marine and continental aquaculture

Aquaculture is the fastest growing food-production industry because of the increasing demand for food fish consumption. It represents around 44 percent of total fish production globally (Assefa and Abunna, 2018). This increased growth of production is achieved despite the challenges in the aquaculture environment. Among the limiting factors, infectious diseases are a primary constraint to aquaculture production, being responsible for severely impeding socio-economic development in many countries in the world. Economic impact is related with the high mortality rates that produce, the costs of treatments or the decreased growth rates of diseased and convalescent fish (Austin and Austin, 2016). Therefore, to reduce the impact of the fish diseases, it is necessary to apply prevention and control strategies. Among them, the performance of pathological studies is one of the main forms to guarantee the profitability of the industry and ensure the welfare of the cultured animals and the quality and healthiness of the products offered to the consumer. Other biological control strategies include the use of fishes and eggs from pathogen free suppliers, clean feed or water treatments to avoid the entrance and scaping of pathogens from the aquaculture facility.

1.1.1. Non-bacterial fish diseases

1.1.1.1. *Virus*

The global expansion of finfish aquaculture and the subsequent improvements in fish health surveillance have led to the discovery of several viruses. Many of these diseases are listed as notifiable by the World Organization for Animal Health (OIE). The virus associated to pathologies that cause significant mortalities in fish belong to the families *Rhabdoviridae*, *Orthomyxoviridae*, *Alloherpesviridae*, *Iridoviridae* and *Nodaviridae*. Some of the most important viral diseases include: i) the infectious haematopoietic

necrosis (IHN) that affects salmonids; ii) the viral haemorrhagic septicaemia that affects rainbow trout and turbot; iii) the viral nervous necrosis (VNN) that cause substantial mortality in some fish species including barramundi, turbot, sea bass, parrotfish, or red spotter grouper among others; iv) the spring viremia of carp (SVC) affects common carp and the ornamental koi carp; v) the infectious salmon anaemia (ISA) which is an emerging disease of Atlantic salmon; vi) the koi herpesvirus disease (KHD) is an emerging disease that cause infection in cyprinid species including common carp and koi carp; vii) the epizootic haematopoietic necrosis produce mortalities in rainbow trout, perch, and some species of amphibians and reptiles and lastly, viii) the red sea bream iridoviral disease cause disease in red sea bream and other 31 marine fish species belonging mainly to the order Perciformes and Pleuronectiformes (Walker and Winton, 2010; Crane and Hyatt, 2011; Muñoz-Atienza, 2015).

1.1.1.2.*Fungal infections*

Fungal infections in fish are generally considered secondary to some other factor or pathogen, due to water quality problems, poor conditions, skin lesions and bacterial or parasitic disease. The main fungal diseases that affect continental and marine fish species are the ichthyophoniasis caused by *Ichtyophonus hoferi* and the branchiomycosis originated by *Branchiomyces sanguinis* and *Branchiomyces demigrans* (Rodríguez-Gutiérrez et al., 2001).

1.1.1.3.*Parasitical infection*

The main parasitic infections affecting continental and marine fish species are caused by a wide group of organisms, including protozoans and metazoans. The protozoans are single celled parasites whose movement is provided by pseudopodia, flagella or cilia (Roberts and Janovy, 2005). The endo-protozoans produce diseases as the white spot disease produced by *Ichtyophthinus multifilis*

that produce extensive damage in the gills and skin of cold and warm water fish species; the marine velvet disease is produced by the dinoflagellates *Amyloodinium* and *Oodinium*; the costia disease caused by ectoparasite *Ichthyobodo* or the trichodiniasis produced by *Tricodina* sp. that mainly affects salmonids, carps and tilapia. The most important diseases caused by ecto-protozoans,¡ include the diseases produced by *Ceratomyxa shasta*, a myxozoan parasite of salmonids; or the whirling diseases in salmonids, produced by *Myxobolus cerebralis*.

The metazoan ectoparasites include diseases produced by monogenean trematodes of the genera *Gyrodactylus* and *Dactylogyrus*, that cause irritation, haemorrhages and erosion of the gills. The most significant metazoan endoparasites include digenea trematodes belonging to the genera *Diplostomulum*, *Clinostomum*, *Sanguinicola* or *Bucephalus*; cestodes of the genus *Proteocephalus*, *Bothriocephalus*, and *Triaenophorus* or some crustacean parasites, which include several species of copepods (*Lernae* sp.), branchiurans isopods (*Argulus* sp.), amphipods or barnacles (Abowei et al., 2011; Rodríguez-Gutierrez et al., 2001).

1.1.2. Bacterial fish diseases

Bacterial diseases represent the major impediment to aquaculture given that bacteria can survive in aquatic environments independently of their hosts. Several bacterial species belonging to at least 13 genera (*Aeromonas*, *Chryseobacterium*, *Edwardsiella*, *Flavobacterium*, *Francisella*, *Lactococcus*, *Moritella*, *Photobacterium*, *Piscirickettsia*, *Pseudomonas*, *Renibacterium*, *Streptococcus*, *Tenacibaculum*, *Vibrio* and *Yersinia*) have been described as pathogenic for the majority of the existing fish taxonomic groups (Austin and Austin, 2016; Toranzo et al., 2005). However, only a relatively small number

are responsible of important economic losses in aquaculture (Toranzo et al., 2005). These pathogenic microorganisms are usually present in wild fish populations; however, they rarely cause mortality due to the lack of stressful conditions that usually occur in the culture facilities (Toranzo et al., 2005). There has been a steady increase in the number of bacterial species associated with fish diseases, with new pathogens regularly recognised in the literature (Austin and Austin, 2016). The most important bacterial diseases affecting marine and freshwater culture fish are shown in Table 1.

Table 1. Main bacterial diseases affecting fish worldwide (Austin and Austin, 2016).

Bacterial agent	Disease	Host
Aeromonas hydrophila	Haemorragic septicaemia	Freshwater fish species
Aeromonas salmonicida subsp. *salmonicida*	Furunculosis	Salmonids, turbot, Senegalese sole, cod, lamprey
Edwarsiella tarda	Edwarsiellosis	Freshwater fish species, turbot
Flavobacterium psychrophilum	Rainbow trout Fry syndrome	Salmonids, eel, carp, tench, ayu
Lactococcus garviae	Lactococcosis	Yellowtail, eel, salmonids
Moritella viscosa	Winter ulcer	Atlantic salmon
Mycobacterium marinum	Mycobacteriosis	Seabass, turbot, Atlantic salmon
Pasteurella skyensis	Pasteurellosis	Atlantic salmon
Piscirickettsia salmonis	Piscirickettsiosis	Salmonids
Photobacterium damselae subsp. *damselae*	Photobacteriosis	Turbot, rainborw trout, sea bass, yellow tail
Photobacterium damselae subsp. *piscicida*	Photobacteriosis (Pasteurellosis)	Seabass, seabream, sole, yellowtail, striped bass
Pseudomonas anguilliseptica	Pseudomonadiasis Winter disease	Eel, gilthead seabream, turbot, ayu, black spot sea bream,
Renibacterium salmoninarum	Bacterial kidney disease (BKD)	Salmonids
Streptococcus iniae	Streptococcosis	Yellowtail, founder, seabass, barramundi
Streptococcus parauberis	Streptococcosis	Turbot
Tenacibaculum discolor	Tenacibaculosis	Turbot, sole
Tenacibaculum maritimum	Tenacibaculosis	Turbot, sole, salmonids, seabream, seabass, flounder
Tenacibaculum soleae	Tenacibaculosis	Sole, sea bass
Vibrio anguillarum	Vibriosis	Salmonids, turbot, sea bass, eel, ayu, cod, seabream
Vibrio ordalii	Vibriosis	Salmonids
Vibrio salmonicida	Vibriosis	Atlantic salmon, cod
Vibrio vulnificus	Vibriosis	Eels
Yersinia ruckeri	Yersiniosis	Salmonids

The bacterial diseases caused by Gram-stain-negative bacteria; especially typical furunculosis, tenacibaculosis, flavobacteriosis and vibriosis; are more

frequent and more important for marine and continental aquaculture industry. Therefore, the main PhD research work will be focussed in the study of these pathogens. The economic impact of these pathogenic bacteria in the aquaculture industry has led to the increased interest in the study of their biochemical, physiological, molecular, chemotaxonomic and proteomic characterization. A summary of these Gram-stain negative pathogens that produce diseases in freshwater and marine fish is presented below.

1.1.2.1.*Aeromonadaceae*

The species of the genus *Aeromonas*, are widely distributed in aquatic environments and are isolated from water, healthy and diseased fish, food products, animal and human faeces and other clinical and environmental samples (Figueras, 2005). *Aeromonas* species can be divided in two phenotypically different groups; the first group comprises mesophilic, motile and non-pigmented bacteria which are heterologous phenotypically and mainly associated with human clinical infections; and the second group includes psychrophilic, non-motile and pigmented strains, which are mainly fish pathogens and represent a homogeneous group, with no biotypes, serotypes or genotypes identified (Austin and Austin, 2016; Beaz-Hidalgo and Figueras, 2012; Toranzo et al., 2005). In the last group, the subspecies *A. salmonicida* subsp. *salmonicida* is the causative agent of "typical" furunculosis, a disease with a worldwide distribution which results in mass death and devasting losses in the aquaculture industry (Austin and Austin, 2016). Forunculosis mainly affects salmonid fish in fresh and marine water, however, it also affects a variety of non-salmonid fish (Austin and Austin, 2016; El Morabit et al., 2004; Magariños et al., 2011; Toranzo and Barja, 1992). The main signs of disease are lethargy, skin hyperpigmentation, the presence of the typical furuncles or ulcers in the skin, exophthalmia, septicaemia or anaemia (Figure 1) (Austin and

Austin, 2016; Toranzo et al., 2005; Wiklund and Dalsgaard, 1998). However, fish affected by furunculosis do not always show these clinical symptoms (Noga, 2010).

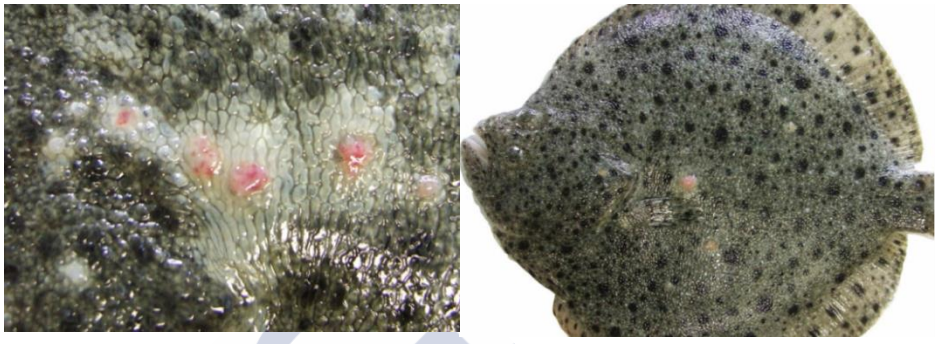

Figure 1. Clinical signs produced by typical furunculosis caused by *Aeromonas salmonicida* subsp. *salmonicida*. Images taken from Coscelli et al. (2014).

Many strains isolated from diseased fish do not present the phenotypic characteristics described for *A. salmonicida* subsp. *salmonicida*¸ which has led to the term "atypical strains" that produce "atypical furunculosis" (Table 2). Atypical *A. salmonicida* include three subspecies, *masoucida*, *smithia*, and *achromogenes* that cause ulcerative and systemic infections in a wide variety of fish, with clinical signs indistinguishable from those associated to typical furunculosis or other diseases produced by motile *Aeromonas* species (Austin and Austin, 2016; Beaz-Hidalgo and Figueras, 2012; Noga, 2010). The differential characteristic of the *A. salmonicida* subspecies are presented in Table 2.

Table 2. Differential characteristics of *Aeromonas salmonicida* (Data from Austin and Austin, 2016)

Character	*Aeromonas salmonicida* subsp.			
	achromogenes	***masoucida***	***salmonicida***	***smithia***
Production of:				
Brown, diffusible pigment	-	-	+	-
Arginine dihydrolase	+	+	v	-
H_2S	-	+	v	+
Lysine decarboxylase	-	+	v	-
Gluconate oxidation	-	+	-	-
Methyl red test	-	+	v	-
Voges Proskauer (VP) reaction	-	+	-	-
Degradation of:				
Aesculin	-	+	v	-
Blood	-	+	+	-
Casein	+	-	+	+
Gelatine	-	+	+	+
Tweens	+	+	+	-
Growth on				
MacConkey agar	+	+	+	-
Production of acid from				
Arabinose	-	+	+ (slow)	ND
Galactose	+	+	+	-
Maltose	+	+	+	-
Mannitol	-	+	+	-
Sucrose	+	+	-	v
Trehalose	+	+	+	-

+, positive results; -, negative results; V, variable result; ND, not done

Diseases caused by motile *Aeromonas* are mainly produced by *A. hydrophila*. However, the recent advances in molecular biology and biotechnology have allowed the discover of new species as *Aeromonas tecta* (Demarta et al., 2008), *Aeromonas aquariorum* (Martinez-Murcia et al., 2008) and *Aeromonas piscicola* (Beaz-Hidalgo et al., 2009), or to the association of other known species to fish diseases (Beaz-Hidalgo and Figueras, 2012). The differential characteristics of the different fish pathogenic *Aeromonas* species are shown in Table 3.

Table 3. Presumptive key tests for the phenotypic differentiation of different *Aeromonas* species [data from Martínez-Murcia et al. (2008) and Beaz-Hidalgo et al. (2009)].

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
β-haemolysis	V	+	+	+	+	-	V	V	+	V	+	ND	+	V
Brown pigment	V	-	-	-	-	-	-	-	-	+	-	-	-	-
VP	V	V	+	+	V	-	-	-	+	-	-	+	V	+
ODC	-	-	-	-	+	-	-	V	-	-	-	-	-	-
LDC	V	+	+	+	+	+	-	+	+	-	-	+	V	-
Glucose (gas)	V	+	+	+	+	V	V	+	+	-	V	+	+	+
Hydrolisis of														
Elastin	V	+	+	+	-	-	-	-	-	-	-	ND	ND	V
Aesculin	+	+	+	+	+	-	V	V	-	V	V	+	V	-
Arbutin	+	+	ND	+	+	-	-	V	-	+	+	+	+	+
Acid from														
Glycerol	+	+	+	+	+	+	+	+	+	V	-	+	+	-
Sucrose	+	+	+	+	+	+	V	+	-	+	V	+	-	V
L-arabinose	+	+	-	V	-	-	-	V	-	+	V	-	-	V
D-cellobiose	V	V	-	-	V	+	-	+	-	+	+	-	-	-
Salicin	V	V	+	V	+	-	-	-	-	V	V	+	V	+
D-sorbitol	+	-	V	-	-	-	-	-	-	-	-	-	-	-
Lactose	+	-	-	V	V	-	+	-	-	V	+	-	-	-
Utilization of **L-lactate**	-	-	-	+	-	-	-	V	-	+	-	-	ND	-

Taxa are identified as: 1, *A. salmonicida*; 2, *Aeromonas bestiarum*; 3, *A. piscicola*; 4, *A. hydrophila*; 5, *Aeromonas veronii*; 6, *Aeromonas sobria*; 7, *Aeromonas encheleia*; 8, *Aeromonas allosacharophila*; 9, *Aeromonas jandaei*; 10, *Aeromonas media*; 11, *Aeromonas eucrenophila*; 12, *A. aquariorum*; 13, *A. tecta*; 14, *Aeromonas trota*. +, 85–100 % of strains positive; V, 16–84 % of strains positive; -, 0–15 % of strains positive; ND, no data available. ODC, ornithine decarboxylase; LDC, lysine decarboxylase.

1.1.2.2.*Flavobacteriaceae*

The family *Flavobacteriaceae* includes many species that are present in extremely diverse habitats, as fresh and marine aquatic environments, soils, foods, beverages as well as human and veterinary hospitals (Bernardet and Nakagawa, 2006). Flavobacteria are mostly saprophytic in terrestrial and aquatic habitats (Bernardet et al., 2002), although many species are pathogenic for different organisms, including plants, invertebrates, amphibians, reptiles, birds, mammals and fish (Bernardet and Bowman, 2006). The fish pathogens of this family are included within the genera *Flavobacterium*, *Flexibacter*, *Chryseobacterium* or *Lacinutrix*.

Bernardet et al. (2002) published the minimal standards (Table 4) for describing new taxa within the family *Flavobacteriaceae* and in the last years the family has rapidly grown from <20 genera to > 100 described genera (Loch and Faisal, 2015).

Table 4. Minimal standards proposed by Bernardet et al., (2002) for the description of new genera in the Family *Flavobacteriaceae*

Phenotypic	Determination of pigments (carotenoids or flexirubin-type pigments), gliding motility, salinity requirements, growth at different temperatures, capnophilic metabolism, growth on MacConkey agar, on B-hydroxybutyrate, hydrolysis of aesculin, DNA, casein, dextran, gelatine, starch, urea, production of cytochrome oxidase,
Chemotaxonomic	Determination of fatty acid methyl esters, Whole-cell protein analysis, menaquinones
Genetic	Determination of DNA base composition, DNA-DNA hybridization, 16S rRNA sequencing

***Flavobacterium* spp.**

Flavobacterial diseases in fish are produced by multiple species within the family *Flavobacteriaceae* (Table 5) and are responsible of important losses in fish stocks worldwide (Austin and Austin, 2016; Bernardet et al., 2002; Loch and Faisal, 2015). All *Flavobacterium* species are Gram-stain negative rods, non-motile or present gliding motility and contain flexirubin and/or carotenoid type pigments (Bernardet et al., 2002). The most important flavobacterial diseases are produced by *F. psychrophilum*, causative agent of bacterial cold-water disease; *Flavobacterium columnare*, the etiological agent of columnaris disease; and *Flavobacterium branchiophilum*, the causative agent of bacterial gill disease (Loch and Faisal, 2015). In addition to these pathogens, other species as *Flavobacterium johnsoniae*, *Flavobacterium hydatis* and *Flavobacterium succinicans* have been occasionally associated with disease in fish (Austin and Austin, 2016; Bernardet et al., 2002; Loch and Faisal, 2015). More recently, several *Flavobacterium* spp. have been isolated from diseased salmonids in America and Europe. Some species as *Flavobacterium chilense* or *Flavobacterium araucananum* have been recovered in mixed cultures with *F. psychrophilum* from salmonids cultured in Chile (Kampfer et al., 2012). In Spain, the species *Flavobacterium oncorhynchi*, *Flavobacterium plurextorum*, *Flavobacterium tructae* and *Flavobacterium piscis* were recovered from the liver, gills or kidney of farmed diseased farmed rainbow trout (Zamora, 2015). Three proposed *Flavobacterium* species (*Flavobacterium collinsi*, *Flavobacterium branchiarum* and *Flavobacterium branchiicola*) have also been recovered from farmed rainbow trout in Spain, tough no sign of disease was reported in fish (Zamora, 2015). In addition to all these fish-associated *Flavobacterium* species, there are other *Flavobacterium*-like bacteria partially characterized or unidentified species that have been periodically isolated from diseased fish (Loch and Faisal, 2015).

Tabla 5. Fish associated *Flavobacterium* species

Species	Source	Reference
Flavobacterium araucananum	Atlantic Salmon, Chile	Kampfer et al., 2012
Flavobacterium branchiophilum	Yamame, Japan.	Bernardet et al., 1996
Flavobacterium chilense	Rainbow trout, Chile	Kampfer et al., 2012
Flavobacterium columnare	Chinook Salmon, USA	Bernardet et al., 1996
Flavobacterium hydatis	Salmon, EEUU	Bernardet et al., 1996
Flavobacterium spartansii	Chinook Salmon, USA	Loch and Faisal, 2014a
Flavobacterium succinicans	Chinook Salmon, USA	Bernardet et al., 1996
Flavobacterium johnsoniae	Pasture grass, UK	Bernardet et al., 1996
Flavobacterium oncorhynchi	Rainbow trout, Spain	Zamora, 2015
Flavobacterium plurextorum	Rainbow trout, Spain	Zamora, 2015
Flavobacterium tructae	Rainbow trout, Spain	Zamora, 2015
Flavobacterium piscis	Rainbow trout, Spain	Zamora, 2015
Flavobacterium collinsii	Rainbow trout, Spain	Zamora, 2015
Flavobacterium branchiarum	Rainbow trout, Spain	Zamora, 2015
Flavobacterium branchiicola	Rainbow trout, Spain	Zamora, 2015

Flavobacteria associated with fish are fastidious and only grown on nutrient poor media (Cepeda et al., 2004), and sometimes there are overgrowth by other less fastidious bacteria (Loch and Faisal, 2015). On the other hand, identification of these bacteria is a challenge since there is a lack of rapid and specific diagnostic protocols available to detect and identify many fish associated flavobacteria outside of those that are commonly associated with fish disease as *F. psychrophilum* or *F. columnare* (Cepeda and Santos, 2000; Toyama et al., 1996).

***Tenacibaculum* spp.**

Tenacibaculosis (formerly marine flexibacteriosis) is an ulcerative disease that limits the culture of many marine fish worldwide (Austin and Austin, 2016; Avendaño-Herrera et al., 2006; Toranzo et al., 2005). The infection produces external clinical signs as ulcers, and necrosis in the skin, eroded mouth, rotten fins, shallow skin lesions and pale internal organs (Figure 2). A systematic

disease can be also established involving different internal organs (Avendaño-Herrera et al., 2006; Santos et al., 1999; Toranzo et al., 2005).

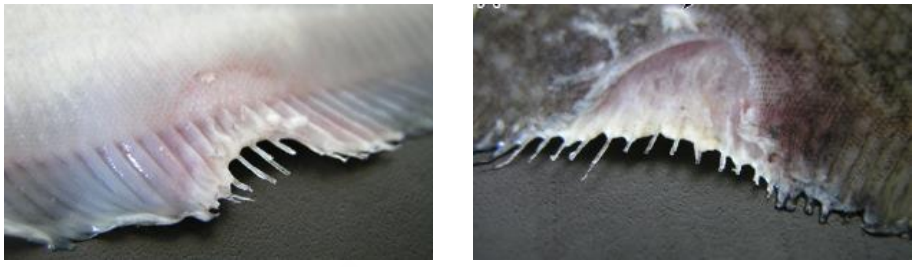

Figure 2. Clinical signs produced by tenacibaculosis in diseased *sole Solea senegalensis*. Image taken from Vilar et al. (2012).

The main causative agent of the disease is the filamentous bacteria *T. maritimum* (formerly *Flexibacter maritimus*) (Avendaño-Herrera et al., 2006; Santos et al., 1999; Toranzo et al., 2005), but other filamentous gliding species belonging to the genus *Tenacibaculum*, including *T. soleae*, *T. discolor*, *Tenacibaculum gallaicum*, *Tenacibaculum dicentrarchi* and "*Tenacibaculum finnmarkense"* have been isolated from diseased fish and oyster and proved to be virulent under experimental conditions (Avendaño-Herrera et al., 2016; Burioli et al., 2018; López et al., 2010; Pineiro-Vidal et al., 2007, 2008a, 2008b, 2012; Småge et al., 2018). Isolation of *Tenacibaculum* species from fish tissues is difficult due to the slow growth of these bacteria and the overgrowth by other bacteria coexisting in tissue samples (Handlinger et al., 1997). *Tenacibaculum* species are phenotypically homogeneous, which facilitates their identification based on the analysis of some morphological and physiological characteristics (Table 5) (Avendaño-Herrera et al., 2006, 2016; Bernardet et al., 2002; Bridel et al., 2018; Olsen et al., 2017; Pineiro-Vidal et al., 2007, 2008a, 2008b; 2012; Småge et al., 2016, 2018; Suzuki et al., 2001; Toranzo et al., 2005). The identification of some *Tenacibaculum* species is also possible using PCR

protocols (Avendaño-Herrera et al., 2004a, Cepeda et al., 2003; García-González et al., 2011). Serological studies have demonstrated the existence of antigenic heterogeneity within *Tenacibaculum* species and the lack of serological relationship among them, which suggest that vaccination against *T. maritimum* will most likely not protect fish against the other *Tenacibaculum* fish-associated species (Pazos et al., 1997; Piñeiro-Vidal, 2008). Molecular characterization of *T. maritimum* using ribotyping and Random Amplification of Polymorphic- DNA PCR (RAPD-PCR) have also demonstrated genetic heterogeneity within this species (Pazos, 1997; Avendano-Herrera et al., 2004).

Table 6. Differential characteristics of the type strains of *Tenacibaculum* species associated to fish diseases. Data from Wakabayashi et al. (1986); Suzuki et al. (2001); Hansen et al. (1992); Bernardet et al. (2002); Piñeiro-Vidal et al. (2008 a,b, 2012); Småge et al. (2016).

Characteristic	***T. maritimum***	***T. ovolyticum***	***T. discolor***	***T. gallaicum***	***T. soleae***	***T. dicentrarchi***	***"T. finnmarkense"***
Temperature range (ºC)	15-34	4-25	14-38	14-38	14-30	4-30	2-20
pH range	5.9-8.6	6-10	6-8	6-8	5-10	6-8	4-9
Degradation of Tween 80	+	+	-	-	-	-	-
Starch hydrolysis	+	+	-	-	-	-	-
Proline utilization	-	-	+	+	-	-	+
Glutamate utilization	-	-	+	+	-	-	+
Growth with seawater (%)[a]	30-100	70-100	30-100	30-100	55-100	30-100	50-100
Trypsin	+	ND	+	+	-	-	ND
α-Chymotrypsin	+	ND	+	+	-	-	-

[a]100 = full-strength seawater; ND, No data available. +, 85–100 % of strains positive; -, 0–15 % of strains positive.

***Chryseobacterium* sp.**

Members of the genus *Chryseobacterium* are Gram-stain negative straight rods. Bacteria do not have flagella, gliding motility or swarming growth and they present a non-diffusible flexirubin-type pigment. The genus *Chryseobacterium* includes several species that have been described as fish pathogens as *Chryseobacterium balustinum*, *Chryseobacterium scophthalmum*, or *Chryseobacterium joostei* (Bernardet et al., 2005). More recently *Chryseobacterium piscicola* was reported to produce mortalities in salmonids (Ilardi et al., 2009); *Chryseobacterium chaponense* was isolated from diseased Atlantic salmon in Chile (Kampfer et al., 2011) and *Chryseobacterium aahli* was first described from systemically-infected trout (*Salvelinus namaycush*) and brown trout (*S. trutta*) in North America (Loch and Faisal, 2014b). In Spain, the species *Chryseobacterium viscerum* and *Chryseobacterium oncorhynchi* were isolated from tissues of diseased rainbow trout (*O. mykiss*) (Zamora, 2015). A number of *Chryseobacterium* spp. that are frequently associated with human infections or human consumables has been recently recovered from fish. For instance, *Chryseobacterium shigense*, which was originally isolated from a beverage in Japan, and was recovered from farmed rainbow trout undergoing multiple disease outbreak in Spain (Zamora, 2015). Presumptive identification of a *Chryseobacterium* sp. is based upon phenotypic characters, fatty acid profiling and sequence and phylogenetic analysis (Bernardet et al., 2002). Recently, proteomic techniques based on MALDI-TOF mass spectrometry have been used to identify *Chryseobacterium* spp. of relevance in aquaculture (Pérez-Sancho et al., 2018).

***Lacinutrix* spp.**

The genus *Lacinutrix* belongs to the Family *Flavobacteriaceae* and was recently described and characterized by Bowman and Nichols (2005). *Lacinutrix* species are present in marine environments and have been isolated from algae and marine sediments, but they have also been associated with marine organisms as commensal or opportunistic pathogens (Bowman and Nichols, 2005; Kim et al., 2017; Lasa et al., 2015; Nedashkovskaya et al., 2008; Srinivas et al., 2013). From all the species described, only *Lacinutrix venerupis* has been reported as a fish pathogen, producing disease in sea bream *Sparus aurata* and European sea bass *Dicentrarchus labrax*, and being virulent for Senegalese sole *Solea senegalensis* (López et al., 2017).

1.1.2.3. *Vibrionaceae*

Vibrios are Gram-negative bacteria, ubiquitous in marine and estuarine ecosystems and aquaculture facilities. *Vibrio* spp. can be found free-living in the water column, as part of biofilm, or in association with the host (Thompson, 2004). Many *Vibrio* spp. are bacterial pathogens responsible for mortality in marine cultures of several fish species worldwide. Vibriosis is caused by a number of *Vibrio* spp. as *V. anguillarum*, *V. ordalii*, *V. salmonicida*, *V. vulnificus*, *Vibrio harveyi*, *Vibrio parahaemolyticus* or *Vibrio alginolyticus* (Austin and Austin, 2016; Toranzo et al., 2005). Among these, *V. anguillarum*, *V. salmonicida* and *V. vulnificus* are the main *Vibrio* pathogens for fish (Austin and Austin, 2016; Toranzo et al., 2005). In general, clinical signs produced by *Vibrio* species include lethargy, tissue necrosis, body malformation, muscle opacity, anemia, ascitic fluids or petechial haemorrhages in the muscle (Austin and Austin, 2016; Chatterjee and Haldar, 2012).

V. anguillarum is the causative agent of classical vibriosis, that cause a typical haemorrhagic septicaemia in several cold and warm water fish species commercially important (Austin and Austin, 2016; Toranzo et al., 2005). Clinical signs include a generalized septicaemia with haemorrhage on the fins, exophthalmia and corneal opacity. A total of 23 O-serotypes (O1-O23) have been described within this species (Toranzo et al., 2005), although only three (O1, O2 and O3) have been associated with fish diseases. The remaining serotypes are environmental strains (O4-O23). The serotype O1 is antigenically homogeneous, contrary to the serotypes O2 and O3 which display antigenic heterogeneity and the existence of two subgroups within each serotype, named O2a and O2b and O3A and O3B (Santos et al., 1995). *V. ordalii* produce haemorrhagic septicaemia infection, although bacteraemia develops later than infection with *V. anguillarum*. This pathogen is antigenically homogeneous with no serotypes been described. *V. salmonicida* is the aetiological agent of the "Hitra disease" which produce severe anaemia and haemorrhages in salmonids and cod (Toranzo et al., 2005).

For prevention of typical vibriosis a licenced bacterin (GAVA-3) covering the three antigenic entities of *V. anguillarum* that cause more mortality is commercially available (Toranzo et al., 1997). Vaccines are also available for the prevention of diseases produced by *V. ordalii*, *V. salmonicida* or *V. vulnificus* (Toranzo et al., 2005).

1.2. Diagnosis and epidemiological studies of bacterial diseases

Diagnosing aquatic animal diseases by the gross observation of the clinical signs is almost impossible due to fishes live in water and move fast, which makes them impossible to visualize closely and inspect them for any clinical deviations (Assefa and Abunna, 2018). Thus, rapid and sensitive diagnostic methods that allow pathogen detection even in subclinical infected or apparently

healthy hosts are extremely important for health management and control of infectious diseases (Altinok and Kurt, 2016). Rapid molecular-based methods have become important tools that allow diagnosing diseases through the detection of the pathogen in lethal or non-lethal fish samples (Assefa and Abunna, 2018). The rapid detection and identification of a pathogen reduces the risk of spread of the pathogen and helps in the establishment of appropriate antibiotic treatments (Altinok and Kurt, 2016).

Furthermore, typing of microbial pathogens, or identifying bacteria at the strain level is important for the establishment of effective prevention and control strategies, for the study of population dynamics and for epidemiological surveillance of bacterial infections (Wolska and Szweda, 2012).

1.2.1. Phenotypic identification methods

For many years, the phenotypic characterization of bacterial strains represented the only identification approach, although the methodology always leaves uncertainties and discrepancies of interpretation and limited reproducibility. In many cases, phenotypic characteristics are not enough to differentiate taxa and must be accompanied by a genotypic analysis (Donelli et al. 2013). Phenotypic characterization includes the study of the morphological (cell shape, flagella, dimension, form of colonies…), biochemical (enzymatic activities, gas production and compound utilization) and physiological (growth temperature, pH, salts concentration…) characteristics as well as the antimicrobial susceptibility profiles of the bacterial strains (Austin and Austin, 2016; Buller, 2014). In the last years, many automated identification systems as the API (API 20E, API ZYM, API 20NE, API 50 CH…), BIOLOG or MINITEK systems have been applied for the identification of fish pathogens. Identification is carried out by using the database provided by the manufacturer. However, the lack of information about veterinary pathogens in the current

databases and the existence of false positive and negative reactions for several tests, have limited their use (Buller, 2014). On the other hand, classical bacteriological methods are appropriate for the detection of easily cultured bacteria, but the identification of many pathogens is delayed or impeded by rather fastidious growth and weak reactivity in biochemical tests. For instance, Santos et al. (1993) analysed 32 isolates of *A. salmonicida* using the API-20E system, and only the 41% of the strains were correctly identified. More recently, other authors reported discordancies between classical biochemical tests and API-20E system in the identification of *Vibrio* and *Aeromonas* strains (Israil et al., 2003). The failure to recognize some strains could raise important epidemiological problems. On the other hand, analysis of the enzymatic activities of *Tenacibaculum* species by API-ZYM have demonstrated the biochemical homogeneity of these species (Buller, 2004; Piñeiro-Vidal, 2008). However, the use of this method is problematic for the differentiation of the different fish-associated *Tenacibaculum* species, since the enzymatic profiles are very similar (Piñeiro-Vidal et al., 2007).

1.2.2. Serological methods

Different immunological techniques are routinely used in fish diagnosis, for antigenic characterization to select strains for vaccine formulation and to detect antibodies to specific pathogens in the serum of animal as an indicator of previous exposure to the microorganism or infection (Adams and Thompson, 2011; González and Santos, 2009; Santos et al. 1995). Immunological assays are based on a highly specific reaction between an antibody (Ab) and an antigen (Ag). Specificity and sensitivity of the binding reaction are determined by the type of Ab, namely polyclonal or monoclonal. Polyclonal antibodies raised in rabbits are frequently used for diagnosis of diseases and serotyping of strains, although present some limitations including product standardization, cross

reaction, limited quantity and inability to discriminate antigen at epitope level. These limitations could be overcome by using monoclonal antibodies which are widely used in the diagnosis, serotyping, and for the monitorization of the immune response of different fish species (Romstad et al., 2012; Kim et al., 2015). The application of immunological methods is limited due to the lack of standardized protocols, the need of skilled operators, the limited sensitivity of the methods to detect the pathogens in environmental samples and sub-clinical disease and that the antigens on the pathogen may be altered and no longer recognized by the antibody (Adams and Thompson, 2011; González and Santos, 2009). Different qualitative or quantitative immunological methods have been developed for detection of antigens or antibodies specific to bacterial diseases for clinical and veterinary medicine.

1.2.2.1. *Agglutination test*

Agglutination tests are used to analyse an unidentified microorganism against known antisera. Bacterial agglutination tests may be performed on a slide, in tubes or in microtiter tray wells. The slide agglutination is the most used test and is performed by mixing antigen and serum on a plate and the reading of the presence of agglutination (visible precipitation of Ab-antigen complexes, by macroscopic examination), usually after 1-2 minutes incubation (Figure 3). Agglutination techniques are widely used for the identification and serotyping of non-auto-agglutinating fish pathogens as *A. salmonicida, A. hydrophila, V. anguillarum, F. psychrophilum, T. maritimum, P. damselae* subsp. *piscicida, Y. ruckeri, E. tarda,* and *R. salmoninarum* (Santos et al., 1991; Santos et al., 1995; González et al., 2004a; Romalde et al., 1995; Mata et al., 2002; González and Santos, 2009; Pazos, 1997). Agglutination kits are commercially available for the rapid preliminary screening of important fish pathogens as *A. salmonicida,*

Y. ruckeri, *V. anguillarum* or *P. damselae* subsp. *piscicida* (BIONOR™ MONO-AQUA Test system, Bionor, Spain).

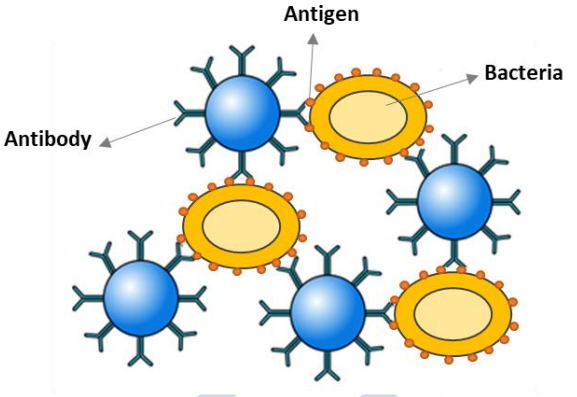

Figure 3. The agglutinnation test is based on the sedimentation of the complexes formed by the specific antibodies (Ab) and the antigen (Ag) present on the bacterial surface. Modified from González (2010).

1.2.2.2. *Immunofluorescence assays.*

Direct and indirect fluorescent antibody techniques are simple, sensitive and rapid methods in fish diagnostics. In these assays, specific monoclonal or polyclonal antibodies conjugated with a fluorescent dye are used as the reagent. In a direct fluorescent antibody test (FAT) the pathogen-specific Ab is labelled with the dye while in the indirect fluorescent antibody test (IFAT) a secondary species-specific Ab is labelled with the fluorescent dye instead of the pathogen-specific Ab (Figure 4) (González, 2010). The disadvantages on the use of these techniques is that a fluorescent or confocal microscope and specialized staff is needed to visualize the results (Adams and Thompson, 2011). Immunofluorescence assays have been used for the diagnosis of bacterial kidney disease (BKD), for the detection of *T. maritimum* in tissues of diseased sea bass,

or for the detection of *F. psychrophilum* in samples of water from fish farms (Adams et al., 1995; Madetoja and Wiklund, 2002; Yardimci and Timur, 2016).

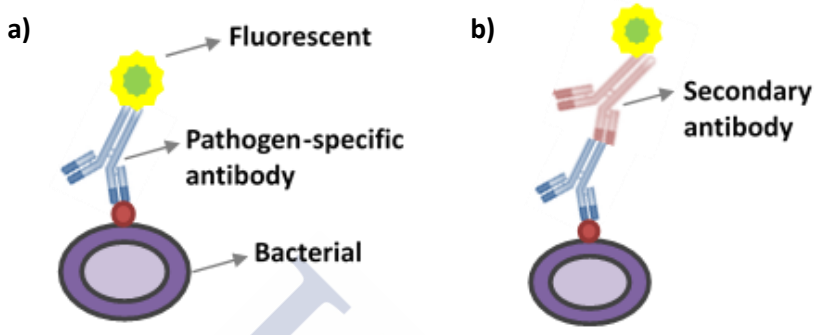

Figure 4. Schematic representation of direct fluorescent antibody test (FAT) (a) and indirect fluorescent antibody test (IFAT) (b). Modified from González (2010).

1.2.2.3. *Immunohistochemistry*

Immunohistochemistry (IHC) allows the detection of the pathogen by incubating the section of tissue with a pathogen specific antibody (Adams and Thompson, 2011). Then, the antibody-antigen complex is bound by a secondary enzyme-conjugated Ab that in the presence of chromogen forms a coloured deposit at the sites of antibody-antigen binding, which could be observed using light microscopy. IHC has been applied for diagnosis of tenacibaculosis (Faílde et al., 2013) or pasteurelloris (Abu-Elala et al., 2015). IHC has also been used to visualize and study the pathology associated with infections produced by *T. maritimum* (Faílde et al., 2013).

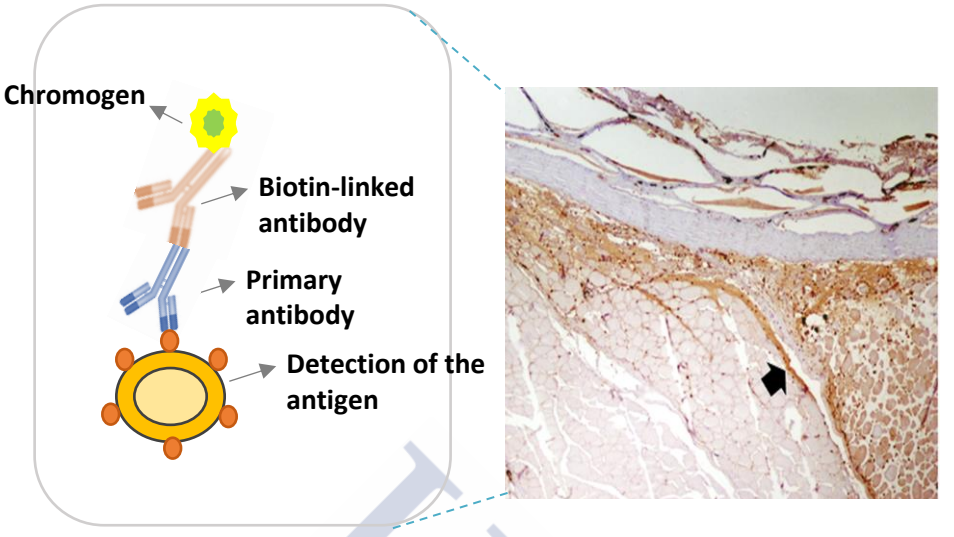

Figure 5. Schematic representation of the immunohistochemistry (IHC) method for detection of pathogens in cells or tissues and immunohistochemical detection of *T. maritimum* in Senegalese sole. Image taken from Faílde et al. (2013)

1.2.2.4. *Enzyme-linked immunosorbent assays (ELISA)*

ELISA is useful for detecting and quantifying pathogens during clinical disease or the host antibody response in tissues or serum of diseased or vaccinated fish (Romstad et al., 2012). The most used ELISA method for the detection of fish pathogens is the sandwich ELISA. In this assay the samples are added to the ELISA plate that is coated with an antigen-specific Ab (called captured Ab) and, after washing to remove unbound material, a secondary antigen-specific Ab is added (detection Ab). In a direct format an enzyme is conjugate to the secondary antibody, while in the indirect format the second pathogen-specific antibody is not conjugated, and a third enzyme-conjugated species-specific antibody is added to obtain the colour reaction after the addition of substrate. ELISA is highly sensitive and allows the high throughput and can be automated. ELISA methods are useful for the diagnosis of diseases produced

by *A. salmonicida* (Hiney et al., 1994; González et al., 2004b), *P. damselae* subsp. *piscicida* (Romalde et al., 1999), *V. anguillarum* (González et al., 2004b), *Y. ruckeri* (Smith et al., 1987) or *F. psychrophilum* (Mata and Santos 2001).

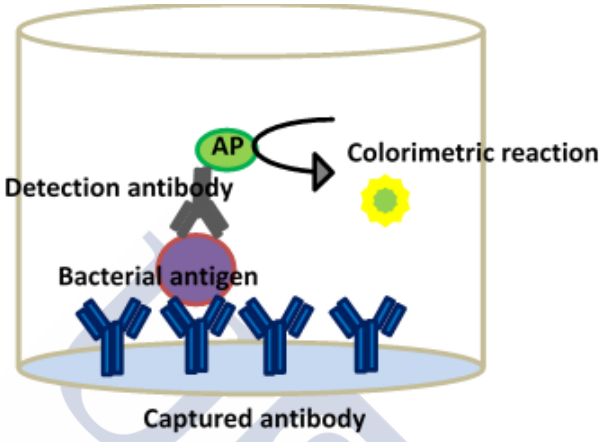

Figure 6. Sandwich ELISA assay. The capture antibody (Ab) fixed on the surface of the well binds to the bacterial antigen (Ag). The detection Ab is then incubated with the sample and the signal is detected by a colorimetric reaction

1.2.2.5. *Dot-blot*

This technique is routinely applied for the detection, identification and serotyping of samples of bacterial cultures and fish tissues. The sensitivity of this method is similar to ELISA-based assays (González et al., 2004a) . Dot-blot has been applied for identification and serotyping of *V. anguillarum* (Santos et al., 1995), *Tenacibaculum* sp. (Avendaño-Herrera et al., 2004b; Pazos, 1997; Piñeiro-Vidal, 2008), *F. psychrophilum* (Mata and Santos, 2001) or *Aeromonas salmonicida* (El Morabit et al., 2004).

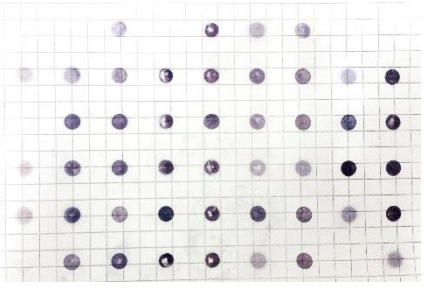

Figure 7. Dot-blot assay of *A. salmonicida* strains (Fernández-Álvarez C., unpublished data). The antigen fixed on the surface of the nitrocellulose paper is incubated with the detection antibody, a second enzyme-conjugated species-specific antibody and the substrate which signal is subsequently detected by a colorimetric reaction.

1.2.3. Molecular methods: identification and typing

Genotypic methods are those focused on the detection of DNA and RNA nucleic acids of the pathogens. At present, these methods dominate the diagnosis of diseases and taxonomic studies.

