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Diagnosis, prevention and control of emerging and re-emerging diseases in aquaculture

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DIAGNOSIS, PREVENTION AND CONTROL OF EMERGING AND RE-EMERGING DISEASES IN AQUACULTURE

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AUTORIZACIÓN DEL DIRECTOR / TUTOR DE LA TESIS

Diagnosis, prevention and control of emerging and re-emerging diseases in aquaculture

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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

A la abuela Maruja

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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 which are presented below:

Articles

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Torres-Corral, Y., Santos, Y. (2019). Identification and typing of *Vagococcus salmoninarum* using genomic and proteomic techniques. Journal of fish diseases, 42 (4), 597-612. Doi: 10.1111/jfd.12967.

Torres-Corral, Y., Santos, Y. (2021). Clonality of *Lactococcus garvieae* isolated from rainbow trout cultured in Spain: a molecular, immunological, and proteomic approach. Aquaculture, 545, 737190. Doi: 10.1016/j.aquaculture.2021.737190

Torres-Corral, Y., Santos, Y. (2022). Predicting antimicrobial resistance of *Lactococcus garvieae*: PCR detection of resistance genes versus MALDI-TOF protein profiling. Aquaculture, 553, 738098. Doi: 10.1016/j.aquaculture.2022.738098

Torres-Corral, Y., Fernández-Álvarez, C., Santos, Y. (2019). High-throughput identification and quantification of *Vagococcus salmoninarum* by SYBR Green I-based real-time PCR combined with melting curve analysis. Journal of fish diseases, 42(10), 1359-1368. Doi: 10.1111/jfd.13053.

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Torres-Corral, Y., González-Barreiro, O., Riaza, A., Santos, Y. (2022). Establishment of different challenge models for *Aeromonas salmonicida* subsp. *achromogenes* in turbot and sole. Aquaculture, 555, 738261. Doi: 10.1016/j.aquaculture.2022.738261

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Resumo

Nas últimas décadas, o aumento anual mundial do consumo de peixe duplicou o crecemento demográfico, poñendo de manifesto que o sector acuícola é un dos sectores gandeiros con maior taxa de crecemento. Na actualidade, os alimentos procedentes do medio acuático representan o 17 % da produción mundial de carne comestible. Non achega unicamente proteína animal, senón que ademais conteñen micronutrientes biodispoñibles e ácidos graxos esenciais que non se atopan facilmente nos alimentos terrestres. A acuicultura converteuse, polo tanto, nun sector fundamental para contribuír á seguridade alimentaria e nutricional a nivel mundial. Neste escenario, xerouse a estratexia da Granxa á Mesa, que persegue políticas que proporcionen aos cidadáns alimentos seguros, nutritivos e alcanzables que apoien aos agricultores, gandeiros, acuicultores e pescadores coa sustentabilidade dos alimentos ao longo de toda a súa cadea de valor. A estratexia da Granxa á Mesa deberá abarcar cada paso da cadea para subministrar alimentos, dende a produción ata o consumo, e impulsar os obxectivos da Economía Circular.

Neste contexto, as enfermidades infecciosas na acuicultura son unha ameaza constante para a produción e, por conseguinte, para a seguridade alimentaria. Os gromos de enfermidades infecciosas causan grandes perdas económicas debido á redución da produtividade, á mala calidade dos produtos acuáticos e aos custos dos tratamentos. Ademais, as enfermidades en acuicultura supoñen un grave risco para a saúde humana pola existencia de axentes potencialmente zoonóticos e polo consumo de produtos acuícolas infectados.

As bacterias Gram-negativas son os principais microorganismos patóxenos que afectan aos peixes. Isto é consecuencia de que moitos destes microorganismos cohabitan no hábitat natural dos organismos acuáticos sen causar enfermidades; con todo, hai condicións que predispoñen ao desenvolvemento da enfermidade, como o baixo nivel de osíxeno disolto, os problemas de calidade da auga, o estrés no manexo ou os traumatismos. Entre elas, resaltan as enfermidades causadas por *Aeromonas* sp. e *Vibrio* sp., responsables de septicemias hemorráxicas, con hemorraxias no peritoneo, na parede corporal e nas vísceras dos peixes enfermos.

As infeccións por cocos Gram-positivos ou " estreptococose" son tamén consideradas unha das principais enfermidades emerxentes e re-emerxente que afectan aos peixes. Estas infeccións foron introducidas en Europa a mediados dos anos 80, e considéranse un complexo de enfermidades similares causadas por diferentes xéneros e especies capaces de inducir danos no sistema nervioso central caracterizados por exoftalmia e meningoencefalitis. Nestas infeccións, a temperatura da auga considérase un factor que predispón para a aparición da enfermidade e adoitan estar producidas por *Vagococcus* sp., *Lactococcus* sp. e *Streptococcus* sp.