1.2.3.1. *PCR-identification methods*

Since its initial development in the early 1980s the polymerase chain reaction (PCR) has become one of the most commonly used techniques in molecular biology and has been adapted to a vast range of fields including DNA cloning, gene expression, and the diagnostic of infectious diseases, among others. In the last years, considerable efforts have been made to adapt PCR to the detection of fish pathogens in aquaculture and to discriminate between non-pathogenic and pathogenic strains (Buller, 2014). These tests have advantages over conventional methods, such as sensitivity, specificity and accuracy, allowing the identification of non-cultivable or slow-growing microorganisms. Moreover, the high sensitivity of PCR allows the detection of pathogens shortly

after infection and even before the onset of the disease, which facilitates the early establishment of specific treatments and management of the disease. There are many types of PCR techniques such as RT-PCR, real-time PCR, nested PCR, multiplex PCR…etc. The high popularity of this technique is reflected in the high number of published PCR protocols for the detection of the most problematic pathogenic bacteria that affects fish and shellfish production. Many of the PCR protocols for the detection of fish pathogens are based on the amplification of ribosomal genes, mainly 16S and 23S (Avendaño-Herrera et al., 2018; Cepeda et al., 2003; Cepeda and Santos, 2000; Fringuelli et al., 2012; García-González et al., 2011) which are present in all eubacteria. Alternatively, some other genes, as β´DNA-dependent RNA polymerase (*rpoC*) (Strepparava et al., 2014); the ferric siderophore receptor (*fstA*) (Beaz-Hidalgo et al., 2008a), the outer membrane protein assembly factor *bamB* (Carraro et al., 2018) or transmembrane transcription regulators as *ToxR* genes (González et al., 2004b) have been selected as targets for primer design.

1.2.3.2. *Genotyping methods*

In the last years, several DNA banding pattern-based methods, which classify the bacteria according to the size of the fragments generated by PCR amplification, by digestion of genomic DNA with restriction enzymes or a combination of both have been frequently used for typing pathogenic bacteria (Wolska and Szweda, 2012). The techniques based on the use of restriction enzymes include the restriction fragments length polymorphism (RFLP) analysis of a PCR-amplified fragment of the 16S rRNA gene (RFLP-PCR) and the ribotyping, which is based on the analysis of sequences of a rRNA operone (Kostman et al., 1992). Ribotyping has been used for the characterization and epidemiological discrimination of several fish pathogenic species (Farto et al., 1999; Pazos, 1997; Romalde et al., 1999).

Among the PCR-based methods, the random amplified polymorphic DNA (RAPD) assay and the repetitive elements sequence-based PCR (Rep-PCR) procedures are powerful tools for genotyping studies (Figure 8).

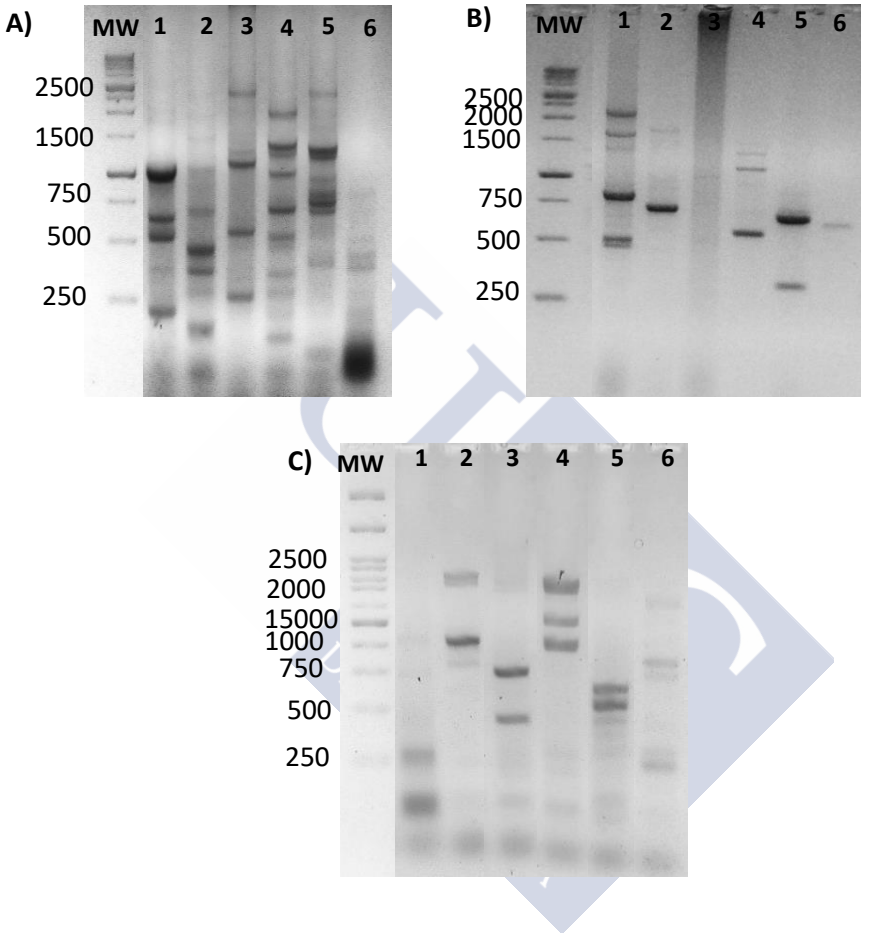

Figure 8. Electrophoretic analysis of different fish- associated *Tenacibaculum* species using REP-PCR (**a**), RAPD (primer 2) (**b**), and ERIC-PCR (**c**). Published in: Fernández-Álvarez and Santos (2018).

RAPD uses oligonucleotide (9 to 10 bases in length) primers with arbitrary sequence, which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate

amplification of regions of the bacterial genome. Rep-PCR is based on primers that amplify repeated sequences of high homology and a high degree of evolutionary conservation in the bacterial genome (Versalovic et al., 1991) as the REP sequences (REP-PCR) (38 bp) and the ERIC sequences (ERIC-PCR) (123 bp). RAPD and Rep-PCR techniques are rapid and ease-to-use and have been used to genotype a diverse group of bacteria isolated from humans, animals and environment (Avendano-Herrera et al., 2004; Beaz-Hidalgo et al., 2008b; Sachdeva and Virdi, 2004). In this sense, genotypic analysis of *A. salmonicida* subsp. *salmonicida* using RAPD, REP and ERIC-PCR revealed a high genomic homogeneity within this species (Beaz-Hidalgo et al., 2008b; Miyata et al., 1995). Although genetic variability has been reported for the fish pathogen *F. psychrophilum*, strains of this species could not have been differentiated according to the isolation source and region by using ERIC, REP and RAPD-PCR (Saticioglu et al., 2018; Valdebenito and Avendaño-Herrera, 2009). On the other hand, RAPD analysis demonstrated the existence of genetic heterogeneity among *T. maritimum* strains associated with the host species and the O-serotypes described (Avendaño-Herrera et al., 2004c). Similarly, Vaseeharan et al. (2008) reported that RAPD analysis of *V. anguillarum* strains produced a unique RAPD profile for each location studied, suggesting that this species is genetically heterogeneous. Although all these techniques are relatively cheap, rapid, and easy to perform, the reproducibility of the Rep-PCR is much higher than that of the RAPD-PCR.

1.2.3.3. *Multi-locus sequence analysis (MLSA)*

MLSA was first introduced in 1998 as a microbial typing method for epidemiological population genetic studies of pathogenic bacteria (Maiden et al., 1998). In MLSA studies, partial sequences of multiple genes coding for proteins with conserved functions (housekeeping genes) are used to generate phylogenetic trees and subsequently deduce phylogenies (Glaeser and Kämpfer, 2015). MLSA was first used and validated for strains of *Neisseria meningitidis* for epidemiological studies (Glaeser and Kämpfer, 2015). In the last years, the use of MLSA has been extended and it has been successfully used for the genotyping and genetic differentiation of bacterial fish pathogenic species and to estimate the evolutionary relationship among isolates recovered from diseased fish worldwide (Avendaño-Herrera et al., 2016; Frisch et al., 2018; Habib et al., 2014; Olsen et al., 2017). Several authors have used MLSA for the identification and differentiation of fish pathogenic or environmental *Tenacibaculum* species isolated from different fish species in Norway, Canada or Chile (Avendaño-Herrera et al., 2016; Frisch et al., 2018; Habib et al., 2014; Olsen et al., 2017). Similar studies have been performed using other fish pathogens as *Flavobacterium* sp. (Ashrafi et al., 2015; Van Vliet et al., 2016) or *Vibrio* sp. (Pérez-Cataluña et al., 2016).

1.2.4. Proteomic and chemotaxonomic methods as a tool for diagnosis and epidemiological studies.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has emerged as a powerful tool for microbial characterization, strain typing, epidemiological studies or for the monitoring the spread of antibiotic-resistant bacteria (Singhal et al., 2015). Moreover, the simple sample preparation required and the rapid rate at which results can be acquired have made MALDI-TOF-MS approaches very popular (Singhal et al.,

2015). MALDI-TOF-MS operates with unique proteomic fingerprints acquired by the desorption of specific protein biomarkers from the bacteria (Chalupová et al., 2014) and identification is carried out by comparison of the mass fingerprint to the protein databases (Figure 9). In recent years, great efforts have been made to increase the taxonomic resolution of the technique. Bioinformatic approaches have leveraged the ever-increasing amount of publicly available proteomic data to construct robust protein databases facilitating the genus, species and strain identification through the detection of biomarkers (Shingal et al., 2015). Application of MALDI-TOF-MS has allowed the characterization of clinical (Assiss et al., 2017; Benagli et al., 2011; Cheng et al., 2015; Dieckmann et al., 2008, 2010; Ilina et al., 2009) and environmentally (Popovic et al., 2017) relevant bacteria, but also of virus (Calderaro et al., 2014), fungus (Becker et al., 2014) and multicellular organisms as nematodes (Perera et al., 2005). In the last decades, several protocols have been developed for the identification and differentiation of closely-related bacterial fish pathogens as *P. damselae*, *Flavobacterium* sp., *Vibrio* sp., *Aeromonas* sp., *Streptococcus* sp. or *Piscirickettsia* sp. (Assis et al., 2017; Benagli et al., 2012; Dieckmann et al., 2010; López-Cortés et al., 2017; Pérez-Sancho et al., 2016, 2017).

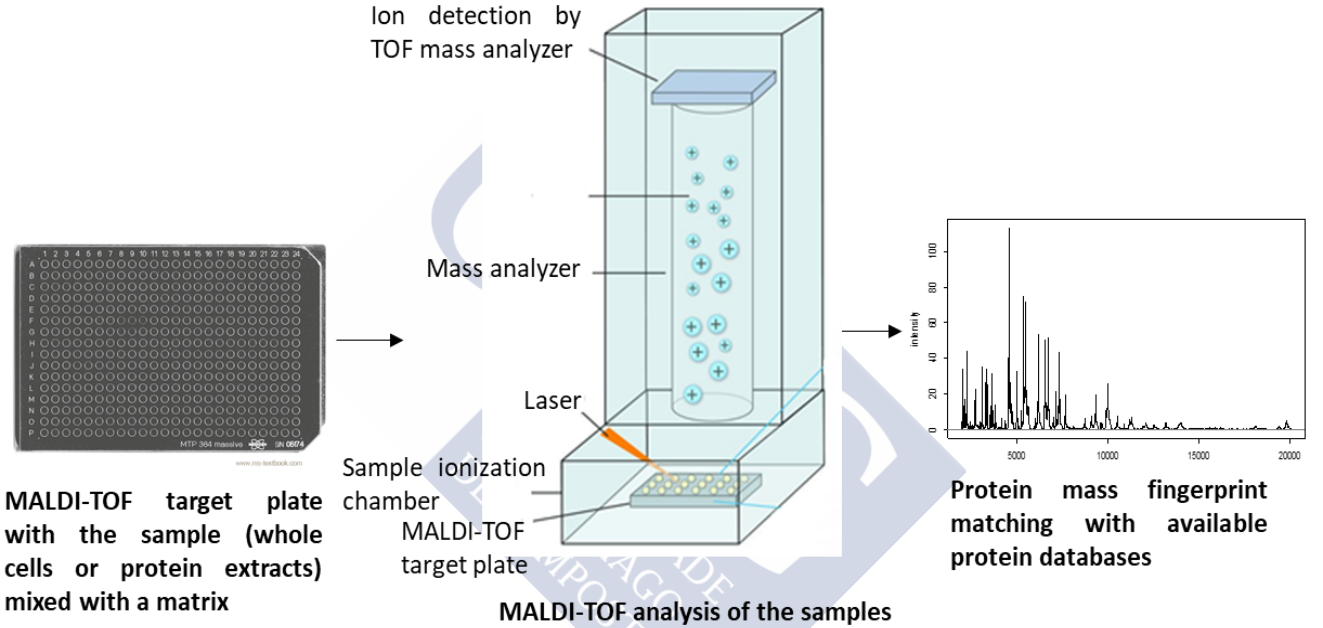

Figure 9. Schematic diagram showing the work-flow in a MALDI-TOF mass spectrometry

Chemotaxonomic methods allow classifying microorganisms based on differences and similarities in chemical constituents of bacteria, including fatty acids, polar lipids, lipopolysaccharide, menaquinones, naphthoquinones, ubiquinone's, mycolic acids, peptidoglycan, polyamines, teichoic and teichuronic acids and isoprenoid quinones. Among these techniques, gas chromatographic analysis of the cellular fatty acids is the most used technique for taxonomic classification of microorganisms. In the last decades large databases have been constructed for the identification and classification of unknown samples considering the presence and relative concentration of the fatty acid methyl esters (FAMEs) (Sasser, 1990; David et al., 2008). Reliable chemotaxonomic data exist for different Gram-stain positive bacterial fish pathogens (Williams et al., 1990). Regarding Gram-stain negative pathogens, the FAMEs profile has been successfully applied in taxonomic studies for the identification of different bacterial species; *Flavobacterium* (Bernardet et al., 2002; Zamora, 2015), *Aeromonas* (Bektas et al., 2007) or *Tenacibaculum* (Piñeiro-Vidal et al., 2008); or subspecies, as *P. damselae* subsp. *piscidida* (Romalde et al., 1994). On the other hand, Piñeiro-Vidal et al. (2008) suggested that fatty acids could be used as chemotaxonomic biomarkers for epidemiological studies in *Tenacibaculum* species isolated from different sources. Although the chemotaxonomic characterization is necessary for the description of new bacterial species, the use of fatty acid analysis for routine identification of bacteria is limited because the results obtained depend largely on growth conditions and the procedure is expensive compared to other available genomic and proteomic procedures.

Overall, the application of prevention and control strategies in aquaculture and in other animal production sectors require rapid and sensitive diagnostic methods, which facilitates prompt and efficient implementation of control measures and subsequently a sustainable future for aquaculture. As stated

above, different conventional and modern methodologies have been applied for these purposes (Table 7). The use of DNA-based methods have demonstrated their applicability for identification and classification of microorganism and have been stablished as effective diagnostic tools in clinical microbiology. These DNA-based methods present many advantages over conventional and serological methods in terms of higher specificity and sensitivity, more rapidity and reasonable costs (Table 7). However, automation in proteomics technology as MALDI-TOF mass spectrometry, has increased its throughput and potential use for a number of microbiological purposes like strain typing, epidemiological studies, identification of microbes inhabiting a particular ecosystem or detection of antibiotic resistance. The respective advantages and disadvantages of all these methods are listed in Table 7.

Tabla 7. Microbial detection methods used in clinical microbiology [Modified from Shingal et al. (2015)].

Detection method	Advantages	Disadvantages
Culture-based methods and phenotypic characterization	• Inexpensive	• Time-consuming process • Lack of reproducibility • Confusion in the interpretation of the results
Immunological-based methods	• Faster than culture-based methods • Can detect both contaminating organisms and their toxins	• Less specific, sensitive and rapid than genomic-based methods • Require large amounts of antigen • Developed for only a small number of microorganisms
Molecular based methods	• Culturing the samples is not required • Specific, sensitive, rapid and accurate • Reduced risk of contamination • Detection of several pathogens simultaneously	• A highly precise thermal cycler is needed • Trained laboratory personnel required
MALDI-TOF mass spectrometry	• Fast • Accurate • Less expensive than molecular and immunological-based detection methods • Easy to interpret results	• High initial cost of the MALDI-TOF equipment

1.3. Approaches for prevention and control of bacterial fish diseases

Disease is the result of a disturbance in the balance between host, pathogen and environment. Thus, strategies to prevent and control bacterial diseases in fish farms should take into account all the different aspects implied in the pathogen-host-environment relationship to ensure a long term protection of fish (Defoirdt et al., 2011) (Figure 10).

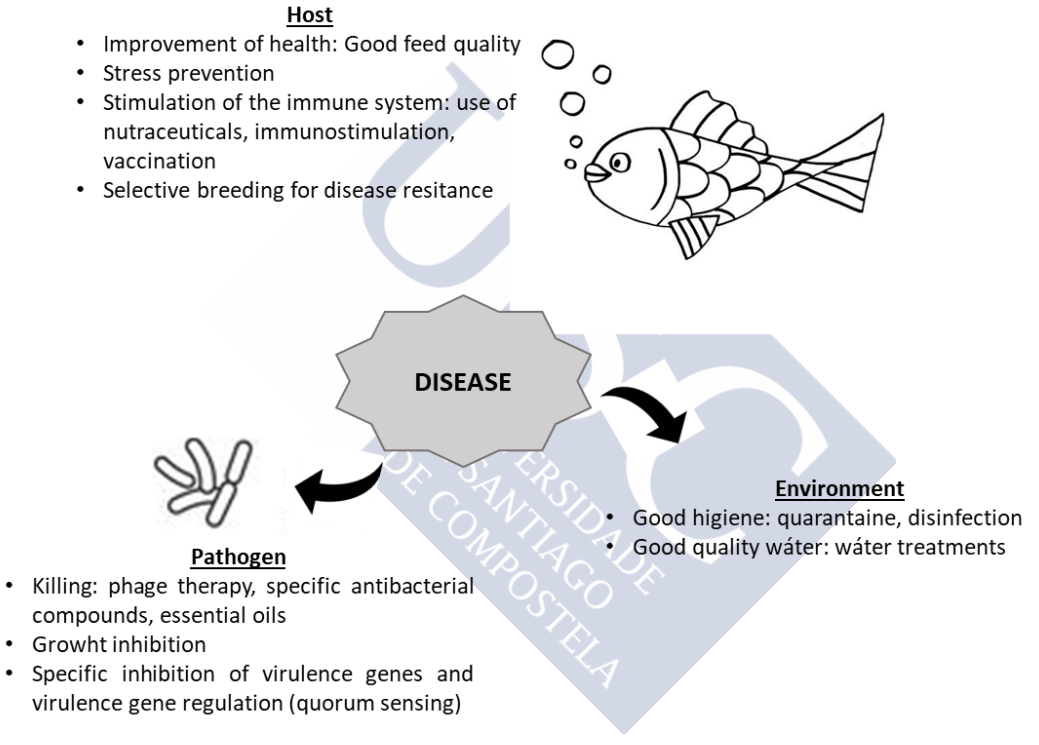

Figure 10. Strategies to prevent and control bacterial diseases in aquaculture taking into account the different aspects of the pathogen-host-environment continuum. Modified from Defoirdt et al. (2011).

The measures for the prevention and control of bacterial fish diseases, include the use of improved husbandry/management practices, control of movement, genetically resistant stock, water disinfection biological control, vaccination, dietary supplements, non-specific immunostimulants, probiotics,

plant-based compounds (essential oils, plant extracts, algae and their metabolites) and antimicrobial compounds. A combination of all these strategies together with sensitive and specific diagnostic tests will be valuable to assure healthy of fish.

1.3.1. Vaccination

Vaccination plays an important role in the prevention and control of infectious diseases and currently there are a number of vaccines commercially available for use in fish (Sommerset et al., 2005). The majority of aquatic vaccines are delivered by injection, which is the most effective method when compared to oral or immersion deliveries (Plant and LaPatra, 2011). Most bacterial vaccines used in aquaculture are inactivated vaccines. The USA licensed the first fish vaccine in 1976; and orally administered killed preparation of *Y. ruckeri* which induced protection against enteric redmouth. Later, *V. anguillarum* formalin-inactivated whole cells were administered via immersion for the prevention of typical vibriosis successfully. The licensed bacterin (GAVA-3) protects against the main three serotypes of *V. anguillarum* responsible for most epizootics (O1, O2a, O2b) (Santos et al., 1991). Moreover, different polyvalent oil-adjuvanted vaccines which combine *V. anguillarum* with other pathogens as *V. ordalii*, *V. salmonicida*, *A. salmonicida* or *M. viscosa* are also commercially available for application to salmonids (Toranzo et al., 2009). For preventing typical furunculosis caused by *A. salmonicida* subsp. *salmonicida*, the best results of protection have been reported with injection with mineral or non-mineral oil-adjuvanted bacterin (Plant and LaPatra, 2011; Sommerset et al., 2005; Toranzo et al., 2009). A tenacibaculosis vaccine "ICTHIOVAC-TM" developed by our group is currently on the market to prevent turbot mortalities produced by *T. maritimum* and is effective by bathing turbot of 1 to 2 g, followed by a booster injection when they attain to 20 to 30 g

(Avendaño-Herrera et al. 2006; Pazos, 1997; Santos et al., 1999; Santos et al., 2005; Toranzo et al., 2005). Vaccination with live vaccines has been tested experimentally, however, safety problems should be resolved before using these vaccines in aquaculture industry. At present, only an *Edwardsiella ictaluri* attenuated live vaccine has been licensed in the USA.

The production of effective fish vaccines present some limitations due to the scarce knowledge about the immune system of different fish species as well as the lack of information regarding the antigens with immunogenic potential (Sommerset et al., 2005). Development of an effective fish vaccine involves the understanding of the fish immune system, the rapid identification of the specific antigen, proper formulation of the antigen, and lastly, the application of an effective delivery method that causes minimal stress to the fish.

1.3.2. Disinfectants and antiseptics

Disinfection, quarantine of the affected aquaculture facilities and stamping out procedures are an essential part of the prevention of infectious diseases in aquaculture facilities and are part of the biosecurity plans. Disinfection is used to prevent entry or exit of pathogens and its propagation in the aquaculture facilities. Disinfection methods used in aquaculture include chemical procedures, physical procedures or combinations of both. Chemical procedures include treatment of water, equipment, materials and surfaces with oxidizing agents (chloramine-T, iodophores, peroxygen compounds, chlorine dioxide and ozone), pH modifying agents (acids or alkalis), biguanides (chlorhexidine) and ammonium quaternary compounds. Disinfection by physical methods is based on the use of ultraviolet irradiation, desiccation or heat treatment (OIE, 2018)

On the other hand, bacterial infections are the major cause of fish egg mortality and the occurrence of deformed larvae at early stages of life (Overton

et al., 2010). Thus, a wide range of chemical agents have been used as antiseptics in aquaculture for the treatment of fish eggs as chloramine B and T, ozone, hydrogen peroxide, iodophors, formalin, hydrogen peroxide, paracetic acid and bronopol (Barnes et al., 2002; Branson, 2002; Can et al., 2012; Escaffre et al., 2001; Grasteau et al., 2015; Grotmol et al., 2003; Katharios et al., 2007; Meinelt et al., 2015; Overton et al., 2010; Straus et al., 2012; Treasurer et al., 2005). During the intensive production of fish, surface disinfection of eggs is a critical control measure to prevent surface transmission of diseases from broodstock to their offspring, to reduce the problems of cross contamination between egg batches and to reduce the bacterial growth that occur in intensive production systems (Overton et al., 2010). Several authors have reported *in vitro* antimicrobial activity of antiseptics on bacterial pathogens as well as the *in vivo* impact of the antiseptic treatment on fish eggs. For instance, Grasteau et al. (2015) showed that treatment with bronopol, hydrogen peroxide or Incimaxx Aquatic® (a mix of peroctanoïc acid, peracetic acid and hydrogen peroxide) were effective against *F. psychrophilum* and did not affet the eggs/fry viability. Kumagai and Nawata (2010) used pre-fertilization disinfection with iodophor treatment to prevent the *F. psychrophilum* vertical transmission in eggs of *O. masou*, *Salvelinus pluvius*, *O. kisutch* and *O. mykiss*. Similarly, Chalupniki and Ketola (2011) proved that iodine immersion (50 mg/L) of Atlantic salmon eggs reduced the numbers of surface bacteria (*Aeromonas* sp., *Citrobacter* sp…) by 97.4% and did not significantly affect the survival of the eggs.

1.3.3. Use of antimicrobials in aquaculture: use and abuse

Antimicrobial agents are substances that have the capacity to kill or inhibit the growth of microorganisms. Antimicrobials can be derived from natural sources or have synthetic origins and should be non-toxic to the host, allowing their use as chemotherapeutic agents for the treatment of bacterial infectious

diseases (Romero et al., 2012). The use of antimicrobials can be categorized as therapeutic, prophylactic or metaphylactic (Romero et al., 2012). Antimicrobial drugs may have different types of chemical structures and act on different parts of bacterial machinery. In general, antibiotics exert a bactericidal (the antibiotic kills the bacteria) or a bacteriostatic effect (the antibiotic stops bacterial multiplication) (Table 8). The use of antimicrobial compounds for the treatment of bacterial diseases in aquaculture started in 1946, when Gutsell (1946) applied sulphonamides for the control of furunculosis. Then, sulphonamides potentiated with trimethoprim were developed, and they are still used in aquaculture farms. After sulphonamides, the range of antibiotic-like compounds increased including chloramphenicol, oxytetracycline, kanamycin, furans and quinolones (Austin, 2017).

Currently, the use of antibacterial agents in food animal species, including fish, is controlled by regulations, particularly in Europe and the USA. The Food and Drug administration (FDA) of the USA authorises the use of oxytetracycline, florfenicol, and sulfadimethoxine/ormetprim in aquaculture, while the Spanish Agency of Medicines and Medical Devices (SAMMD) allows the use of flumequine, oxytetracycline and florfenicol for oral and bathing treatment.

Table 8. Diferent mechanisms of action of antibiotics and examples (Romero et al. 2012).

Mechanisms of action of antibacterial agents		Examples of antibiotics
Interference with cell wall synthesis	β-lactams	Cephalosporins, carbapenems, monobactams
	Glycopeptides	Vancomycin, teicoplanin
Proteins synthesis inhibition	Bind to 50 ribosomal subunit	Macrolides, chloranphenicol, clindamycin, linezolid, quinuspristin-dalfopristin
	Bind to 30 ribosomal subunit	Aminoglycosides, tetracyclines
Interference with nucleic acid synthesis	Bind to bacterial isolecyl-tRNA synthetase	Mupirocin
	Inhibit DNA synthesis	Fluoroquinolones
	Inhibitir RNA synthesis	Rifampin
Inhibition of metabolic pathway		Sulfonamides, folic acid analogues
Disruption of bacterial membrane structure		Polymyxins, daptomycin

To control fish bacterial diseases, feeding infected fish with formulated feed mixed with antibiotics is a general practice. However, the use of medicated feed is usually expensive and may be ineffective because numerous diseases produce inappetence in fish (Austin, 2017). Antimicrobials may be also added directly to the water. The extensive use of antimicrobial compounds has led to the emergence and spread of antimicrobial-resistant bacteria in the environments and the transfer of resistance to human and veterinary pathogens, the accumulation of tissue residues and their implications for human health (Austin, 2017; Romero et al., 2012; Miller and Harbottle, 2018). Moreover, administration of drugs to the water together with the drugs not metabolized and excreted by the fish result in environmental disturbance of the microbiota

(Assefa and Abunna, 2018; Romero et al., 2012). For all these reasons, the use of antibiotics in aquaculture is no more a primary treatment option.

In the last years there have been several reports about the isolation of fish pathogens resistant to different antimicrobials as *A. salmonicida*, *A. hydrophila*, *E. ictaluri*, *E. tarda*, *Y. ruckeri*, *P. damselae* subsp. *piscicida*, *F. pyschrophilum*, *Streptococcus* sp. or *V. anguillarum* (Adams et al., 1998; Akinbowale et al., 2006; Giraud et al., 2018; Michel et al., 2003; Miller and Harbottle, 2018; Osman et al., 2017; Rhodes et al., 2000; Trudel et al., 2016).

Quinolones (oxolinic acid, flumequine, and enrofloxacin…), tetracyclines (oxytetracycline) and phenicols (florfenicol, chloramphenicol…) have been reported to be selective for a variety of antimicrobial resistance genes which tend to be present in mobile genetic elements that favour their dissemination as transposons, plasmids, or integrons (Miller and Harbottle, 2018; Miranda et al., 2013). Quinolone resistance is due to chromosomal mutations in topoisomerases genes (*gyrA*, *gyrB*, *parC* and *parE*) that reduce drug accumulation by decreasing uptake or increasing efflux (Ruiz, 2003). However different plasmid-associated quinolone resistance mechanisms have been also identified (Jacoby, 2005). Different point mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and/or *parC* genes have been detected in quinolone-resistant strains of *V. anguillarum*, *A. salmonicida* or *F. psychrophilum* (Alcaide et al., 2010; Avendano-Herrera et al., 2004; Kim et al., 2011; Miranda et al., 2013; Rodkhum et al., 2008). Tetracycline resistance has been found to be mainly mediated by one or more of the *Tet* family of proton-dependent efflux pumps or via ribosomal protection by cytoplasmic proteins found in Gram-negative bacteria (Miranda et al., 2013). The spread of *tet* genes in explained by their location on plasmids and transposons (Chopra and Roberts, 2001). Several *tet* determinants (*tet(A)*, *tet(B)*, *tet(D)*, *tet(E)*, *tet(G)*, *tet(H)*, *tet(L)*, *tet(M)*,

tet(O), *tet(Q)*, *tet(S)*, and *tet(W)*, *tet(34)*, and *tet(35)* have been found in fish pathogens (*A. salmonicida*, *E. tarda*; *Vibrio* sp.) from different geographical areas and fish species (Akinbowale et al., 2006; Kim et al., 2011; Miranda et al., 2003; Rhodes et al., 2000; Schmidt et al., 2001; Trudel et al., 2016). Mechanisms of resistance to florfenicol or chloramphenicol include specific and non-specific drug transporters, RNA methyltransferases and specific hydrolases (Schwarz et al., 2004). The chloramphenicol resistance mechanism more frequently found is enzymatic inactivation of the drug via chloramphenicol acetyltransferases (CATs) (Schwarz et al., 2004). Most studies of florfenicol resistance have reported the occurrence of the *florR* gene, which code for efflux proteins that export florfenicol out of the cell (Schwarz et al., 2004). Several authors have reported the occurrence of strains of the species *A. salmonicida*, *A. hydrophila* or *E. tarda* carrying plasmids harbouring *florR* and *cat* that could be transferable to other pathogens (Miranda et al., 2013).

1.3.4. Probiotics

Probiotics are live microorganisms not pathogenic for fish that when administered to host in adequate amounts confer a health benefit on the host (FAO and WHO, 2001). For selection of probiotic strains there are several criteria that must be considered as: host origin, the safety of the strain, production of antimicrobial substances, resistance to commercially available antimicrobials, ability to stimulate host immune response, or efficient competition with pathogens for intestinal mucosa adhesion sites, absence of virulence factors or production of toxins (FAO, 2016; Muñoz-Atienza, 2015; Assefa and Abunna, 2018). Probiotics for aquaculture exert a beneficial effect on the host through: i) the modification of the microbiota of the fish and the environment; ii) the increase in the efficiency of the assimilation of the feed and its nutritional value; iii) the increase in the resistance of the fish host against

infectious diseases and iv) the improve in the quality of the aquatic environment in which the host is developed (Verschuere et al., 2000). During the last years, several authors have reported the ability of some probiotic bacteria to inhibit the growth of bacterial fish pathogens. For instance, Korkea-aho et al. (2011) demonstrated the *in vitro* antimicrobial activity of *Pseudomonas* M174 against *F. psychrophilum*. Moreover, rainbow trout fed with *Pseudomonas* M174 and challenged with *F. psychrophilum* showed lower levels of mortality than control fish and presented stimulation of some parameters of the immune system. Similarly, Burbank et al. (2012) also isolated bacterial probiotic candidates belonging to the genus *Aeromonas*, *Citrobacter*, *Lysinibacillus*, *Staphylococcus* or *Enterobacter*, from the gastrointestinal tract of rainbow trout that showed inhibitory activity against *F. psychrophilum*. Later, LaPatra et al. (2014) reported stimulation of the innate and adaptative immunity and induction of protection against *F. psychrophilum* after injection with a probiotic *Enterobacter* sp. Muñoz-Atienza et al. (2014) also reported the antimicrobial activity of lactic acid bacteria against strains of *T. maritimum* and *V. splendidus* and the stimulation of immunity-related genes in turbot when administered probiotics by bath. Overall, probiotics represent a new prophylactic strategy in human and veterinary medicine, including fish, molluscs and crustaceans, being considered as an alternative for the control of pathologies in aquaculture.

1.3.5. Plant-based methods

The use of plant products for aquaculture disease management is a more sustainable alternative to chemotherapy in aquaculture. The steps for the use of medicinal plants in aquaculture are shown in Figure 11. Plants possess a complex chemical composition which displays varied biological activities which make plants suitable for the treatment of multifactorial diseases. Therapies based on plant-products are cost effective, eco-friendly and possess

little risk for development of resistance due to the high diversity of plant extract molecules (Awad and Awaad et al., 2017; Harikrishnan et al., 2011; Srivastava et al., 2014; Reverter et al., 2017). Many products from the plant origin have been reported to have various properties like anti-stress, growth promotion, appetite stimulation, immune system enhancement, and to have antibacterial, antiviral and anti-parasitic (protozoans, monogeneans) properties in fish and shrimp aquaculture (Awad and Awaad et al., 2017; Harikrishnan et al., 2011; Reverter et al., 2017). These activities are due to the presence of various bioactive compounds such as phenols, sulphur, terpenoids, alkaloids, flavonoids, and saponins and essential oils (Harikrishnan et al., 2011; Jana et al., 2018; Sivaram et al., 2004).

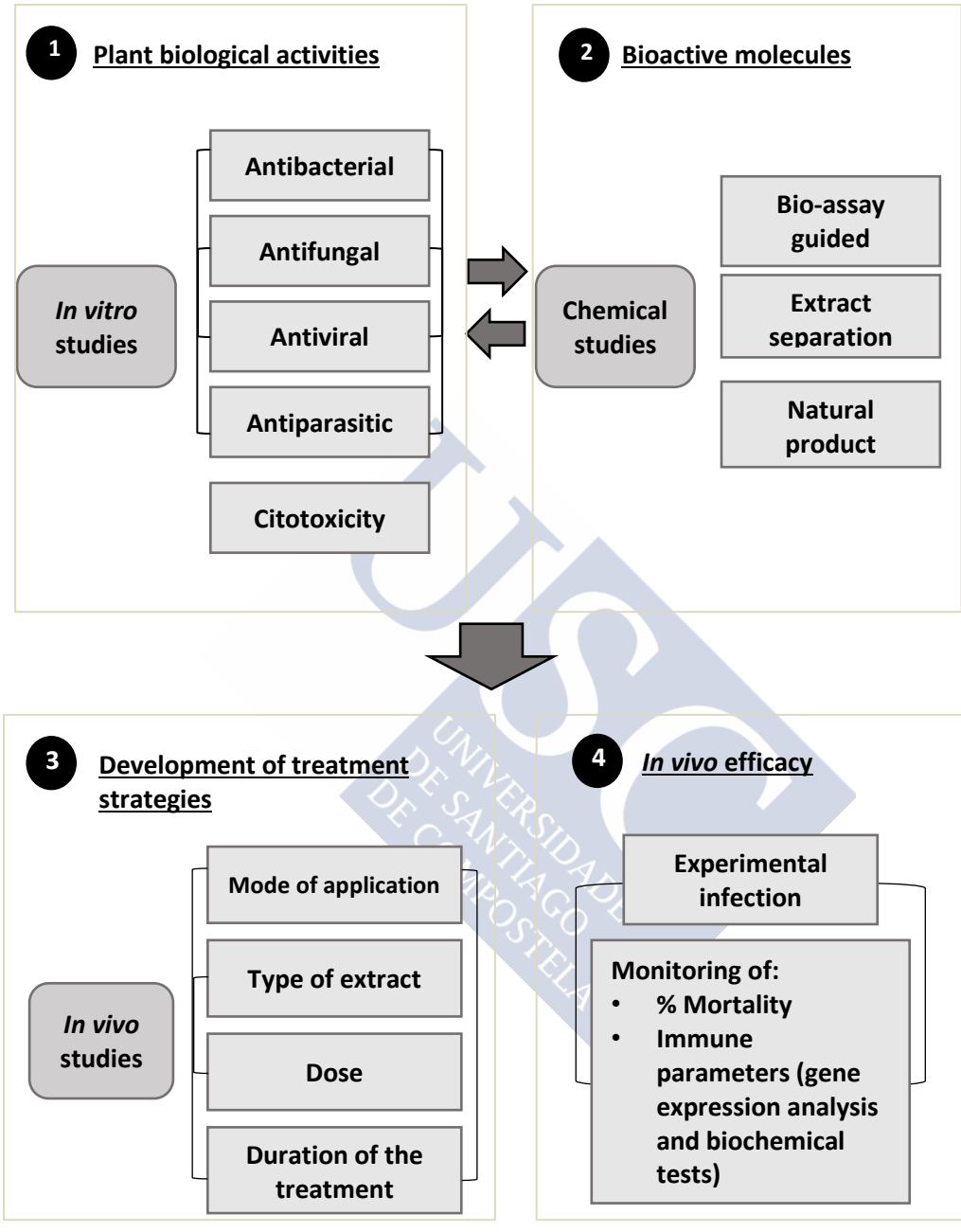

Figure 11. Diagram of the steps for the use of medicinal plants in aquaculture. [Modified from Reverter et al. (2017)]

Several authors have shown the potential of medicinal plants against a wide range of marine pathogens. Antibacterial properties of medicinal plants have been widely studied in fish pathogens. Plant-based compounds exhibit antibacterial activies by different mechanisms including: the degradation of the cell wall, the damage of the cytoplasmatic membrane and the membrane proteins, the increase of the permeability of the membrane, the reduction in the intracellular ATP pool, the inhibition of the enzyme secretions or the interference with the cell signalling mechanisms of quorum sensing pathway (Chouhan et al., 2017; Nazzaro et al., 2013; Szabó et al., 2010; Yang et al., 2015). Abundant *in vitro* studies reporting antibacterial activity of numerous plants against both Gram-stain-negative and positive bacteria have been published (Hammer et al., 1999). For instance, the inhibitory effect of several herbal plants including garlic (*Allium sativum*), mentha (*Mentha piperita*), green tea (*Camellia sinensis*), fennel (*Foeniculum vulgare*), eucalyptus (*Eucalyptus camaldulensis*), sweet orange (*Citrus sinensis*), thyme (*Thymus vulgaris*), *Litsea cubeba*, ginger (*Zingiber oficinale*) or aloe vera (*Aloe barbadensis*) has been demonstrated (Ahmad et al., 2011; Ankri and Mirelman, 1999; Awad and Awaad et al., 2017; Bulfon et al., 2014; Chouhan et al., 2017; Debbarma et al., 2012; Hammer et al., 1999; Harikrishnan et al., 2011; Nguyen et al., 2016; Sutili et al., 2015; Thanigaivel et al., 2015).

Plant-extracts can be administrated to fish through oral (dietary administration) or injection (intraperitoneal or intramuscular) route (Awad and Awaad et al., 2017; Harikrishnan et al., 2011). To date, the positive effects of the administration of several plants have been observed in the resistance of fish against bacterial diseases and in the immune system and growth performance of fish and shrimp (Adel et al., 2016; Ahmad et al., 2011; Awad and Awaad et al., 2017; Devakumar and Chinnasamy, 2017; Harikrishnan et al., 2011; Heidarieh et al., 2013; Navarrete et al., 2010; Nguyen et al., 2016; Nya and Austin, 2009,

2011; Sivaram et al., 2004; Thanigaivel et al., 2015). Medicinal plants and their products can act as immunostimulants modulating the immune response by enhancing both the specific and non-specific defense mechanisms of fish (Figure 12). Thus, disease outbreaks in commercial fisheries may be controlled by the enhancement of innate immunity through the application of natural immunostimulants.

The immune-protective effect of plant-based products is produced through the enhancement of innate immune parameters as lysozyme, complement, antiprotease, reactive oxygen species, reactive nitrogen species, phagocytosis, respiratory burst activity, total haemocytes and adaptative immune parameters as antibody titre, bactericidal activity, hemagglutination, neutrophil against bacterial, fungal, viral and parasitic diseases (Figure 12) (Ardó et al., 2008; Awad and Awaad et al., 2017; Baba et al., 2016; Harikrishnan et al., 2011; Nya and Austin, 2011; Sivaram et al., 2004; Yogeshwari et al., 2015).

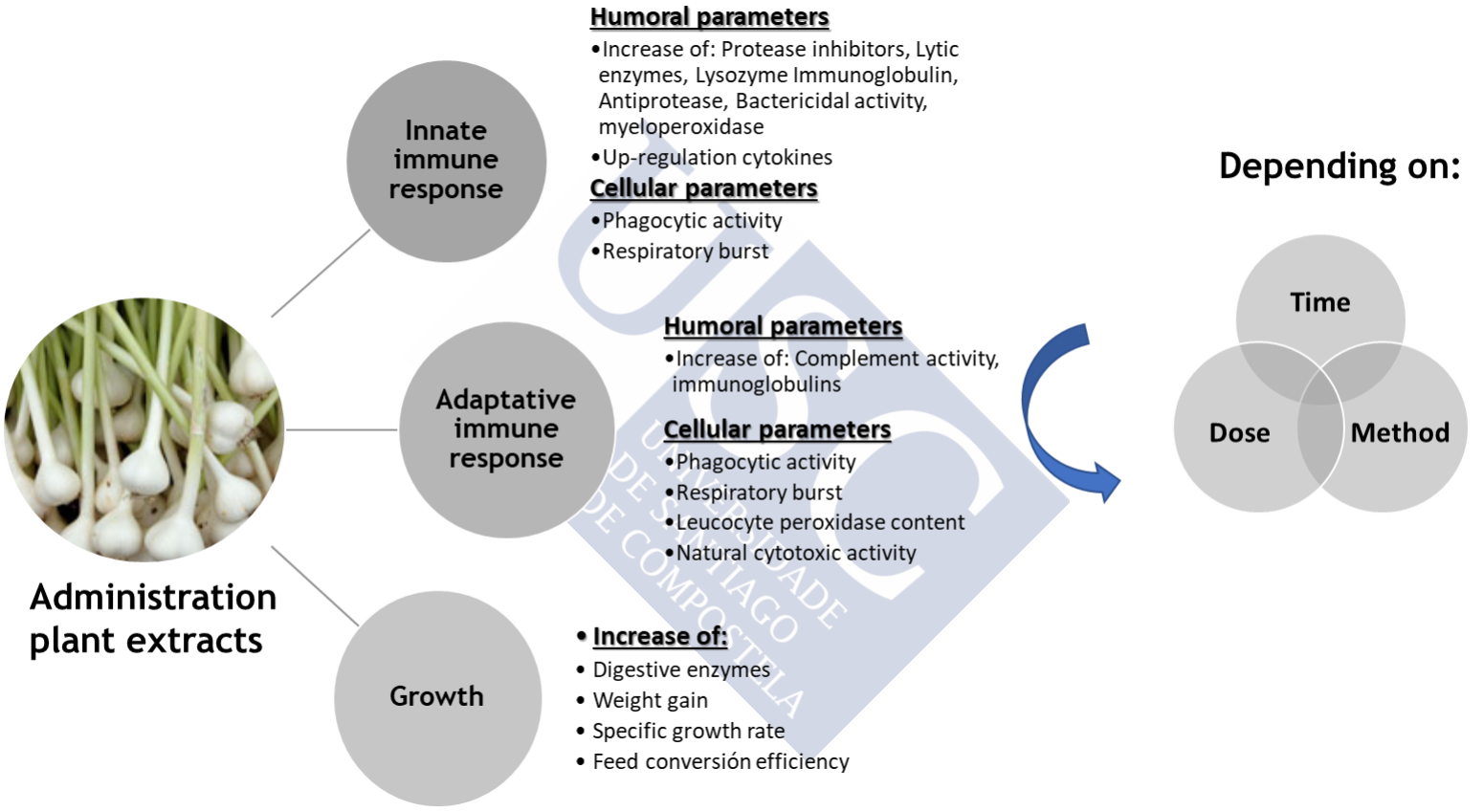

Figure 12. Mode of action of medicinal plants and their products on the modulation of the specific and non-specific defense mechanisms of fish

Several authors have studied the modulation of the immune system of fish using *in vitro* and *in vivo* studies (Harikrishnan et al., 2011). In this sense, extracts derived from *Ocimum sanctum* positively enhanced the antibody response and disease resistance in *Oreochromis mossambicus* against *A. hydrophila* (Logambal et al., 2000). *Allium sativum* enriched diets also increased the serum bactericidal activity, phagocytosis, haematocrit, respiratory burst, lysozyme and protease activities in rohu *Labeo rohita*, rainbow trout *O. mykiss* and Nile tilapia *Oreochromis niloticus* and increased resistance against infections produced by *A. hydrophila* (Aly and Mohamed, 2010; Nya and Austin, 2011, 2009; Sahu et al., 2007). Administration of diets supplemented with Siberian gingseng exhibited improved non-specific immunity (phagocytic activity and lysozyme activity in the serum and mucus) and also showed increased resistance to *E. tarda* and *V. anguillarum* infections (Won et al., 2008). The effect of the administration of these immunostimulants in aquaculture depend on varios parameters as time, dosage, method of administration and the physiological conditions of the fish (Harikrishnan et al., 2011). Thus, these factors need to be taken into account for and effective use of these plant-based compounds. The research on plant-based compounds to prevent and control fish diseases has increased to identify and develop safe dietary supplements that enhance the life activity, health, and immune systems of farm fish (Harikrishnan et al., 2011; Sivaram et al., 2004; Yogeshwari et al., 2015).