O éxito da xestión da saúde dos organismos acuáticos comeza coa prevención das enfermidades máis que co tratamento. O obxectivo principal é aplicar o enfoque "Unha Saúde" para lograr unha produción acuícola sostible e segura. Os principios de "Unha Saúde" pretenden involucrar a múltiples sectores e disciplinas para lograr resultados beneficiosos para a saúde e o benestar das persoas, os animais e a contorna que comparten. A observación diaria do comportamento dos peixes e o coñecemento integrado das interaccións entre os patóxenos, o hóspede e o medio ambiente, permiten detectar problemas de forma temperá cando se producen, de modo que se pode realizar un diagnóstico e seguimento antes de que a maior parte da poboación enferme.

A correcta identificación e seguimento do axente etiolóxico é un dos principais factores para unha boa xestión da enfermidade. Historicamente, os diagnósticos dos peixes afectados e as identificacións dos axentes patóxenos realizábanse mediante o illamento bacteriano e a caracterización fenotípica dos microorganismos illados. Con todo, os diagnósticos baseados nestas análises microbiolóxicos tardan polo menos un día e a miúdo non proporcionan unha identificación fiable do patóxeno. Por iso, cada vez é máis necesario o uso de ferramentas analíticas alternativas para a identificación rápida e precisa das especies bacterianas que son difíciles de discernir mediante as técnicas clásicas, así como para a discriminación entre cepas estreitamente relacionadas, co fin de mellorar a xestión da enfermidade.

O serotipado dos illados bacterianos realizábase con fins diagnósticos, epidemiolóxicos, e para a selección de cepas para o desenvolvemento de vacinas para previr as enfermidades nos peixes. Esta técnica xeralmente é máis rápida, sensible e específica que os métodos de diagnóstico baseados no cultivo dos microorganismos. Con todo, o serotipado ten unha capacidade limitada para discernir entre algúns illados e, a necesidade de obter antisoros dos animais constitúe unha limitación importante.

A identificación e tipificación de microorganismos baseada na amplificación do ADN bacteriano tamén é un método utilizado habitualmente na investigación médica e biolóxica. Os métodos baseados na amplificación do ADN bacteriano proporcionan identificacións específicas das especies e información sobre o parentesco xenético das cepas, a fonte de infección e a virulencia das cepas, así como a distribución xeográfica e do hóspede. Os métodos de tipificación baseados en patróns de bandas de ADN demostraron previamente a súa eficacia na caracterización de especies bacterianas Gram-positivas. Algúns destes métodos son a electroforese en xel de campo pulsado (PFGE), o ADN polimórfico amplificado ao azar (RAPD), o ribotipado e a amplificación de secuencias de ADN repetitivas de función descoñecida, entre as que se inclúen as secuencias repetitivas palindrómicas extraxénicas (secuencias REP) e as secuencias consenso repetitivas intraxénicas de enterobacterias (secuencias ERIC), sendo as secuencias REP as máis utilizada no estudo de gromos.

Nos últimos anos, a espectrometría de masas de desorción/ ionización asistida por unha matriz con detección de masas por tempo de voo (EM-MALDI-TOF) xurdiu como un enfoque prometedor para o diagnóstico, a discriminación entre cepas ambientais estreitamente relacionadas ou a identificación de microorganismos raros ou menos

frecuentes, os cales son difíciles de discriminar coas técnicas clásicas. En comparación con outros métodos convencionais ou moleculares, a EM-MALDI-TOF é un método de diagnóstico sinxelo e de baixo custo, o cal permite obter resultados nuns poucos minutos tras o cultivo do microorganismo. Ademais, trátase dun método de diagnóstico non dirixido, de alto rendemento e que ten facilidade de automatización. Na espectrometría de masas MALDI-TOF xérase un espectro de masas do microorganismo, o cal é traducido electrónicamente a unha lista de picos expresados como unha relación entre a súa masa/carga e a abundancia relativa da mostra, e é diferente para cada microorganismo. Este patrón único, producido polas proteínas e outras biomoléculas da mostra analizada, cotexarase co espectro dun microorganismo coñecido, previamente depositado nunha base de datos. Tendo en conta que cada espectro contén picos de masa únicos para cada xénero, especie e mesmo cepa, este espectro funcionará como unha pegada dactilar que pode ser cotexada para identificar un microorganismo. Con todo, EM-MALDI-TOF tamén demostrou ser valiosa para a caracterización de microorganismos para os que non existen espectros de masas de referencia a través do uso de análise multivariantes (análise de conglomerados o cluster ou análise de compoñentes principais) dos datos de masas proteómicas.

Tras a correcta identificación e caracterización dos patóxenos presentes nas instalacións acuícolas, é precisa unha adecuada xestión do gromo infeccioso. Para controlar os gromos infecciosos utilizáronse de forma frecuente produtos químicos e medicamentos. Con todo, estes métodos serven para gañar tempo para os peixes e permitirlles superar as infeccións oportunistas, pero non substitúen a unha cría e seguimento adecuado. Ademais, o uso destes medicamentos ten repercusións negativas sobre o medio ambiente e sobre a saúde humana; como son a propagación de bacterias resistentes a antimicrobianos e a transferencia de xenes de resistencia a patóxenos humanos e de animais. Neste contexto, as vacinas son consideradas un novo enfoque ecolóxico para previr e controlar as enfermidades dos peixes, polo que nalgunhas zonas do mundo produciuse unha transición do uso de fármacos ao uso de vacinas.

Por todo o exposto anteriormente, a presente tese doutoral centrouse en resolver algúns dos principais problemas relacionados coas enfermidades producidas por patóxenos bacterianos dos xéneros *Streptococcus*, *Lactococcus*, *Vagococcus*, *Aeromonas* e *Vibrio*. Os obxectivos específicos establecidos na presente tese foron:

1. Avaliación das técnicas moleculares, serolóxicas e proteómicas para a identificación, tipificación e seguimento das bacterias patóxenas dos peixes.

2. Análise de perfís fenotípicos, xenotípicos e proteómicos para determinar a susceptibilidade antimicrobiana e a resistencia a múltiples fármacos das bacterias patóxenas dos peixes.

3. Desenvolvemento de métodos moleculares para a identificación e detección cuantitativa das bacterias patóxenas dos peixes e a súa diferenciación de especies estreitamente relacionadas.

4. Estudo da eficacia e seguridade de novas vacinas para a prevención de enfermidades bacterianas en peixes.

No **Capítulo 3**, descríbese o potencial de diferentes métodos de diagnóstico para a rápida diferenciación e tipificación de cepas bacterianas illadas de peixes con signos de estreptococose. Tipificáronse e caracterizaron cepas patóxenas de peixes pertencentes aos xéneros *Streptococcus*, *Vagococcus* e *Lactococcus* mediante métodos convencionais (métodos bioquímicos e sistemas API), serolóxicos (aglutinación en portaobxectos e ensaio dot- blot), moleculares (REP e ERIC- PCR), antixénicos (inmunoblot), proteómicos (EM- MALDI- TOF) e quimiotaxonómicos (análise de ésteres metílicos de ácidos graxos). Ademais, a potencial virulencia das cepas bacterianas avaliouse mediante infeccións experimentais de peixes.

A identificación e tipificación de cepas de *Streptococcus parauberis* e *Streptococcus iniae* illadas de peixes (**artigo nº 1**) realizouse de forma comparativa mediante métodos serolóxicos (aglutinación en portaobxectos e ensaio dot- blot), moleculares (REP-PCR) e proteómicos (EM-MALDI-TOF). Este estudo demostrou que todas as técnicas testadas son útiles como ferramentas de diagnóstico, xa que son capaces de diferenciar as cepas de *Streptococcus* segundo a súa posición taxonómica. Ademais, demostrouse variabilidade antixénica, proteómica e xenética dentro da especie *S. parauberis*. Por outra banda, as cepas de *S. iniae* mostraron ser antixénica, proteómica e xenéticamente homoxéneas. Os resultados proteómicos e serolóxicos permitiron confirmar a existencia de novos potenciais serotipos en cepas de *S. parauberis* illadas de rodaballo en España (definidas como serotipo III) e cepas illadas de mostras de leite dun animal con mastite (definida como serotipo IV). Así mesmo, atopáronse biomarcadores proteicos específicos de xénero e/ou especie para as cepas de *Streptococcus* analizadas mediante EM-MALDI-TOF. Tamén se observaron perfís de ADN e patróns proteicos específicos de serotipo e subserotipo para as cepas de *S. parauberis*. Atopouse unha boa correlación entre os métodos serolóxicos, proteómicos e moleculares testados no **artigo nº 1** para a diferenciación e tipificación de especies de *Streptococcus*.