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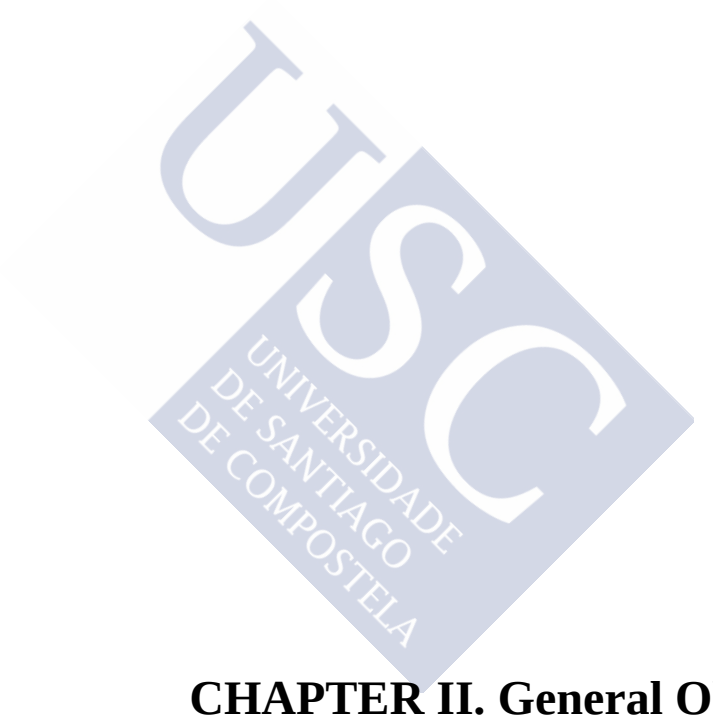

CHAPTER II. General Objectives

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Infectious diseases are a primary constraint to aquaculture production, being responsible for severe losses, impeding both economic and socio-economic development in many countries in the world. The expansion and diversification of the aquaculture industry based on movements of live aquatic animals and animal products have accelerated the accidental spread and incursion of diseases into new populations and geographic areas. Some of the most threatening bacterial diseases caused by Gram-stain-negative pathogens occurring in fish cultured in marine and continental farming worldwide include furunculosis, flexibacteriosis, flavobacteriosis and vibriosis. The development of rapid and sensitive diagnostic protocols for the detection of the microorganism in diseased and carrier fish and farm waters is crucial for the establishment of effective control measures that reduce or eliminate the impact of the infectious disease in farmed fish. The study of the proteomic, genetic or serological diversity (intra- and inter- species) of fish pathogens is also of great importance when considering targeted prevention and control strategies. The systematic application of antimicrobials for treating diseased fish as well as prophylactic measure has prompted in the appearance of bacterial strains resistant to these compounds. This worrying situation has led to the development of new eco-friendly alternatives to prevent and control bacterial disease outbreaks without selecting resistance genes. This PhD work has focused on resolving the current concerns of bacterial fish diseases produced by *Tenacibaculum* sp., *Aeromonas* sp., *Flavobacterium* sp. and *Vibrio* sp. The specific objectives of this work were:

1. Determination of the taxonomic position and study of the virulence of Gram-stain-negative bacteria causing diseases in farmed fish species.

2. Development of highly sensitive molecular methods for the detection and identification of fish pathogens and its differentiation from genetically closely related species.

3. Evaluation of proteomic, serological and molecular methods for the typing of fish pathogenic bacteria and for epidemiological studies.

4. Phenotypic and molecular characterization of the resistance to antimicrobials commonly used in aquaculture of fish pathogenic bacteria.

5. Evaluation of the antimicrobial activity of natural and chemical compounds and their effect on the resistance of fish against bacterial diseases of great impact in the aquaculture sector.

To carry out these objectives the following reference strains (Table 1) obtained from culture collections and clinical strains of *Tenacibaculum* spp. *Aeromonas salmonicida*, *Flavobacterium sp.* and *Vibrio anguillarum* isolated from clinical specimens (Tables 2, 3, 4 and 5) were used:

Table 1. Reference strains used in the present work

Strain	Species	Origin
ATCC 33658	*A. salmonicida* subsp. *salmonicida*	*Salmo salar*, USA
ATCC 14174	*A. salmonicida* subsp. *salmonicida*	*Salvelinus fontinalis*, USA
NCIMB 2261	*A. salmonicida* subsp. *salmonicida*	*S. salar*, Scotland
CECT 895	*A. salmonicida* subsp. *achromogenes*	*Salmo trutta*, USA
CECT 4238	*A. salmonicida* subsp. *achromogenes*	*S. trutta*, Scotland
CECT 4239	*A. salmonicida* subsp. *achromogenes*	*Phoxinus phoxinus*, Norway
CECT 896	*A. salmonicida* subsp. *masoucida*	*Oncorhynchus masou*, Japan
CECT 5179	*A. salmonicida* subsp. *smithia*	*Rutilus rutilus*, UK
CECT 5752	*A. salmonicida* subsp. *pectinolytica*	Water from a cistern, Spain
CECT 5753	*A. salmonicida* subsp. *pectinolytica*	Water of a river, Argentina
CECT 4330	*Aeromonas hydrophila*	Water from an eel farm, Spain
CECT 839	*Aeromonas hydrophila*	Milk with fishy odour, Unknown
CECT 4222	*Aeromonas caviae*	Sewage, Unknown
CECT 837	*Aeromonas sobria*	Fish, France
CECT 4227	*Aeromonas bestiarum*	Fish, France
CECT 7443	*Aeromonas piscicola*	*Salmo salar*, Spain
CECT 7444	*Aeromonas piscicola*	*Oncorhynchus mykiss*, Spain
CECT 4232	*Aeromonas media*	Fish farm effluent, UK
CECT 4199	*Aeromonas allosaccharophila*	*Anguilla Anguilla*, Spain
CECT 4995	*Aeromonas popoffii*	Water plant, Belgium
CECT 4255	*Aeromonas trota*	Human stool, India
CECT 4224	*Aeromonas eucrenophila*	Fresh water fish, Germany
CECT 4246	*Aeromonas veronii*	Infected frog, Unknown

CECT 5864	*Aeromonas molluscorum*	*Donax trunculus*, Spain
CECT 4228	*Aeromonas jandeii*	Human faeces, USA
CECT 4342	*Aeromonas encheleia*	European eels, Spain
NCIMB 2034	*Edwarsiella tarda*	Human faeces, USA
CECT 849	*Edwarsiella tarda*	Human faeces, USA
ATCC 43921	*Lactococcus garviae*	Bovine mastitis, Unknown
NCIMB 1900	*Vibrio pelagius*	Seawater, USA
ATCC 25922	*Escherichia coli*	Clinical isolate, Unknown
CECT 899	*Pseudomonas anguilliseptica*	*Anguilla japonica*, Japan
CECT 958	*Streptococcus suis*	Pig, Unknown
NCIMB 2154	*Tenacibaculum maritimum*	*Pagrus major*, Japan
NCIMB 2158	*Tenacibaculum maritimum*	*Solea solea*, UK
CECT 7292	*Tenacibaculum soleae*	*S. senegalensis*, Spain
DSM 18842	*Tenacibaculum discolor*	*S. senegalensis*, Spain
DSM 18841	*Tenacibaculum gallaicum*	Seawater turbot tank, Spain
NCIMB 14598	*Tenacibaculum dicentrarchi*	*Dicentrarchus labrax*, Spain
DSM 18103	*Tenacibaculum ovolyticum*	*Hippoglossus hippoglossus*, Norway
HFJ^T	*Tenacibaculum finnmarkense*	*S. salar*, Norway
NCIMB 13384	*Flavobacterium psychrophilum*	*O. mykiss*, Denmark
NCIMB 13383	*Flavobacterium psychrophilum*	*O. mykiss*, Denmark
NCIMB 1947	*Flavobacterium psychrophilum*	*Oncorhynchus kisutch*, USA
NCIMB2282	*Flavobacterium psychrophilum*	*Oncorhynchus kisutch*, USA
ATCC 35035	*Flavobacterium branchiophilum*	*Oncorhynchy masou*, Japan
NCIMB12056	*Flavobacterium flevense*	Water, Netherlands
NCIMB 2277	*Flavobacterium succinicans*	*O. tshawytscha*, USA
CECT 7796	*Flavobacterium collinsi*	*O. mykiss*, Spain
CECT 7844	*Flavobacterium plurextorum*	Eggs of rainbow trout; Spain
CECT 7678	*Flavobacterium oncorhynchi*	*O. mykiss*, Spain
CECT 7909	*Flavobacterium piscis*	*O. mykiss*, Spain
CECT 5015	*Flavobacterium johnsonieae*	Grass, UK
DSM 24789	*Flavobacterium branchiophilum*	*O. masou*, Japan

CECT 7791	*Flavobacterium tructae*	*O. mykiss*, Spain
ATCC 43305	*Vibrio anguillarum*	*O. mykiss*, Denmark
ATCC 14181	*V. anguillarum*	*Salmo trutta*, USA
ATCC 43306	*V. anguillarum*	*Gadus morhua*, Denmark
ATCC 43307	*V. anguillarum*	*O. mykiss*, Denmark
ATCC 43308	*V. anguillarum*	*G. morhua*, Denmark
ATCC 43310	*V. anguillarum*	*G. morhua*, Denmark
ATCC 43311	*V. anguillarum*	*A. anguilla*, Denmark
ATCC 43312	*V. anguillarum*	*G. morhua*, Denmark
ATCC 43313	*V. anguillarum*	*G. morhua*, Denmark
ATCC 43314	*V. anguillarum*	*G. morhua*, Denmark
CECT 8573	*Lacinutrix venerupis*	*Ruditapes decussatus*, Spain
ATCC 33209	*Renibacterium salmoninarum*	*Oncorhynchus tshawytscha*, USA
DSM 6631	*Streptococcus parauberis*	Milk, Unknown
CECT 7363	*Streptococcus iniae*	*Inia geoffrensis*, Unknown
CECT 5810	*Vagococcus salmoninarum*	*O. mykiss*, Australia
CECT 521	*Vibrio alginolyticus*	*Trachurus trachurus*, Japan
CECT 630	*Vibrio proteolyticus*	*Limnoria tripunctata*
CECT 627	*Vibrio diazotrophicus*	*Strongylocentrotus dreobrachiensis*, Canada
CECT 525	*Vibrio harveyi*	*Talorchestia* sp., USA
CECT 4638	*Vibrio slophthalmi*	*Scophthalmus maximus*, Spain
CECT 511	*Vibrio parahaemolyticus*	Human, Japan
CECT 526	*Vibrio natriegens*	Marsh mud, USA
CECT 631	*Vibrio tubiashii*	*Mercenaria mercenaria*, USA
CECT 955	*Yersinia ruckeri*	*Salmo gairdneri*, USA

ATCC American Type Culture Collection (USA), CECT Spanish Type Culture Collection (Spain), DSM Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany), NCIMB National Collection of Industrial and Marine Bacteria (UK).

Table 2. Clinical isolates of *Aeromonas salmonicida* used in the present work

Strain	Species	Origin	Year
AsV09.1.15	*A. salmonicida* subsp. *salmonicida*	*Oncorhynchus mykiss*, Spain	2015
AsV09.2.15	*A. salmonicida* subsp. *salmonicida*	*O. mykiss*, Spain	2015
AsV09.3.15	*A. salmonicida* subsp. *salmonicida*	*O. mykiss*, Spain	2015
AsV09.4.15	*A. salmonicida* subsp. *salmonicida*	*O. mykiss*, Spain	2015
IAs57.1	*A. salmonicida* subsp. *salmonicida*	*Scophthalmus maximus*, Spain	2001
1.50	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	ND
ClAS08.4	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2008
L3R	*A. salmonicida* subsp. *salmonicida*	*Petromyzon marinus*, Spain	2000
L9R1	*A. salmonicida* subsp. *salmonicida*	*P. marinus*, Spain	2000
L9R2	*A. salmonicida* subsp. *salmonicida*	*P. marinus*, Spain	2000
L3S1	*A. salmonicida* subsp. *salmonicida*	*P. marinus*, Spain	2000
L10S1	*A. salmonicida* subsp. *salmonicida*	*P marinus*, Spain	2000
IAs06.1	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2006
IAs06.3	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2006
IAs06.5	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2006
IAs07.1	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
IAs07.7	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
IAs07.13	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
IAs07.17	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
IAs07.20	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
IAs07.24	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
IAs07.26	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2007
SK112/06	*A. salmonicida* subsp. *salmonicida*	*Salmo salar*, Spain	2006
TAC7.2.1	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2001
SK366/09	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2009
SK267/09	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2009
SK181/12	*A. salmonicida* subsp. *salmonicida*	*D. labrax*, Spain	2012
SK164/12	*A. salmonicida* subsp. *salmonicida*	*D.labrax*, Spain	2012

SK50/17 47	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2017
SK37/18 TK5	*A. salmonicida* subsp. *salmonicida*	*Sparus aurata*, Spain	2018
SK37/18 TK7	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK37/18 TK25	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK37/18 Abs	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK43/18	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK43/18 (2)	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK23/18 L-1	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK23/18 L-5	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK23/18	*A. salmonicida* subsp. *salmonicida*	*S. aurata*, Spain	2018
SK152/18	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Spain	2018
SK19/08	*A. salmonicida* subsp. *salmonicida*	*Salmo trutta*, Spain	2008
MT416	*A. salmonicida* subsp. *salmonicida*	*S. salar*, Scotland	ND
MT004	*A. salmonicida* subsp. *salmonicida*	*S. salar*, Scotland	1980
T096 7.1	*A. salmonicida* subsp. *salmonicida*	*S. maximus*, Portugal	ND

ND, Not determined

Table 3. Clinical isolates of *Tenacibaculum* used in the present work

Strain	Species	Origin	Year
	***Tenacibaculum maritimum* strains**		
Lyl-1	*T. maritimum*	*Pagrus major*, Japan	1985
FCP394	*T. maritimum*	*P. major*, Japan	ND
GBF 8601	*T. maritimum*	*Paralychthys olivaceous*, Japan	1986
DBA 4a	*T. maritimum*	*Seriola quinqueradiata*, Japan	1986
LL01 8.3.8	*T. maritimum*	*Solea senegalensis*, Spain	2001
JIP10/97	*T. maritimum*	*Scophthalmus maximus*, France	1997
JIP31/99	*T. maritimum*	*S. maximus*, France	1999
Tm Chile	*T. maritimum*	*S. maximus*, Chile	1998
LPV7.1	*T. maritimum*	*S. maximus*, Spain	1995
LC11.8.16.1	*T. maritimum*	*S. senegalensis*, Spain	2011
LL08.5.4.2	*T. maritimum*	*S. senegalensis*, Spain	2008
TM 3.35	*T. maritimum*	*S. maximus*, Spain	ND
LC12.10.1.2.	*T. maritimum*	*S. senegalensis*, Spain	2012
LC07.10.1.2	*T. maritimum*	*S. senegalensis*, Spain	2007
RQ07.10.7.12.1	*T. maritimum*	*S. maximus*, Spain	2007
RQ07.8.2.2	*T. maritimum*	*S. maximus*, Spain	2007
RQ07.7.3.1	*T. maritimum*	*S. maximus*, Spain	2007
RQ06.9.1.1	*T. maritimum*	*S. maximus*, Spain	2006
Itm07.3	*T. maritimum*	*S. maximus*, Spain	2007
Itm07.5	*T. maritimum*	*S. maximus*, Spain	2007
Itm06.4	*T. maritimum*	*S. maximus*, Spain	2006
Bx09.8.17.3	*T. maritimum*	*S. senegalensis*, Spain	2009
TM 3.77	*T. maritimum*	*S. senegalensis*, Spain	ND
LL05.6.1.2	*T. maritimum*	*S. senegalensis*, Spain	2005
ELM06.3.1	*T. maritimum*	*S. senegalensis*, Spain	2006
ELM8.6.8.12	*T. maritimum*	*S. senegalensis*, Spain	2008
P2R	*T. maritimum*	*Solea solea*, Spain	ND
TM 3.46	*T. maritimum*	*S. solea*, Spain	ND
TM 3.3	*T. maritimum*	*S. maximus*, Spain	ND
ELL07.1.1.1	*T. maritimum*	*S. senegalensis*, Spain	2007

ELL07.1.1.3	*T. maritimum*	*S. senegalensis*, Spain	2007
RL06.6.5.1	*T. maritimum*	*S. senegalensis*, Spain	2006
LC10.10.2.2	*T. maritimum*	*S. senegalensis*, Spain	2010
LC10.10.1.3	*T. maritimum*	*S. senegalensis*, Spain	2010
LC10.11.9.1	*T. maritimum*	*S. senegalensis*, Spain	2010
LC11.8.16.1	*T. maritimum*	*S. senegalensis*, Spain	2011
LC12.11.2.2	*T. maritimum*	*S. senegalensis*, Spain	2012
RV12.11.1.1	*T. maritimum*	*S. maximus*, Spain	2012
RV06.10.2.1	*T. maritimum*	*S. maximus*, Spain	2006
RV07.7.6.1	*T. maritimum*	*S. maximus*, Spain	2007
ELM06 8.3.1	*T. maritimum*	*S. senegalensis*, Spain	2006
ELM06.8.4.1	*T. maritimum*	*S. senegalensis*, Spain	2006
ELM068.5.1	*T. maritimum*	*S. senegalensis*, Spain	2006
ELM06 3.3.1	*T. maritimum*	*S. senegalensis*, Spain	2006
ELM06 3.4.1	*T. maritimum*	*S. senegalensis*, Spain	2006
ELM06 3.5.1	*T. maritimum*	*S. senegalensis*, Spain	2006
	***Tenacibaculum soleae* strains**		
TS11	*T. soleae*	*S. rhombus*, Spain	2006
567/06.1	*T. soleae*	*D. labrax*, Spain	2006
567/06.2	*T. soleae*	*D. labrax*, Spain	2006
567/11	*T. soleae*	*D. labrax*, Spain	2011
TS50	*T. soleae*	*S. rhombus*, Spain	2004
TS216	*T. soleae*	*Dicologoglossa cuneata*, Spain	2006
CLT07.1	*T. soleae*	*S. maximus*, Spain	2007
TS410	*T. soleae*	*S. senegalensis*, Spain	2006
TS462	*T. soleae*	*D. cuneate*, Spain	2006
TS467	*T. soleae*	*D. cuneate*, Spain	2006
TS469	*T. soleae*	*S. senegalensis*, Spain	2006
TS3	*T. soleae*	ND	ND
TS47	*T. soleae*	*S. rhombus*, Spain	2004
	***Tenacibaculum discolor* strains**		
Td3.88	*T. discolor*	*S. maximus*, Spain	2003
TdA35.2	*T. discolor*	Seawater from turbot tanks, Spain	2003

TdA36.1	*T. discolor*	Seawater from turbot tanks, Spain	2003
TdRI45.1	*T. discolor*	*S. maximus*, Spain	ND
TdR016	*T. discolor*	*S. maximus*, Spain	ND
TdA36.9	*T. discolor*	Seawater from turbot tanks, Spain	2003
LC06 11.5.1	*T. discolor*	*S. senegalensis*, Spain	2006
Td3.31	*T. discolor*	*S. maximus*, Spain	2003
	***Tenacibaculum gallaicum* strains**		
TgA13.3	*T. gallaicum*	Seawater from turbot tank, Spain	2003
TgR006.0.1	*T. gallaicum*	*S. maximus*, Spain	2000
TgA35.1	*T. gallaicum*	Seawater from turbot tank, Spain	2003
TgA38.1	*T. gallaicum*	Seawater from turbot tank, Spain	2003

ND, Not determined

Table 4. Clinical isolates of *Flavobacterium* used in the present work

Strain	Species	Origin	Year
	***Flavobacterium psychrophilum* strains**		
TW104/13	*F. psychrophilum*	*Salmo salar*, UK	2013
TW105/06	*F. psychrophilum*	*S. salar*, UK	2006
TW105/13	*F. psychrophilum*	*S. salar*, UK	2013
TW131/13	*F. psychrophilum*	*S. salar*, UK	2013
TW132/13	*F. psychrophilum*	*S. salar*, UK	2013
TW135/13	*F. psychrophilum*	*S. salar*, UK	2013
TW139/06	*F. psychrophilum*	*S. salar*, UK	2006
TW140/13	*F. psychrophilum*	*S. salar*, UK	2013
TW199.06	*F. psychrophilum*	*S. salar*, UK	2006
TW29/13	*F. psychrophilum*	*S. salar*, UK	2013
TW30/13	*F. psychrophilum*	*S. salar*, UK	2013
TW54/14	*F. psychrophilum*	*S. salar*, UK	2014
TW77/12	*F. psychrophilum*	*S. salar*, UK	2012
TW84/13	*F. psychrophilum*	*S. salar*, UK	2013
TW91/13	*F. psychrophilum*	*S. salar*, UK	2013
TW94/12	*F. psychrophilum*	*S. salar*, UK	2012
TW69/15	*F. psychrophilum*	*S. salar*, UK	2015
TW72/15	*F. psychrophilum*	*S. salar*, UK	2015
03-398-1	*F. psychrophilum*	*Oncorhynchus kisutch*, USA	2003
03-449-5	*F. psychrophilum*	*O. kisutch*, USA	2003
CSF259/93	*F. psychrophilum*	*Oncorhynchus mykiss*, USA	1993
1545/09	*F. psychrophilum*	*O. mykiss*, Finland	2009
FEFR04	*F. psychrophilum*	*O. mykiss*, Switzerland	2004
FEFR07	*F. psychrophilum*	*O. mykiss*, Switzerland	2007
JIP09/06	*F. psychrophilum*	*O. mykiss*, France	2006
JIP08/09	*F. psychrophilum*	*O. mykiss*, France	2009
TG28/86	*F. psychrophilum*	*O. mykiss*, France	1986
MT2699	*F. psychrophilum*	*O. mykiss*, Scotland	ND

MT2747	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT2749	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT2853	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT3002	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT3399	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT3529	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT2747	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT2902	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT3399	*F. psychrophilum*	*O. mykiss*, Scotland	ND
MT3900	*F. psychrophilum*	*O. mykiss*, Scotland	ND
389.09	*F. psychrophilum*	Unknown	2009
463.96	*F. psychrophilum*	*O. mykiss*, USA	1996
AA3.1	*F. psychrophilum*	*Anguilla anguilla*, Spain	1993
AFTC P-3	*F. psychrophilum*	*Oncorhynchus tshawytscha*, USA	2000
B8059	*F. psychrophilum*	*O. mykiss*, Spain	2016
F130/03	*F. psychrophilum*	ND	2003
RBR4.04	*F. psychrophilum*	*O. mykiss*, Spain	2004
RBT14.6.16.1	*F. psychrophilum*	*O. mykiss*, Spain	2016
Riobo10.5	*F. psychrophilum*	*O. mykiss*, Spain	2010
229	*F. psychrophilum*	*O. mykiss*, Spain	2000
TROW201	*F. psychrophilum*	*O. mykiss*, Spain	2000
TROW385	*F. psychrophilum*	*O. mykiss*, Spain	2000
EC98-305-5402K	*F. psychrophilum*	*O. kisutch*, USA	1998
YUMBO	*F. psychrophilum*	*O. mykiss*, Spain	2000
FP313/92	*F. psychrophilum*	Unknown	1992
RBR2.04	*F. psychrophilum*	*O. mykiss*, Spain	2004
RBT4.1.04	*F. psychrophilum*	*O. mykiss*, Spain	2004
Riobo1.12	*F. psychrophilum*	*O. mykiss*, Spain	2012
SK127/14	*F. psychrophilum*	*O. mykiss*, Spain	2014
Riobo10.5	*F. psychrophilum*	*O. mykiss*, Spain	2010
RBR03.4	*F. psychrophilum*	*O. mykiss*, Spain	2003

RBR1	*F. psychrophilum*	*O. mykiss*, Spain	2010
RBR1-06	*F. psychrophilum*	*O. mykiss*, Spain	2006
Riobo1	*F. psychrophilum*	*O. mykiss*, Spain	2008
Riobo10.2	*F. psychrophilum*	*O. mykiss*, Spain	2010
Riobo4-04	*F. psychrophilum*	*O. mykiss*, Spain	2004
Riobo10508	*F. psychrophilum*	*O. mykiss*, Spain	2008
Riobo9.13	*F. psychrophilum*	*O. mykiss*, Spain	2013
Other *Flavobacterium* species			
SK320/13	*Flavobacterium sp.*	*O. mykiss*, Spain	2013
SK363/13	*Flavobacterium sp.*	*O. mykiss*, Spain	2013
RBT11.05.16.3	*Flavobacterium sp.*	*O. mykiss*, Spain	2016
Riobo 18.10.12.5	*Flavobacterium sp.*	*O. mykiss*, Spain	2012
Riobo 18.10.13.6	*Flavobacterium sp.*	*O. mykiss*, Spain	2013
ICH29.01.18.1	*Flavobacterium sp.*	*O. mykiss*, Spain	2018
ICH29.01.18.5	*Flavobacterium sp.*	*O. mykiss*, Spain	2018
ICH29.01.18.6	*Flavobacterium sp.*	*O. mykiss*, Spain	2018
F.C.V.	*Flavobacterium sp.*	*O. mykiss*, Spain	2000

ND, not determined

Table 5. Clinical isolates of *Vibrio anguillarum* used in the present work

Strain	Species	Origin	Year
	***Vibrio anguillarum* strains**		
R-82	*V. anguillarum*	*S. maximus*, Spain	1983
I.VA09.07.3	*V. anguillarum*	*S. maximus*, Spain	2009
Ila10.1	*V. anguillarum*	*S. maximus*, Spain	2010
Ila10.3	*V. anguillarum*	*S. maximus*, Spain	2010
Ila10.4	*V. anguillarum*	*S. maximus*, Spain	2010
Ila10.5	*V. anguillarum*	*S. maximus*, Spain	2010
CLa290609	*V. anguillarum*	*S. maximus*, Spain	2009
CUVA16.03.1	*V. anguillarum*	*D. labrax*, Spain	2016
CUVA16.03.2	*V. anguillarum*	*D. labrax*, Spain	2016
CUVA16.03.3	*V. anguillarum*	*D. labrax*, Spain	2016
CUVA16.03.4	*V. anguillarum*	*D. labrax*, Spain	2016
SK606/06	*V. anguillarum*	*S. maximus*, Spain	2006
SK265/14 B8	*V. anguillarum*	Spain	2014
SK43/14	*V. anguillarum*	Spain	2014
SK165/15	*V. anguillarum*	*S. aurata*, Spain	2015
SK198/15	*V. anguillarum*	*D. labrax*, Spain	2015
SK21/15 B9	*V. anguillarum*	*D. labrax*, Spain	2015
SK11/14 J8	*V. anguillarum*	*D. labrax*, Spain	2014
SK11/14 J12	*V. anguillarum*	*D. labrax*, Spain	2014
Ila10.2	*V. anguillarum*	*S. maximus*, Spain	2010

CHAPTER III.

Determination of the taxonomic position and study of the virulence of Gram-stain-negative bacteria causing diseases in farmed fish species.

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Article nº1. Clara Fernández-Álvarez, Daniel Gijón, Mireya Álvarez, Ysabel Santos (2016). First isolation of *Aeromonas salmonicida* subspecies *salmonicida* from diseased sea bass, *Dicentrarchus labrax* (L.), cultured in Spain. *Aquaculture Reports* 4:36-41. DOI: 10.1016/j.aqrep.2016.05.006.

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First isolation of *Aeromonas salmonicida* subspecies *salmonicida* from diseased sea bass, *Dicentrarchus labrax* (L.), cultured in Spain.

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https://www.sciencedirect.com/science/article/pii/S2352513416300461Abstract

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Supplementary Material

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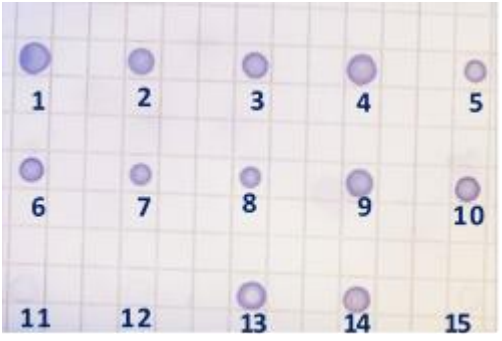

Figure 1S. Dot-Blot analysis of the *A. salmonicida* strains included in this study. Strains: 1, *A. salmonicida* subsp. *salmonicida* SK164/12.1; 2, *A. salmonicida* subsp. *salmonicida* SK164/12.2; 3, *A. salmonicida* subsp. *salmonicida* SK164/12.3; 4, *A. salmonicida* subsp. *salmonicida* SK164/12.4; 5, *A. salmonicida* subsp. *salmonicida* SK181/12.1; 6, *A. salmonicida* subsp. *salmonicida* SK181/12.2; *A. salmonicida* subsp. *salmonicida* SK181/12.3; *A. salmonicida* subsp. *salmonicida* SK181/12.4; *A. salmonicida* subsp. *salmonicida* ATCC33658; *A. salmonicida* subsp. *salmonicida* T096 7.1; 11-12, *A. salmonicida* subsp. *achromogenes* ATCC33659; 13-14, *A. salmonicida* subsp. *masoucida* ATCC27013; 15, *A. salmonicida* subsp. *smithia* ATCC49393.

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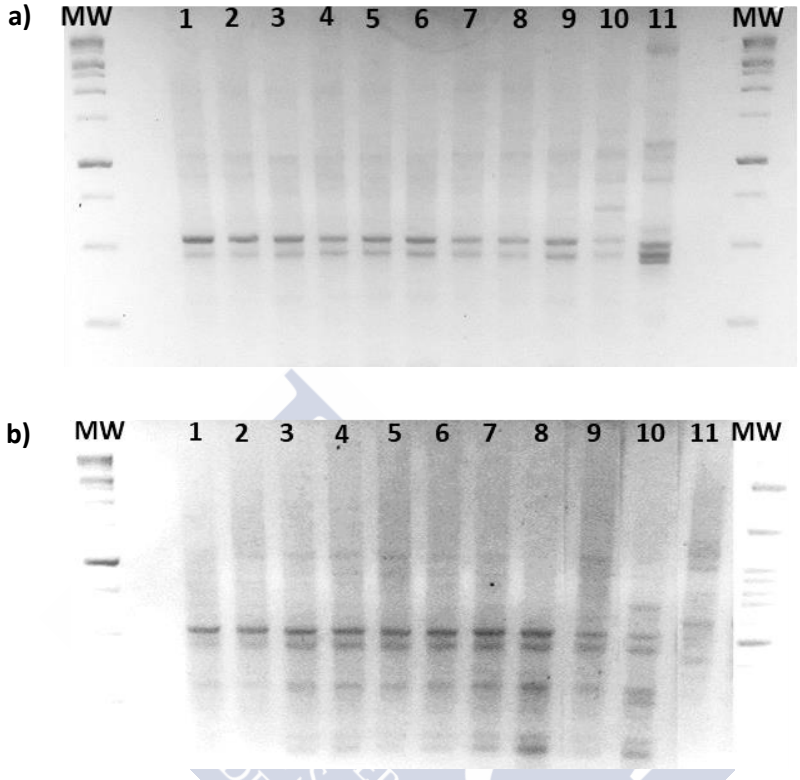

Figure 2S. Genetic profiles obtained from the analysis of typical and atypical *A. salmonicida* strains by ERIC-PCR (a) and REP-PCR (b). Lanes 1, *A. salmonicida* subsp. *salmonicida* SK 164/12.1; 2, *A. salmonicida* subsp. *salmonicida* SK 164/12.2; 3, *A. salmonicida* subsp. *salmonicida* SK 181/12.1; 4, *A. salmonicida* subsp. *salmonicida* SK 181/12.2; 5, *A. salmonicida* subsp. *salmonicida* ATCC33658; 6, *A. salmonicida* subsp. *salmonicida* TO96 7.1; 7, *A. salmonicida* subsp. *salmonicida* MT416; 8, *A. salmonicida* subsp. *salmonicida* AsV09.2.15; 9, *A. salmonicida* subsp. *achromogenes* ATCC33659; 10, *A. salmonicida* subsp. *masoucida* ATCC27013; 11 *A. salmonicida* subsp. *smithia* ATCC49393.

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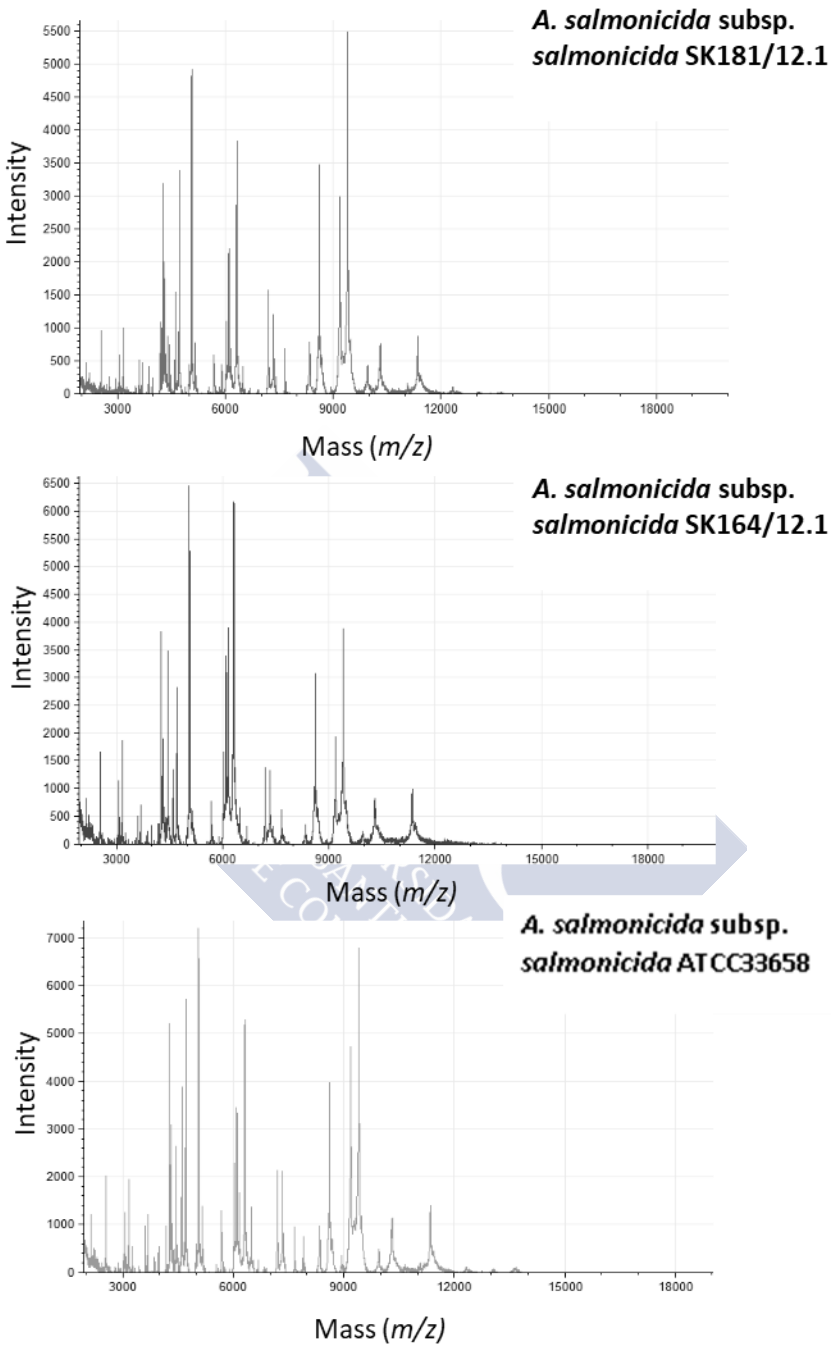

Figure 3S. Proteomic profiles of the strains a) *A. salmonicida* subsp. *salmonicida* ATCC33658, b) *A. salmonicida* subsp. *salmonicida* SK181/12.1 and c) *A. salmonicida* subsp. *salmonicida* SK164/12.1 obtained by MALDI-TOF-MS.

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Article nº2. Clara Fernández-Álvarez, Ysabel Santos (2018). First isolation and characterization of *Lacinutrix venerupis* isolated from *Trachurus trachurus*, an emerging pathogen for wild and cultured fish. Sent for publication in: *Research in Veterinary Science***.**

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First isolation and characterization of *Lacinutrix venerupis* isolated from *Trachurus trachurus*, an emerging pathogen for wild and cultured fish

Clara Fernández-Álvarez and Ysabel Santos

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Abstract

The aim of the present study was to characterize two Gram-stain negative bacterial strains isolated from diseased mackerel *Trachurus trachurus* in 2017. Basing on the results obtained from the biochemical and chemotaxonomic characterization, the isolates were identified as *Lacinutrix* spp. The partial sequencing of the 16S rRNA gene confirmed these results, allowing to identify the strains as *Lacinutrix venerupis*. The highest similarity of the 16S rRNA sequence was obtained with the type strain *L. venerupis* CECT 8573^T (99.1%), while other species showed similarities of 98% (*L. jangbogonensis*) and 97% (*L. algicola or L. mariniflava*). Molecular characterization by REP- and ERIC-PCR and proteomic characterization by MALDI-TOF-MS found genetic heterogeneity between the mackerel strains and the type strain CECT 8573^T. Virulence for turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*) and Senegalese sole (*Solea senegalensis*) of the isolate AqJ1.17 was assessed under experimental conditions. No mortalities were recorded after intra-peritoneal injection with high doses of bacteria ($1x10^9$ CFU/ml). Thus, further studies are necessary to dilucidated the impact of this bacterial species as a fish pathogen.

Keywords: *Lacinutrix venerupis*, *Trachurus trachurus*, characterization, fatty acids, sequencing

1. Introduction

The genus *Lacinutrix* belongs to the Family *Flavobacteriaceae* and was first described and characterized by (Bowman and Nichols, 2005). This genus currently consists of 8 described species including; *Lacinutrix copepodicola* (Bowman and Nichols, 2005), *Lacinutrix algicola* (Nedashkovskaya et al., 2008), *Lacinutrix mariniflava* (Nedashkovskaya et al., 2008), *Lacinutrix himadriensis* (Srinivas et al., 2013), *Lacinutrix jangbogonensis* (Lee et al., 2014), *Lacinutrix undariae* (Park et al., 2015), *Lacinutrix venerupis* (Lasa et al., 2015) and *Lacinutrix chionocetis* (Kim et al., 2017). All these bacterial species are present in marine environments and have been isolated from algae and marine sediments (Lasa et al., 2015), but also have been associated with marine organisms as commensal or opportunistic pathogens. In particular, *L. venerupis* was first isolated from reared healthy clams *Venerupis decussata* and *Venerupis philippinarum* in Spain (Lasa et al., 2015). Lately, López et al. (2017) reported the first isolation of *L. venerupis* associated with disease outbreaks in sea bream *Sparus aurata* and European sea bass *Dicentrarchus labrax* and proved their pathogenic potential for Senegalese sole. However, the importance of this bacterial species as a fish pathogen for other economically important cultured fish species is still unknown.

In the present study, the isolation and characterization of two strains of *Lacinutrix venerupis* isolated in 2017 from diseased mackerel *Trachurus trachurus* is presented. Information about the biochemical, physiological, chemotaxonomic, molecular, and proteomic characteristics as well as a virulence test of the isolates are reported.

2. Materials and Methods

2.1. Collection of fish and bacterial identification

During June 2017 a natural outbreak affected juveniles of *Trachurus trachurus* (12±1 cm body length) captured in the sea and maintained in an Aquarium facility located in the Northwest of Spain. Main clinical signs of diseased fish included eroded tail and fins and haemorrhages in eyes and body surface. Ten fish were randomly collected during the mortality episode to determine the presence or absence of fish pathogens. Gram-stained smears prepared from surface lesions were examined using a light microscope. For bacteriological analysis, samples from liver, kidney and spleen from fish were cultured on Marine Agar (MA), Trypticase Soy Agar supplemented with 1% NaCl (TSA-1); Tiosulphate Citrate Bile Salts Sucrose Agar (TCBS) and *Flexibacter maritimus* medium (FMM) (Pazos et al., 1996) and incubated at 18 ± 1 and 25 ± 1 °C for 24–96 h. Pure bacterial cultures were recovered on FMM agar plates from all fish analysed. Bacteria recovered from fish were transferred onto MA and incubated at 25± 1 during 2-3 days for further characterization. Pure stock cultures of the strains were stored at -30° C in Microbank™ tubes (Prolab Diagnostics, Ontario, Canada).

2.2. Phenotypic characterization

Bacterial isolates were characterized following the minimal standards proposed by Bernardet et al. (2002) using morphological, physiological and biochemical tests as well as fatty acid analysis. The type strain of *Lacinutrix venerupis* CECT 8573^{T} was cultured and biochemically characterized at the same time and conditions with the isolates under study for comparative purposes. The following tests were carried out: Gram staining, oxidase, catalase, cell morphology and motility, oxidation/fermentation test, Voges-Proskauer reaction, indole and utilization of citrate. The production of flexirubin-type

pigment was determined as previously described (Reichenbach et al., 1980). Growth at pH 4-10 (at unit intervals) was assessed in AM broth; pH was adjusted using 1M NaOH and 1 M HCl. The temperature range for growth was determined on MA plates incubated at 4, 18, 25, 32 and 37 ºC for 7 days. Salinity tolerance was tested in nutrient agar broth containing 0.8, 1.0, 3.0, 5.0, 7.0 and 10.0 % (*w/v*) NaCl. Degradation of casein (1 %), gelatine (1 %), starch (1 %), aesculin and Tween 80 (1 %) were evaluated on MA plates (Suzuki et al., 2001). The absence of growth after 1 month of incubation was scored as a negative result. Other enzymic activities were evaluated in the API ZYM system (BioMérieux Madrid, Spain) following the instructions provided by the manufacturer except that sterile seawater was used as the suspension medium and the incubation at 25ºC. Susceptibility to antimicrobials was evaluated by the agar diffusion tests following the procedures described by the Clinical and Laboratory Standards Institute (CLSI, 2006) on FMM agar plates. The antimicrobial agents and the concentrations used were ampicillin (10 µg), O/129 (pteridine, 150 µg), amoxicillin (25 µg), florfenicol (30 µg), flumequine (30 µg), oxytetracycline (30 µg), thrimethoprim-sulfamethoxazole (25 µg) and novobiocin (30 µg). All the antimicrobials were obtained from Oxoid (Thermo Scientific, USA).

For fatty acid analysis, strains AqJ1.17 and AqJ2.17 were grown on MA for 48 h at 25 ºC. Extraction of fatty acid methyl esters were performed according to the standard procedures of the Microbial Identification System (MIDI; Microbial ID) (Sasser, 1990) using the MIDI Sherlock version 6.1 and TSBA6 method (MIDI, 2008).

2.3. PCR analysis

Based on the growth and the morphological characteristics of the bacterium isolated, a disease produced by a flavobacteria was suspected. Thus, DNA from

pure cultures of the strains AqJ1.17 and AqJ2.17 extracted using the InstaGene matrix system (Bio-Rad, California, USA) were subjected to PCR amplification for the detection of *T. maritimum* and *T. soleae* using the specific primers and protocols previously described (García-González et al., 2011). All amplifications were carried out in a Mastercycler Epgradient thermal cycler (Eppendorf, Hamburg, Germany) and PCR products were electrophoresed in a 1% (*w/v*) agarose gel during 1h at 100V, stained with 5µL of Redsafe nucleic acid staining solution (20.000x) (iNtRON Biotechnology) and visualized using an ultraviolet light transilluminator (Bio-Rad UV Transilluminator 2000). Reactions lacking DNA or including DNA from the type strains of *T. soleae* CECT7292 and *T. maritimum* NCIMB2154 were used as negative and positive controls, respectively.

2.4. Sequencing

The complete 16S rRNA sequencing was performed using the universal primers 616V: 5´-AGA GTT TGA TYM TGG CTC AG -3´ and 699R: 5´-RGG GTT GCG CTC GTT -3´ as described by Arahal et al. (2008). The PCR products was submitted to the Spanish Collection of Type Cultures (CECT, Valencia, Spain) for sequencing analysis using an ABI 3730xl sequencer (Applied Biosystems) and the same primers. Nucleotide Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) software was used to retrieve the sequences most closely related to the complete sequence of 16S rRNA gene of the strain AqJ1.17. The partial sequences of the 16S rRNA gene of the strain isolated from *T. trachurus* were aligned and compared with the sequences of other related *Lacinutrix* spp. and flavobacteria using the Clustal Omega software. MEGA version 6.06 was used to build the neighbour-joining (NJ) and maximum-parsimony (MP) tree with 1000 bootstrap replicates. The genetic

distances for the neighbour-joining analysis were calculated using Kimura's two parameter model.

2.5. REP- and ERIC-PCR analysis

The repetitive extragenic palindromic (REP) and the repetitive intergenic consensus sequences (ERIC) were analysed for all the strains included in this study basically as described. The primer sequences REP1 (5′-NNHRCGYCGNCATCMGGC-3′) and REP2 (5′- RCGYCTTATCMGG CCTAC-3′) ERIC1 (5′-ATGTAAGCTCCTGGGGATT CAC-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) described by Versalovic et al. (1991) were used. All the PCR amplifications were performed in a Mastercycler Ep Gradient thermal cycler (Eppendorf, Hamburg, Germany). For REP-PCR an initial denaturation at 95 °C for 7 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min and extension at 65 °C for 8 min and a final extension at 65 °C for 8 min were used. For ERIC-PCR analysis an initial denaturation of 7 min at 95 °C followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 52 °C, extension at 65 °C for 8 min, and a final extension for 8 min at 65 °C were applied. Ten microliters of each PCR product were analysed by gel electrophoresis (100 V for 90 min) on a 1.5% w/v agarose gel in TAE 1× (Tris 0.04 M, EDTA 0.001 M, pH 8) electrophoresis buffer, and stained with RedSafe Nucleic Acid Staining Solution (20,000×) (iNtRON Biotechnology, Seongnam-Si, Korea). A 1-kb DNA ladder (Fermentas, Madrid, Spain) was included as a molecular weight marker. PCR products were visualized using an ultraviolet light transilluminator (UV Transilluminator 2000, Bio-Rad, California, USA). For analysis, all gels were photographed and analysed using the Quantity One software v. 4.6.5 (Bio-Rad, California, USA) for comparison of the REP-PCR and ERIC-PCR profiles within and among the strains under study.

2.6. Proteomic characterization by MALDI-TOF mass spectrometry

Proteins from the mackerel isolates AqJ1.17 and Aq2.17 and the type strain *L. venerupis* CECT8573 were extracted by using formic acid (Sigma-Aldrich, St. Louis, USA) before analysis by MALDI-TOF mass spectrometry, as previously described. Mass spectra raw data were processed using the statistical package for mass spectrometry data analysis MALDIquant (Gibb and Strimmer, 2012). The Mass-Up software (Mass-Up, Vigo, Spain) (López-Fernández et al., 2015) was used for spectra processing and for the detection of the characteristic mass peaks.

2.7. Pathogenicity assay

Fish were obtained from farms located in Galicia (Spain). The assays were carried out at the aquarium facilities of the University of Santiago de Compostela. Pathogenicity assays were carried out by intraperitoneal injection of turbot (average body weight of 27±7 g), sea bass (average body weight of 80±5 g) and Senegalese sole (average body weight of 20±2 g) following the methodology described by Santos et al. (1991). Turbot were reared in separate 100 l tanks, aerated and supplied with sand-filtered seawater at 16±1 ºC. Fish were fed on commercial diet during the experiments. Before challenge experiment, fish were subjected to bacteriological, virologic and parasitological analysis to verify their health status. The strain AqJ1.17 isolated from mackerel was selected for virulence assays. Bacterial suspensions were prepared, adjusted to 10^{10} cells ml^{-1} (McFarland scale tube 3) and serially ten-fold diluted using saline solution (0.9% NaCl). Colony forming units (CFU) were enumerated by the plate dilution method by seeding bacterial cell suspensions onto MA agar plates and counting the bacterial colonies produced. For challenge, fish were anaesthetised by immersion in tricaine methane sulfonate (MS-222, Sigma) (60 mg L^{-1}, Neiffer and Stamper, 2009) and intraperitoneally injected with 0.1 ml

of bacterial suspensions, containing $1x10^{10}$ to $1x10^{5}$ CFU ml^{-1} (10 fish per dose). Infected fish were kept in tanks on the conditions above described. Fish mortalities were considered caused by the inoculated strain, only if the bacterium was recovered in pure culture from liver, kidney and spleen of dead or dying fish.

3. Results

3.1. Phenotypic analysis

Phenotypically, the isolates from mackerel were Gram-stain-negative, non-motile rods, catalase and oxidase positive. Colonies were round and yellow, but flexirubin pigments were not produced. Voges-Proskauer and indole reactions were negative. Strains were positive for arginine di-hydrolase but negative for lysine and ornithine decarboxylases. Hydrolysis of aesculin, Tween 20, tyrosine, and starch were detected but not for casein. Growth occurred between 4 and 32ºC, from 0.5 to 10% NaCl, and from pH 5 to 10. Bacteria did not grow on TSA-1, nutrient agar, or MacConkey agar. The comparative between the phenotypic characteristics of the mackerel isolates and the type strain CECT8573 are showed in Table 1. With the API ZYM system, the strains were positive to phosphatase alkaline, phosphatase acid, naphthol-AS-BI-phosphohydrolase, leucine and valine arylamidase, but activities for all of the enzymes related to the metabolism of carbohydrates are absent.

Table 1. Comparative phenotypic characteristics of the isolates used in this study

Characteristic	***L. venerupis* AqJ1.17**	***L. venerupis* AqJ2.17**	***L. venerupis* CECT8573^T**
Colony form	Round	Round	Round
Colony colour	Yellow	Yellow	Yellow
Gram	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Motility	-	-	-
Flexirrubin pigment production	-	-	-
Voges-Proskauer	-	-	-
Indole	-	-	-
Congo red	-	-	-
Growth at			
4-32ºC	+	+	+
37ºC	-	-	-
pH4	-	-	-
pH5 – pH10	+	+	+
pH11	-	-	-
0.5-5% NaCl	+	+	+
5-10%	-	-	+
Trypticase soy agar	-	-	-
Nutrient agar	-	-	-
Marine agar	+	+	+
MacConkey agar	-	-	-
Hydrolysis of			
Aesculin	+	+	+
Casein	-	-	-
L-tyrosine	+	+	+
Starch	+	+	-
Tween 20	+	+	+
Arginine decarboxylase	+	+	+
Lysine decarboxylase	-	-	-
Ornithine decarboxylase	-	-	-

+, positive result; -, negative result.

The antimicrobial susceptibility tests showed that the isolates AqJ1.17 and AqJ2.17 were resistant to all antimicrobial agents used, except to florfenicol (Table 2). The reference strain was sensitive to florfenicol and potentiated sulphonamide and resistant to the remaining antimicrobial tested.

Table 2. Antimicrobial susceptibility patterns of isolates of *L. venerupis* from *T. trachurus* and *L. venerupis* CECT8573^T

Antimicrobials	AqJ1.17	AqJ2.17	CECT 8573^T
Amoxycillin	R	R	R
Ciprofloxacin	R	R	R
Trimethoprim-Sulfametoxazole	R	R	S
Ampicillin	R	R	R
Oxytetracycline	R	R	R
Norfloxacin	R	R	R
Pteridine	R	R	R
Florfenicol	S	S	S

R, resistant; S, susceptible; I, intermediate

The cellular fatty acid profile of the isolates AqJ1.17 was dominated by $C_{15:0}$ iso 3OH (22.72%), $C_{15:0}$ iso (16.09%) and $C_{15:1}$ iso G (16.09%). The fatty acid profiles obtained for the strains AqJ1.17 was similar to the profile of the type strain of *L. venerupis* CECT8573^T reported by Lasa et al. (2015), although there were differences in the proportions of some fatty acids (Table 3).

Table 3. Fatty acid composition (%) of the strains of *L. venerupis* isolated in this study and the type strains *L. venerupis* CECT8573T (Lasa et al. 2015)

Fatty acid	**Percentage (%)**	
	AqJ1.17	***L. venerupis* CECT 8573^{T}**
$C_{15:0}$ iso 3OH	22.72	12.3
$C_{15:0}$ iso	16.09	21.2
$C_{15:1}$ iso G	16.05	22.9
Sum in Feature 3	13.06	8.56
$C_{17:0}$ iso 3OH	10.62	15.0
$C_{16:0}$ iso 3OH	10.52	7.6

*Summed feature 3 contains C16:1 ω6c/ C16:1 ω7c that cannot be separated by gas chromatography using the microbial identification system.

3.2. Molecular characterization

The analysis by PCR of the bacterial isolates did not give the expected PCR product specific for *T. soleae* or *T. maritimum*.

Comparative BLAST analysis of the partial sequences of the 16S rRNA gene of the bacteria under study revealed 99.1% sequence similarity with the type strains of *L. venerupis* CECT 8573^{T} isolated from clams in Spain (GenBank accession number NR_145942.1) and the strain *L. venerupis* DOK2-8 isolated from marine sediment in Korea (GenBank accession number MG493235.1) (Lim et al., 2018). The phylogenetic tree grouped the bacteria isolated from mackerel with the type strain of *L. venerupis* 8573^{T}, forming a clade separated from other *Flavobacteria* species (Figure 1). Thus, results obtained from the partial sequencing of the 16S rRNA gene clearly showed that the isolates under study belong to the *Lacinutrix venerupis* species (Figure 1).

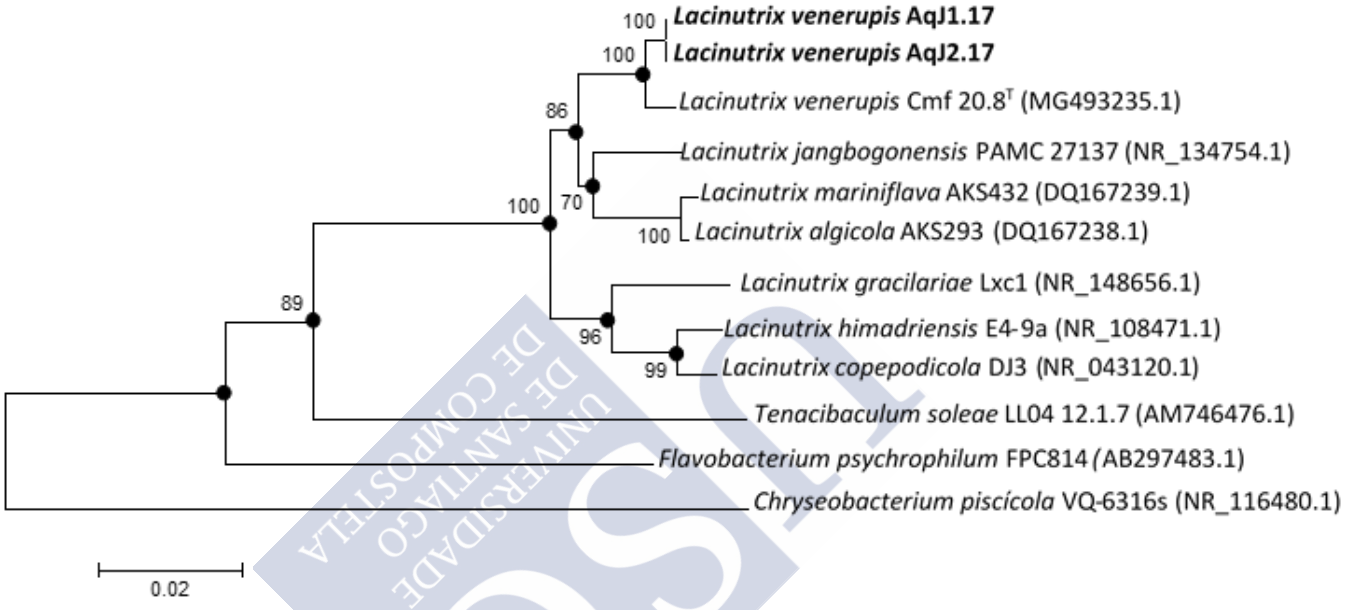

Figure 1. Phylogenetic tree based on partial 16S rRNA gene sequence. The tree was constructed using the neighbour-joining method using the Mega 6.06 software. The figure represents the evolutionary relationship between the *L. venerupis* isolated in this study and other genetically related flavobacteria species. The accession numbers in the GenBank database of the 16S rRNA sequences of the bacterial strains are shown in parenthesis after scientific names. Solid circles indicate that the corresponding nodes are also recovered using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and maximum-parsimony (MP) methods. The numbers of the nodes are showing the levels of bootstrap resampling based on 1000 replicates. Bar, 0.02 nucleotide substitutions per site.

Genetic characterization by REP and ERIC-PCR yielded reproducible patters and gave an appropriate number of bands (Figure 2a, b). The strains isolated from mackerel showed genetic profiles with a high level of similarity. The REP-PCR profile consisted of 5-6 bands with molecular weight ranging from 250 to 750 bp (Figure 2a) and the ERIC-PCR profile consisted of 4 bands ranging from 150 to 700 bp (Figure 2b). However, both the ERIC and REP profiles of the type strain of *L. venerupis* CECT 8573^{T} differed in the number, size and intensity of some of the bands produced. Thus, the ERIC fingerprints presented 6 bands ranging from 300 to 750 and the REP profiles 7 bands ranging from 150 to 750 as presented in Figure 2.

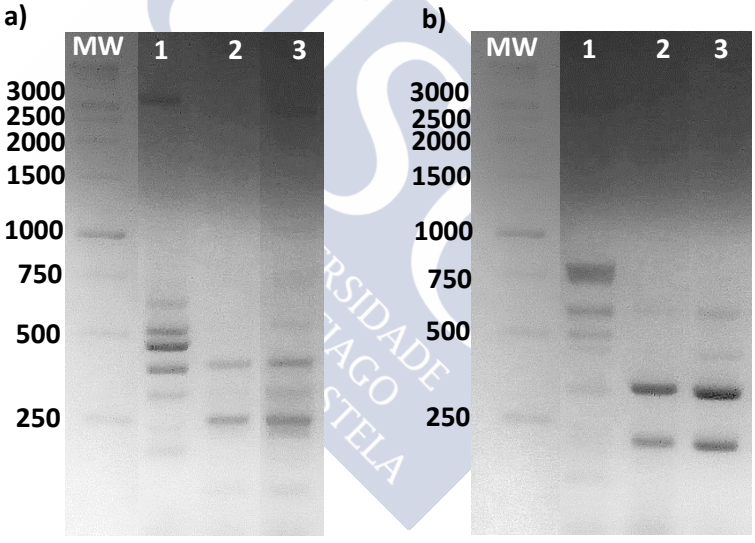

Figure 2. a) REP and b) ERIC profiles obtained for the mackerel isolates. MW, molecular weight marker (GeneRulerTM 1 kb DNA Ladder, Fermentas); 1, *Lacinutrix venerupis* CECT 8573T; 2-3, *L. venerupis* AqJ1.17 and AqJ2.17.

3.3. Proteomic characterization

The protein extracts of the *L. venerupis* strains AqJ1.17, AqJ2.17 and CECT8573 were analysed by MALDI-TOF mass spectrometry. None of the

isolates were identified by the Bruker database of clinical and environmental bacteria. The spectra obtained for the strains analysed showed good resolution with several peaks (60 to 65 mass peaks) at *m/z* ranging from 2077.82 to 13596.92. From all the mass peaks detected, 13 were present in the two mackerel strains and the type strain CECT8573, which could be used as biomarkers for the detection of *Lacinutrix* species (2081.65, 2121.94, 2911.08, 4648.58, 4766.22, 5016.25, 5617.22, 5705.75, 7220.91, 8491.97, 9295.24, 10029.79, 11230.68). The mass protein profiles of the two strains analysed are present in Figure 3.

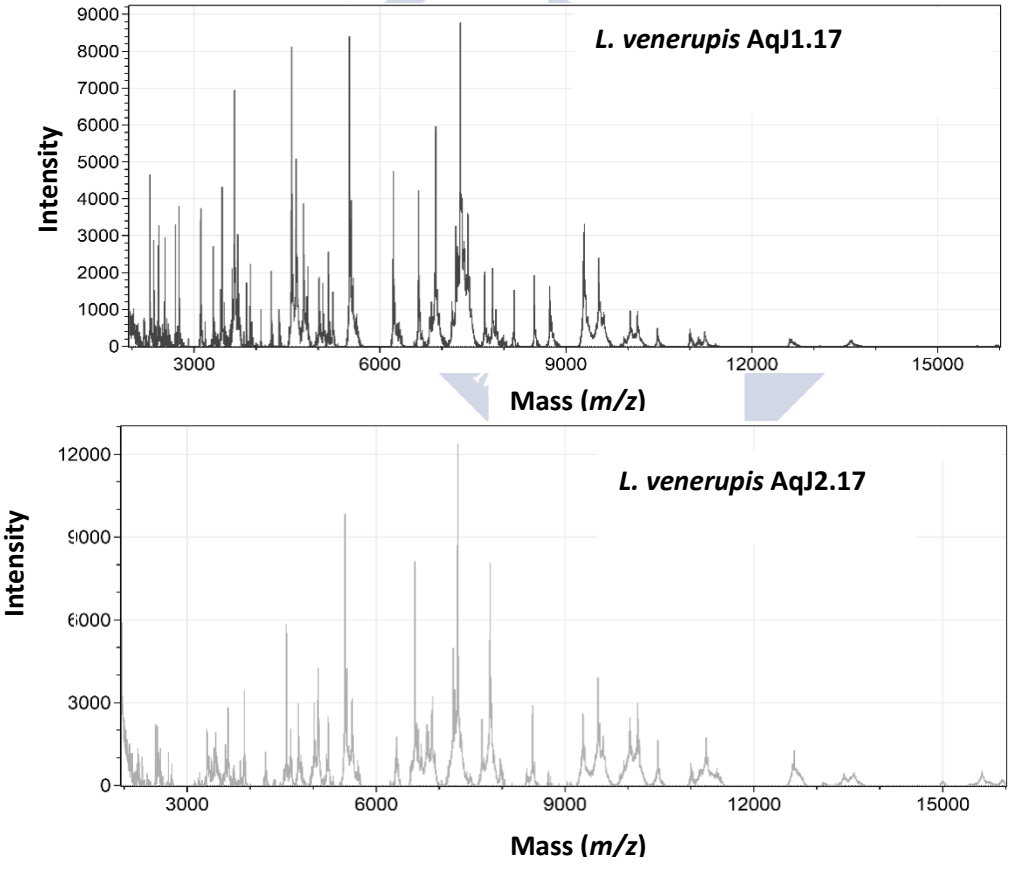

Figure 3. Protein profiles obtained from MALDI-TOF analysis of the strains AqJ1.17 and AqJ2.17.

3.4. Pathogenicity assay

Strain AqJ1.17 isolated from diseased mackerel *T. trachurus* was used for intraperitoneal experimental challenge of turbot. No mortalities of turbot were recorded at any of the doses used (10^4 –10^9 CFU/fish) after 20 days of experimental infection. None of the control fish died during the experiment.

4. Discussion

The present study reports the isolation, characterization and identification of two bacterial isolates recovered from diseased wild *T. trachurus*. Bacteriological analysis of samples of kidney, spleen, and liver allowed to isolate yellow colonies in pure culture on FMM agar plates. Characterization of the isolates by using phenotypic, molecular and chemotaxonomic techniques allowed to identify the bacteria as *Lacinutrix venerupis*. *L. venerupis* was first isolated from clams, and lately was first described as the cause of disease in reared sea bream and sea bass (López et al., 2017). However, limited information is available on the pathogenic potential for fish of the bacterial species belonging to the genus *Lacinutrix*

The phenotypic characteristics of the *L. venerupis* strains isolated in this study were similar to those reported for the type strain *L. venerupis* CECT8573^T (Lasa et al., 2015). However, some discrepancies were found in the phenotypic characteristics of the strains isolated in this study and the *L. venerupis* strains isolated from clams and sea bass and sea bream previously reported (Lasa et al., 2015; López et al., 2017). Thus, further comparative studies should be conducted for a better understanding of the intra-species diversity among commensal or pathogenic strains. Moreover, this study confirmed that there are several phenotypic characteristics that could be considered to discriminate

between the different species of the genus *Lacinutrix* as the hydrolysis of aesculin or Tween 80 (Lasa et al., 2015).

Lacinutrix species are present in marine environments and may be associated with marine organisms as commensal or opportunistic pathogens. Antibiotic resistance patterns observed in the present study suggest that some *Lacinutrix* spp. strains could act as reservoir or transmitters of resistant determinants to other pathogenic, commensal or environmental bacteria. Since, some authors have suggested that *Lacinutrix* species could be acting as opportunistic pathogens producing disease only under certain conditions (López et al., 2017), the best procedure to prevent the emergence of these diseases would be the reduction of biotic and abiotic stress.

Regardless some differences in the phenotypic characteristics, the partial sequencing of the 16S rRNA gene of the isolates under study shared 99.1% homogeneity with previously identified *L. venerupis* strains isolated from clams and marine sediments (Lasa et al., 2015; Lim et al., 2018). These similarity values were higher than the limit of 98.7% of intraspecific variability described by Stackebrandt and Ebers (2006). Moreover, cluster analysis by using the neighbour-joining and maximum parsimony methods confirmed these results since the isolates from mackerel were grouped together with reference strain CECT8573^{T} included in the study.

The repetitive elements sequence-based PCR generate DNA profiles that could be used for the identification and discrimination of bacterial species and/or strains (Versalovic et al., 1991). In this sense, ERIC and REP-PCR methodologies have been successfully used for the typing and discrimination of different species of the Family *Flavobacteriaceae*, as *Tenacibaculum* spp. and *Flavobacterium* spp. (Valdebenito and Avendaño-Herrera, 2009; Barony et al., 2015). In this study, the bacteria isolated from mackerel showed a high level of

similarity. However, their genetic profiles were completely different to those obtained with the type strain of *L. venerupis* CECT 8573^T using the same culture conditions, DNA extraction methods, and PCR conditions. These results suggest the existence of genetic heterogeneity within the species *L. venerupis*. In this sense, future studies should focus on the study of the genetic heterogeneity within and among the *Lacinutrix* species from different origins as well as for the search of differences between the environmental, commensal and pathogenic strains. Analysis of the profiles of FAMEs revealed differences in the proportion of the presence of the cellular fatty acids between the *L. venerupis* isolated in this study, and those profiles reported previously (Lasa et al., 2015; López et al., 2017). These differences could be attributed to the use of different culture and analysis conditions.

Proteins of the mackerel isolates were characterized using MALDI-TOF mass spectrometry. However, the strains could not be identified due to that the spectra of these microorganisms are not included in the available Bruker databases. A high number of mass peaks (46 mass peaks) were found to be common in the spectra of the two *L. venerupis* strains analysed. However, to find specific peaks that allow the identification of the species *L. venerupis*, further studies should be made analysing the MALDI-TOF profiles of *L. venerupis* strains from different sources and fish species as well as other *Lacinutrix* species.

Virulence test showed that the isolate AqJ1.17 was not virulent for turbot after intra-peritoneally injection with 10^9 CFU per fish. Previous studies reported that *L. venerupis* could produce significant mortalities (80-100% mortality within 4 days) in Senegalese sole fry, but only with high doses of bacteria of 10^7 CFU per fish (López et al., 2017). These results are in concordance with those described by López et al. (2017), who suggests that the

microorganism could be present in healthy fish as an opportunistic pathogen and produce diseases only under certain conditions. Thus, further studies will be necessary to determine the importance of this species as a fish pathogen.

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Isolation and characterization of *Flavobacterium psychrophilum*-like bacteria from diseased rainbow trout and eggs (*Oncorhynchus mykiss*) in Spain.

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Abstract

A total of nine *F. psychrophilum*-like strains isolated from diseased fish and trout eggs were characterized using phenotypic (biochemical and API systems), molecular (REP-PCR and ERIC-PCR) and proteomic (MALDI-TOF mass spectrometry) analysis in order to investigate the diversity of the *Flavobacterium* species present in rainbow trout (*Oncorhynchuss mykiss*) farms in Spain. Reference strains of the species *Flavobacterium psychrophilum*, *Flavobacterium branchiophilum Flavobacterium flevense*, *Flavobacterium johnsoniae*, *Flavobacterium oncorhynchi*, *Flavobacterium tructae*, *Flavobacterium piscis* or *Flavobacterium plurextorum* were used for comparative purposes and for identification of the isolates under study. The *Flavobacterium psychrophilum*-like strains under study showed similar phenotypic characteristics, but they were completely different to those showed by *F. psychrophilum*, *F. branchiophilum* or *F. flevense*. ERIC and REP techniques were effective for typing and differentiating all the *Flavobacterium* isolates under study. MALDI-TOF analysis and Biotyper 3.1 software did not allow identifying any of the isolates under study. Genetic and proteomic analysis showed high similarity between the isolates FCV.00 and *F. tructae* CECT7791 and RBT11.05.16.3 and *F. collinsi* CECT7796, respectively.

Virulence studies suggested that some of these isolates could be considered as a potential threaten for rainbow trout farming. Further studies basing on the sequencing of the 16S rRNA gene and the analysis of the fatty acids will allow classifying these bacteria and determining the risks for trout culture.

Keywords: *Flavobacterium* sp., characterization, ERIC-PCR, REP-PCR, MALDI-TOF-MS

1. Introduction

The aquaculture industry is the fastest-growing food sector in the world; as a consequence, problems for controlling fish infections are also increasing. During the last decade, flavobacterial infections have become a major problem in cultured rainbow trout and Atlantic salmon in many countries in Europe (Zamora et al., 2012; Zamora et al., 2013; Zamora et al., 2014), America (Faisal et al., 2017; Ilardi and Avendaño-Herrera, 2008) and Asia (Verma and Rathore, 2013). The genus *Flavobacterium* includes a physiologically diverse and widely distributed microorganisms that have been isolated from soil, water, plants and food products as fish, meat, poultry or milk (Bernardet and Bowman, 2006). Flavobacteria are commonly isolated from fresh water environments related with aquaculture activities as well as from the skin, gills, and eggs of fish (Bernardet and Bowman, 2006). Most flavobacterial disease outbreaks in freshwater fish species are attributed to the species *Flavobacterium psychrophilum*, *Flavobacterium columnare* or *Flavobacterium branchiophilum*, which are responsible for important economic losses in the aquaculture industry. However, in the recent years, several *Flavobacterium* spp. have been reported in association with fish disease. Thus, species as *Flavobacterium succinicans*, *Flavobacterium chilense*, *Flavobacterium johnsoniae*, *Flavobacterium*

oncorhynchi, *Flavobacterium tructae*, *Flavobacterium piscis* or *Flavobacterium plurextorum* have been occasionally isolated from diseased rainbow trout fry that presented clinical symptoms consistent with infections produced by *F. psychrophilum* worldwide (Kampfer et al., 2012; Loch and Faisal, 2015; Zamora et al., 2014, 2013, 2012). These species have been designated with the generic name *F. psychrophilum*-like species due to the high similarity in the macroscopic and biochemical characteristics among them and the species *F. psychrophilum* (Pérez-Sancho et al., 2017). Several molecular or serological protocols have been developed for the identification of the *Flavobacterium* sp. most commonly associated with fish disease as *F. psychrophilum*, *F. columnare* or *F. branchiophilum* (Darwish et al., 2004; Cepeda and Santos, 2000; Panangala et al., 2007; Strepparava et al., 2014; Wiklund et al., 2000; Toyama et al., 1996; Santos and Mata, 2001). However, there is a lack of specific diagnostic reagents available to detect and identify many fish-associated flavobacteria (Loch and Faisal, 2015).

In the present study, the phenotypic, genotypic and proteomic characterization of nine *F. psychrophilum*-like isolates recovered during the period of 2011 to 2017 from trout eggs and from diseased farmed trout (*Oncorhynchus mykiss*) presenting clinical sign of flavobacteriosis were performed.

2. Material and Methods

2.1. Isolation of the bacterial strains and culture conditions

Diseased rainbow trout and trout eggs were sent to the Department of Microbiology and Parasitology of the University of Santiago de Compostela for routine bacteriological analysis. Samples of kidney, spleen and liver from fish were cultured on FLP agar medium (Cepeda et al., 2004) and trypticase soy agar supplemented with 1% NaCl (TSA-1) and incubated at 18 and 25ºC for 2-3

days. The trout eggs were examined for the extra and intra-ovum presence of *Flavobacterium psychrophilum*. Untreated eggs or eggs macerated with sterile scissors were incubated in FLP broth for 2 weeks. Enriched eggs samples were spread onto FLP agar and incubated for up to 2 weeks. Bacterial colonies displaying a yellow colour, characteristic of flavobacteria, were transferred onto FLP agar plates and grown at 18ºC for 2-3 days for further characterization. All the *F. psychrophilum*-like isolates recovered, and their origins are indicated in Table 1. Moreover, strains belonging to the species *Flavobacterium collinsi*, *Flavobacterium plurextorum*, *Flavobacterium oncorhynchi*, *Flavobacterium piscis*, *Flavobacterium johnsoniae*, *Flavobacterium branchiophilum*, *Flavobacterium tructae*, *Flavobacterium flevense* and *F. psychrophilum* from the Spanish Type Culture Collection (CECT), the German Collection of Microorganisms and Cell Cultures (DSMZ), and National Collection of Marine and Industrial Bacteria (NCIMB) were also included in this study for comparative purposes. Pure cultures of these isolates were stored at -30ºC in FLP broth with glycerol (15%) until analysis.

2.2. *F. psychrophilum* PCR assay

The yellow pigmented colonies of the isolates were consistent with the presumptive diagnosis of infections caused by *F. psychrophilum*. To confirm the identification of the isolates a PCR assay was carried out. Thus, DNA from pure bacterial cultures was extracted using Insta-Gene matrix (Bio-Rad, Madrid, Spain). PCR amplifications were performed using the specific primers for *F. psychrophilum* and the protocol described by Cepeda and Santos (2000). All amplifications were carried out in a Mastercycler Epgradient thermal cycler (Eppendorf, Hamburg, Germany) and PCR products were electrophoresed in a 1% (*w/v*) agarose gel during 1h at 100V, stained with 5µL of Redsafe nucleic acid staining solution (20.000x) (iNtRON Biotechnology) and visualized using

an ultraviolet light transilluminator (Bio-Rad UV Transilluminator 2000). Reactions lacking DNA or including DNA from the reference strain of *F. psychrophilum* NCIMB13384 were used as negative and positive controls, respectively. The presence of a band with a size of 1089 bp was considered as a positive result for *Flavobacterium psychrophilum*.

Table 3. *Flavobacterium* strains included in this study

Bacteria	**Source**	**Year of isolation**
Isolates characterized in this study		
SK320/13	*Oncorhynchus mykiss*	2013
SK363/13	*O. mykiss*	2013
RBT11.05.16.3	*O. mykiss*	2016
Riobo 18.10.12.5	*O. mykiss*	2011
Riobo 18.10.13.6	*O. mykiss*	2012
ICH29.01.18.1	*O. mykiss*	2017
ICH29.01.18.5	*O. mykiss*	2017
ICH29.01.18.6	*O. mykiss*	2017
FCV.00	*O. mykiss*	2000
Reference strain		
F. collinsi CECT 7796	*O. mykiss*	2016
F. plurextorum CECT 7844	Eggs of rainbow trout	2014
F. oncorhynchi CECT 7678	*O. mykiss*	2012
F. piscis CECT7909	*O. mykiss*	2014
F. johnsoniae CECT 5015	Root surface of pasture grass	1996
F. branchiophilum DSM 24789	*Oncorhynchus masou*	1996
F. tructae CECT 7791	*O. mykiss*	2014
F. flevense NCIMB 12056	Fresh water	1975
F. psychrophilum NCIMB 13384	*O. mykiss*	1990

CECT, Spanish Type Culture Collection (Spain), DSMZ, German Collection of Microorganisms and Cell Cultures, NCIMB National Collection of Industrial and Marine Bacteria (UK).

2.3. Phenotypic characterization

Isolates were characterized using conventional phenotypic tests proposed by (Bernardet et al. 2002). The biochemical reactions tested in this study included: Gram-staining, cell morphology, production of catalase and oxidase, gliding motility, production of flexirubin-type pigments, absorption of Congo red and oxidation/fermentation reactions. Hydrolysis of DNA, gelatine, casein, starch, Tween 20 and L-tyrosine were determined using Nutrient Agar (NA) as basal medium (Zamora et al., 2012). Growth in FLP agar medium was assessed at 4, 18, 25, 32 and 37ºC. Isolates were also evaluated for their halotolerance by its growth on nutrient agar containing concentrations of NaCl of 0, 1, 3, 5 and 6.5%. Growth on MacConkey agar, nutrient agar, Marine agar, blood agar, Trypticase Soy Agar (TSA) and Simmons´ citrate agar was determined. The isolates were further characterized using the API-ZYM system (BioMérieux, Madrid, Spain) following the instructions provided by the manufacturer.

Susceptibility to antimicrobials was evaluated by the agar diffusion tests following the procedures described by the Clinical and Laboratory Standards Institute document M42-A (CLSI, 2006). The antimicrobial agents and the concentrations (µg/disc) used were ampicillin (25), florfenicol (30), flumequine (30), oxytetracycline (30), trimethoprim-sulfamethoxazole (25). The type strain *A. salmonicida* ATCC33658 was used as quality control strain in all tests.

2.4. ERIC-PCR and REP-PCR

All the strains included in this study were analyzed using the repetitive extragenic palindromic PCR (REP-PCR) and the enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) using the primers, procedures and conditions previously described (Versalovic et al., 1991). PCR amplifications were performed in a Mastercycler Epgradient thermal cycler

(Eppendorf, Hamburg, Germany). PCR products were analysed by gel electrophoresis (100 V for 90 min) on a 2% *w/v* agarose gel in TAE 1× (Tris 0.04 M, EDTA 0.001 M, pH 8) electrophoresis buffer, and stained with RedSafe Nucleic Acid Staining Solution (20,000×) (iNtRON Biotechnology, Seongnam-Si, Korea). A 1-kb DNA ladder (Fermentas, Madrid, Spain) was included as a molecular weight marker. PCR products were visualized using an ultraviolet light transilluminator (UV Transilluminator 2000, Bio-Rad, California, USA). The gels were photographed and analysed using the Quantity One software v. 4.6.5 (Bio-Rad, California, USA) for comparison of the REP-PCR and ERIC-PCR profiles of all the strains analysed. Similarity between the profiles of the different isolates was calculated with the Dice coefficient (Sd) (Dice, 1945). Cluster analysis and dendrograms were performed using the unweighted pair-group method with arithmetic averages (UPGMA).

2.5. MALDI-TOF analysis

Proteomic analysis of the strains isolated in the study was performed using MALDI-TOF mass spectrometry analysis. Proteins from the *Flavobacterium* strains used in this study were extracted by using formic acid (Sigma-Aldrich, St. Louis, USA) before analysis by MALDI-TOF mass spectrometry. The peak lists generated from each strain were analysed using BioTyper3.1 software (Bruker Daltonics, GmbH) and results were expressed using the criteria proposed by the manufacturer. For interpretation of the results, score values following the MALDI Biotyper analysis were considered: i) score values ≥ 2.000, identification at species level with high confidence; score values = 1.999-1.800, identification with low confidence; and score values ≤1.799, non-reliable identification. Mass spectra raw data were processed using the statistical package for mass spectrometry data analysis MALDIquant (Gibb and Strimmer, 2012). The Mass-Up software (Mass-Up, Vigo, Spain) (López-Fernández et al.,

2015) was used for spectra processing and to determine and compare the representative common peak masses of each strain. Similarities were calculated using curve-based (Pearson Product Moment Correlation Coefficient, PPMCC), as well as band-based (Jaccard similarity coefficient) measures and single-linkage. For cluster analysis IBM SPSS statistics V22 was used.

Principal component analysis (PCA) was performed on binary spectra data using the application of XLSTAT software package (Addinsoft, New York, USA). The principal components can be represented in a three-dimensional space and give a concise and simplified description of the variance present in the data set. From the analysis of this graphical representation, it is possible to identify groups of samples showing a similar (samples close on the graph) or a different (samples far in the graph) behaviour.

2.6. Pathogenicity assay

Fish used in this study were obtained from a farm located in Galicia (Spain). The assays were carried out at the aquarium facilities of the University of Santiago de Compostela. Pathogenicity assays were carried out by intraperitoneal injection of rainbow trout (average body weight of 27±7 g). Rainbow trout were maintained in 100 l tanks and supplied with aerated freshwater at 16 ±1ºC. Fish were fed on commercial diet during the experiment. Before challenge experiment, fish were subjected to bacteriological analysis in order to verify their health status. The strains RBT11.5.16.3 and ICH29.01.18.1 were selected for the virulence assay. Bacterial cultures were suspended in saline solution (0.9% *w/v*), adjusted to 10^9 cells ml^{-1} (McFarland scale tube 3) and serially ten-fold diluted. Colony forming units (CFU) were enumerated by the plate dilution method by seeding bacterial cells suspensions onto FLP agar plates and counting the bacterial colonies produced. For challenge, fish were anaesthetised by immersion in tricaine methane-sulfonate (MS-222, Sigma) (60

mg l^{-1}) (Neiffer and Stamper, 2009) and intraperitoneally injected with 0.1 ml of bacterial suspensions, containing 1×10^9, 1×10^8 and 1×10^7 CFU ml^{-1} (10 fish per dose). Infected fish were kept in tanks on the conditions above described. Fish mortalities were considered caused by the inoculated strain, only if the bacterium was recovered in pure culture from liver, kidney and spleen of dead or dying fish.

3. Results

3.1. Phenotypic characterization

All bacteria isolated from rainbow trout formed smooth, shiny, circular with regular edges and yellow-pigmented colonies on FLP, which leads to the presumptive diagnosis of infection by *F. psychrophilum*. However, none of the isolates gave the specific amplicon of 1089 bp, specific for *F. psychrophilum* using the primers described by Cepeda et al. (2000). Phenotypically, all the isolates were short rods, Gram-stain-negative, non-motile bacteria, cytochrome oxidase and catalase negative and were non-fermentative. All *F. psychrophilum*-like isolates produced flexirrubin-type pigment but did not absorb Congo red. Bacteria grew on TSA-1, nutrient agar, blood agar, but not on MacConkey and Marine agar. Bacteria were able to grow at 4, 18 and 25ºC, but not at 37ºC; and grew on nutrient agar containing concentrations up to 1% NaCl. Casein was hydrolysed by all the strains studied; however, differences were shown for the hydrolysis of starch, tyrosine and Tween 20 (Table 2). With the API-ZYM system, all the *Flavobacterium* isolates showed activity for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and n-acetyl-β-glycosaminidase. All isolates had negative reactions for esterase C4, lipase C14, β-glucuronidase, and α-fucosidase. Variable results were obtained for the cystine arylamidase, alpha-chymotrypsin, β-galactosidase, β -glucosidase, α -

mannosidase, esterase lipase (C8) and alpha-galactosidase. The antimicrobial susceptibility tests showed that all strains were susceptible to flumequine (except *F. branchiophilum*) and resistant to trimethoprim-sulfamethoxazole (except *F. branchiophilum*) and ampicillin. Variable results were found for florfenicol and oxytetracycline (Table 2).

Table 2. Differential characteristics of the *Flavobacterium* strains analysed

Phenotypic characteristic	**Bacterial strains**									
	1	**2**	**3**	***4***	***5***	***6***	***7***	***8***	***9***	***F. p.-like n=9***
Hydrolysis of agar	-	-	-	-	-	-	-	+	-	-
Growth at 32ºC	-	-	+	+	+	+	-	+	-	-
Simmons Citrate	+	-	-	-	-	+	+	+	-	v
Hydrolysis Starch	+	-	-	+	+	-	+	-	-	v
Hydrolysis Tyrosine	+	+	+	-	-	+	+	+	+	v
Hydrolysis Tween 20	-	+	-	-	+	-	-	-	+	v
Susceptibility to										
Flumequine	S	S	S	S	S	R	S	S	S	S
SXT	R	R	R	R	R	S	R	R	R	R
Ampicillin	R	R	R	R	R	R	R	R	R	R
Florfenicol	S	S	S	R	R	S	S	R	S	v
Oxytetracycline	R	R	R	S	S	R	R	S	R	v

1, *F. collinsi* CECT 7796; 2, *F. pluretorum* CECT 7844; 3, *F. oncorhynchi* CECT 7678; 4, *F. piscis* CECT 7909; 5, *F. johnsoniae* CECT 5015; 6, *F. branchiophilum* DSM 24789; 7, *F. tructae* CECT 7791; 8, *F. flevense* NCIMB 12056; 9, *F. psychrophilum* NCIMB 13384.

3.2. REP-PCR and ERIC-PCR

Genetic characterization of the 17 *Flavobacterium* strains using REP-PCR and ERIC-PCR produced profiles that varied in both the number and the size of the bands produced (Figure 1a, b). Moreover, molecular profiles were highly reproducible allowing comparison between isolates tested on different days.

REP-PCR analysis yielded 4 to 12 bands depending on the isolate and the size of the DNA fragments ranged from 158 to 3288 bp (Figure 1a). The profile of the isolate FCV.00 appeared to be identical to that of the strain *F. tructae* CECT 7791, since they shared five bands of 359, 454. 782, 860 and 1802 bp in the REP profiles. The molecular profiles of the isolate RBT11.05.16.3 were identical to that of the strain *F. collinsi* CECT 7796, sharing eight bands of 203, 229, 492, 712, 1066, 1268,1802 and 3075 bp in the REP-profiles. On the other hand, the three strains isolated from trout eggs (ICH29.01.18.1; ICH29.01.18.5, ICH29.01.18.6) showed identical REP profiles and shared eleven bands of 203.34, 229.80, 353.07, 712.52, 986.52, 1181.05, 1268.98, 1531.41, 1436.75, 1802.81, 3075.25 bp. The isolates SK320/13 and SK363/13 also showed the same REP pattern with bands of 229.80, 271.76, 384.11, 554.17, 653.20, 860.00, 1331.19, 1531.47, 1632.87 (Figure 1a).

According to these results, cluster analysis of the REP-PCR results distributed the strains in different clusters (Figure 2a). Cluster I grouped the *F. psychrophilum*-like strains SK320/13 and SK363/13, but they were not clustered with any of the reference strains tested in the study. Cluster II grouped together the isolate FCV.00 with the reference strains *F. tructae* CECT 7791; while cluster III grouped the strain *F. collinsi* CECT 7796 with the strain RBT11.05.16.3, very closely with the strains ICH29.01.18.1, ICH29.01.18.5 and ICH29.01.18.6 isolated from eggs. Cluster IV grouped the rest of the reference strains included in the study as well as the isolates Riobo 18.10.13.6

and Riobo 18.10.12.5, which were closer to *F. flevense* NCIMB 12056 (Figure 2a).

ERIC-PCR profiles yielded a lower number of bands comparing to REP analysis (3 to 6 bands depending on the isolate) with sizes of the DNA fragments ranging from 177 to 3848 bp (Figure 1b). As occurred in the REP analysis, the isolate FCV.00 showed identical ERIC profile than the strain *F. tructae* CECT 7791, sharing five bands of 326, 445, 491, 970 and 977 bp. Besides, the ERIC profile of the isolate RBT11.05.16.3 was identical to that of the strain *F. collinsi* CECT 7796 as they shared six common bands of 177, 265, 326, 424, 803, 970, 3848 bp (Figure 1b). The strains isolated from eggs (ICH29.01.18.1, ICH29.01.18.5 and ICH29.01.18.6) also showed the same ERIC profile with bands of 265.65, 424.44, 727.30 and 3200.85 bp. As in the REP profile, the isolates SK320/13 and SK363/13 also showed identical ERIC profile with bands of 326.10, 491.58 and 629.29 bp (Figure 1b).

Cluster analysis of the ERIC profiles allowed to distribute the strains in different groups (Figure 2b). Moreover, some clusters were coincident with the results obtained in the REP analysis. Thus, cluster I grouped the FCV.00 and the strain *F. tructae* CECT 7791, as expected by their identical molecular profiles. Cluster II grouped the isolates SK320/13 and 363/13 that were almost identical and the isolate Riobo 18.10.13.6. Cluster III grouped the strains *F. flevense* NCIMB and *F. oncorhynchi* CECT 7678; while cluster IV clustered the isolate Riobo 18.10.12.5 with the reference strain *F. johnsoniae* CECT 5015. As occurred with the REP analysis, the isolate RBT11.05.16.3 was closely related with the reference strain *F. collinsi* CECT 7796, and with the strains isolated from eggs, forming the cluster V (Figure 2b). The reference strains of *F. psychrophilum*, *F. piscis*, and *F. plurextorum* formed separated groups in the cluster.

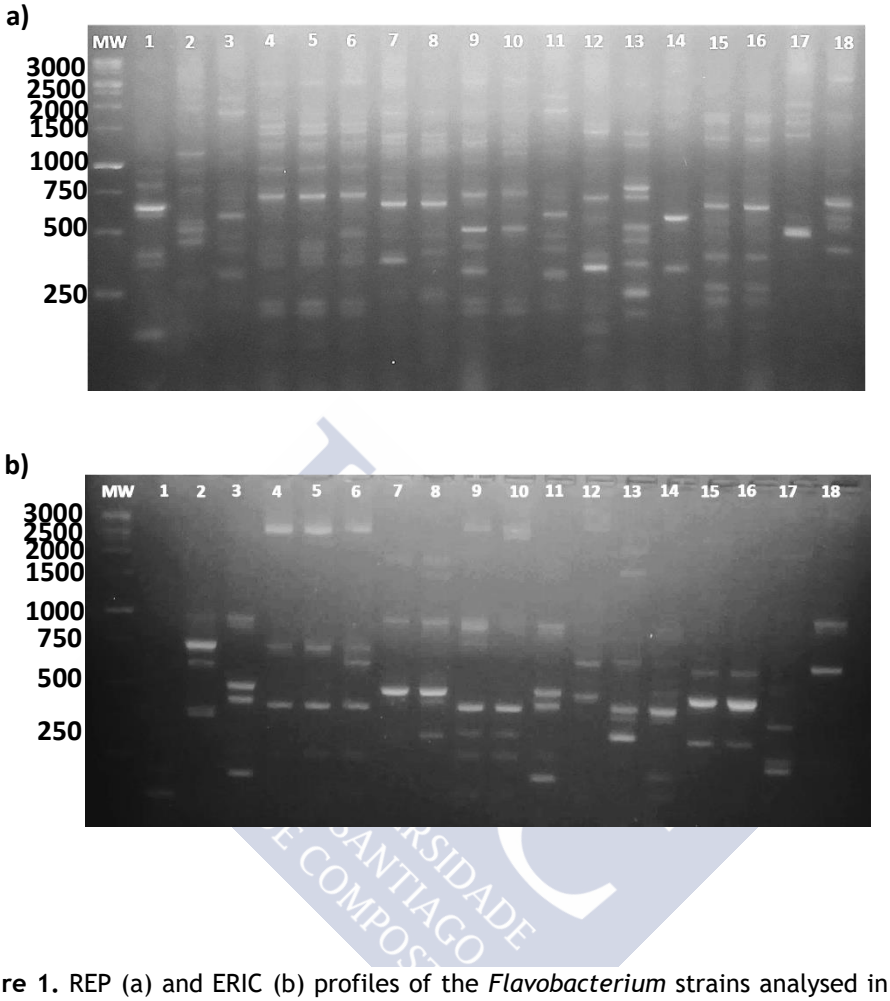
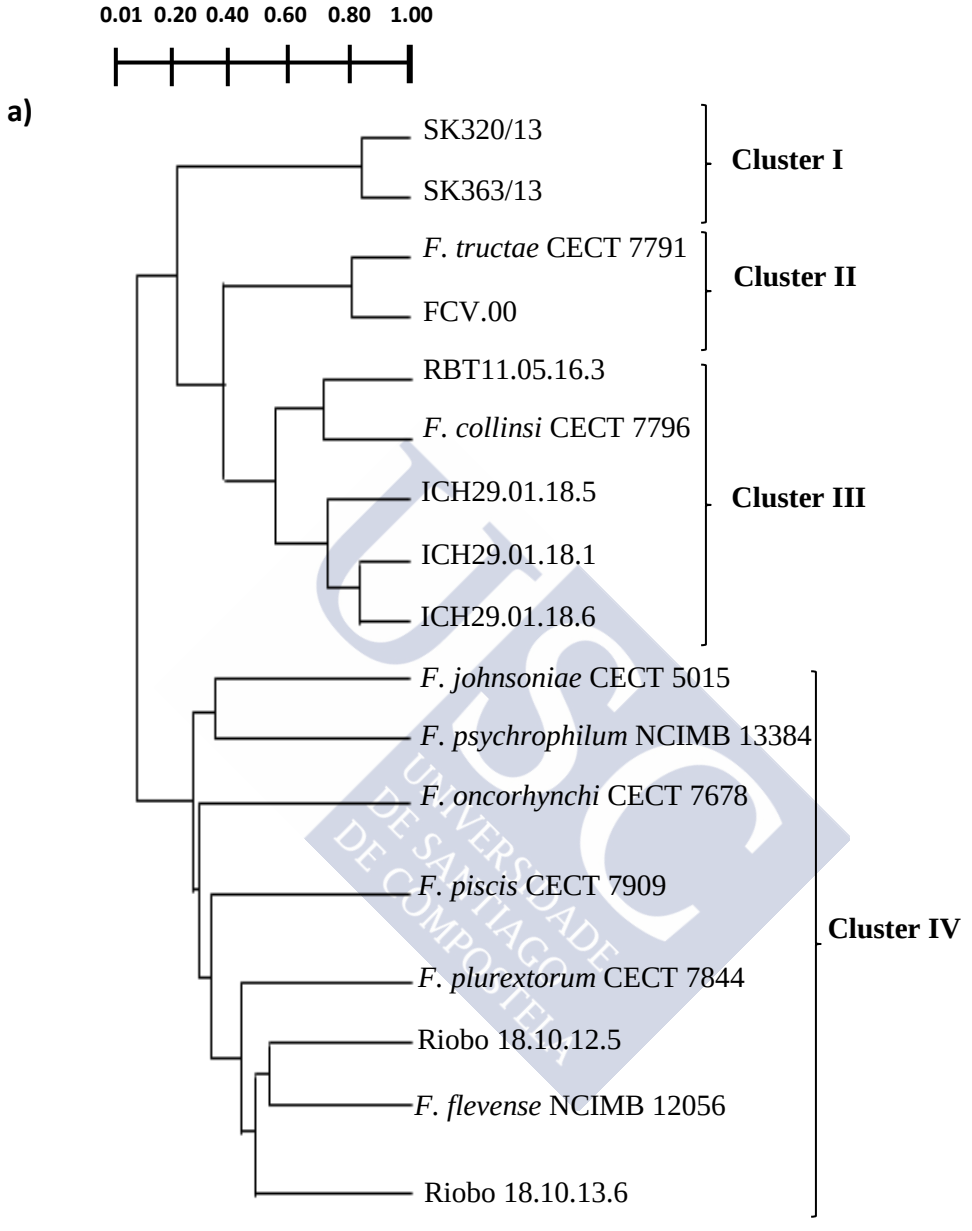


Figure 1. REP (a) and ERIC (b) profiles of the *Flavobacterium* strains analysed in this study. MW, molecular weight marker; 1, *F. psychrophilum* NCIMB 13384; 2, *F. flevense* NCIMB 12056; 3, *F. piscis* CECT 7909; 4, ICH29.01.18.1; 5, ICH29.01.18.6; 6, ICH29.01.18.5; 7, FCV.00; 8, *F. tructae* CECT 7791; 9, *F. collinsi* CECT 7796; 10, RBT11.05.16.3; 11, *F. piscis* CECT 7909; 12, Riobo 18.10.13.6; 13, Riobo 18.10.12.5; 14, *F. johnsoniae* CECT 5015; 15, SK363/13; 16, SK320/13; 17, *F. oncorhynchi* CECT 7678; 18, *F. plurextorum* CECT 7844.


0.01 0.20 0.40 0.60 0.80 1.00
a)
SK320/13
SK363/13
Cluster I
F. tructae CECT 7791
FCV.00
Cluster II
RBT11.05.16.3
F. collinsi CECT 7796
ICH29.01.18.5
ICH29.01.18.1
ICH29.01.18.6
Cluster III
F. johnsoniae CECT 5015
F. psychrophilum NCIMB 13384
F. oncorhynchi CECT 7678
F. piscis CECT 7909
F. plurextorum CECT 7844
Riobo 18.10.12.5
F. flevense NCIMB 12056
Riobo 18.10.13.6
Cluster IV

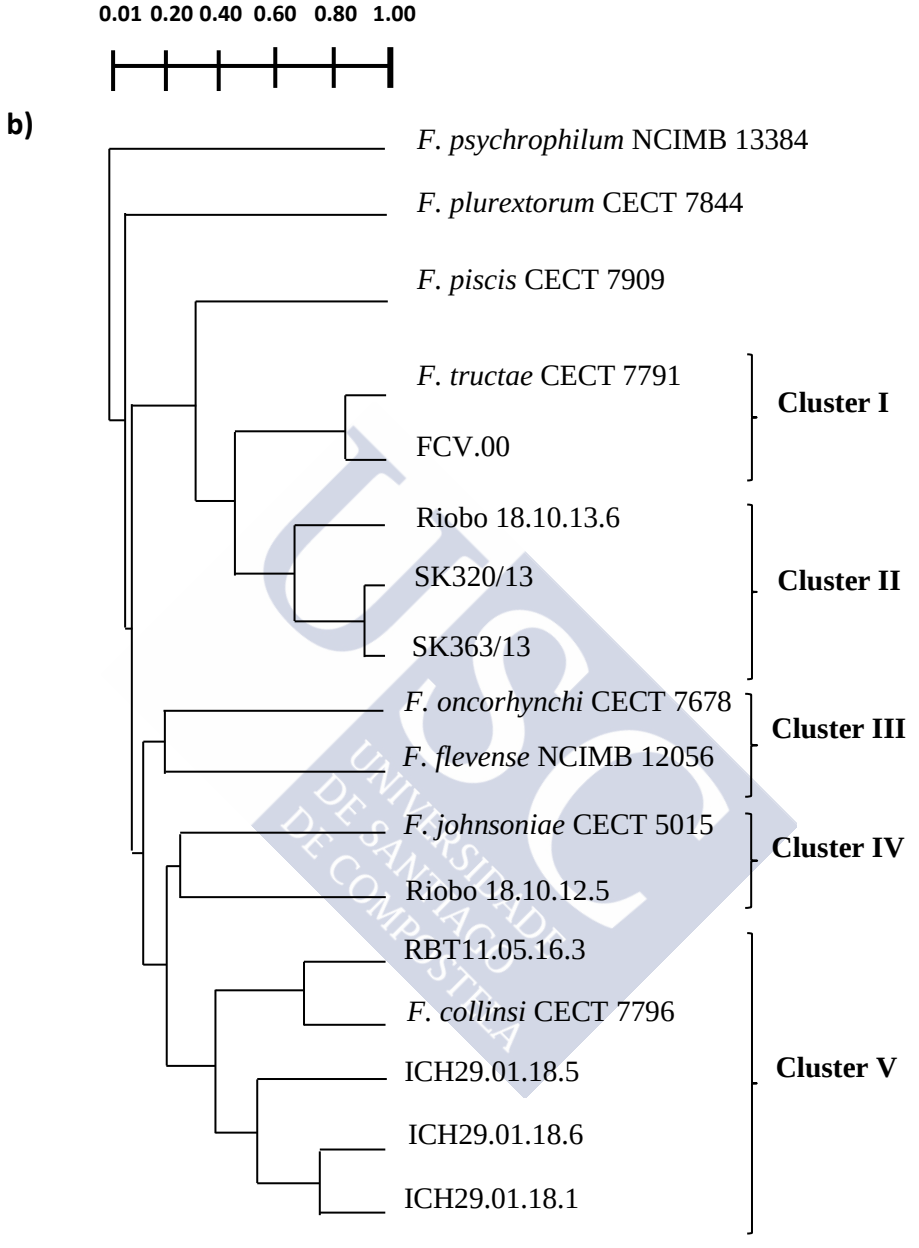


Figure 2. Dendrogram showing genetic relatedness of 17 strains of *Flavobacterium* sp. determined by analysis of REP-PCR (a) and ERIC-PCR (b) fingerprint patterns using Dice similarity coefficient and UPGMA cluster method. The scale above the dendrogram indicates the relative distance used in the clustering analysis.

3.3. Proteomic characterization

The analysis of the generated mass spectra profiles using BioTyper software v3.1 did not allow identification of the unknown *Flavobacterium* strains included in this study or produced misidentification. Some of the strains were misidentified as *F. oncorhynchi* or *F. saccharophilum* with score values between 1.700 - 1.999; while other strains showed scores < 1.700 and were considered to have no identification profiles (Table 3). Only the strains of the species *F. plurextorum*, *F. oncorhynchi*, *F. piscis* and *F. johnsoniae* were correctly identified to the species level (score values > than 2). Spectra obtained by MALDI-TOF-MS of each *Flavobacterium* strain were analysed using the Mass-Up software. Reproducible and informative mass spectra were acquired for all the *Flavobacterium* strains tested under the defined conditions, with good resolution and a large number of peaks (40-60 peaks in the *m/z* range corresponding to 2-15 kDa).

Table 3. BioTyper identification of the *Flavobacterium* strains analysed

Bacteria under study	Biotyper identification	Score
SK320/13	Not identified	-
SK363/13	Not identified	-
RBT11.05.16.3	*Flavobacterium saccharophilum*	1.901
Riobo 18.10.12.5	*Flavobacterium oncorhynchi*	1.761
Riobo 18.10.13.6	Not identified	-
ICH29.01.18.1	Not identified	-
ICH29.01.18.5	*Flavobacterium saccharophilum*	1.792
ICH29.01.18.6	*Flavobacterium saccharophilum*	1.743
FCV.00	*Flavobacterium saccharophilum*	1.700
F. collinsi CECT 7796	*Flavobacterium saccharophilum*	1.901
F. plurextorum CECT 7844	*Flavobacterium plurextorum*	2.318
F. oncorhynchi CECT 7678	*Flavobacterium oncorhynchi*	2.209
F. piscis CECT7909	*Flavobacterium piscis*	2.238
F. johnsoniae CECT 5015	*Flavobacterium johnsoniae*	2.062
F. branchiophilum DSM 24789	*Microbacterium liquefaciens*	2.247
F. tructae CECT 7791	*Flavobacterium saccharophilum*	1.707
F. flevense NCIMB 12056	*Microbacterium liquefaciens*	2.023

Cluster analysis using the Mass-Up software and the 17 *Flavobacterium* strains under study, demonstrated that *F. psychrophilum*, *F. branchiophilum* and *F. flevense* were clearly separated from another *F. psychrophilum*-like strains or *Flavobacterium* species (Figure 3). As in the molecular analysis, the strain FCV.00 and the reference strain of *F. tructae* CECT 7791 grouped together (Figure 3) and were closely related with the strains SK363/13 and SK320/13. The strains RBT11.05.16.3, ICH29.01.18.1, ICH29.01.18.5 and ICH29.01.18.6 showed a high similarity with the reference strain *F. collinsi* CECT 7796; while the strains Riobo 18.10.12.5 and Riobo 18.10.13.6 could not be grouped with any of the reference strains analysed. The strains *F. oncorhynchi* CECT 7678 and *F. plurextorum* CECT 7844 also were closely related (Figure 3).

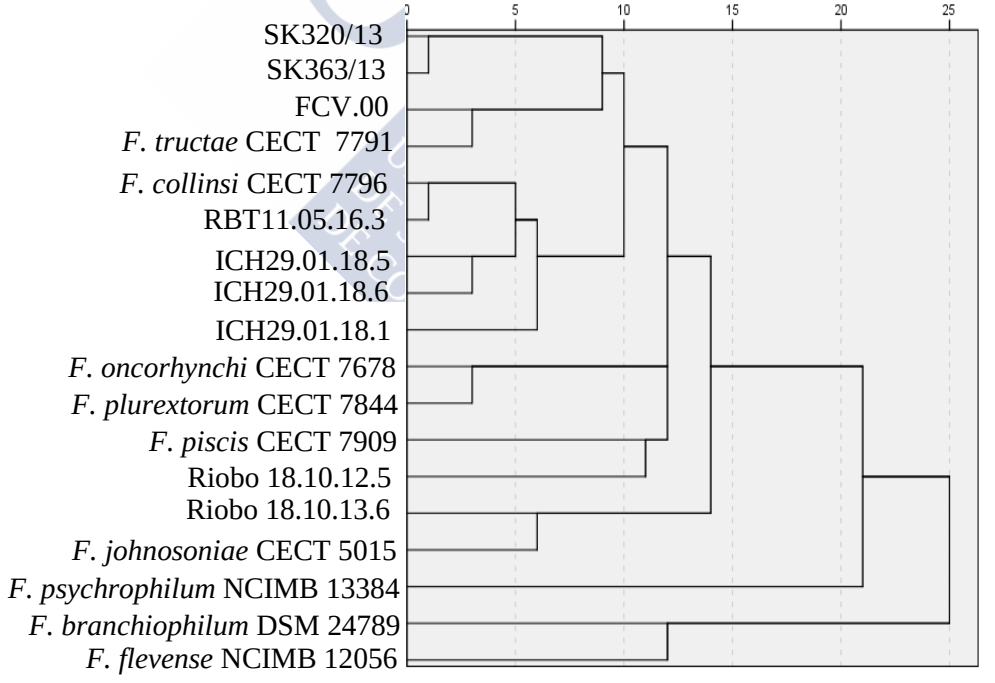
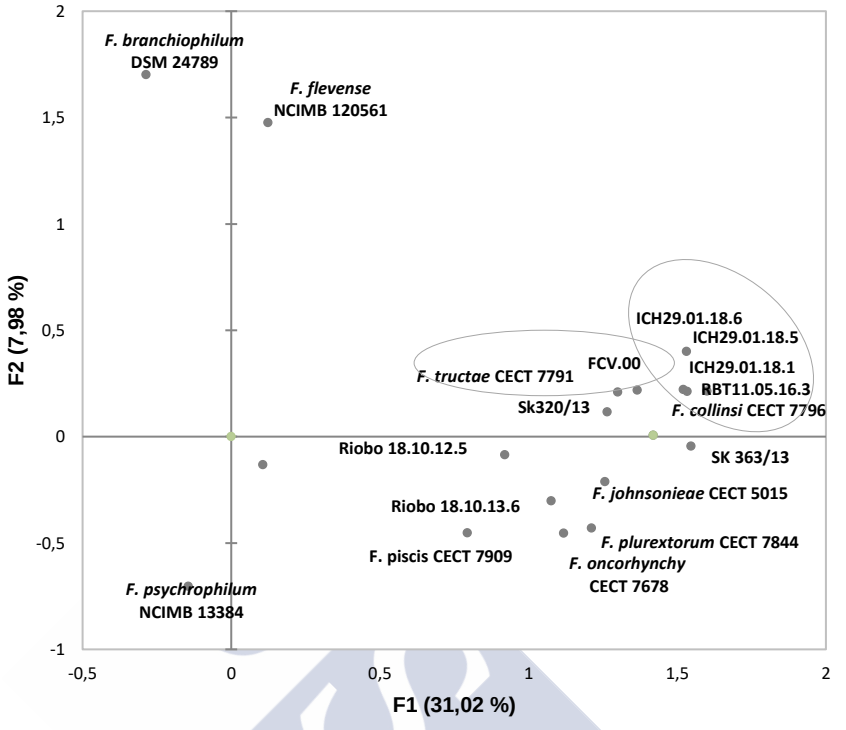


Figure 3. Phylo-proteomic tree of the *Flavobacterium* strains used in this study using Pearson Product Moment Correlation Coefficient (PPMCC). The scale above the dendrogram indicates the relative distance used in the clustering analysis.

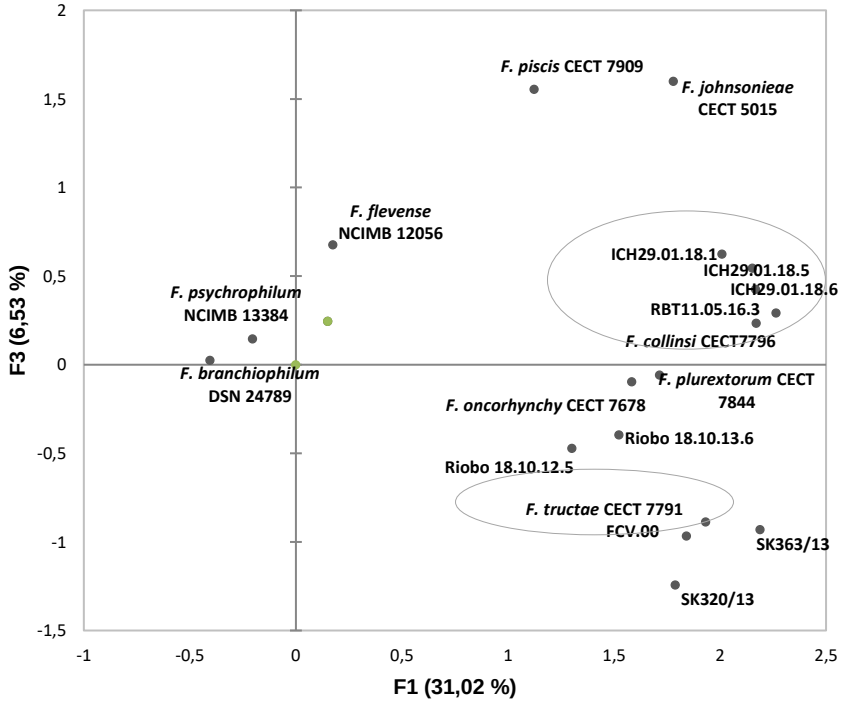
Analysis of the principal components (PCA) was performed on the binary data of the presence or absence of the mass peaks obtained from the proteomic analysis of the *Flavobacterium* species. The score plots of the first 3 PCs from PCA are shown in Figure 4. The strains of *F. psychrophilum*, *F. branchiophilum* and *F. flevense* were clearly separated from the other *F. psychrophilum*-like strains (Figure 4) as indicated by the cluster analysis. The strains FCV.00 and *F. tructae* were grouped together in the PCA. The reference strain *F. collinsi* was also clustered together with the strain RBT11.05.16.3 isolated from trout and with all the strains isolated from trout eggs (ICH29.01.18.1, ICH29.01.18.5 and ICH29.01.18.6) (Figure 4), as occurred in the molecular analysis and the proteomic cluster analysis.

The contributions of PC1, PC2, and PC3 to the generation of profiles in a percentage plot of the variance explained were approximately 31.02%, 7.97%, and 6.53%. To simplify the analysis, the variables (*m/z* values) with their absolute loading values (either PC1 or PC2) greater than 1 were selected for further analysis by PCA. The number of these peaks (n=93) was lower than using the entire peak mass list (n=316), thus, simplifying the analysis. The obtained graphic was highly similar to the one showed in the PCA using the entire mass peaks (Data not shown).

a)


F. branchiophilum DSM 24789
F. flevense NCIMB 120561
ICH29.01.18.6
ICH29.01.18.5
FCV.00
F. tructae CECT 7791
ICH29.01.18.1
RBT11.05.16.3
Sk320/13
F. collinsi CECT 7796
Riobo 18.10.12.5
SK 363/13
F. johnsonieae CECT 5015
Riobo 18.10.13.6
F. plurextorum CECT 7844
F. piscis CECT 7909
F. oncorhynchy CECT 7678
F. psychrophilum NCIMB 13384
F2 (7,98 %)
F1 (31,02 %)

b)


F. piscis CECT 7909
F. johnsonieae CECT 5015
F. flevense NCIMB 12056
ICH29.01.18.1
ICH29.01.18.5
ICH29.01.18.6
F. psychrophilum NCIMB 13384
RBT11.05.16.3
F. collinsi CECT7796
F. branchiophilum DSN 24789
F. plurextorum CECT 7844
F. oncorhynchy CECT 7678
Riobo 18.10.13.6
Riobo 18.10.12.5
F. tructae CECT 7791
FCV.00
SK363/13
SK320/13
F3 (6,53 %)
F1 (31,02 %)

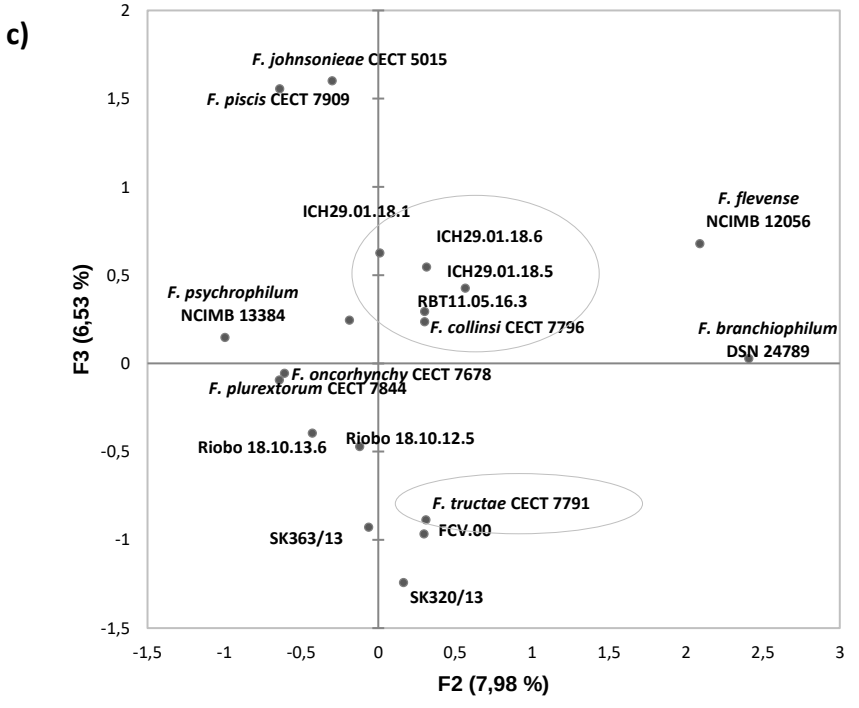


Figure 4. Principal component analysis of the binary data of the presence and absence of mass peaks obtained by MALDI-TOF-MS of the *Flavobacterium* strains under study. Multiple principal components are plotted: **a)** PC1 vs. PC2, **b)** PC2 vs. PC3 and **c)** PC1 vs. PC3. The diagrams show the distribution of the isolates tested.

3.4. Pathogenicity assay

The pathogenicity assay demonstrated that the *F. psychrophilum*-like strain RBT11.05.16.3 was virulent for rainbow trout, causing 80 to 100% of fish mortality with the doses evaluated (10^7 and 10^8 CFU/fish). The bacterium was recovered in pure cultures from liver, kidney and spleen of all moribund and dead fish. However, the strain ICH29.01.18.1 isolated from trout eggs was not virulent for rainbow trout at any of the doses used (10^6 to 10^8 CFU/fish) after 20

days of experimental infection. None of the control fish died during both experiments.

4. Discussion

Flavobacterium spp. are important fish pathogens and are a persistent threat to the aquaculture industry. In this study, nine *F. psychrophilum* -like strains isolated from 2011 to 2017 from diseased fish and eggs were characterized. Several authors have reported fish diseases with unidentified or partially characterized yellow-pigmented *F. psychrophilum*-like species (Ilardi and Avendaño-Herrera, 2008; Loch and Faisal, 2014; Loch and Faisal, 2015). All the isolates tested in this study, showed morphological and biochemical characteristics that conform to *Flavobacterium* genus (Bernardet et al., 2002). However, common biochemical tests employed did not allow differentiating the phenotypically similar *F. psychrophilum*-like under study to species level. Antibiotic susceptibility analysis showed that all the *Flavobacterium* sp. were resistant to ampicillin and to trimethoprim-sulfamethoxazole and variable results were obtained when flumequine, florfenicol or oxytetracycline were used. Antimicrobial sensitivity data is imperative for the treatment of fish in case that disease outbreak associated with a *Flavobacterium* sp. occur in an aquaculture farm. Analysis of *Flavobacterium* species using molecular typing techniques as ERIC and REP-PCR have demonstrated that these techniques are not able to differentiate strains according to isolation source and geographic region (Saticioglu et al., 2018; Valdebenito and Avendaño-Herrera, 2009). However, in the present study we demonstrated that these techniques could be used to differentiate or to identify *Flavobacterium* species by comparing the molecular profiles obtained for the unknow isolates with those of the reference strains. Thus, the strains FCV.00 could be tentatively identified as *F. tructae* and the strain RBT11.05.16.3 as *F. collinsi*. On the other hand, the strains

isolated from eggs ICH29.01.18.1, ICH29.01.18.5 and ICH29.01.18.6 showed ERIC and REP profiles similar to that of the species *F. collinsi.* This indicates that these strains could be closely related to this species or could represent a new species. However, to confirm the identification of a *Flavobacterium* isolate it is necessary other studies based on DNA-DNA hybridization as well as the phylogenetic analysis derived from 16S rRNA sequencing (Bernardet et al., 2002).

In this study, MALDI-TOF-MS was used for the differentiation and discrimination of microorganisms of the family *Flavobacteriaceae*. This proteomic technique has been previously used for this purpose (Pérez-Sancho et al., 2017). Most of the species of the genus *Flavobacterium* described for now are not included in the Bruker database of clinical and environmental bacteria. As a consequence, most of the strains under study were misidentified or not identified using the BioTyper 3.1 software. Previous reports have described that MALDI-TOF-MS correctly differentiate *F. psychrophilum* from other *F. psychrophilum*-like species (Pérez-Sancho et al., 2017). However, its ability to differentiate among *F. psychrophilum*-like species is limited. In this study, two mass peaks previously described that are present in the spectra of *F. psychrophilum*-like but absent in *F. psychrophilum* were also found (mass peaks *m/z* 6170 and 9241) (Pérez-Sancho et al., 2017). On the other hand, the mass peak at *m/z* 3124 specific for *F. piscis* described by Pérez-Sancho et al. (2017), was also found in our study. As previously described, the isolates of *F. plurextorum* and *F. oncorhynchi* showed high levels of similarity in their mass spectra (Pérez-Sancho et al., 2017). These high levels of similarity among the MALDI spectra of the *Flavobacterium* species could explain the problems of misidentification among the isolates. Thus, MALDI-TOF could be used for the differentiation of *F. pyschrophilum*-like strains from *F. psychrophilum* but not to define their taxonomic identity.

PCA has been previously used for the discrimination of bacteria (AlMasoud et al. 2014), including *Flavobacterium* species (Pérez-Sancho et al., 2017). In this study, PCA was performed using the binary peak matrix as previously reported (AlMasoud et al. 2014). The species *F. psychrophilum, F. branchiophilum* and *F. flevense* were clearly separated from the other *Flavobacterium* strains included in this study, as also reported by Pérez-Sancho et al. (2017). The species *F. oncorhynchi* and *F. plurextorum,* clustered together as described (Pérez-Sancho et al., 2017), indicating that MALDI analysis and PCA may not be useful for the differentiation of these species. The strains isolated from eggs used in the study were grouped together with strains isolated from fish in the molecular and protemic analysis, indicating that these methods are not useful for the discrimination of bacteria isolated from different sources (eggs or fish). However, further studies using a higher number of strains isolated from fish and eggs should be carried out to clarify the usefulness of these molecular and proteomic techniques for epidemiological purposes.

In this study, virulence tests showed that the isolate RBT11.05.16.3 isolated from fish produced 80 to 100% mortality in rainbow trout with doses of bacteria of 10^7 and 10^8 CFU per fish. However, strain ICH29.01.18.1 isolated from trout eggs was not virulent for rainbow trout even after intra-peritoneally injection of 10^8 CFU per fish. The aetiology of diseases caused by other *Flavobacterium* spp. is less well known than pathologies produced by *F.psychrophilum*, *F. columnare* or *F. branchiophilum*. Some authors have suggested that some *Flavobacterium* species could act as saprophytic or commensal organisms and may act as opportunistic fish pathogens (Bernardet and Bowman, 2006).

Lastly, although 16S rRNA sequencing analysis is needed; biochemical, genetic and proteomic results (cluster and PCA) are in accordance. Results suggest that the strains FCV.00 and RBT11.05.16.3 may be identified as *F. tructae* and *F. collinsi*, respectively. On the other hand, the strains isolated from

eggs (ICH29.01.18.1, ICH29.01.18.5 and ICH29.01.18.6) were highly similar to the strains *F. collinsi* and RBT11.05.16.3. The results obtained for the rest of the strains analysed were not consistent indicating that these strains could likely represent novel flavobacterial taxa.

The present study elucidates the diversity of flavobacteria that are associated with both diseased, apparently healthy fish or fish eggs in aquaculture systems in Spain. This suggests that diseases produced by *F. psychrophilum*-like strains are likely to become more frequent in the future.

5. References

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Supplementary Material

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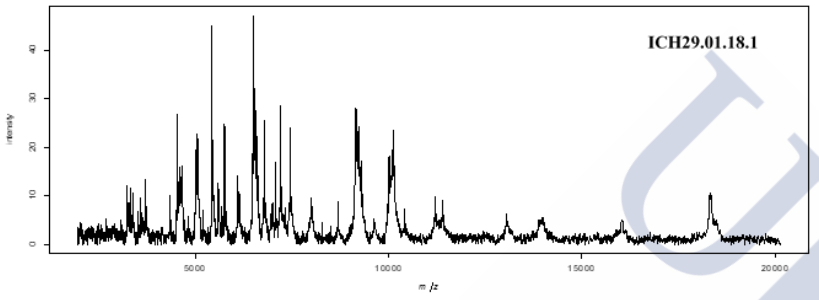
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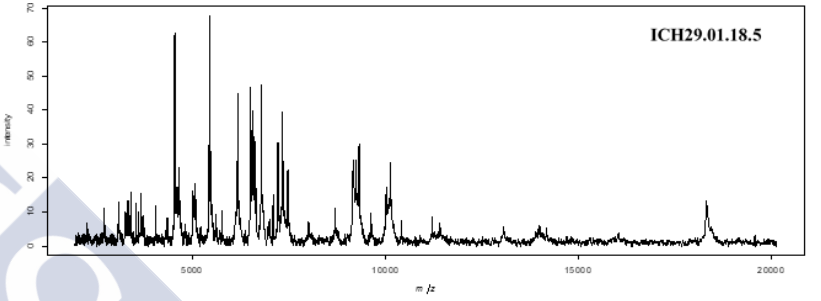
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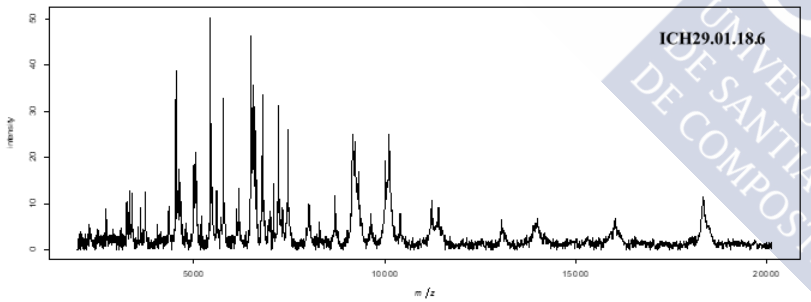
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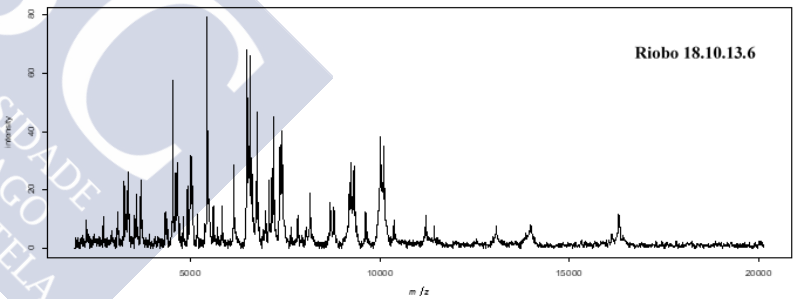
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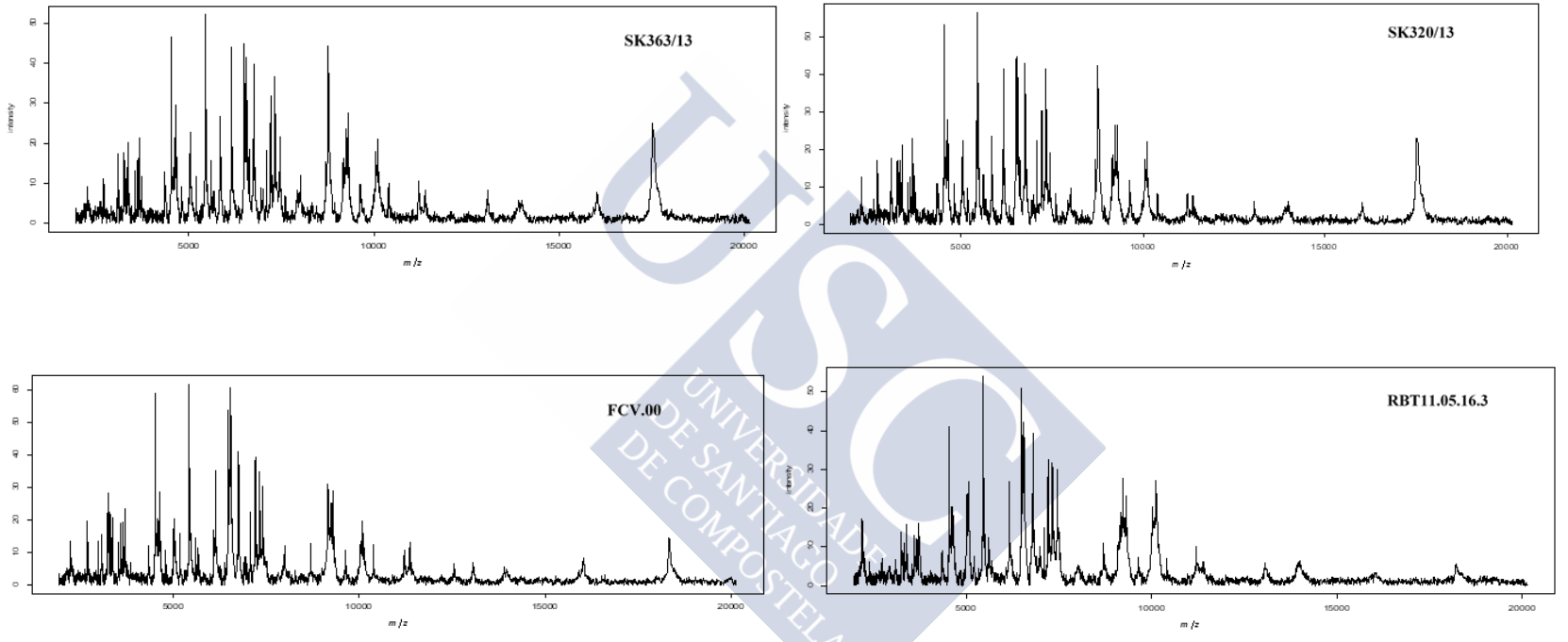

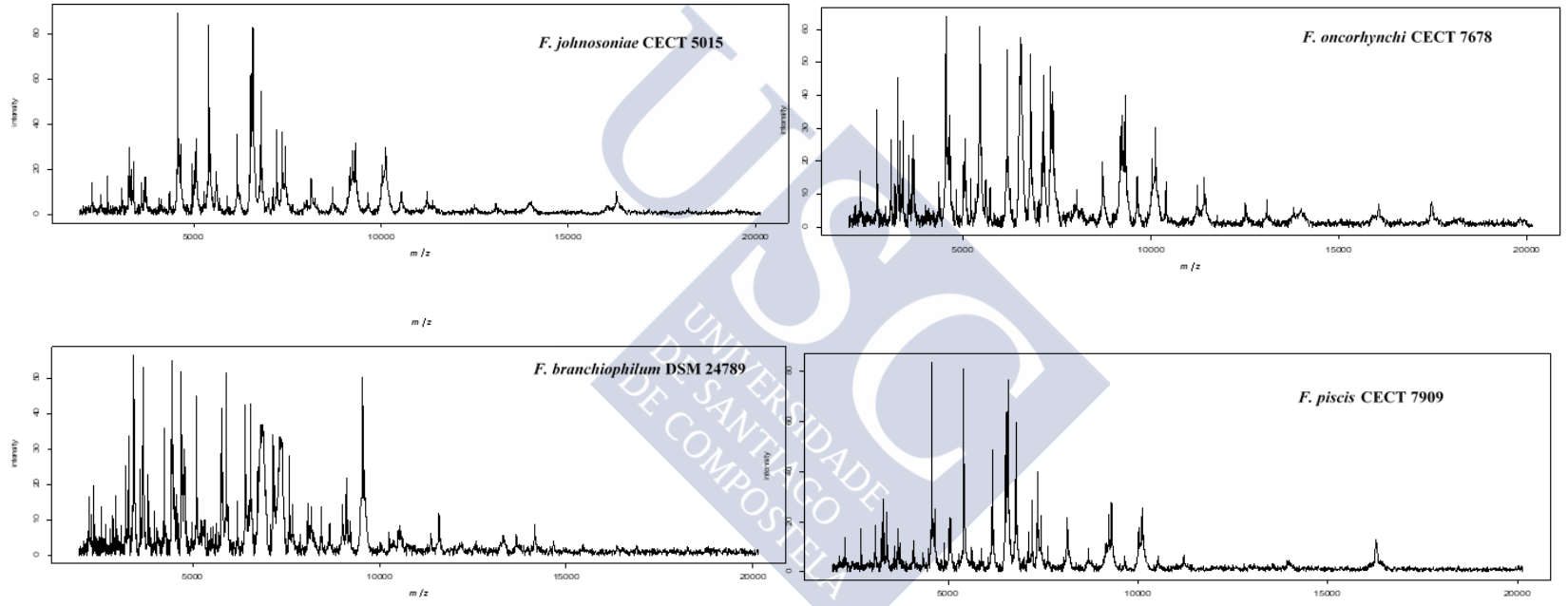
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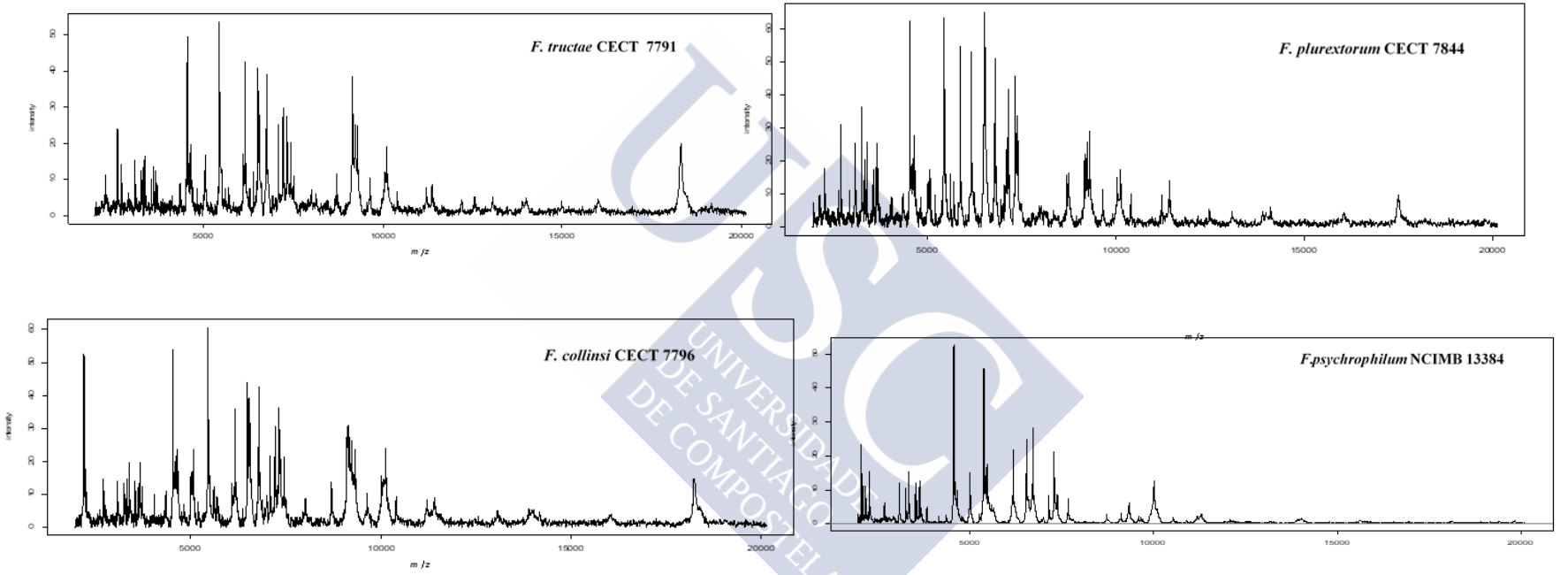

Figure 1S. MALDI-TOF spectra of the *Flavobacterium* strains analysed in this study

CHAPTER IV.

Development of highly sensitive molecular methods for the detection and identification of fish pathogens and its differentiation from genetically closely related species.

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Article nº4. Fernández-Álvarez C., González S.F., Santos Y. (2017). Development of a SYBR Green I real-time PCR assay for specific identification of the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida*. *Applied Microbiology and Biotechnology* 100(24):10585-10595. DOI: 10.1007/s00253-016-7929-2.

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Development of a SYBR Green I real-time PCR assay for specific identification of the fish pathogen *Aeromonas salmonicida* subspecies *salmonicida*

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https://link.springer.com/article/10.1007%2Fs00253-016-7929-2

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nº5. Fernández-Álvarez C., González S.F., Santos Y. (2019). Quantitative PCR coupled with melting curve analysis for rapid detection and quantification of *Tenacibaculum maritimum* in fish and environmental samples. *Aquaculture* 498:289-296. DOI: 10.1016/j.aquaculture.2018.08.039

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Quantitative PCR coupled with melting curve analysis for rapid detection and quantification of *Tenacibaculum maritimum* in fish and environmental samples

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https://www.sciencedirect.com/science/article/pii/S0044848617323864

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CHAPTER V.

Evaluation of proteomic, serological and molecular methods for the typing and epidemiological study of pathogenic fish bacteria.

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Article nº6. Clara Fernández-Álvarez, Yolanda Torres-Corral, Nancy Saltos-Rosero, Ysabel Santos (2017). MALDI-TOF mass spectrometry for rapid differentiation of *Tenacibaculum* species pathogenic for fish. *Applied Microbiology and Biotechnology* 101(13) 5377-5390. DOI: 10.1007/s00253-017-8324-3.

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MALDI-TOF mass spectrometry for rapid differentiation of *Tenacibaculum* species pathogenic for fish.

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https://link.springer.com/article/10.1007%2Fs00253-017-8324-3

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Article nº7. Clara Fernández-Álvarez, Yolanda Torres-Corral, Ysabel Santos (2018). Comparison of serological and molecular typing methods for epidemiological investigation of *Tenacibaculum* species pathogenic for fish. *Applied Microbiology and Biotechnology* 102(6):2779-2789. DOI: 10.1007/s00253-018-8825-8

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Comparison of serological and molecular typing methods for epidemiological investigation of *Tenacibaculum* species pathogenic for fish

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https://link.springer.com/article/10.1007%2Fs00253-018-8825-8

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Article nº8. Clara Fernández-Álvarez, Yolanda Torres-Corral, Ysabel Santos (2018). Use of ribosomal proteins as biomarkers for identification of *Flavobacterium psychrophilum* by MALDI-TOF mass spectrometry. *Journal of Proteomics* 170:59-69. DOI: 10.1016/j.jprot.2017.09.007

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Use of ribosomal proteins as biomarkers for identification of *Flavobacterium psychrophilum* by MALDI-TOF mass spectrometry

Clara Fernández-Álvarez, Yolanda Torres-Corral, Ysabel Santos

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CHAPTER VI.

Phenotypic and molecular characterization of the resistance of fish pathogenic bacteria to antimicrobials commonly used in aquaculture.

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Phenotypic and molecular identification of antimicrobial resistance in *Aeromonas salmonicida* subsp. *salmonicida* isolated in Spain.

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Abstract

Aeromonas salmonicida subsp. *salmonicida* is the aetiological agent of typical furunculosis, an important disease in the salmonid and non-salmonid fish farming industry worldwide. The development of antimicrobial resistance by typical *A. salmonicida* is a concern because management of outbreaks of furunculosis often requires the use of antimicrobials. In this study, a preliminary assessment of the occurrence of resistance to antimicrobials in the fish pathogen *A. salmonicida* isolated from a variety of aquaculture species in Spain was performed. Thus, twenty-seven strains of *A. salmonicida* isolated from 2000 to 2018 were tested for sensitivity to six antimicrobial agents using the disk diffusion assay. The Minimal Inhibitory Concentrations (MIC) and the Minimal Bactericidal Concentration (MBC) were determined by the broth microdilution method. Resistance to trimethoprim/sulfamethoxazole (66.6% of the strains), oxytetracycline (55.5% of the strains) and chloramphenicol (33.3% of the strains) was determined according to either disk diffusion and/or MIC assays. Resistance to florfenicol (7.40% of the strains) and enrofloxacin (3.70% of the strains) was less common, while resistance to flumequine was not present in the strains analysed. Multiple resistance was observed in the 66.6% of the strains

analysed. Similar results were obtained for both assays for all the antimicrobials tested. However, minor errors (intermediate susceptibility by disk diffusion and resistant or susceptible by broth microdilution methods) were obtained for oxytetracycline, enrofloxacin and chloramphenicol. Molecular identification of antimicrobial resistance genes of the *A. salmonicida* subsp. *salmonicida* strains by PCR showed that the gene *sul1*; that confers resistance against trimethoprim/sulfamethoxazole; appeared to be the most prevalent (66.6% of the isolates), followed by the gene *tet(A)* (55.5% of the strains); that confers resistance to oxytetracycline; and the *cat* gene (33.3% of the strains); that confers resistance to chloramphenicol. Lastly, the genes *tet(E)* and *flor* were only present in the 7.40% of the strains. This study provides an update on the antimicrobial resistance on *A. salmonicida* subsp. *salmonicida* isolated in Spain. All the results obtained suggest that this fish pathogen is an important reservoir of drug resistance genes and should be monitored more extensively.

1. Introduction

Aeromonas salmonicida subsp. *salmonicida* is the causative agent of "typical" furunculosis, that causes economically important losses in the farming of salmonids in fresh and marine waters. This disease produce a chronic or acute haemorrhagic septicaemia, often with extensive liquefactive necrosis (Toranzo et al., 2005). A variety of inhibitory agents have been applied with varying degrees of success for the treatment of furunculosis; including chloramphenicol, oxytetracycline, flumequine, oxalinic acid, and potentiated sulphonamides (Austin and Austin, 2012). Within Spain, three compounds, namely flumequine, oxytetracycline and florfenicol are currently approved by the Spanish Agency of Medicines and Medical Devices (AEMPS) for fisheries use in the treatment of diseased fish. In addition to systematic application of these compounds to

treat diseased fish, prophylactic use of antimicrobials in aquaculture is widespread for the prevention of *Aeromonas*-related diseases (Patil et al., 2016). The indiscriminate use of antimicrobials has led to the emergence of antimicrobial-resistant bacteria, which represent a serious concern because of the risk of transfer of their antimicrobial-resistance genes to human pathogens via the food chain (Akinbowale et al., 2006; Cabello, 2006; Radhouani et al., 2014; Sapkota et al., 2008). These antimicrobial resistance genes are usually located on mobile genetic elements including conjugative plasmids, class 1 integrons, IS elements, transposons, or genomic islands carrying single or multidrug resistance genes and gene cassettes (Patil et al., 2016; Piotrowska and Popowska, 2015). These mobile elements harbour multiple antimicrobial resistance determinants which may be transferred and spread within bacterial communities (Blair et al., 2015). Resistance to oxytetracycline or to a combination of trimethoprim/sulfamethoxazole has been reported to be encoded by transferable plasmids within the genus *Aeromonas*; while several classes of tetracycline resistance determinants have described on R-plasmids within this species (Adams et al., 1998; DePaola et al., 1988; Rhodes et al., 2000).

In this study, we carried out a preliminary evaluation of the occurrence of resistance to antimicrobials in *Aeromonas salmonicida* subsp. *salmonicida* isolated from 2000 to 2018 from a variety of aquaculture and wild species and regions in Spain. For this purpose, the susceptibility of isolates of *A. salmonicida* to different antimicrobials frequently used in aquaculture worldwide as oxytetracycline, florfenicol, trimethoprim/sulfamethoxazole, chloramphenicol, flumequine and enrofloxacin (some of which are registered for use in Spain); was determined using the disk diffusion and/or broth microdilution assays. Moreover, the molecular identification of antimicrobial-resistant genes was examined by polymerase chain reaction (PCR) in all the isolates of *A. salmonicida* included in the study.

2. Material and Methods

2.1. Microorganisms tested and culture conditions

The bacterial strains tested included 28 clinical isolates of *Aeromonas salmonicida* subsp. *salmonicida* isolated from different fish species and regions (Table 1). Reference strains used in this study were obtained from the American Type Culture Collection (ATCC). Stock bacterial cultures were stored frozen at -30ºC in Microbank™ commercial medium (Pro-Lab Diagnostics) until use. Bacterial strains were cultured on Mueller-Hinton agar plates for 24-48 h at 18ºC. The identity of the bacterial strains was confirmed using the PCR methods previously described (Beaz-Hidalgo et al., 2008). The strain of *A. salmonicida* ATCC33658 was used as quality control in all assays.

Table 1. *A. salmonicida* strains used in this study

Reference strains	**Source of isolation**	**Year**
ATCC33658	Atlantic Salmon	-
ATCC14174	Brook trout (USA)	-
Clinical isolates		
A. salmonicida (*n*=4)	Rainbow trout, Spain	2009
A. salmonicida (*n*=2)	Sea bass, Spain	2012
A. salmonicida (*n*=1)	Sea lamprey, Spain	2000
A. salmonicida (*n*=1)	Rainbow trout, Spain	2008
A. salmonicida (*n*=1)	Turbot, Spain	2006
A. salmonicida (*n*=3)	Turbot, Spain	2001
A. salmonicida (*n*=2)	Turbot, Spain	2009
A. salmonicida (*n*=1)	Fario trout, Spain	2008
A. salmonicida (*n*=9)	Seabream, Spain	2018
A. salmonicida (*n*=1)	Turbot, Spain	2018
A. salmonicida (*n*=1)	Turbot, Spain	2017

ATCC American Type Culture Collection (USA)

2.2. Disk diffusion test

Susceptibility profiles of the fish pathogenic *A. salmonicida* isolates to various antimicrobials normally used in aquaculture were evaluated by the disk diffusion method using 24 h cultures of all strains as recommended by the Clinical and Laboratory Standards Institute document M42-A and M49-A (CLSI, 2006). The antimicrobial agents and the concentrations (μg $disc^{-1}$) used were: oxytetracycline (30), enrofloxacin (5), trimethoprim-sulfamethoxazole (25), chloramphenicol (30), flumequine (30) and florfenicol (30). The entire surface of the Mueller-Hinton agar plates was inoculated by streaking with the swab containing the inoculums adjusted to a concentration of $1\text{-}2x10^9$ colony forming units (CFU ml^{-1}) (OD_{620}). Agar plates inoculated with *A. salmonicida* subsp. *salmonicida* were incubated at 18 ºC. After incubation (24-48 h), the sensitivity of the bacterium to the antimicrobial was determined by measuring the diameters of the inhibition zone (mm). Assays were performed in triplicate, and inhibition zones were expressed in millimetres (average ± SD). The strains were divided in sensitive, intermediate or resistant following the criteria described by Miller et al. (2006) and the CLSI (2010)

2.3. Determination of minimum inhibitory concentration (MIC)

The inhibitory activities of the antimicrobials oxytetracycline, enrofloxacin and chloramphenicol were screened against all *A. salmonicida* isolates using the microdilution assay, following the procedures described by the Clinical and Laboratory Standards Institute (CLSI, 2006). The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antimicrobial that significantly inhibited the growth of the bacteria tested. The micro-dilution method in culture broth was performed using 96-well plates (Becton Dickinson Labware Europe, Francia). A stock solution of all antimicrobials was prepared in Mueller-Hinton broth at an initial concentration

of 1024 µg ml^{-1}, and two-fold dilutions with concentrations varying from 512 µg ml^{-1} to 0.0312 µg ml^{-1} were tested. The culture medium was used as negative control. In positive-control wells any antimicrobial was included. Each test and growth control wells were inoculated with 10 µl of the bacterial suspension of *A. salmonicida* subsp. *salmonicida* adjusted to a concentration of 1-2x10^9 CFU ml^{-1} (OD_{620}). All experiments were performed in triplicate and then averaged. The plates were incubated for 48-72 h at 18ºC. Bacterial growth was measured by optical density (Microplate Reader, Model 680, Bio-Rad) at OD_{620}. The strains were divided in sensitive, intermediate or resistant following the criteria described by Miller et al. (2006) and the CLSI (2010).

2.4. Determination of minimum bactericidal concentration (MBC)

The MBC was determined by culture and spectrophotometric methods. All bacterial strains of *A. salmonicida* subsp. *salmonicida* were used in this assay. For culture method, the initial inoculum, the dilution representing the MIC and at least two concentrations above the MICs were serially diluted and plated to enumerate to viable cells. The cultures were incubated at 18 or for 48–72 h and the antimicrobial concentration that cause a reduction of the 99.9% of the original inoculums was considered the MBC. The reduction of number of viable cells was also measured by a colorimetric assay based on the reduction of a tetrazolium salt (MTT method). Briefly, viable bacteria present in the wells were quantified by the addition of 10 µl of [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, 5 mg ml^{-1}), which is reduced in proportion to the number of viable bacteria present. The optical density was read at 620 nm 15 min later on a spectrophotometer (Microplate Reader Model 680, BioRad). Experiments were carried out in triplicate. To determine the bactericidal or bacteriostatic capacity of the nutraceutical, the ratio MBC/MIC was used. If the ratio MBC/MIC was lower than 4, the effect was considered as bactericidal. If

the ratio MBC/MIC was higher than 4, the effect was determined as bacteriostatic.

2.5. DNA isolation and quantification

Total nucleic acid was extracted from bacterial pellets using the commercial system InstaGene Matrix (Bio-Rad, California, USA). The concentration of purified DNA was determined using the fluorimeter Qubit® 2.0 and the Qubit® double-stranded DNA (dsDNA) BR assay kit (Invitrogen, California, USA) according to the manufacturers´ manual. The DNA was kept at -30ºC until subsequent use in the PCR analyses.

2.6. Detection of tetracycline resistance determinants

DNA samples from bacterial isolates resistant to antimicrobials were screened for resistance genes known to be present in *A. salmonicida* subsp. salmonicida: *tet(A)*, *tet(G)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(H)*, *cat*, *sul1*, *sul2* and *flor*. The primers used have been previously described (Schmidt et al., 2001; Trudel et al., 2016) and are indicated in Table 2. PCR amplifications were performed in a final volume of 25 µl containing 4 µl of 5 x Phire Reaction Buffer (Thermo Scientifics, Massachusetts, USA), 0.4 µl of 10 µM dNTP mixture (200µM each) (Thermo Scientifics), 0.4 µl of Phire Hot Start II DNA polymerase (Thermo Scientifics, Massachusetts, USA), 400 nM of each primer, 1 µl of DNA template and sterile water. Reactions lacking DNA template (no template control) were used as negative controls. The PCR conditions were an initial denaturation step of 98 °C for 5 min, followed by 30 cycles of denaturation at 98 °C for 30s, annealing at 60ºC for 30s, extension at 72 °C for 30s and a final extension at 72 °C for 5 min. Amplifications were performed in a Mastercycler Epgradient thermal cycler (Eppendorf, Hamburg, Germany). PCR products were electrophoresed in 1.5 % agarose gel and visualized by staining with RedSafe

nucleic acid staining solution (20000x) (iNtRON Biotechnology, Seongnam-Si, Korea). A 1 kb and 100 bp DNA ladder (Fermentas, Madrid, Spain) was included as a molecular weight marker.

Table 2. Primer sequences used in this study.

Target	Primer	Nucleotide sequence (5´–3´)	Amplicon size (bp)	Reference
tet(A)	tetA F1	GTAATTCTGAGCACTGTCGC	957	Schmidt et al. (2001)
	tetA R1	CTGCCTGGACAACATTGCTT		
tet(C)	tetA(C)-F1	CTGTAGGCATAGGCTTGGTTAT	629	Trudel et al. (2016)
	tetA(C)-R1	CTGTCCTACGAGTTGCATGATA		
tet(D)	tetA(D)-F1	ATTACACTGCTGGACGCGAT	1124	Schmidt et al. (2001)
	tetA(D)-R1	CTGATCAGCAGACAGATTGC		
tet(E)	tetA(E)-R1	GATGTCACACCTGAGGAATCC	351	Trudel et al. (2016)
	tetA(E)-F1	TCCGAATAAAACCCATAATGTTGC		
tet(G)	tetA(G)-F1	GGTTCGCATCAAACCATTCG	460	Trudel et al. (2016)
	tetA(G)-R1	GCTTAGATTGGTGAGGCTCG		
cat	cat-F1	CTATTTTGACAATACGCCCTGC	448	Trudel et al. (2016)
	cat-R1	CTTCCCAAACGTAAATATCGGC		
floR	flor-F1	TTGAGCCTCTATATGGTGATGC	632	Trudel et al. (2016)
	flor-R1	GTTGTCACGATCATTACAAGCG		
sul1	sul1-F1	GGGCTACCTGAACGATATCC	550	Trudel et al. (2016)
	sul1-R1	CTAGGCATGATCTAACCCTCG		
sul2	sul2-F1	ATCATCTGCCAAACTCGTCG	449	Trudel et al. (2016)
	sul2-R1	TTCTTGCGGTTTCTTTCAGC		

3. Results

3.1. Antimicrobial susceptibility profile

Twenty-seven strains of fish pathogenic *A. salmonicida* subsp. *salmonicida* isolates were tested for their susceptibility against six antimicrobial agents (oxytetracycline, enrofloxacin, florfenicol, chloramphenicol, trimethoprim-sulfamethoxazole, flumequine) using the disk diffusion assay. The average diameter of the zones of inhibition obtained for the *A. salmonicida* strains for each of the antimicrobials tested are showed in Table 2. Inhibition zone diameters for the disk diffusion assay ranged from 0 to 45 mm for oxytetracycline, for enrofloxacin ranged from 17 to 48 mm and for florfenicol the inhibition zones ranged from 0 to 45 mm. Dimeters from 0 to 30 mm were obtained for trimethoprim-sulfamethoxazole, diameters from 0 to 47 mm were obtained for chloramphenicol, and finally, inhibition zones from 20 to 45 mm were obtained for flumequine after 48 h of incubation period (Table 3).

Table 3. Results obtained from the disk diffusion method

Parameter	OT	FLOR	ENR	SXT	CHL	FLU
Mean diameter (mm)	16.03	34.95	29.08	15.38	11.75	30.88
Standard deviation (mm)	17.58	12.56	8.36	12.90	13.93	6.78
Coefficient of variation (%)	1.09	0.36	0.28	0.83	1.18	0.22
Maximum (mm)	45	55	48	30	47	49
Minimum (mm)	0	0	17	0	0	24

OT, oxytetracycline; ENR, enrofloxacin; FLOR, florfenicol; CHL, chloramphenicol; SXT, trimethoprim-sulfamethoxazole; FLU, flumequine.

Considering these results, the *A. salmonicida* strains were classified as resistant, intermediate or sensitive to the antimicrobials following the criteria described by Miller et al. (2006), the CLSI (2006) (documents M42-A, M49-A and M11-A6) and the EUCAST (2018) (Figure 1 and Table 4).

Following the disk diffusion results and the criteria selected, more than half (66.67%) of all the isolates were resistant to trimethoprim-sulfamethoxazole. Tetracycline resistance was found in 55.5% of the isolates; while resistance to chloramphenicol and florfenicol was found in 33.3% and 7.4% of the isolates, respectively. The 100% of the strains were susceptible to flumequine; while for enrofloxacin, the 88.8% of the strains were susceptible and the 11.1% showed intermediate susceptibility (Figure 1). Of the 28 isolates tested, only 5 (18.51%) were sensitive to all six antimicrobial agents tested. From all the strains, 3 isolates (11.1%) were resistant to just one antimicrobial agent, fifteen isolates (55.55%) were resistant to two antimicrobials, while two strains (7.4%) were resistant to three antimicrobials and one strain (3.7%) to four antimicrobials.

Table 4. Epidemiologic cut-off values for diameters of the zones of inhibition used in this study (CLSI, 2006; EUCAST, 2018; Miller et al., 2006)

Antimicrobial (µg/disk)	**Diameter of zone of inhibition (mm)**		
	Sensitive	**Intermediate range**	**Resistant**
Oxytetracycline (30)	≥28	24-27	≤23
Florfenicol (30)	≥31	ND	≤30
Trimethoprim-sulfamethoxazole (25)	≥28	27-24	≤23
Enrofloxacin (5)	≥23	22-17	≤16
Flumequine (30)	≥20	19-17	≤16
Chloramphenicol (30)	≥18	13-17	≤12

ND, Not determined.

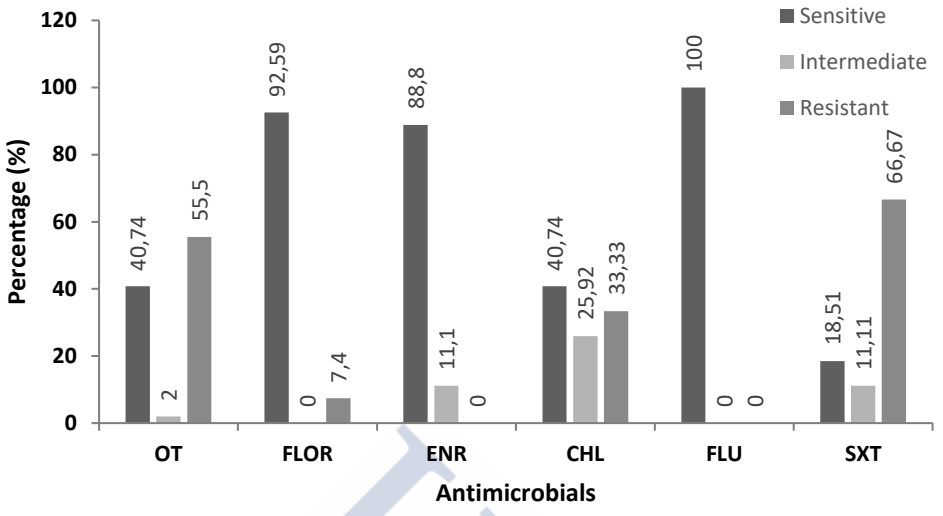

Figure 1. Distribution of the *A. salmonicida* strains according to their susceptibility to the antimicrobials analysed by the disk diffusion assay. OT, oxytetracycline; FLOR, florfenicol; ENR, enrofloxacin; CHL, chloramphenicol; FLU, flumequine; SXT, trimethoprim-sulfamethoxazole.

3.2. Determination of the MIC and MBC

Considering the results obtained in the disk diffusion assay, the MICs of the oxytetracycline, enrofloxacin and chloramphenicol were determined by the broth dilution methods. The *A. salmonicida* strains were divided in sensitive, intermediate or resistant following the criteria described by Miller et al. (2006) and the CLSI (2006) (documents M42-A, M49-A and M11-A6) and the results are showed in Table 5. Oxytetracycline MICs of resistant *A. salmonicida* strains ranged from 32 μg ml^{-1} to 128 μg ml^{-1}, whereas susceptible strains presented MICs lower than 0.5 μg ml^{-1}. The strains that were resistant to oxytetracycline in the disk diffusion assay were also resistant using the broth microdilution method (55.5% of the strains). However, the strains that showed intermediate susceptibility to oxytetracycline in the disk diffusion assay, were sensitive accordingly to the MICs (≤4 μg/ml) obtained (Table 5). Chloramphenicol MICs

of resistant *A. salmonicida* strains ranged from 256 µg ml^{-1} to 512 µg ml^{-1}, whereas susceptible strains showed MICs ranging from 0.125 µg ml^{-1} to 0.5 µg ml^{-1}. The strains that showed intermediate susceptibility to chloramphenicol in the disk diffusion assay were sensitive in the broth dilution assay; while the number of resistant strains were the same in both studies. For enrofloxacin, one strain that showed intermediate sensitivity to enrofloxacin in the disk diffusion assay was resistant to this antimicrobial, with a MIC ≥ 4 µg/ml. The rest of the strains showed MIC values for enrofloxacin ranging from 0.25 µg ml^{-1} to 0.5 µg ml^{-1}. Considering the ratio of MBCs and MICs (MBC/MIC<4), the oxytetracycline, chloramphenicol and enrofloxacin showed a bactericidal activity against the *A. salmonicida* strains tested.

Taking into account both disk diffusion and MIC results, three isolates (11.1%) were resistant to just one antimicrobial agent, fifteen isolates (59.25%) were resistant to two antimicrobials, two strains (7.4%) were resistant to three antimicrobials and one strain (3.7%) to five antimicrobials.

Table 5. MIC values obtained for the *A. salmonicida* strains by the broth microdilution method

Antimicrobial		% strains
Oxytetracycline	S (≤4 µg/ml)	44.5
	I (8 µg/ml)	-
	R (≥16 µg/ml)	55.5
Chloramphenicol	S (≤1-2 µg/ml)	66.6
	-	-
	R (≥2 µg/ml)	33.3
Enrofloxacin	S (≤ 0.5 µg/ml)	96.29
	I (1-2 µg/ml)	-
	R (≥ 4 µg/ml)	3.70

S, sensitive; I, intermediate; R, resistant according to the epidemiologic cut-off values for MICs used in this study (CLSI, 2006; EUCAST, 2018; Miller et al., 2006).

3.3. Detection of resistance determinants by real-time PCR

All the *A. salmonicida* strains were analysed by PCR to determine the presence of the antimicrobial resistance genes *tet(A)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(G)*, *tet(H)*, *cat*, *sul1*, *sul2* and *flor*. The gene *sul1* appeared to be the most prevalent and was detected in 66.6% (18/27) of the total isolates. This gene was present in all the strains designed as resistant by the disk diffusion assay. The *tet(A)* gene was detected in all the strains resistant to oxytetracycline (55.5%, 14/27); while the *tet(E)* gene was only detected in 7.4 % (2/27) of the strains. The *cat* gene was detected in the 33.3% (9/27) of the strains analysed; no sensitive or intermediate strain presented this gene. Lastly, the *flor* gene was detected in 7.4% (2/27 of the strains) (Figure 2). The resistance genes *tet(C)*, *tet(D)*, *tet(G)*, *tet(H)*, and *sul2* were never detected in the strains analysed.

A total of 7 resistance patterns were identified (Figure 3) among the *A. salmonicida* strains analysed. None of the resistance genes were detected in the strains that were susceptible or that showed intermediate susceptibility to the antimicrobials analysed by the disk diffusion and/or broth microdilution methods.

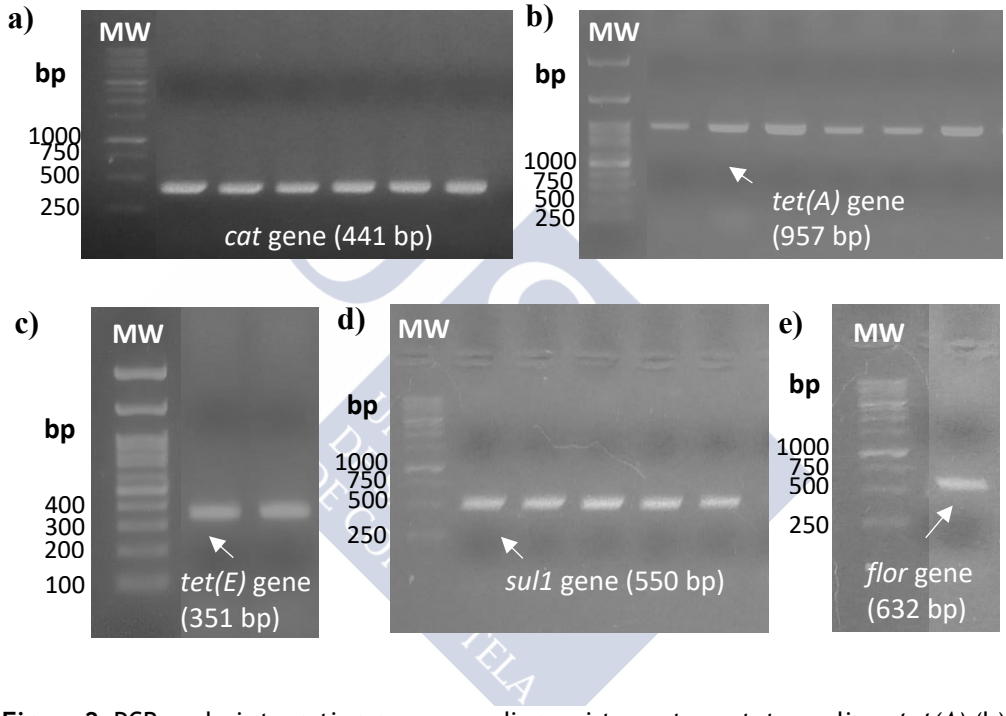

Figure 2. PCR analysis targeting genes encoding resistance to oxytetracyclines *tet(A)* (b) and *tet(E)* (c), sulphonamides *sul1* (d), chloramphenicol cat (a) and florfenicol flor (e). MW, GeneRuler DNA Ladder (Fermentas, Madrid, Spain). The sizes of the various bands in the molecular size marker are indicated in each panel.

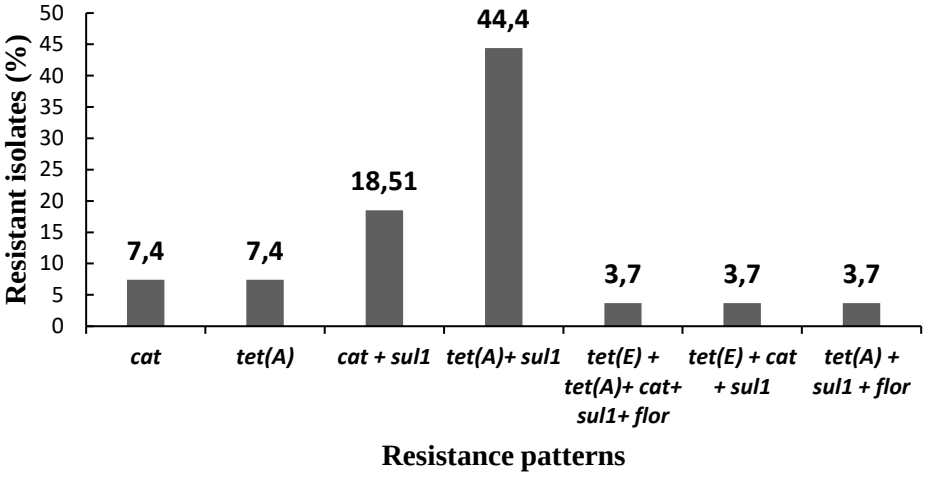

Figure 3. Molecular resistance patterns obtained for the *A. salmonicida* isolates

4. Discussion

Aeromonas species have been proposed as a potential indicator of antimicrobial susceptibility for aquatic environment (Baron et al., 2017; Usui et al., 2016; Varela et al., 2016). In this study, a preliminary assessment of the occurrence of resistance to antimicrobials in the fish pathogen *A. salmonicida* isolated in Spain was performed. The susceptibility of thirty strains of *A. salmonicida* isolated from 2000 to 2018 against six antimicrobial agents (oxytetracycline, florfenicol, enrofloxacin, chloramphenicol, flumequine, trimethoprim-sulfamethoxazole) using the disk diffusion assay and the broth microdilution method. Similar results were obtained for both assays for all the antimicrobials tested. However, minor errors (intermediate susceptibility by disk diffusion and resistant or susceptible by broth microdilution methods) were obtained for oxytetracycline (2 strains, 7.4%), enrofloxacin (3 strains, 11.1%) and chloramphenicol (7 strains, 25.92%). These results suggest that

susceptibility tests should be carried out by the microdilution methods, which is more reliable (Meyer et al., 2011).

In the current study, fish pathogenic *Aeromonas salmonicida* isolates demonstrated a high frequency of resistance to the potentiated sulphonamide trimethoprim-sulfamethoxazole (66.6% of the strains), oxytetracycline (55.5% of the strains) and chloramphenicol (33.3% of the strains) using the disk diffusion or the broth microdilution method. Resistance to these antimicrobials has been commonly reported previously on *A. salmonicida* (Adams et al., 1998; Akinbowale et al., 2006; Schmidt et al., 2001; Trudel et al., 2016) and other fish pathogens as *Flavobacterium psychrophilum* (Hesami et al., 2010; Van Vliet et al., 2016). Contrary, all the *A. salmonicida* strains analysed were susceptible to flumequine and only one strain was resistant to enrofloxacin using the disk diffusion assay and/or broth microdilution method. The widespread use of quinolones against *Aeromonas* infections; as acid oxalinic, flumequine or enrofloxacin; has led to the increase of the number of quinolone-resistant strains in the last years. Resistance to oxolinic acid and flumequine have been described in *Aeromonas* species (Giraud et al., 2018). However, as occurred in our study, low levels of resistance to enrofloxacin have been found in *A. salmonicida* isolated in other geographic areas (Kim et al., 2011; Guo et al., 2014). Contrary, other fish pathogens as *Tenacibaculum maritimum* has experienced a rapid appearance of resistance to enrofloxacin caused by chromosomal mutations in the gyrase genes (Baquero, 1990).

One of the aims of this study was to evaluate the occurrence of genes that encode for resistance to chloramphenicol (*cat*), oxytetracycline (*tet(A), tet(C), tet(D), tet(E), tet(G), tet(H*), florfenicol (*flor*) and sulfamethoxazole (*sul1* and *sul2*). Based on the results obtained by PCR, *sul1* gene was the most common antimicrobial resistance gene detected in the Spanish isolates. However, the *sul2*

gene was not present in any of the isolates resistant to sulphonamides evaluated. Molecular analysis of Canadian isolates of *A. salmonicida* also showed that the most common antimicrobial resistance gene was *sul1* (33% of the strains analysed) (Trudel et al., 2016). Among the oxytetracycline-resistant strains, 14 of the isolates harboured *tetA* genes and two isolates harboured *tetE* gene. A similar proportion among these two genes (7:1) was found among resistant *A. salmonicida* strains isolated in Korea (Kim et al., 2011). The appearance of resistance is likely due to the intensive use of this antimicrobial (Miller et al., 2016), which have led to a selective pressure and the subsequent spread of oxytetracycline-resistant isolates. Previous studies reported that the half-life of residues of oxytetracycline in fish farms sediments range from 9 to 419 days, indicating that under anoxic conditions, the antimicrobial may be very persistent (Björklund et al., 1990). Some studies have revealed that when oxytetracycline medicated feed is used to treat fish in a recirculating aquaculture system, antimicrobial residues accumulate in fish tissue, water, biofilter sand and sediments (Bebak-Williams et al., 2002). These results are alarming since oxytetracycline is one of the three authorised by the Spanish Agency of Medicines and Medical Devices (AEMPS) as an antibacterial to treat many bacterial infections in terrestrial and aquatic animals. Chloramphenicol has been used to treat furunculosis, however due to its severe toxicity, appearance of resistance and the safety concern, the use of this antimicrobial was strictly prohibited for animal use in many countries in the mid-1990s. Despite this fact, the 33.3% of the strains isolated between 2000 and 2018 analysed presented the *cat* gene. Only two isolates of *A. salmonicida* were found to be resistant to florfenicol; and both strains harboured the *flor* gene. Previous studies have also suggested that chloramphenicol resistance is more frequent and express at higher levels than florfenicol resistance (Michel et al., 2003).

Molecular analysis by PCR showed that many *A. salmonicida* isolates presented up to five resistance genes. These results show that fish pathogenic *Aeromonas salmonicida* act as reservoir of antimicrobial resistance genes (Trudel et al., 2016), as other environmental *Aeromonas* (Biyela et al., 2004). Further studies following the antimicrobial susceptibility trend over a long period of time will be crucial to monitor antimicrobial resistance dissemination in the environment. These worrying results shows that it is urgent to develop and apply alternative strategies to prevent and control of bacterial disease outbreaks without selecting resistance genes. In this sense, probiotics, immunostimulants or plant-based compounds could represent a promising approach alternative to antimicrobials.

5. References

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CHAPTER VII.

Evaluation of the antimicrobial activity of new natural and chemical compounds and their effect on the resistance against bacterial fish diseases.

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Screening of the antibacterial activity of chemical and plant-based products against *Aeromonas salmonicida* subsp. *salmonicida* and *Vibrio anguillarum*

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Abstract

Furunculosis produced by *Aeromonas salmonicida* and vibriosis produced by *Vibrio anguillarum* are some of the most threatening diseases affecting marine and freshwater fish species. The use of commercial antimicrobial drugs for the treatment of these diseases has led to the emergence of antibiotic-resistant bacteria worldwide. In this study, the antibacterial activity of two commercially available products was tested. The agents studied in this work were the Bronopol (2-bromo-2-nitro-1,3 propanediol) which is a chemical with antimicrobial and antifungal activity and the plant-based nutraceutical Liptosa-P203 (Lípidos Toledo S.A, Liptosa). The potential antimicrobial activities of these compounds and its applicability for the management of bacterial outbreaks in fish farming was evaluated by determining the Minimum Inhibitory Concentration (MIC) against 20 strains of *A. salmonicida* subsp. *salmonicida* and 30 strains of *V. anguillarum* isolated from diseased fish. *V. anguillarum* strains showed MIC values ranging from 4.57 to 9.15 ppm of Bronopol and of 292.96-585.92 ppm of Liptosa-P203. *A. salmonicida* strains presented MIC values of 9.15-36.62 ppm for Bronopol and 292.96-585.92 ppm of Liptosa-

P203. Both agents showed detectable killing effect on both bacterial species within 1 and 4 hours at concentrations of 4x MIC of Liptosa-P203 and Bronopol, respectively.

Keywords: Bronopol, Liptosa-P203, *Aeromonas salmonicida*, *Vibrio anguillarum*, antibacterial

1. Introduction

Aquaculture industry has grown more rapidly than other food production sectors, however, it is limited by unpredictable mortality due to negative effect of high stocking rates and bacterial diseases. A significant challenge for fish farming is infectious diseases produced by Gram-stain-negative bacteria. Typical furunculosis produced by *Aeromonas salmonicida* subsp. *salmonicida* and vibriosis caused by *Vibrio anguillarum* are some of the most threatening diseases. Fish under intensive production conditions are exposed to antibiotics, which are used as prophylactic agents and also for non-therapeutic purposes as like growth promoters or food additives (Sanchez-Martínez, Pérez-Castañeda, Rábago-Castro, Aguirre-Guzmán & Vázquez-Sauceda, 2008; Reda, Ibrahim, Ahmed & El-Bouhy, 2013; Marti, Huerta, Rodríguez-Mozaz, Barceló, Marcé & Balcázar, 2018). The indiscriminate use of commercial antimicrobial drugs has led to the emergence of antibiotic-resistant bacteria worldwide, including in the causative agents of furunculosis and vibriosis (Inglis et al., 1993; Pedersen et al., 1995; Akinbowale et al., 2006; Cabello, 2006; Sapkota et al., 2008; Radhouani et al., 2014). The global concerns about drug-resistance and antibiotic residues in human and animal medicine have forced the search of new alternative preventive and therapeutic strategies.

Proper cleaning and disinfection play a vital role in protecting food animals from endemic and zoonotic diseases, and thus indirectly protecting human health. A range of disinfectants are allowed for decontamination in fish farming (eggs, ponds and equipment) but also for the treatment and protection of fish against infectious diseases. These are iodoforms, glutaraldehyde, hydrogen peroxide, iodine, ozone and Bronopol (Escaffre, Bazin & Bergot 2001; Branson 2002; Katharios, Agathaggelou, Paraskevopoulos & Mylonas 2007; Wagner, Oplinger, Arndt, Forest & Bartley 2009; Can, Karacalar, Saka & Firat 2012; Grasteau, Patrick Daniel & Valérie Chesneau 2015). Biocides are also used as animal feed preservatives, with the aim of protecting feed against deterioration caused by microorganisms. However, the application of some disinfectants has been found to be harmful in marine environment; and could represent a risk for public health due to: i) the accumulation of disinfectants in fish and shellfish may contaminate predator and human consumers and ii) the pollution of natural ecosystems (Cengizler et al., 2017; Cui et al., 2011).

A promising source of alternative bactericidal agents to control bacterial infections in aquaculture are plant-derivate essential oils, plant extracts and natural herb products (Hammer, Carson & Riley 1999; Debbarma, Kishore, Nayak & Kannuchamy 2012; Starliper, Ketola, Noyes, Schill, Henson, Chalupnicki & Dittman 2015). Some of these natural products have been screened for their antioxidant and antimicrobial properties against clinically important bacteria and have also been reported to stimulate the immune system of fish (Baba, Acar, Öntaş, Kesbiç & Yılmaz 2016; Brum, Pereira, Owatari, Chagas, Chaves, Mouriño & Martins 2017).

Bronopol (2-bromo-2-nitro-1,3 propanediol) is a broad-spectrum fungicide and bactericide indicated for the treatment and control of bacterial and fungal infections in fish eggs and in farmed salmon or rainbow trout (Branson, 2002;

Oono et al., 2007; Grasteau et al., 2015). The antimicrobial potential of Bronopol has been demonstrated against marine and freshwater bacteria isolated from eggs and fish including species of *Vibrio*, *Moritella*, *Pseudomonas*, *Tenacibaculum* or *Flavobacterium* species (Birkbeck et al., 2006; Grasteau et al., 2015). Liptosa-P203 is a commercially available plant-based nutraceutical and is recommended as a diet additive to promote the care and integrity of the skin and the epithelial cells (Lípidos Toledo S.A., Liptosa, Madrid, Spain).

The present study aimed to evaluate and compare the bactericidal activity of the chemical product Bronopol (2-bromo-2-nitro-1,3 propanediol) and the plant-based additive Liptosa-P203 (Liptosa, Madrid, Spain) against the fish pathogens *V. anguillarum* and *A. salmonicida* subsp. *salmonicida*. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-killing curves of both products against *V. anguillarum* and *A. salmonicida* subsp. *salmonicida* are presented.

2. Materials and Methods

2.1.Products tested

The chemical Bronopol (Bronopol, 2-bromo-2-nitro-1,3 propanediol) (stock concentration of 500000 ppm) and the natural product Liptosa-P203 (Lípidos Toledo S.A., Liptosa, Madrid, Spain) (stock concentration 300000 ppm) which is a plant-based nutraceutical based on vegetable fatty acids and essential oils were evaluated in the present study.

2.2.Bacterial strains and growth conditions

The bacterial strains tested include clinical isolates of *Aeromonas salmonicida* subsp. *salmonicida* (*n*=20 strains) and *V. anguillarum* (*n*=30 strains) isolated from different fish species. Reference strains used in this study were obtained from the American Type Culture Collection (ATCC). The

serotype, geographical area of isolation and host species are indicated in Table 1. Stock bacterial cultures were stored frozen at -30ºC in Microbank™ commercial medium (Pro-Lab Diagnostics) until use. Bacterial strains were cultured on Mueller-Hinton agar plates for 24-48 h at 25ºC (*V. anguillarum* strains) and 18ºC (*A. salmonicida* subsp. *salmonicida*). The identity of the bacterial strains was confirmed using PCR methods (Gonzalez, Osorio & Santos 2003; Fernández-Álvarez, González & Santos 2016).

Table 1. *Vibrio anguillarum* strains used in this study

Reference strains	**Sero type**	**Source of isolation**	**MIC (ppm) Bronopol**	**MIC (ppm) Liptosa-P203**
ATCC14181	O2	Unknown	4.57	292.96
ATCC43306	O2	Cod (Denmark)	4.57	292.96
ATCC43307	O3	Trout (Denmark)	9.15	292.96
ATCC43308	O4	Cod (Denmark)	4.57	585.93
ATCC43309	O5	Cod (Denmark)	4.57	292.96
ATCC43310	O6	Cod (Denmark)	4.57	585.93
ATCC43311	O7	Eel (Denmark)	9.15	292.96
ATCC43312	O8	Cod (Denmark)	4.57	292.96
ATCC43313	O9	Cod (Denmark)	4.57	292.96
ATCC43314	O10	Cod (Denmark)	9.15	292.96
Clinical isolates				
V. anguillarum (*n*=2)	O1	Turbot (Spain)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O2	Turbot (Spain)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O2	Sea bream (Spain)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=4)	O1	Sea bass (Spain)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=2)	O1	Unknown, (Spain)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	-	*Morone saxatilis*, USA	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O1	Striped bass (USA)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O2B	Striped bass (USA)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O1	Rainbow trout (Denmark)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=2)	O2	Rainbow trout (Denmark)		
V. anguillarum (*n*=1)	O2B	Turbot (Spain)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O3	Sea bass (France)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O3	Ayu (Japan)	4.57-9.15	292.96-585.93
V. anguillarum (*n*=1)	O3	Japanese eel (Japan)	4.57-9.15	292.96-585.93

ATCC American Type Culture Collection (USA)

Table 2. *A. salmonicida* strains used in this study

Reference strains	Source of isolation	MIC (ppm) Bronopol	MIC (ppm) Liptosa-P203
ATCC33658	Atlantic Salmon (USA)	39.06	292.96
ATCC14174	Brook trout (USA)	39.06	585.93
Clinical isolates			
A. salmonicida (*n*=2)	Sea bass (Spain)	36.62-73.24	292.96-585.93
A. salmonicida (*n*=1)	Sea lamprey (Spain)	36.62-73.24	292.96-585.93
A. salmonicida (*n*=3)	Turbot (Spain)	36.62-73.24	292.96-585.93
A. salmonicida (*n*=1)	Atlantic Salmon (Scotland)	36.62-73.24	292.96-585.93
A. salmonicida (*n*=3)	Rainbow trout (Spain)	36.62-73.24	292.96-585.93

ATCC American Type Culture Collection (USA)

2.3.Antibacterial susceptibility test

2.3.1. Determination of minimum inhibitory concentration (MIC)

The MIC was calculated using the agar dilution method and the broth microdilution assay following the procedures described by the Clinical and Laboratory Standards Institute (CLSI, 2006). The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the antibiotic that significantly inhibited the growth of the bacteria tested. Minimum inhibitory concentration (MIC) and minimum bactericidal (MCB) concentration were carried out using 24 h old cultures of all strains. Inoculum prepared in sterile saline solution (NaCl 0.9% *w/v*) and adjusted to contain approximately 1-2x10^9 colony forming units (CFU/ml) (OD_{620}) was used for all antibacterial susceptibility assays.

Agar dilution method

For the agar dilution method, Mueller-Hinton agar plates containing increasing concentrations of Liptosa-P203 or Bronopol (concentrations varying from 5000 ppm to 1 ppm) were used. A stock solution of the compounds was prepared at an initial concentration of 10000 ppm in Mueller-Hinton broth. Inoculum (1µl) of each bacterial suspension was spotted directly onto the agar plates that had incorporated the different concentrations of Bronopol and Liptosa-P203 using a multipoint inoculator. Mueller-Hinton agar plates without the tested compounds were used as positive growth control. Tests were performed in duplicate. The agar plates were dried at room temperature and incubated for 24-48h at 18 or 25ºC depending on the requirements of the bacteria.

Broth micro-dilution method

The broth microdilution method was performed using 96-well plates (Becton Dickinson Labware Europe, Francia). A stock solution of both Bronopol and Liptosa-P203 products was prepared in Mueller-Hinton broth at concentration of 10000 ppm. Then, the stock solutions were two-fold diluted from 5000 to 0.61 ppm in MH broth, reaching a final volume of 100 µl in each well. The culture medium was used as negative control. Positive control wells did not include any tested compounds. Each test and growth control wells were inoculated with 10 µl of the bacterial suspension of *A. salmonicida* subsp. *salmonicida* or *V. anguillarum* strains containing approximately 1-2x10^9 colony forming units (CFU/ml). All experiments were performed in triplicate and then averaged. The plates were incubated for 48-72 h at 18ºC or 25ºC following the requirement of the bacteria tested. Bacterial growth was measured by optical density (Microplate Reader, Model 680, Bio-Rad) at OD_{620}.

2.3.2. Determination of minimum bactericidal concentration (MBC) assay

The MBC was determined by spectrophotometry and culture methods. All bacterial strains of *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* were used in this assay. For culture methods the initial inoculum, the dilution representing the MIC and at least two of the more concentrated test product dilutions were plated and enumerated to determine viable CFU/ml by using the plate dilution. The cultures were incubated at 18 or 25ºC (depending on the requirement of the bacteria) for 48–72 h and the antimicrobial concentration that causes a reduction of the 99.9% of the original inoculums was considered the MBC. The reduction of number of viable cells was also measured by a colorimetric assay based on the reduction of a tetrazolium salt (MTT method). Briefly, viable bacteria present in the wells were quantified by the addition of 10 µL of [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, 5 mg/ml), which is reduced in proportion to the number of viable bacteria present. The optical density was read at 620 nm 15 min later on a spectrophotometer (Microplate Reader Model 680, BioRad). Experiments were carried out in triplicate. To determine the bactericidal or bacteriostatic capacity of the nutraceutical, the ratio MBC/MIC was used. If the ratio MBC/MIC was lower than 4, the effect was considered as bactericidal. If the ratio MBC/MIC was higher than 4, the effect was determined as bacteriostatic.

2.4. Time-kill curve analyses

The time-killing curve of Bronopol and Liptosa-P203 was determined using three representative strains of *V. anguillarum* (ATCC 43306, ATCC14181, and ATCC43307) and three strains of *A. salmonicida* subsp. *salmonicida* (ATCC 33658, ATCC14174 and NCIMB2261). Time–killing assays were performed in triplicate by inoculating $1x10^6$ CFU/ml of the bacterial strains tested into 3 ml of fresh Mueller–Hinton broth. Bronopol and Liptosa-P203 concentrations of

1x, 2x and 4x the MIC for each strain were added. Bacterial growth was measured at 1, 2, 3, 4 and 5h post-inoculation, by measuring the absorbance (A_{620}) and by the drop count techniques (Miles, Misra & Irwin 1938) on Mueller-Hinton agar plates and incubating for 24-48h at temperatures required by the bacterium tested. Tubes with the broth medium and the inoculum were used as positive growth controls and tubes without the inoculum and the tested compounds were used as negative controls.

3. Results

3.1 *In vitro* inhibitory effect

The antimicrobial activity of Bronopol and Liptosa-P203 against causative agents of typical vibriosis and furunculosis diseases was evaluated by the agar dilution and broth microdilution methods. Both products showed high inhibitory effect against the strains of *V. anguillarum* and *A. salmonicida* subsp. *salmonicida*. The results obtained demonstrated that Bronopol possess strong antibacterial effects against the fish pathogens tested, with MIC values of 4.57-9.16 ppm for the strains of *V. anguillarum* and a MIC of 9.16-36.62 ppm for the strains of *A. salmonicida* (Table 1 and 2). Liptosa-P203, showed the same MIC values of 292.96-585.92 ppm for strains of both pathogens (Table 1 and 2). The results revealed homogeneity in the inhibitory concentrations of both products, regardless their serotype and source of isolation (Table 1 and 2).

Considering the ratio of MBCs and MICs (MBC/MIC<4), the products tested (Bronopol and Liptosa-P203) exerted a bactericidal effect against the strains of *A. salmonicida* subsp. *salmonicida* and *Vibrio anguillarum* tested.

3.2. Time-killing assay

Exposure of *A. salmonicida* and *V. anguillarum* to Bronopol and Liptosa-P203 (at 1x, 2x, 4x MIC values) reduced both the absorbance of broth cultures and bacterial counts when compared to bacterial cells exposed to MH broth medium (positive control) (Figure 1 and 2). Both Bronopol and Liptosa-P203 showed bactericidal activity at any of the concentrations used in the time-killing assay against *V. anguillarum* and *A. salmonicida* subsp. *salmonicida* strains (Figure 1 and 2).

For *V. anguillarum* treated with Bronopol, the reduction in both absorbance and CFU/ml started after 2 h and >99.99% of bacteria were killed within 4 h with 1x, 2x, and 4x MIC (4.57, 9.15 and 18.31 ppm) (Figure 1a). Treatment of *V. anguillarum* cultures with Liptosa-P203 produced reduction of the bacterial concentration with the 1x MIC (292.96 ppm) at 3 h, while treatment with 2x and 4x MIC (585.92 and 1171.84 ppm) killed the >99.99% of bacteria at 1 h (Figure 2a).

Treatment of *A. salmonicida* suspension with Bronopol reduced both the absorbance and CFU/ml after 30 min and >99.99% of bacteria were killed within 2h (Figure 1b) with the 1x, 2x and 4x MIC. On the other hand, treatment with Liptosa-P203 produced reduction of the bacterial concentration with the 1x MIC (292.96 ppm) at 4 h, while treatment with 2x and 4x MIC (585.92 and 1171.84 ppm) killed the >99.99% of bacteria at 4 and 1 h, respectively (Figure 2b).

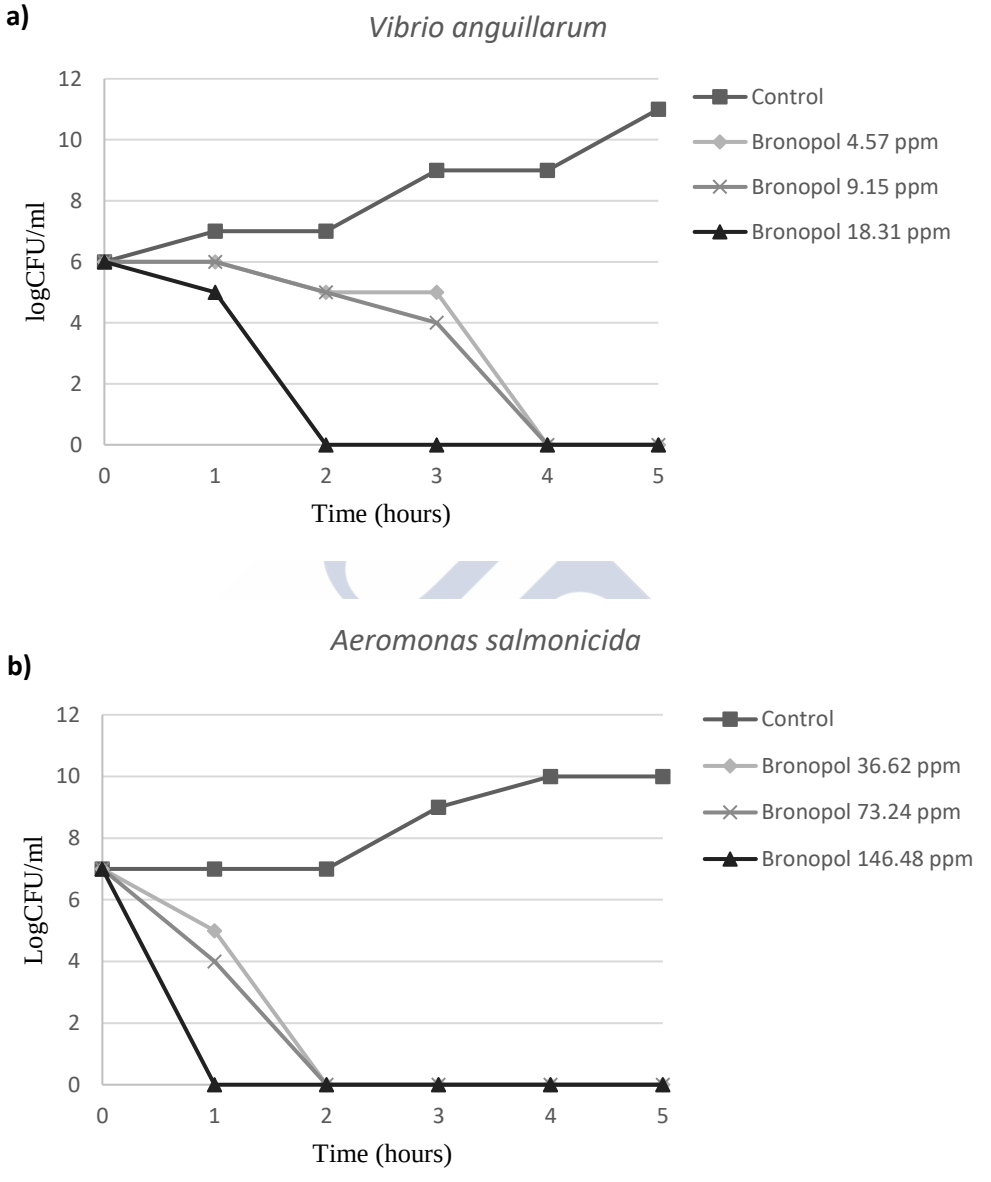

Figure 1. Time-killing curves at 4x, 2x and 1x the minimum inhibitory concentration (MIC) of Bronopol assayed on the strain *Vibrio anguillarum* ATCC14181 (a) and the strain of *Aeromonas salmonicida* ATCC33658 (b). CFU, colony forming units

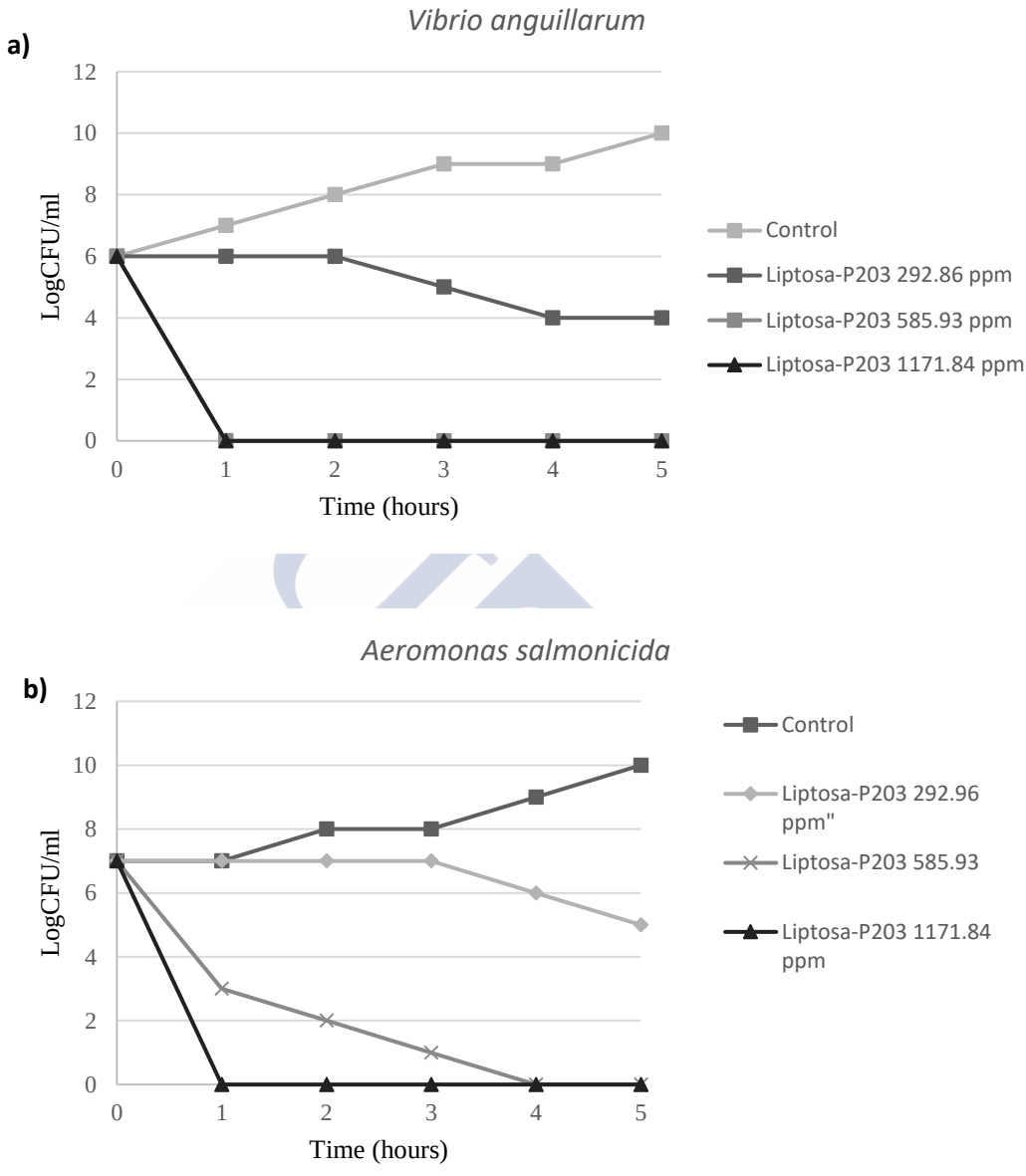

Figure 2. Time-killing curves at 4x, 2x and 1x the minimum inhibitory concentration (MIC) of Liptosa-P203 assayed on the strain *Vibrio anguillarum* ATCC14181 (a) and the strains of *Aeromonas salmonicida* ATCC33658 (b). CFU, colony forming units.

4. Discussion

In the present study, the susceptibility of clinical isolates of *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* against two commercially available compounds; Bronopol and the plant-based product Liptosa-P203 (Liptosa S.A) was assessed. The antimicrobial activity was quantified by MIC, MBC and time-kill assays.

Infections produced by multidrug-resistant bacteria pose an important problem for aquaculture industry. Many bacterial strains have been reported to be resistant to standard antibiotics. Thus, in the last years, the searching for new broad-spectrum antimicrobials to treat bacterial fish diseases has led toward plant extracts and biologically active compounds isolated from plants.

The agar dilution and broth microdilution methods showed that both products had significant *in vitro* antibacterial activity against the pathogens tested. For strains of *V. anguillarum* isolated from different fish species and belonging to different serotypes (O1-O10) the Bronopol MIC values ranged from 4.57-9.15 ppm. These results are in accordance with those described by Birkbeck et al. (2006), who reported MICs of 4 µg/ml of Bronopol for *V. anguillarum* strains isolated from turbot. Similarly, Bronopol showed MIC values of 9.15-36.62 ppm for *A. salmonicida* strains isolated from different fish species and geographical areas. Torkildsen et al. (2003) tested this compound against 10 marine bacteria isolated from *Pecten maximus* rearing system. Bacteria belonged to the genera *Vibrio, Pseudomonas*, *Aeromonas* and *Alteromonas*, and the MIC values of Bronopol ranged from 10.7 to 26.7 µg/ml.

Time-killing assays showed that >99.99% of *V. anguillarum* cells were killed within 2h using Bronopol at concentrations of 4x MIC (18.31 ppm) and >99.99% of *A. salmonicida* cells within 1h at concentrations of 4x MIC (156.24

ppm). The killing efficiency of Bronopol for *V. anguillarum* and *A. salmonicida* seemed to be better than those described for *T. ovolyticum* (>99.99% of bacteria within 2h with 200 µg/ml Bronopol) (Birkbeck et al., 2006) or *Escherichia coli* (>90% of bacteria within 1h with 100 µg/ml Bronopol) (Shepherd, Waigh & Gilbert 1988). Shepherd et al. (1988) established that the bactericidal action of Bronopol could occur through the catalytic oxidation of accessible thiols that is responsible for the growth inhibition and generation of free radicals causing cell death.

The searching for new broad-spectrum antimicrobials to treat bacterial fish diseases has led toward plant extracts and biologically active compounds isolated from plants. In this study, we evaluated the antimicrobial action of the commercially available plant-based additive Liptosa-P203 (Liptosa S.A.), which is composed by fatty acids and essential oils. Although at higher concentrations than Bronopol, Liptosa-P203 showed a high antimicrobial activity (292.96-585.92 ppm) against *V. anguillarum* and *A. salmonicida* isolates. Moreover, the results revealed homogeneity in the inhibitory concentrations of the product, regardless the source of isolation of the strains. Interestingly, Liptosa-P203 was able to kill the 99.99% of cells of *V. anguillarum and A. salmonicida* in only 1h at concentrations of 4x MIC (1171.84 ppm), suggesting that this product could be an alternative for the control of the diseases caused by both pathogens. Future studies should be carried out to determine the efficacy of Liptosa-P203 under field conditions.

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Dietary administration of a commercial nutraceutical: antimicrobial activity and effect in the resistance against *Flavobacterium psychrophilum* infections on rainbow trout

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Abstract

This study describes the *in vitro* antibacterial activity of the commercial nutraceutical Liptofry, manufactured by Lipidos Toledo S.A. (Liptosa), and the effect of Liptofry-supplemented diets in the modulation of immune-related-genes and on the resistance of *Oncorhynchus mykiss* to bacterial infection. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration methods (MBC) of the nutraceutical was tested against the fish pathogens *Vibrio anguillarum*, *Aeromonas salmonicida* subsp. *salmonicida* and *F. psychrophilum* using the agar and broth dilution methods. The strongest inhibitory effect was shown with *F. psychrophilum* isolates, with MIC_{90} and MBC values of 64 µg ml^{-1}. To evaluate the effect of Liptofry-supplemented diets on disease resistance, rainbow trout fingerlings were fed diets containing 0 (control diet) and 0.5 % (experimental diet) of the nutraceutical for 115 days and challenged with a virulent strain of *F. psychrophilum*. The cumulative mortality of fish fed with the nutraceutical-supplemented diet was significantly lower ($p<0.05$) than that for fish fed control diets. The immune response was

investigated by transcriptome analysis of kidney, spleen, skin and intestine using real-time RT-PCR analysis (qPCR). Organs from fish fed nutraceutical-supplemented diets and fish fed control diet were sampled at 0- and 115-days post-feeding. Administration of nutraceutical-supplemented diets for 115 days appeared to be sufficient to stimulate immune parameters of fish. Genes encoding tumour necrosis factor-α (TNF-α), complement (C5a) and complement receptor (rC5a) were over-expressed in spleen and skin in fish fed Liptofry-supplemented diet compared to control fish, while immunoglobulin M (IgM) gene expression was only increased in intestine from fish fed nutraceutical-additives. Our results indicated that the use of diets supplemented with the nutraceutical Liptofry confers protection against bacterial diseases produced by *F. psychrophilum*.

Keywords: Nutraceuticals, immunostimulation, gene expression, *Flavobacterium psychrophilum*

1. Introduction

Aquaculture industry has grown more rapidly than other food production sectors, however, it is limited by unpredictable mortality due to negative effects of high stocking rates and diseases produced by different bacterial pathogens as *Flavobacterium psychrophilum*, *Vibrio anguillarum* or *Aeromonas salmonicida* subsp. *salmonicida*. Antibiotics have been traditionally used as a prophylactic measure to control pathogenic outbreaks as well as for non-therapeutic purposes like growth promoters or food additives (Reda et al., 2013). However, the over-use of commercial antimicrobials has led to the selection of antibiotic-resistant bacteria, immunosuppression and the presence of antibiotic residues in the

aquatic environment and in resultant seafood products, which could be potentially harmful to public health (FAO, 2003; Sapkota et al., 2008). Disease prevention through vaccines has been considered as an effective alternative to control bacterial and viral infections. Although there have been significant advances in the development of vaccines for fish, the specificity of the protection induced, and the high vaccination costs have forced the search for others prevention and therapeutic strategies that enhance fish resistance against infectious diseases and improve growth and feed efficiency for the development of a sustainable aquaculture (Reverter et al., 2014).

A promising source of alternative antibacterial agents to control bacterial infections in aquaculture are essential oils, medicinal plants and natural herb products (Hammer et al., 1999). Some of these plant-based products have been screened for their antioxidant and antimicrobial properties against bacterial pathogens of great importance in human clinical (Kitzberger et al., 2007) and for the aquaculture industry (Nguyen et al., 2016; Roohi Fatima et al., 2016; Schrader, 2010; Thanigaivel et al., 2015). Moreover, the dietary administration of herbal-based nutraceuticals is known to have an important role in disease control through the enhancement of the fish innate immune system. The term "nutraceutical" is referred to any substance administered through diet that provides health benefits, including the prevention and treatment of disease (DeFelice, 1995). To date, the positive effects of several herbal plants including garlic (*Allium sativum*), mentha (*Mentha piperita*), green tea (*Camellia sinensis*), fennel (*Foeniculum vulgare*), thyme (*Thymus vulgaris*), ginger (*Zingiber oficinale*) or aloe vera (*Aloe barbadensis*) have been observed in the immune system of fish, in the resistance against bacterial diseases and growth performance of fish and shrimp (Adel et al., 2016; Ahmad et al., 2011; Devakumar and Chinnasamy, 2017; Harikrishnan et al., 2011; Heidarieh et al., 2013; Navarrete et al., 2010; Nguyen et al., 2016; Nya and Austin, 2011, 2009;

Sivaram et al., 2004; Thanigaivel et al., 2015). This immune-protective effect is produced through the enhancement of the antibody response, the phagocytic activity, the serum lysozyme levels and the serum bactericidal activity of fish (Bricknell and Dalmo, 2005; Harikrishnan et al., 2011; Nya and Austin, 2009; Peddie et al., 2002). Nutraceuticals have also been used to reduce stress associated to high density culture conditions, as well as for growth and appetite promotion. Their action is mainly due to active principles such as alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids, and essential oils (Harikrishnan et al., 2011; Sivaram et al., 2004).

In particular, garlic, *Allium sativum*, has been proven to inhibit the growth of several bacterial species (Gull et al., 2012; Nya and Austin, 2009; Seong Wei and Musa, 2008), fungus (Burian et al., 2017; Pai and Platt, 1995), virus (Weber et al., 1992) and parasites (Gaafar, 2012). Moreover, garlic has also been associated to the induction of anti-stress protection, the improvement of the growth performance, and the stimulation of the immune system of fish (Ghehdarijani et al., 2016; Nya and Austin, 2009). Resistance conferred by garlic-supplemented diets against pathogens has been reported in different fish species infected with *Aeromonas hydrophila* (Nya and Austin, 2009; Thanikachalam et al., 2010), *Edwardsiella tarda* (Abraham and Ritu, 2015) or *Streptococcus iniae* (Guo et al., 2015). The protective activity induced by garlic-supplemented diets in fish include an increase in the number of erythrocytes and leucocytes, haematocrit, phagocytic activity, respiratory burst, lysozyme, anti-protease and bactericidal activities of rainbow trout (Nya and Austin, 2009; Thanikachalam et al., 2010).

Liptofry, is an herbal-garlic-based nutraceutical commercially available and manufactured by Lípidos Toledo S.A (Liptosa, Madrid, Spain). The main objectives of the present study were i) to investigate the *in vitro* antibacterial

activity of the nutraceutical Liptofry against the fish pathogens *F. psychrophilum*, *V. anguillarum* and *A. salmonicida* subsp. *salmonicida*, ii) to assess the *in vivo* effect of Liptofry-supplemented diets on resistance of rainbow trout (*Oncorhynchus mykiss*) against diseases produced by *F. psychrophilum*, and iii) to evaluate the effect of diets supplemented with the nutraceutical on the modulation of the expression of immunity-related genes.

2. Material and methods

2.1. Nutraceutical

The nutraceutical evaluated in this study is the commercially available known as Liptofry manufactured by Lípidos Toledo S. A. (Liptosa, Madrid, Spain). This product is a vegetal-derived supplement consisting of 10% garlic, 4% nucleotids, anti-caking agents 33%, flavouring mixture based on botanical herbs (33%), and preservatives 20%.

2.2. *In vitro* evaluation of the antimicrobial activity of the nutraceutical Liptofry

2.2.1. Bacterial strains

The antimicrobial activity of the nutraceutical was tested against thirty strains of *Flavobacterium psychrophilum*, fifteen strains of *Vibrio anguillarum* and ten strains of *A. salmonicida* subsp. *salmonicida* isolated from different fish species and geographical areas (Table 1). Microorganisms were obtained from the NCIMB (National Collection of Industrial and Marine Bacteria), the ATCC American Type Culture Collection (USA), CECT Spanish Type Culture Collection (Spain) and from our own collection. *F. psychrophilum* strains were cultured on FLP agar plates (Cepeda *et al*. 2004) and *A. salmonicida* and *V. anguillarum* strains on Muller-Hinton agar plates and incubated for 24-48 h at 18ºC (*F. psychrophilum* and *A. salmonicida*) or 25ºC (*V. anguillarum)*. Bacteria

were kept frozen at -30ºC in the commercial medium MicrobankTM (Pro-Lab Diagnostics) until use.

Table 1. Bacterial strains used in this study

Flavobacterium psychrophilum		
Reference strains	**Serotype**	**Source of isolation**
NCIMB13384	O2a	Rainbow trout (Denmark)
NCIMB13383	O3	Rainbow trout (Denmark)
NCIMB1947	O1	*O. kitsutch* (USA)
NCIMB2282	O1	*O. kitsutch* (USA)
Clinical isolates		
F. psychrophilum (n=1)	O1	Coho salmon (USA)
F. psychrophilum (n=1)	O2a	Coho salmon (USA)
F. psychrophilum (n=1)	O2a	Rainbow trout (France)
F. psychrophilum (n=4)	O2a	Rainbow trout (Spain)
F. psychrophilum (n=4)	O2b	Rainbow trout (Spain)
F. psychrophilum (n=8)	O2b	Salmo salar (UK)
F. psychrophilum (n=1)	O2	Rainbow trout (Switzerland)
F. psychrophilum (n=2)	O3	Rainbow trout (USA)
F. psychrophilum (n=4)	O2	Rainbow trout (Scotland)
Vibrio anguillarum		
Reference strains	**Serotype**	**Source of isolation**
ATCC14181	O2	Unknown
ATCC43306	O2	Cod (Denmark)
ATCC43307	O3	Trout (Denmark)
ATCC43308	O4	Cod (Denmark)
ATCC43309	O5	Cod (Denmark)
ATCC43310	O6	Cod (Denmark)
ATCC43311	O7	Eel (Denmark)
ATCC43312	O8	Cod (Denmark)
ATCC43313	O9	Cod (Denmark)
ATCC43314	O10	Cod (Denmark)
Clinical isolates		
V. anguillarum (*n*=4)	O1	Turbot (Spain)
V. anguillarum (*n*=7)	O2	Turbot (Spain)
V. anguillarum (*n*=1)	O2	Cod (Denmark)
V. anguillarum (*n*=1)	O2	Striped bass (USA)
V. anguillarum (*n*=1)	O2B	Cod (Denmark)
V. anguillarum (*n*=1)	O2B	8606-9 3/2
V. anguillarum (*n*=2)	O3	Sea bass (France)

V. anguillarum (*n*=1)	O3	Ayu (Japan)
V. anguillarum (*n*=1)	O3	Japanese eel (Japan)
A. salmonicida subsp. salmonicida		
Reference strains	**Source of isolation**	
ATCC33658	Atlantic Salmon (USA)	
ATCC14174	Brook trout (USA)	
Clinical isolates		
A. salmonicida (*n*=2)	Sea bass (Spain)	
A. salmonicida (*n*=1)	Sea lamprey (Spain)	
A. salmonicida (*n*=3)	Turbot (Spain)	
A. salmonicida (*n*=1)	Atlantic Salmon (Scotland)	
A. salmonicida (*n*=3)	Rainbow trout (Spain)	

ATCC, American Type Culture Collection (USA); NCIMB, National Collection of Industrial and Marine Bacteria (UK)

2.2.2. Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was determined by the agar and broth dilution methods, following the procedures described by the Clinical and Laboratory Standards Institute (CLSI, 2006).

For the agar dilution method, FLP and MH agar plates containing increasing concentrations (0.016, 0.032, 0.064, 0.125, 0.25, 0.75, 1.25, 1.75 and 3 mg extract ml^{-1}) of the nutraceutical Liptofry were used. The antimicrobial stock solution was previously prepared using DMSO as solvent. Inoculum was prepared in sterile saline solution (NaCl 0.9% *w/v*) from 24 h bacterial cultures and adjusted to an OD_{620} of 0.08. 1µl of each bacterial suspension was spotted directly onto the agar plates with the different nutraceutical concentrations incorporated using a multipoint inoculator. FLP and TSA-1 agar plates without the antimicrobial compound were used as growth controls. The assays were performed in triplicate. The plates were dried at room temperature and incubated at 18 ºC for 48-72 h.

For broth microdilution assay, 96-well microplates (Becton Dickinson Labware Europe, Francia) were used. For *F. psychrophilum* the nutraceutical was initially dissolved in DMSO at a concentration of 1024 µg ml^{-1} and two-fold diluted in FLP broth medium with concentrations varying from 512 µg ml^{-1} to 2 µg ml^{-1} in 100 µl. For *A. salmonicida* subsp. *salmonicida* and *V. anguillarum* and initial stock of 10,000 µg ml^{-1} was prepared and serially diluted in MH broth medium with concentrations varying from 5000 µg/ml to 39 µg/ml. Each test and growth control wells were then inoculated with 10µl of a bacterial suspension (OD_{620} of 0.08) of all the strains of *F. psychrophilum*, *A. salmonicida* subsp. *salmonicida* or *V. anguillarum* tested. Positive control wells did not include any nutraceutical. The culture medium plus DMSO was used as negative control. All experiments were performed in triplicate and then averaged. The plates were incubated for 48-72 h following the temperature requirements of the bacteria tested. Bacterial growth was measured by optical density at OD_{620} using a Microplate Reader (Model 680, Bio-Rad). The minimum inhibitory concentration (MIC) was considered the lowest concentration of the substance that inhibited the visual bacterial growth. Results were expressed as the MIC value which inhibits 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates tested.

2.2.3. Determination of the Minimum Bactericidal Concentration

The Minimum Bactericidal Concentration (MBC) was determined by sub-culturing samples from the wells with concentrations above the MIC on FLP or MH agar plates and using a colorimetric assay based on the reduction of a tetrazolium salt (MTT method). Briefly, viable bacteria present in the wells were quantified by the addition of 10 µL of [3(4,5-dimethylthiazol-2- yl)-2,5-diphenyltetrazolium bromide] (MTT, 5 mg ml^{-1}), which is reduced in proportion to the number of viable bacteria present. The optical density was read at 620 nm 15 min later on a spectrophotometer (Microplate Reader Model 680, BioRad).

Experiments were carried out in triplicate. Results were expressed as the MBC value which kills 50% (MBC_{50}) and 90% (MBC_{90}) of the isolates tested.

To determine the bactericidal or bacteriostatic capacity of the nutraceutical, the ratio MBC/MIC was used. If the ratio MBC/MIC was lower than 4, the effect was considered as bactericidal. If the ratio MBC/MIC was higher than 4, the effect was determined as bacteriostatic.

2.3. Experimental challenge

2.3.1. Experimental diet

The usefulness of the nutraceutical Liptofry (Liptosa S.A.) as a protection measure against infections produced by *F. psychrophilum* was tested by its inclusion on diets for rainbow trout fingerlings. The basal diet (control diet) was composed by fish meal, fish oil, wheat gluten, wheat, yeast, krill meal, vitamins and minerals. The proximate composition of the basal diet is indicated in Table 2. For feeding experiments, an experimental diet was prepared by supplementation of the basal diet with 0.5 % of the nutraceutical Liptofry (5 kg t^{-1} diet). All the ingredients were blended thoroughly in a mixer, made pellets, air-dried, ground and sieved to produce a suitable crumble.

Table 2. Proximate composition of the basal diet

Proximate composition	**Percentage (%)**
Crude protein	64
Crude fat	12
NFE	5
Ashes	11
Fibre	0.5
Gross energy (Kcal/MJ)	4976/20.8

2.3.2. Fish and feeding regime

Healthy rainbow trout (*Oncorhynchus mykiss*) (*n*=4000 fish) (0.1g±0.02 g body weight) from an aquaculture facility of the north of Spain were used in the present study. Fish were divided into two experimental groups (Groups 1 and 2) of 2000 individuals each. Fish of Group 1 were fed with Liptofry-supplemented diets and fish of Group 2 were fed with control diet (control fish). Fish were fed twice a day with the same diets. The rate of food supply of both groups was based on the requirements of the fish, taking as a reference the parameters of size and weight of the specimens and the temperature of the water. Fish remained at the farm during 90 days until its transfer to the aquarium of the Faculty of Biology of the University of Santiago de Compostela (USC) for challenge experiments. Prior to infection, fish were acclimatized for 2 weeks at the aquarium conditions. Fish were maintained in plastic tanks of 300 L using fresh water at 16ºC ± 1 with oxygen content above 8 mg/L. During this time the feeding regime was maintained. The physical chemical characteristics of the experimental water were maintained throughout the experimental period. Before the challenge experiments, 6 fish of each experimental group were subjected to microbiological and molecular analysis as previously described (Cepeda and Santos, 2000) to evaluate their health status.

2.3.3. Experimental infection with *Flavobacterium psychrophilum*

The usefulness of administering feed supplemented with nutraceuticals in the prevention of diseases caused by *Flavobacterium psychrophilum* was evaluated by experimental infection of rainbow trout (average weight of 5.84±1.29 g) using the virulent strain *F. psychrophilum* RBT4.1.04 isolated in 2004 from diseased rainbow trout in the Northwest of Spain. For the assays, 24h bacterial cultures in FLP agar medium were suspended in sterile saline solution at a concentration of 10^9 CFU ml^{-1} (OD_{620}). Colony forming units (CFU) were

enumerated by the plate dilution method by seeding bacterial cell suspensions onto FLP agar plates and counting the bacterial colonies produced.

Fish (*n*=120 individuals) fed Liptofry-supplemented diet (Group 1) and fish fed control diet (Group 2) were used in the experimental infection. Fish were anaesthetised by immersion in tricaine methane sulfonate (MS-222, Sigma) (60mg l^{-1}) (Neiffer and Stamper, 2009) and were intra-peritoneal injected with 0.1 ml of the bacterial suspension, containing 10^9 CFU ml^{-1}, following the methodology described by Santos et al. (1991). Mortality was monitored several times daily for 15 days and dead and dying fish were instantly removed. Fish mortalities were considered caused by the inoculated strain, only if the bacterium was recovered in pure culture from the internal organs of dead or dying fish or if it was detected by PCR in tissues of the dead fish by using the procedure described by Cepeda and Santos (2000). Twenty-five fish from group 1 and 2 were injected with 0.1 ml sterile saline solution, were kept on separate tanks and used as negative controls. The studies presented in this manuscript were approved by the USC Bioethics Committee.

2.4. Gene expression analysis of immunity-related genes by real-time PCR

2.4.1. Sampling for gene expression analysis

For sampling, fish were euthanized by overexposure to MS-222 (400 mg l^1) and necropsied. Samples of kidney, spleen, gut and skin were aseptically removed from 10 fish of each experimental group (group 1 and group 2) before challenge. Fish tissues were pooled, transferred into RNAlater™ (Sigma-Aldrich), pre-stored at 4ºC for 24h and stored at -80ºC for subsequent RNA isolation.

2.4.2. RNA isolation and cDNA synthesis

For RNA isolation from each organ pool, sampled tissues were homogenized manually, by forcing them through a nylon mesh of pore size 100µm, with the aid of a steel sterile spatula and adding Leibovitz´s medium (L15) (BioWhittaker) supplemented with antibiotic (100 IU ml^{-1} of penicillin and 100 µg ml^{-1} of streptomycin, BioWhitakker). Cell suspensions were centrifuged at 3000 rpm during 5 min at 4ºC, and the resulting cellular pellet was used to isolate the RNA. RNA isolation was carried out following the protocol of RNA InstaPure™ System (Eurogentec). Obtained RNA was suspended in diethyl pyrocarbonate (DEPC)-treated water, quantified using the fluorimeter Qubit® 2.0 and the Qubit® RNA BR Assay Kit system according the manufacturer instructions (Invitrogen), and stored at -80ºC until use. Briefly, 1µg of RNA was treated with DNase I, RNase-free system (Thermo Scientifics) to remove genomic DNA traces. 500 ng of RNA were used for cDNA synthesis using NZY First Strand cDNA Synthesis Kit (Nzytech, Portugal). Total RNA was mixed with the components indicated by the manufacturer. Samples were incubated at 25ºC for 10 min and at 50ºC for 30 min in an Eppendor Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The reaction was stopped by heating at 85 ºC for 5 min and the obtained cDNA was treated with RNAase-H at 37ºC for 20 min. Synthesised cDNA was ten-fold diluted in milliQ sterilised water and stored at -80ºC until use for qPCR reaction.

2.4.3. Real time RT-qPCR

To determine the effect of the oral administration of the commercial nutraceutical (Liptofry) on the modulation of the immune system, the transcription levels of the immunity-related genes encoding tumor necrosis factor-α (TNF-α), complement (C5a), complement receptor (rC5a) and immunoglobulin M (IgM) were evaluated by real-time PCR. Elongation factor-

α (EF- α) gene was assessed separately as endogenous housekeeping gene (positive control) and for sample normalization. The primers used in this study (Table 3) were previously designed by Raida et al. (2011). Stocks primers were dissolved in sterile water to 100 µM and then adequately diluted in order to establish the optimal final concentration of primers in the reaction. The specificity and efficiency of qPCR reaction were evaluated by a melt-curve analysis follow up by gel electrophoresis and a single peak was obtained in all cases. The qPCR efficiency was determined both for the target genes and the reference (housekeeping) gene by conducting a standard curve analysis. The real-time PCR reactions were performed in a volume of 25 µl in DNase, RNase-free tubes (Thermo Scientifics, USA), using a MiniOpticon real-time PCR thermal-cycler with CFX Manager™ software detection system (Bio-Rad Laboratories, München, Germany). The reaction contained 12.5 µl of Maxima SYBR Green qPCR Master Mix (2X), no ROX (Thermo Scientifics, USA), 0.5 µl of each primer (200nM), 4 µl of cDNA (ten-fold diluted) and sterilized milli-Q water to complete the reaction. Real-time PCR amplifications were performed in triplicate. The thermal cycling conditions comprised an initial denaturation at 95ºC for 5 min, followed by 40 cycles of 30s at 95ºC and 30s at 60ºC. For all qPCR assays, reactions lacking cDNA template (no template control), as well as reactions including reverse transcriptase minus (RT-) product as template were used as negative controls. Real-time PCR results were analyzed using the CFX Manager™ software detection system (Bio-Rad) and the gene expression was corrected by considering the endogenous control, Elongation factor 1-α, expression in each sample. The relative quantification was calculated using the formula previously described (Pfaffl, 2001). The expression values for control groups were considered as 1, while higher and lower values indicated an increased (up-regulation) and decreased response (down-regulation), respectively.

2.5. Cytotoxicity test

The nutraceutical was assayed for cytotoxicity using Epithelioma Papillosum Cyprini (EPC) and Rainbow Trout Gonads (RTG-2) fish cell lines and Human Epitheloid cervix carcinoma (HeLa) cell line. The EPC and RTG-2 cells were grown in 24 well flat bottom microtiter plate (Sigma-Aldrich, USA) in Leibovitz' medium (L-15, Gibco BRL) supplemented with 2% inactivated (56ºC, 30 min) foetal calf serum (iFCS), 1% penicillin-streptomycin (P/S, Gibco) for 48h at 18 and 25ºC, respectively. The HeLa cells were grown in Dulbecco´s Modified Eagle Medium (DMEM, BioWhittaker®, Lonza) supplemented with 10% FBS, 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37ºC. Cell monolayers were inoculated with 0.1 ml of the nutraceutical tested (final concentrations of 32, 64, 128 and 625 µg ml^{-1}) and incubated following the requirement of the cells. Total or partial destruction of monolayers within a 3-day period was scored as a positive cytotoxic effect. Cell control (cell monolayers inoculated with sterile saline solution with DMSO added and non-inoculated cells) was maintained throughout the experiment.

2.6. Statistical analysis

Differences in survival between fish fed nutraceuticals (Group 1) and control fish (Group 2) after the experimental infection with *F. psychrophilum* were analysed by Chi-square test ($p<0.05$). Significant differences on expression levels of immunity-related genes between different treatments and their respective controls were analyzed by Student´s t test. One-way ANOVA test was used to determine significant differences between treatments followed by Bonferroni´s multiple comparisons as post hoc comparison. All statistical analyses were performed using IBM SPSS Statistics 22.0. Differences were regarded statistically significant when $p<0.05$.

Table 3. Primers used for reference genes and related-immune genes of interest with their GenBank accession numbers, sequence, size of the amplified product and % of efficiency.

Gene	GenBank accesion nº	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size	qPCR efficiency %
IgM	S63348	CTTGGCTTGTTGACGATGAG	GGCTAGTGGTGTTGAATTGG	72	98.4
C5a	AF349001	TGGCAAGGACTTTTTCTGCT	AGCACAGGTATCCAGGGTTG	64	98.5
C5a receptor	AY438032	ACGCACCTTGAGGGTCATT	CAGTGGAAACCAGCACAGG	61	101.9
TNF-α	AJ277604	GGGGACAAACTGTGGACTGA	GAAGTTGTTGCCCTGCTCTG	75	99.9
		House-keeping genes			
β-actina	NM_001124235.1	GCCTCCTCTTCCTCTCTGGA	CTCTCGTTGCCGATGGTGAT	71	100.1
1α-Elongation-factor	AF498320	ACCCTCCTCTTGGTCGTTTC	TGATGACACCAACAGCAACA	63	100.7
r18S	AF308735	GATCCATTGGAGGGCAAGTCT	CGAGCTTTTTAACTGCAGCAACTTT	89	98.7

3. Results

3.1. Antimicrobial activity

The antimicrobial activity of the nutraceutical was tested against strains of *F. psychrophilum*, *V. anguillarum* and *A. salmonicida* subsp. *salmonicida* isolated from different fish species. The results of the present study indicated that the nutraceutical showed variable degrees of antibacterial activity against all the fish pathogens tested.

The minimum inhibitory concentration (MIC) was evaluated by the agar dilution and broth microdilution method. Both assays showed similar results for all the tested bacteria. For *F. psychrophilum* the nutraceutical inhibited bacterial growth at MIC_{90} values of 64 µg ml^{-1} (Table 4). The results revealed homogeneity in the inhibitory concentrations of the nutraceutical for *F. psychrophilum* regardless the serotype and source of isolation of the strains.

For *V. anguillarum* strains, differences on the MIC values were found depending on the serotypes analysed. Using the agar dilution method, strains from serotypes O1, O2a, O2b and O3A and O3B isolated from diseased fish displayed lower MICs (750-1750 µg ml^{-1}) than serotypes typically isolated from environment (O4 to O10) (MICs of 3 mg ml^{-1}). In the broth microdilution method, *V. anguillarum* strains from serotypes O2 were inhibited at MIC_{50} of 625 µg ml^{-1} and MIC_{90} of 1250 µg/ml, while strains from serotype O1 and O3 showed MIC_{90} of 1250 µg ml^{-1} of the nutraceutical.

The less sensitive bacterial species was *A. salmonicida* subsp. *salmonicida* that showed MIC_{90} values of 3000 µg ml^{-1} of the nutraceutical using both agar and broth methods.

Considering the ratio of MBC/MIC<4, the nutraceutical exerted a bactericidal effect against all bacteria tested.

Table 4. MIC (µg ml-1) values obtained for Liptofry

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F. psychrophilum	
Average	60
Range	16-128
CMI_{50}	64
CMI_{90}	64
Vibrio anguillarum	
Average	1208
Range	625-1750
CMI_{50}	1250
CMI_{90}	1250
Aeromonas salmonicida* subsp. *salmonicida	
Average	3000
Range	3000
CMI_{50}	3000
CMI_{90}	3000

MIC_{50} and MIC_{90}, MIC values which inhibits 50% and 90% of the isolates tested, respectively

3.2. Cytotoxicity assay

No loss of cell viability was observed in EPC, RTG-2 and HeLa cell lines when the nutraceutical was used at concentrations up to 625 µg ml^{-1}.

3.3. Effect of the administration of nutraceuticals in the prevention of BCWD

No adverse effect was observed in the fish during the 115-days feeding period before the experimental infection. As above indicated, the nutraceutical exerted the highest antimicrobial activity against strains of the species *F. psychrophilum*, thus, the *in vivo* effect of the administration of the nutraceutical was tested against diseases produced by this fish pathogen.

The mortalities in rainbow trout fingerlings infected with the strain *F. psychrophilum* RBT4.1.04 began after 24 hours of the experiment and stopped 4 days later, in both groups (control fish and fish fed Liptofry-supplemented

diet) (Figure 1). However, cumulative mortalities observed for the two groups was statistically different ($p<0.05$). Fish fed Liptofry-supplemented diets showed an average cumulative mortality of 27% following infection, compared to 62% of fish fed control diet (Figure 1). Thus, the survival of fish fed nutraceutical diets was significantly higher than the control group ($p<0.05$). Non-infected control fish showed 100% of survival. The strain of *F. psychrophilum* inoculated was recovered from head kidney of all infected fish which died in the challenge experiments. Common signs of the disease observed in moribund and died fish included accumulation of liquid in the coelomic cavity, liquefaction of tissues, haemorrhages in the inoculation and surrounding areas, as well as in the muscles.

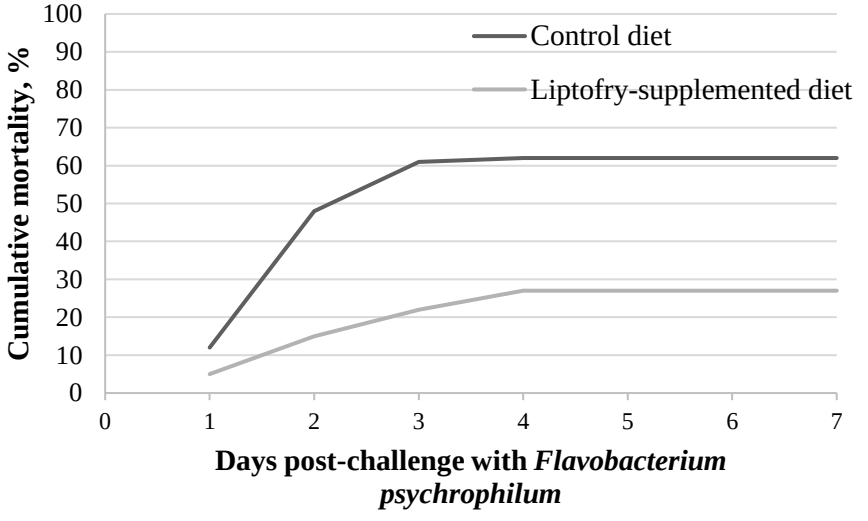

Figure 1. Cumulative mortality of rainbow trout (*Oncorhynchus mykiss*) from group 1 (fish fed Liptofry-supplemented diets, n=120) and from group 2 (control fish, n=120) following challenge with the virulent strain *Flavobacterium psychrophilum* RBT4.1.04.

3.4. Effect of the nutraceutical on the expression of immunity-related genes

The effect of the dietary administration of the nutraceutical on the expression of immunity-related genes was evaluated in different organs (head-kidney, skin, mid-gut and spleen) of rainbow trout. Thus, the transcription levels of the genes encoding TNF-α, C5a, rC5a and IgM were analysed by real-time PCR. The expression of most of selected genes changed significantly in spleen, skin and gut in fish fed Liptofry-supplemented diets in comparison with the control group. The expression of IgM (Figure 2) did not change significantly in the spleen and skin, but its expression was significantly up-regulated ($p<0.05$) in gut of fish fed with Liptofry supplemented diet (2.5-fold increase) (Figure 2). The expression of the genes encoding for C5a and its receptor rC5a (Figure 2), was statistically up-regulated in spleen and skin ($p<0.01$) in fish fed Liptofry supplemented diet, while their expression in gut was maintained constant (Figure 2). The expression levels for TNF-α gene (Figure 2), showed a significant ($p<0.01$) up-regulation in spleen (9-fold increase), skin (8-fold increase) and gut (2-fold increase) in fish fed Liptofry supplemented diet.

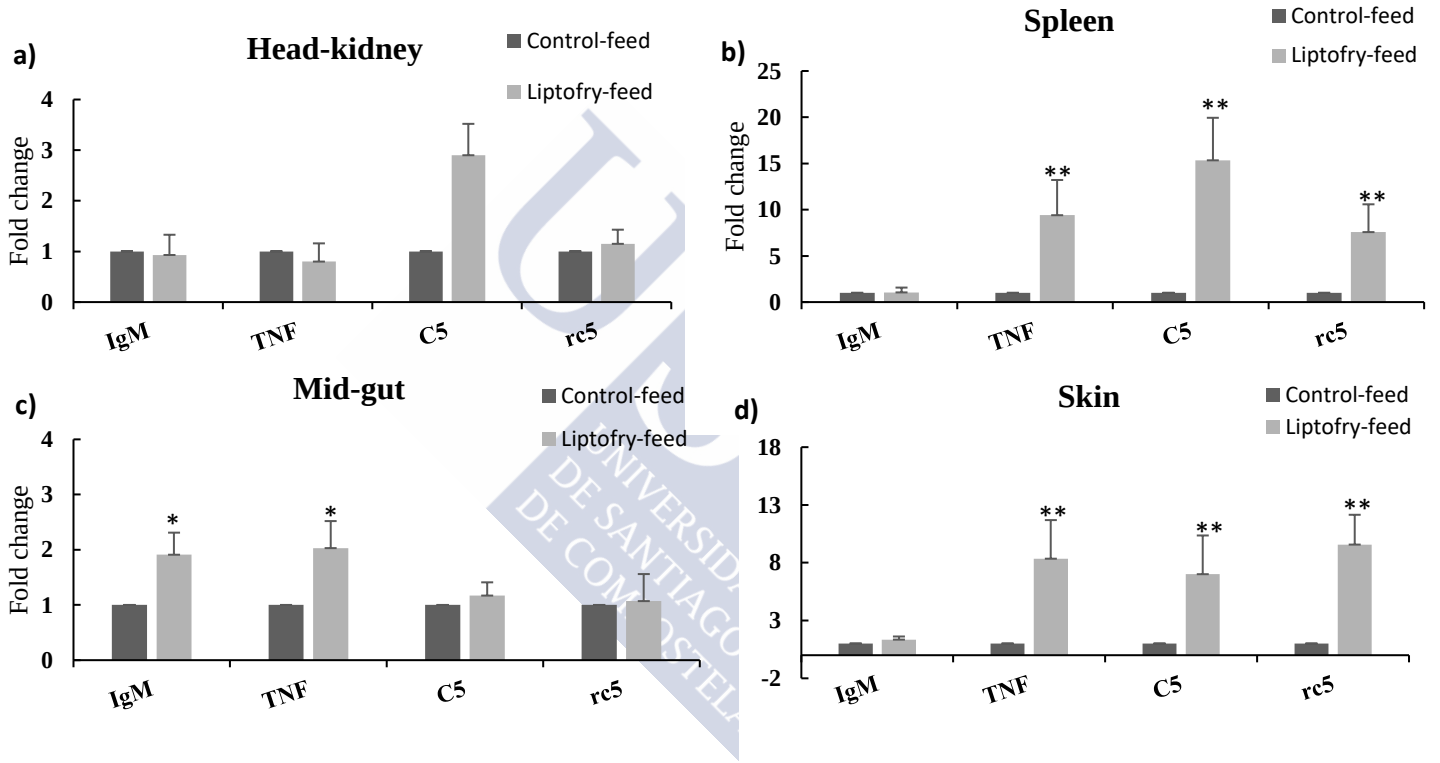

Figure 2. Expression of the immunity-related genes IgM, C5a, rC5a, and TNF-α in head-kidney (a), spleen (b), mid-gut (c) and skin (d) tissues of rainbow trout fed control and Liptofry-supplemented diet analyzed by RT-qPCR. Asterisks denote statistically significant differences compared to control level of 1 (*p < 0.05; **p < 0.01)

4. Discussion

The present study reports the antimicrobial activity of the garlic-based nutraceutical Liptofry (manufactured by Liptosa) as well as the influence of Liptofry-supplemented diets over the resistance and immune response of rainbow trout against BCWD. In our study, *in vitro* assays demonstrated that the product Liptofry displayed the highest bactericidal effect against the bacterial species *F. psychrophilum* (MIC_{90} of 64 µg ml^{-1}); while *Vibrio anguillarum* and *A. salmonicida* subps. *salmonicida* showed MIC_{90} values of 1250 µg ml^{-1} and 3000 µg ml^{-1}, respectively regardless the serotype or source of isolation of the strain. These results are in agreement with the work reported by Lu et al., (2011), that demonstrated that the mode of action of garlic and garlic-derived organosulfur compounds could freely penetrate cell membranes of Gram-stain negative bacteria and combine with a thiol-containing enzyme and/or protein, altering their structures (Lu et al., 2011).

As resistance to commonly used antibiotics has been reported worldwide for these fish pathogens (Hesami et al., 2010; Inglis et al., 1993; Pedersen et al., 1995; Schmidt et al., 2001), Liprofry would represent a good alternative to control bacterial outbreaks. Furthermore, the complex nature of the plant extracts may reduce the spontaneous occurrence of bacterial resistance since multiple simultaneous mutations may be required to overcome the antimicrobial actions of all the active plant components (Nguyen et al., 2016). Citotoxicity assay revealed that the nutraceutical-additive analysed did not affect the viability of fish and human cell lines at the tested concentrations (between 32 and 625 µg ml^{-1}), which confers to Liptofry an additional value.

In our study, diets containing 0.5% of the nutraceutical tested for 105 days produced a significant ($p<0.05$) reduction in the cumulative mortalities of rainbow trout after challenge with *F. psychrophylum* compared to fish fed

control diet. The protective effect of herbs, essential oils, and plant extracts in aquatic animals against other fish pathogens as *Aeromonas hydrophila* (Nya and Austin, 2009; Sutili et al., 2015), *Yersinia ruckeri* (Adel et al., 2016), *Photobacterium damseale* subsp. *piscicida* and *Streptococcus iniae* (Guo et al., 2015) have been reported in previous studies. However, the prevention of BCWD using natural extracts had not been reported before.

Nutrition may have influence on the modulation of the immune system. The use of diet supplements such as probiotics, β-glucans or plant-derived supplements, has been shown to produce changes in the levels of transcription of immunity-related genes (Baba et al., 2018, 2016; Falco et al., 2012; Kim and Austin, 2006; Muñoz-Atienza et al., 2014; Panigrahi et al., 2007; Peddie et al., 2002; Pionnier et al., 2013). In this study, we investigated the expression of immunity-related genes in several organs of rainbow trout (kidney, spleen, gut and skin) after 105 days of feeding nutraceutical-supplemented diets.

The humoral elements of the specific immune system of fish, include different types of immunoglobulins (Magnadóttir, 2006), being the IgM the most dominant in terms in all organs and is essential for immune protection against different pathogens upon different routes of infection. IgM molecules can either be natural, which provide early and broad protection without prior exposure to antigens (innate/non-specific), or produced after exposure to antigens, resulting in an antigen-specific response (adaptive/specific). Despite the significance of IgM in the immune response, there are relatively few studies on changes induced by nutraceuticals and vegetal-derived products in its total levels in systemic and mucosal compartments. In the present study, after 105 days of feeding nutraceuticals, the expression of immunoglobulin M was only up-regulated on the gut of rainbow trout. These results could indicate that Liptofry could promote an inflammatory response in the intestine that may lead to increased protection against fish diseases. Other studies have demonstrated

that diets containing soybean products significantly increase levels of lysozyme and IgM in the mid and distal intestinal mucosa (Krogdahl et al., 2000). These results, confirm that the presence of fish IgM is not limited to the serum, being originated in fish epithelial mucus (Evenhuis and Cleveland, 2012; LaFrentz et al., 2003; Rombout et al., 2011) as well as in the intestinal mucosa (Krogdahl et al. 2001). Nevertheless, no significant changes in IgM gene expression in spleen were obtained in our study. These findings could be explained because the main regulation of immunoglobulin expression takes place in the head-kidney and not in the spleen, as reported by (Raida and Buchmann, (2008). Further studies should be carried out to clarify the relationship between the natural antibody response and resistance and susceptibility to bacterial fish pathogens.

TNF-α are pro-inflammatory cytokines with a variety of functions as cell proliferation, apoptosis, enhance leucocyte migration, phagocytic activity and the expression of other pro-inflammatory cytokines. Our study found up-regulation in the expression of TNF-α gene in gut, spleen and skin of trout fed with nutraceutical-supplemented diet in comparison with fish fed control diet. These results indicated that diets supplemented with nutraceuticals may stimulate the innate immune response of rainbow trout, promoting the release of substances that mediated the inflammatory response. Our results overlap with those described by Baba et al. (2018), who reported an increase of TNF-α in spleen of rainbow trout fed 0.1% dietary olive leaf (*Olea europea* L.). Diets supplemented with green tea (*Camellia sinensis*) (100 mg/kg) also induced TNF-α gene expression in spleen of rainbow trout (Nootash et al., 2013).

C5a is a potent anaphylatoxin generated during complement activation promoting: i) inflammation process by attracting phagocytic cells to the site of the infection (Holland and Lambris, 2002), ii) activation of the respiratory burst in phagocytes and iii) enhancement of expression of TNF-α in monocyte derived

macrophages. In the present study, the administration of nutraceutical-supplemented diet significantly induced the C5a and rC5a expression in spleen, intestine and skin compared to the control group. According to these results, the nutraceutical may stimulate the fish immune response by activating the complement system, which could indicate an activation of inflammatory processes. Other studies reported that feeding Senegalese sole with diets supplemented with soybean resulted in an induction of genes related to complement pathway and their receptors (Montero et al., 2015).

To conclude, the present results revealed that feeding rainbow trout fingerlings with diets supplemented with 0.5% of the nutraceutical Liptofry induce protection against diseases produced by the fish pathogen *F. psychrophilum*. This fact may be due to the combined effect of the high antimicrobial activity of the nutraceutical against *F. psychrophilum* and the ability of nutraceutical-supplemented diets to stimulate parameters of the specific and non-specific immune response of rainbow trout. Further studies should investigate the expression of additional genes for a better understanding of the mechanisms by which nutraceuticals influence over immune system, increasing the resistance against bacterial pathogens.

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CHAPTER VIII. General Discussion

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From an economic perspective, fish farming is an important industry that is considered crucial for the future supply of fish for human consumption. Currently, about 580 aquatic species are farmed all over the world by some of the poorest farmers in developing countries and by multinational companies. As occurs with other animal species, one of the main limiting factors for the development of aquaculture is the appearance of infectious diseases. Cultured fish are susceptible to a great variety of diseases, being bacterial diseases, the main responsible of important economic losses in the intensive cultures throughout the world (Austin and Austin, 2016; Buller, 2004; Toranzo et al., 2005). Moreover, as aquaculture expands and new species are farmed, new diseases are emerging that affects both wild and farmed fish adversely. The rate and extent of emergence can be reduced by the application of biosecurity programmes designed to mitigate the risk factor for disease emergence (Murray and Peeler, 2005).

The general aims of the present work were: I) to determine the taxonomic position and study of the virulence of Gram-stain-negative bacteria causing diseases in different marine and freshwater farmed fish species (Chapter 3), II) the development of molecular diagnostic techniques that are more sensitive and specific and less laborious than the conventional methods, for the identification and/or detection of pathogenic bacteria in the tissues of diseased fish (Chapter 4), III) the evaluation of different serological, molecular and typing methods for the study of the heterogeneity within and among fish pathogenic bacteria (Chapter 5), IV) the characterization of the antimicrobial resistance of bacterial fish pathogens using phenotypic and molecular methods, and the evaluation of new chemical or natural compounds as an alternative for the management of bacterial outbreaks (Chapters 6 and 7).

In the first part of this work (Chapter 3), the taxonomic position and the potential virulence of Gram-stain-negative pathogens isolated from marine (sea bass and mackerel) and freshwater (rainbow trout) farmed fish with clinical signs of disease were determined. Thus, the strains were characterized using different phenotypic (biochemical tests, API systems, chemotaxonomic analysis), serological (Dot-Blot assay), genotyping (ERIC and REP-PCR) and proteomic (MALDI-TOF-MS) methods and/or sequencing of the 16S rRNA gene.

Analyses of the microorganisms isolated from sea bass (SK181/12.1, SK181/12.2, SK181/12.3, SK181/12.4, SK181/12.5, SK164/12.1, SK164/12.2, SK164/12.3, SK164/12.4, SK164/12.5) showed that the strains were very homogeneous at phenotypical, serological, molecular, chemotaxonomic and proteomic levels and were very similar to the reference strains of *A. salmonicida* subsp. *salmonicida* used for comparative purposes. This homogeneity in the *A. salmonicida* subsp. *salmonicida* characters have been previously reported by other authors (Beaz-Hidalgo et al., 2008a; Beaz-Hidalgo and Figueras, 2012.; Magariños et al., 2011). Molecular and serological tests did not differentiate *A. salmonicida* subsp. *salmonicida* from the atypical strains of *A. salmonicida* included in the study; since an unspecific amplification was obtained using the PCR analysis described by Beaz-Hidalgo et al. (2008b) and the anti-*A. salmonicida* serum cross-reacted with the subspecies *A. salmonicida* subsp. *masoucida*. A weak cross-reaction between these two subspecies had been previously described by other researchers using Dot-blot assay (Toranzo et al., 1987), confirming the low specificity and sensitivity levels of this method for the identification of this fish pathogens. The article nº 1 derived from this study described the first isolation and the polyphasic characterization of the fish pathogen *A. salmonicida* subsp. *salmonicida* in farmed sea bass in Spain.

Regarding strains isolated from marine fish *Trachurus trachurus* (article nº 2) (AqJ1.17 and AqJ2.17) were presumptively assigned to the genus *Tenacibaculum* due to their growth and morphological characteristics. However, results did not show positive amplification using primers specific for the detection of *T. maritimum* or *T. soleae* (Cepeda et al., 2003; García-González et al., 2011). To determine if the isolates represented a new taxa within the Family *Flavobacteriaceae* we performed a preliminary polyphasic study using some of the minimal standards recommended by Bernardet et al. (2002): i) phenotypic characteristics, ii) determination of the fatty acid methyl esters, iii) 16S rRNA sequencing. Biochemical tests and analysis of the fatty acids indicated that the isolates belonged to the Genus *Lacinutrix* spp., while the partial sequencing of the 16S rRNA gene allowed us to identify the strains as *Lacinutrix venerupis* (similarity levels of 99.1% with the type strains *L. venerupis* CECT 8573^T) (article nº 2). Sequencing of this gene is very useful for the correct identification of atypical or unusual bacteria isolated from clinical samples (Bosshard et al., 2006; Janda and Abbott, 2007). This bacterial species has only been recently described as a potential fish pathogen for sea bass and sea bream (López et al., 2017).

In a subsequent study, a group of Gram-stain-negative bacteria isolated from fish eggs and rainbow trout that showed clinical signs (ulceration, necrosis or inflammation of the spleen) compatible with Bacterial Cold-Water Disease (BCWD) caused by *F. psychrophilum* (Austin and Austin, 2016; Loch and Faisal, 2015), were characterized. The isolates studied were obtained in FLP medium, one of the culture media commonly used for isolation of *F. psychrophilum* (Cepeda et al., 2004). This fact, together with the colour and morphology of the colonies and the clinical data, made us suspect that the outbreak could be due to this bacterium. However, none of the isolates presented the typical phenotypic characteristics exhibited by *F. psychrophilum* (Bernardet

et al., 2002) or could be identified by PCR using a *F. psychrophilum*-specific PCR protocol (Cepeda and Santos, 2000). The phenotypic characteristics showed by the nine isolates under study were more similar to those described for other *Flavobacterium* species recently described as *F. collinsi*, *F. tructae*, *F. johnosonieae*, *F. plurextorum* or *F. piscis* (Suebsing and Kim, 2012; Zamora, 2015). The differential phenotypic characters that help to differentiate *Flavobacterium* species were reviewed by Bernardet and Bowman (2011). However, *Flavobacterium* species isolated more recently are not included in this review; thus, the use of conventional identification systems was not useful to clarify the taxonomic position of the bacteria isolated from juveniles and eggs of rainbow trout. Similarities among the molecular (ERIC and REP) and the proteomic profiles showed a high proximity among the strains FCV.00 and *F. tructae* CECT 7791 and RBT11.05.16.3 and *F. collinsi* CECT 7796, respectively. However, most of the strains could not be identified using the characterization methods used in this study. The lack of conclusive results derived from this study indicate that more exhaustive studies (16S rRNA sequencing, DNA-DNA hybridization, determination of G+C content of genomic DNA and composition of the fatty acid methyl esters) (Bernardet et al., 2002) should be performed in future studies to determine if these strains could represent a novel flavobacterial taxa. It should also be noted that there are few specific diagnostic methods for lesser-known flavobacteria pathogenic for fish, making identification difficult (Loch and Faisal, 2015).

In order to determine the potential risk to economically relevant fish cultures of the microorganisms characterized in Chapter 3, pathogenicity tests were performed. The results obtained showed that the *A. salmonicida* subsp. *salmonicida* strains isolated from sea bass were virulent for turbot and trout when inoculated by intraperitoneal injection with doses ranging from 2×10^4 to 2×10^7 CFU per fish. The *A. salmonicida* strains under study presented lack of

host specificity, therefore, it would be necessary to apply measures to prevent the spread of these microorganisms to other species susceptible to the disease. Similarly, the strain RBT11.05.16.3, presumptively identified as *F. collinsi* basing on MALDI-TOF, REC and ERIC profiles, was virulent for rainbow trout, causing 80 to 100% of fish mortality with the dose evaluated (1×10^7 and 1×10^8 CFU per fish). These results suggest that these microorganisms could be present in aquaculture facilities and/or in healthy fish as an opportunistic pathogen and produce diseases only under certain conditions (López et al., 2017). Thus, the best procedure to prevent the emergence of new diseases would be the reduction of biotic and abiotic stress in fish. On the other hand, the isolate *L. venerupis* AqJ1.17 was not virulent for turbot, sea bass and Senegalese sole after intraperitoneal injection with high doses of the bacteria (10^9 CFU per fish). Similarly, *F. pyschrophilum*-like strain ICH29.01.18.1 did not produce mortality when it was intra-peritoneally injected in rainbow trout using high concentrations of the bacteria. Further studies will be necessary to determine the importance of these species as fish pathogens.

Traditional diagnostic methods involve the isolation of the pathogen followed by morphological, biochemical, serological and molecular characterization and the analysis of their antimicrobial susceptibility profiles. The main disadvantages of conventional microbiological methods is the time required to isolate the microorganism and to complete the diagnostic, specially, in the case of slow-growing bacteria or fastidious bacteria that requires specific media as *Flavobacterium* or *Tenacibaculum* spp. (Cepeda et al., 2004; Pazos et al., 1996). Furthermore, as above described, some bacteria cannot be differentiated from other bacteria of the same genus by conventional identification methods or commercial multi-test systems, since they present similar phenotypic characteristics (Toranzo et al., 1987; Piñeiro-Vidal, 2008; Beaz-Hidalgo et al., 2010; Zamora, 2015).

The need for rapid, sensitive and specific diagnostic methods, has stimulated the development of molecular techniques such as real-time PCR, DNA-DNA hybridization, or multi-locus sequence analysis (MLSA) that have been applied to the identification of different fish pathogens (Abayneh et al., 2012; Ashrafi et al., 2015; Fringuelli et al., 2012; Gordon et al., 2008; Marancik and Wiens, 2013; Strepparava et al., 2014; Van Vliet et al., 2016). In order to develop species-specific molecular techniques that do not require prior isolation of the microorganism from the diseased fish, the Chapter 4 was focused on the design of diagnostic techniques for the detection of *Aeromonas salmonicida* subsp. *salmonicida* (article nº4) and *Tenacibaculum maritimum* (article nº5) based on the polymerase chain reaction (PCR).

The sequence encoding the 16S rRNA gene has become an essential tool for the development of diagnostic methods based on PCR (Bader and Shotts, 1998; Warsen et al., 2004; Zlotkin et al., 1998). However, the exhaustive comparison of the 16S rRNA gene sequences of the different *A. salmonicida* subspecies (*salmonicida*, *achromogenes*, *masoucida* and *smithia*) available from databases carried out in the present study (article nº 4), demonstrated that the differences between these subspecies were not enough to assure a specific amplification. Therefore, we selected the sequence of the specific gene *aopO* (GenBank accession nº DQ386862.1), located in the low-copy-number *pAsa5* plasmid present in all virulent strains of *A. salmonicida* subspecies *salmonicida* (Dacanay et al., 2006). Specific primers were designed on the basis of the sequence of *aopO* gene, which encodes for a serine/threonine protein kinase of the type III secretion system linked to virulence. The PCR method developed showed high specificity (97.3 %) for *A. salmonicida* subsp. *salmonicida* (article nº 4). At difference to other real-time PCR protocols (Balcazar et al., 2007; Keeling et al., 2013), this assay clearly differentiates *A. salmonicida* subsp. *salmonicida* from the atypical subspecies *A. salmonicida*

subsp. *masoucida* and from other non-related bacteria tested even in mixed cultures and in tissues of diseased fish. However, the protocol was unable to reliably differentiate *Aeromonas salmonicida* subsp. *salmonicida* from one strain of *A. salmonicida* subsp. *smithia* and one strain of *A. salmonicida* subsp. *achromogenes*. This could represent a drawback for this PCR approach; however, these atypical subspecies could be differentiated rapidly from the typical subspecies by serological methods as slide agglutination and dot blot as previously described in Chapter 3. Thus, a combination of both molecular and serological methods would be the best option in order to clearly identify the typical subspecies of *A. salmonicida*. The assay showed a high sensitivity when using pure cultures (1-2 cells per PCR reaction) and tissue samples of infected fish (6-60 cells per PCR reaction) (article nº 4). The PCR method showed a similar or greater sensitivity than other molecular methods previously described for detection of *A. salmonicida* (Balcazar et al., 2007; Keeling et al., 2013) and other bacteria pathogenic for fish (Beaz-Hidalgo et al., 2008b; Cepeda et al., 2003; González et al., 2004).

In the article nº 5 included in Chapter 4 we also described the development of a specific and sensitive real-time PCR protocol for the specific diagnosis of the fish pathogen *T. maritimum*. In this study, the alignment and comparison of variable regions of the 16S rRNA gene sequences from 8 fish pathogenic species of the genus *Tenacibaculum* (*T. maritimum, T. soleae, T. discolor, T. gallaicum, T. dicentrarchi, T. finnmarkense* and *T. ovolyticum*) seemed to be enough for the design of specific primers for *T. maritimum*. The protocol showed 100% specificity for *T. maritimum*, since DNA from other bacteria tested did not show any amplification. In this sense, this is the first qPCR protocol that tested isolates from all the fish-associated *Tenacibaculum* species described until now. The assay allowed the specific detection and quantification of the bacterium in lethal and non-lethal fish samples and seawater samples. Thus, this protocol could be

used for epidemiological studies, monitoring the presence of *T. maritimum* in fish production systems, and to evaluate the quality of waters entering and leaving the aquaculture facilities, which may help to prevent the occurrence of tenacibaculosis outbreaks.

On the other hand, the study of the antigenic, genetic and proteomic diversity of fish pathogens is also of great importance when considering targeted control strategies (including vaccine development) or to recognize factors involved in virulence (Buján et al., 2015; Dumpala et al., 2010; Van Vliet et al., 2016). Different DNA-based (ribotyping, pulsed-field gel electrophoresis, PFGE; multi-locus sequence analysis, MLSA; restriction fragment length polymorphism, RFLP; randomly amplified polymorphic DNA PCR, RAPD-PCR; and repetitive sequence-based, Rep-PCR), proteomic (MALDI-TOF mass spectra, 2-D LC ESI MS/MS, 2-DE MALDI TOF/TOF MS) and serological (slide agglutination and Dot-Blot) typing methods have been applied for the characterization and differentiation of bacterial species pathogenic for fish as well as for epidemiological studies and bacterial source tracking (Abayneh et al., 2012; Ashrafi et al., 2015; Avendano-Herrera et al., 2004; Avendaño-Herrera et al., 2004; Beaz-Hidalgo et al., 2008a; Bernardet et al., 2005; Buján et al., 2015; Dumpala et al., 2010; Maiden et al., 1998; Olivares-Fuster et al., 2007; Rochat et al., 2017; Sachdeva and Virdi, 2004; Saticioglu et al., 2018; Valdebenito and Avendaño-Herrera, 2009)

MALDI-TOF-MS analysis provides a unique mass spectral fingerprint for one microorganism. This method represents a simple, cost-effective, time-saving detection system with high-throughput capability that does not require specific expertise. MALDI-TOF-MS has been applied for the identification of microorganisms from distinct genera and species and to differentiate strains of the same species; for epidemiological studies or for the detection of water and

food-borne pathogens (Benagli et al., 2012; Carbonnelle et al., 2011; Dieckmann et al., 2010; Donohue et al., 2006; Pérez-Sancho et al., 2016, 2017, 2018; Singhal et al., 2015). Specifically, there are few data or no data regarding the proteomic characteristics of the Genus *Tenacibaculum* or *Flavobacterium* (Pérez-Sancho et al., 2017), and few studies in which proteomic profiles had been used for epidemiological purposes in fish diseases.

In Chapter 5, the proteomic tool MALDI-TOF-MS was evaluated as: i) a diagnostic technique for the identification and differentiation of *Tenacibaculum* and *Flavobacterium* species and ii) to evaluate the proteomic variability among strains belonging to different serotypes and isolated from different sources and geographical regions.

Thus, MALDI-TOF profiles of the *Tenacibaculum* species tested (*T. maritimum*, *T. soleae*, *T. discolor*, *T. gallaicum*, *T. dicentrachi* and *T. ovolyticum*) were clearly different between them and showed different species-specific mass peaks that could be used as biomarkers for the rapid diagnosis of tenacibaculosis (article nº 6). However, no specie-specific mass peaks were detected for the species *T. discolor* and *T. gallaicum*. These results are congruent with those reported by Piñeiro-Vidal et al. (2008) that showed that these two species are difficult to discriminate on the basis of the 16S rRNA gene sequences (similarity levels of 97–99%) and only can be differentiated by DNA:DNA hybridization (Piñeiro-Vidal et al., 2008). Likewise to that described by Pérez-Sancho et al. (2017), in the present study (article nº8) the MALDI-TOF-MS proteomic analysis allowed to differentiate *F. psychrophilum* from other *Flavobacterium* species as *F. flevense*, *F. succinicans*, *F. columnare*, *F. branchiophilum* and *F. johnsoniae* by defining species identifying biomarkers and by hierarchical cluster analysis.

All these results demonstrated that MALDI-TOF mass spectrometry represents a powerful tool that can be used by diagnostic laboratories for rapid identification of the fish pathogens belonging to the species *Flavobacterium psychrophilum* or *Tenacibaculum* and its differentiation from other closely-related species. Moreover, as demonstrated in article nº 6, the ability of this technique to produce comparable spectra of *Tenacibaculum* species using different media (FMM or MA) is important for the construction of spectra databases that can be used as a reliable tool for the rapid classification and identification of the unknown bacterial isolates in diagnostic laboratories all over the world. In recent years, great efforts have been carried out to construct protein databases for the identification and for the study of phylo-proteomic relationships between closely related fish bacterial pathogens (Pérez-Sancho et al. 2016, 2017; Assis et al. 2017; López-Cortés et al. 2017). However, despite the importance of reference databases for identification, MALDI-TOF has also proven valuable for the characterization of microorganisms for which no reference mass spectra exist, using cluster analysis of proteomic mass data (Conway et al. 2001; Böhme et al. 2011). Cluster and principal component analysis (PCA) of the mass data also proved to be reliable methods for taxonomy of *Tenacibaculum* and *Flavobacterium* species used in Chapter 5 by comparing similarities and differences in their proteomic profiles, except for the species *T. discolor* and *T. gallaicum.*

MALDI-TOF-MS was also assessed to evaluate the proteomic variability among *Tenacibaculum* (article nº 7) and *Flavobacterium* (article nº 8) strains belonging to different serotypes and isolated from different sources and geographical regions. A comparative analysis of spectra did not reveal any serotype-specific mass peaks for the different serotypes of *T. maritimum*; probably due to the low number of strains belonging to serotypes O2, O3, and O4 used. However, 11 serotype-specific mass peaks were found for the serotype

O1 of *T. soleae* and 8 mass peaks were present in all the *T. soleae* strains belonging to serotype O2. For the species *T. discolor*, analysis of spectra revealed the existence of two specific mass peaks for the O1 serotype. Similarly, for *F. psychrophilum* four mass peaks patterns associated with the serotype O1, O2a, O2b and O3 were found (article nº 8). On the other hand, mass peaks associated to the source of isolation of *Tenacibaculum* species could not be defined (article nº 7); contrary to *F. psychrophilum* whose mass spectra showed different patterns associated with the source of the isolation of the pathogen (salmon or rainbow trout) (article nº 8). All these results suggest that protein biomarkers detected by MALDI-TOF could be used for epidemiological purposes as well as for the detection of different serotypes, therefore, reducing the number of samples that have to be analysed by conventional serotyping techniques, as slide-agglutination test. However, further studies analysing a higher number of strains representatives of pathogenic *Tenacibaculum sp. and F. psychrophilum* isolated from different sources are needed to evaluate the ability of MALDI-TOF for epidemiological studies and to confirm its efficacy as diagnostic method.

Apart from MALDI-TOF-MS, in Chapter 5 other serological (slide-agglutination and Dot-Blot) and molecular (ERIC and REP-PCR) techniques were also evaluated for diagnostic purposes as well as to identify heterogeneity within strains of *T. maritimum*, *T. soleae* and *T. discolor* (article nº 7). Serological studies demonstrated the lack of immunological relationship among all the *Tenacibaculum* species analysed, demonstrating that, despite the low sensitivity described for this method, it represents a rapid and specific diagnostic method which allow distinguishing fish-associated *Tenacibaculum* species (Piñeiro-Vidal, 2008). Moreover, these results suggest that fish vaccinated against *T. maritimum* would not be protected against diseases produced by other *Tenacibaculum* species. In this sense, further studies should be carried out to

clarify if fish immunized with *T. maritimum*-vaccines are cross-protected against other *Tenacibaculum* spp. diseases. Antigenic heterogeneity was found in *T. maritimum* (being the serotype O1 the dominant) as previously described (Avendaño-Herrera et al., 2004; Pazos, 1996; Santos et al., 1999); while at least two serotypes (O1 and O2) were found within *T. soleae* strains, and one for the species *T. discolor* (article nº 7), confirming the existence of antigenic heterogeneity within these species (López et al., 2010; Piñeiro-Vidal, 2008).

Molecular fingerprinting by ERIC and REP-PCR revealed specific profiles for each of the *Tenacibaculum* species analysed *T. maritimum*, *T. soleae* and *T. discolor* (article nº 7). As occurred with the MALDI-TOF analysis, the three species were distributed in separate groups in the two dendrograms generated, demonstrating that both REP-PCR and ERIC-PCR are effective typing methods with high discrimination power. Several other authors have used Rep-PCR genomic fingerprinting as a valuable tool for the identification and classification of bacteria, as well as for molecular epidemiological studies (Aljindan et al., 2018; Asgarani et al., 2015; Beaz-Hidalgo et al., 2008a; Hiett and Seal, 2009; Meacham et al., 2003; Moser et al., 2010; Stephenson et al., 2009; Wilson and Sharp, 2006). However, in the present study slight genomic variability was found within each *Tenacibaculum* species analysed using both REP- and ERIC-PCR. Furthermore, these differences could not be related with the serotype, fish species of isolation or geographical region (article nº 7). Thus, these techniques proved not to be useful for epidemiological investigation and population genetic analysis of *Tenacibaculum* species pathogenic for fish. Other authors have applied other genotyping techniques such as ribotyping or Randomly Amplified Polymorphic DNA PCR (RAPD) for the study of *T. maritimum* (Avendano-Herrera et al., 2004; Pazos, 1997). Thus, RAPD analysis revealed the existence of genetic variability within *T. maritimum* strains isolated from different marine fish and belonging to different O-serotypes (Avendano-Herrera et al., 2004).

Moreover, this technique also allows to differentiate *T. maritimum* from *T. discolor*, *T. gallaicum* and *T. soleae* (Piñeiro-Vidal, 2008). In addition, although this technique is relatively cheap, rapid, and easy to perform, RAPD patterns are notoriously difficult to reproduce from one laboratory to another or when attempting to compare isolates tested on different days. In the last years, multi-locus sequence analysis (MLSA) has been successfully used for the genotyping and genetic differentiation of *Tenacibaculum* and *Flavobacterium* species, for epidemiological studies and to estimate the evolutionary relationship among isolates recovered from diseased fish worldwide (Ashrafi et al., 2015; Avendaño-Herrera et al., 2016; Frisch et al., 2018; Habib et al., 2014; Kayansamruaj et al., 2017; Mun et al., 2013; Olsen et al., 2017). In addition, MLSA provides unambiguous DNA sequence data that can be easily exchanged and compared via web-based databases around the world. MLSA combines PCR and automated DNA sequencing to reduce labour and analysis time, thereby providing discriminatory power comparable or superior to that provided by other PCR-based methods (Lin et al., 2014). Knowledge of the complete genome sequence of fish pathogens will help to improve diagnostics and better understand the biology of these microorganisms, virulence mechanisms or epidemiology (Duchaud et al., 2007; Pérez-Pascual et al., 2017; Rochat et al., 2017; Teramoto et al., 2016; Touchon et al., 2011).

For the prevention and control of bacterial fish diseases, different methods can be used, including the treatment of fish with chemotherapeutics, vaccination, or immunostimulants (probiotics, β-glucans and plant-based products) as well as by using fishes and eggs from pathogen free suppliers, foods free of pathogens, and monitoring the presence of fish pathogens in fish production tanks. The evaluation of the quality of the waters entering and leaving an aquaculture facility, is also important for preventing the introduction of new diseases or the dissemination of fish pathogens. It has been widely

studied that the use of antimicrobial agents in farms leads to the development of antimicrobial-resistant pathogens in fish and the aquatic environment (Blair et al., 2015; Miller and Harbottle, 2018). Currently, some antimicrobial agents commonly used in aquaculture are only partially effective against different fish pathogens due to the emergence of resistant bacteria. Therefore, in the Chapter 6 of the present PhD work research a preliminary assessment of the occurrence of antimicrobial resistant *A. salmonicida* strain in Spain was evaluated (article nº 9). Both disk diffusion and/or broth-microdilution assays demonstrated a high frequency of resistance against trimethoprim-sulfamethoxazole, oxytetracycline and chloramphenicol as previously described (Adams et al., 1998; Akinbowale et al., 2006; Schmidt et al., 2001; Trudel et al., 2016). These results are worrying since oxytetracycline is one of the three antimicrobials authorised by the Spanish Agency of Medicines and Medical Devices (AEMPS) to treat many bacterial infections in terrestrial and aquatic animals. Contrary, all the *A. salmonicida* strains were susceptible to enrofloxacin and flumequine (article nº 9). Molecular characterization of the resistance to antimicrobials showed that *sul1* gene; one of the genes that encodes resistance to sulphonamides; was the most commonly observed in the Spanish isolates of *A. salmonicida*. Similarly, this gene was also found to be the most common antimicrobial resistance gene in Canadian isolates of *A. salmonicida* (Trudel et al., 2016). Among the oxytetracycline-resistant strains, only the genes *tetA* and *tetE* were found in the *A. salmonicida* isolates in a proportion of 7:1 (article nº 9). The same proportion of these genes were found in resistant *A. salmonicida* strains isolated in Korea (Kim et al., 2011). The 30% of the strains analysed presented the *cat* gene (that confers resistance to chloramphenicol) despite the fact that the use of this antibiotic was strictly prohibited for animal use in many countries in the mid-1990s. Only two isolates of *A. salmonicida* were found to be resistant to florfenicol; and both strains harboured the *flor* gene (article nº 9). Previous

studies have suggested that chloramphenicol resistance is more frequent and express at higher levels than florfenicol resistance (Michel et al., 2003). Overall, the results included in Chapter 6 showed that fish pathogenic *A. salmonicida* could act as reservoir of antibiotic resistance genes (Trudel et al., 2016). Thus, it is urgent to develop and apply alternative strategies to prevent and control of bacterial disease outbreaks without selecting resistant bacteria.

For instance, the use of herbal medicines within animal production has been promising for prevention and control of bacterial diseases in fish. Plant-based products are natural and biodegradable, they present antimicrobial activity against different fish pathogens or stimulate the immune system of fish. In the Chapter 7 of the present work the antimicrobial activity of different commercial natural or chemical compounds against the fish pathogens *A. salmonicida, V. anguillarum* and/or *F. psychrophilum* was evaluated. Thus, the potential antimicrobial activity of the nutraceutical Liptosa-P203 (Lípidos Toledo S.A, Liptosa) (based on vegetable fatty acids and essential oils) and the chemical agent Bronopol and its potential applicability for the management of bacterial outbreaks in fish farming was evaluated by determining the Minimum Inhibitory Concentration (MIC) against *A. salmonicida* subsp. *salmonicida* (*n*=20 strains) and *V. anguillarum* (*n*=30 strains) (article nº 10). Results obtained for *V. anguillarum* showed MIC values ranging from 4.57 to 9.15 ppm of Bronopol and of 292.96-585.92 ppm of Liptosa-P203; while *A. salmonicida* strains presented MIC values of 9.15-36.62 ppm for Bronopol and 292.96-585.92 ppm of Liptosa-P203. Moreover, Liptosa-P203 was able to kill the 99.99% of cells of *V. anguillarum* and *A. salmonicida* in only 1h at concentrations of 4x MIC. Similar or slightly higher time-killing values were obtained for the commonly used bactericidal agent Bronopol using 4x MIC concentrations (article nº10). Although future studies should be conducted under field conditions, these results suggest that Liptosa-P203 could be an alternative for the control of the

diseases caused by *A. salmonicida* and *V. anguillarum.* Several other studies have demonstrated that plant-derivate essential oils, plant extracts and natural herb products represent a promising source of alternative bactericidal agents against clinically important bacteria (Baba et al., 2016; 2018; Chouhan et al., 2017; Debbarma et al., 2012; Navarrete et al., 2010; Nazzaro et al., 2013; Starliper et al., 2015; Sutili et al., 2015).

Similarly, in a different study (article nº11) we evaluated *in vitro* the antibacterial activity of the commercial nutraceutical Liptofry, manufactured by Lipidos Toledo S.A. (Liptosa) against the fish pathogens *Vibrio anguillarum*, *Aeromonas salmonicida* subsp. *salmonicida* and *F. psychrophilum* using the agar and broth dilution methods. Liptofry is a commercially available vegetal-derived supplement containing 10% garlic. *In vitro* assays demonstrated that the product Liptofry displayed the highest bactericidal effect against the bacterial species *F. psychrophilum* (MIC_{90} of 64 µg/ml); while *Vibrio anguillarum* and *A. salmonicida* subps. *salmonicida* showed were less sensitive (MIC_{90} values of 1250 µg/ml and 3000 µg/ml, respectively) (article nº11). Several authors have demonstrated the inhibitory effect of garlic, *Allium sativum*, against different bacterial species (Gull et al., 2012; Nya and Austin, 2009; Seong Wei and Musa, 2008).

Due to the high *in vitro* sensitivity of *F. psychrophilum* to Liptofry, it was considered of interest to evaluate its usefulness for the control of bacterial cold-water disease in rainbow trout. Thus, the effect of Liptofry-supplemented diets on disease resistance was tested using rainbow trout fed diets containing 0 (control diet) and 0.5 % (experimental diet) of the nutraceutical and challenged with a virulent strain of *F. psychrophilum*. Results showed that the cumulative mortality of fish fed with the nutraceutical-supplemented diet was significantly lower ($p<0.05$) than that for fish fed control diets. These results indicate that

Liptofry-supplemented diets could be used as an eco-friendly alternative for the prevention and control of diseases produced by *F. psychrophilum*. Resistance conferred by garlic-supplemented diets against pathogens has been reported in different fish species infected with *Aeromonas hydrophila* (Nya and Austin, 2009; Thanikachalam et al., 2010), *Edwardsiella tarda* (Abraham and Ritu, 2015) or *Streptococcus iniae* (Guo et al., 2015).

The protective activity induced by garlic-supplemented diets or other plant-based additives in fish could be due to an increase in the number of erythrocytes and leucocytes, haematocrit, phagocytic activity, respiratory burst, lysozyme, anti-protease and bactericidal activities of rainbow trout (Baba et al., 2018; Harikrishnan et al., 2011; Nya and Austin, 2009; Thanikachalam et al., 2010; Yogeshwari et al., 2015). Some authors have also described that the use of diet supplements (probiotics, β-glucans or plant-derived supplements) could produce changes in the levels of transcription of immunity-related genes (Baba et al., 2016b, 2018; Falco et al., 2012; Kim and Austin, 2006; Muñoz-Atienza et al., 2014; Panigrahi et al., 2007; Peddie et al., 2002; Pionnier et al., 2013). Therefore, the expression of genes TNF-α, C5a, rC5a and IgM in several organs of rainbow trout (kidney, spleen, gut and skin) was investigated after 105 days of feeding Liptofry-supplemented diets. The results showed that genes encoding tumour necrosis factor-α (TNF-α), complement (C5a) and complement receptor (rC5a) were over-expressed in spleen and skin in fish fed Liptofry-supplemented diet compared to control fish, while immunoglobulin M (IgM) gene expression was only increased in intestine from fish fed nutraceutical-additives. These results demonstrated that administration of nutraceutical-supplemented diets for 115 days appeared to be enough to stimulate immune parameters of fish. This immunity stimulation could explain the observed protection of rainbow trout against the experimental infection with *F. psychrophilum*. In conclusion, the nutraceutical Liprofry would represent a good

alternative to control BCWD outbreaks. However, we must take into account that in this study we have only tested one dose (5 kg t^{-1} diet) of the product and one period of treatment (115 days). It has been described that the effect of immunostimulants are species-specific and dose dependent (Harikrishnan et al., 2011; Vallejos-Vidal et al., 2016); thus, further studies should be performed to determine the optimum dose that trigger the maximum immune response in fish (Harikrishnan et al., 2011; Sakai, 1999; Vallejos-Vidal et al., 2016).

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CHAPTER IX. Conclusions

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1. The Gram-stain-negative bacteria isolated from sea bass and mackerel were identified as *Aeromonas salmonicida* subsp. *salmonicida* and *Lacinutrix venerupis* using phenotypic, serological, molecular and proteomic techniques. The strains isolated from trout and trout eggs were presumptively identified as *F. collinsi* and *F. tructae* or assigned to *Flavobacterium* Genus. *A. salmonicida* strains isolated from sea bass could be considered as primary pathogens, while *L. venerupis* and *F. psychrophilum*-like strains were probably opportunistic pathogens.

2. The PCR assays designed for the detection and identification of *A. salmonicida* subsp. *salmonicida* and *T. maritimum* showed high specificity levels (97.3 to 100%) and sensitivity and allowed the identification of the pathogens in pure and mixed bacterial cultures and fish tissue samples of infected fish. Both protocols could be used for epidemiological studies, monitoring the presence of *T. maritimum* and *A. salmonicida* in fish production systems, and to evaluate the quality of waters entering and leaving the aquaculture facilities.

3. Serological, proteomic and genotyping techniques are useful for identification and/or typing of *A. salmonicida*, subsp *salmonicida* and fish-associated *Tenacibaculum and Flavobacterium* strains. Proteomic and serological methods have also potential applicability for epidemiological studies as well as for the selection of strains for the development of vaccines to prevent the tenacibaculosis and flavobacteriosis .

4. Phenotypic and molecular methods demonstrated a high prevalence of resistance to oxytetracycline, potentiated sulphonamides and chloramphenicol among *A. salmonicida* subsp. *salmonicida* strains isolated in Spain.

5. The vegetal-based nutraceuticals Liptofry and Liptosa P-203 (Lípidos Toledo S.A., Liptosa) demonstrated antibacterial activity against *F. psychrophilum* and /or *V. anguillarum* and *A. salmonicida* using both the agar and broth dilution methods. Moreover, administration of Liptofry-supplemented diets increased resistance of rainbow trout against diseases produced by *F. psychrophilum* and induced over-expression of the immunity-related genes encoding the tumour necrosis factor-α (TNF-α), complement (C5a) and complement receptor (rC5a), and immunoglobulin M (IgM).

Patents

1. **Procedure for the detection, quantification and identification of *Aeromonas salmonicida* spp. *salmonicida*.**

Inventors: **Clara Fernández Álvarez**; Santiago Fernández González; Ysabel Santos Rodríguez

Institution: Universidade de Santiago de Compostela

Number of publication: 2 578 367

Date of publication of the concession: 01/12/2016

Patent licensed to: Exopol S.L., Zaragoza , Spain

2. **Procedure for the detection, quantification and identification of *Tenacibaculum maritimum***

Inventors: **Clara Fernández Álvarez**; Ysabel Santos Rodríguez

Institution: Universidad de Santiago de Compostela

Application number: P201730991

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