Para a caracterización de *Vagococcus salmoninarum* (**artigo nº 2**) utilizáronse métodos fenotípicos (métodos bioquímicos, sistemas API e análises de resistencia a antimicrobianos), serolóxicos (aglutinación en portaobxectos e ensaios dot-blot), antixénicos (inmunoblot), xenéticos (RAPD, ERIC e REP- PCR) e proteómicos (espectrometría de masas MALDI-TOF). Os resultados demostraron que as cepas de *V. salmoninarum* foron resistentes á maioría dos antimicrobianos probados e só o 10% das cepas foron sensibles ao florfenicol. As análises de aglutinación en portaobxectos e ensaios dot-blot puxeron de manifesto a existencia dunha gran homoxeneidade antixénica dentro da especie *V. salmoninarum*, e que non existen reaccións cruzadas con outras especies patóxenas de peixes causantes de estreptococose como son *S. parauberis, S. iniae, S. agalactiae, L. garvieae* ou *Carnobacterium maltaromaticum*. Estes resultados foron apoiados pola análise electroforético das proteínas da superficie celular e por inmunoblot. Ademais, a análise xenotípico e proteómico tamén mostrou

unha limitada variabilidade dentro da especie *V. salmoninarum*, permitindo establecer patróns e picos de masa específicos desta especie. Estes resultados indican que os métodos fenotípicos, xenómicos e proteómicos permiten diferenciar rapidamente á especie *V. salmoninarum* das demais especies causantes de estreptococose. Con todo, a EM-MALDI-TOF permite unha tipificación e caracterización rápida e fácil de *V. salmoninarum* sen necesidade dunha base de datos específica.

No **artigo nº 3** descríbese a caracterización fenotípica (ensaios bioquímicos e API), molecular (RAPD e REP- PCR), antixénica (Inmunoblot), proteómica (espectrometría de masas MALDI- TOF), quimiotaxonómica (análise de ésteres metílicos de ácidos graxos) e de virulencia de illados de *L. garvieae*. Neste estudo analizáronse de forma comparativa cepas de *L. garvieae* illadas a partir do ano 2015 de troitas cultivadas, que foran inmunizadas coa vacina Ictiovac LG (Hipra, España) ou coa autovacina combinada contra *L. garvieae* e *Yersinia ruckeri* (Acuipharma Aquaculture Health solutions, España), con cepas de *L. garvieae* illadas de troitas cultivadas en diferentes granxas de España durante o período 2002 a 2014. Este estudo permitiu detectar certa heteroxeneidade fenotípica dentro das cepas de *L. garvieae*. Esta heteroxeneidade foi confirmada mediante técnicas de xenotipado e proteómicas, as cales permitiron separar as cepas de *L. garvieae* illadas de humanos e bovinos das cepas de *L. garvieae* illadas do medio acuático. Por outra banda, os métodos de tipificación por PCR convencional mostraron que ningún dos illados de *L. garvieae* analizados pertencía ao serotipo II e non portaban a agrupación de xenes da cápsula descrito no xenoma ou en plásmidos de cepas xaponesas de *L. garvieae*. No **artigo nº 3**, identificáronse diferentes patróns clonais en *L. garvieae* mediante RAPD e REP-PCR (sete perfiles) e MALDI- TOF (seis patróns), os cales estaban estreitamente relacionados coa fonte de illamento das cepas de *L. garvieae*, pero non coa virulencia destas. Ademais, detectáronse dúas variantes antixénicas da proteína (W1 e W2), que difiren en reactividade no rango de 35 a 70 KDa, utilizando antisoros de coello e de troita. Ambos antisoros detectaron dúas bandas proteicas altamente inmunoxénicas de 30 e 45 KDa. Estes resultados poderían contribuír a unha mellor comprensión da patoxénese da lactococose e axudar á selección de antíxenos para o desenvolvemento de vacinas eficaces.

No **artigo nº 4** realizouse un estudo sobre a susceptibilidade aos antimicrobianos e a resistencia a múltiples fármacos (MDR) de *L. garvieae* illadas de troita, e determinouse a correlación entre o fenotipo de resistencia, o xenotipo e o perfil proteómico. Os resultados do ensaio de susceptibilidade antimicrobiana demostraron a resistencia dos illados ao trimetoprima- sulfametoxazol e á flumequina (100% de resistencia), á enrofloxacina (67%), á oxitetraciclina (44%), á amoxicilina (23%), ao cloranfenicol (18%) e ao florfenicol (13%). A detección mediante PCR identificou varios xenes de resistencia aos antimicrobianos (ARG), sendo o tet(B) o de maior prevalencia (85%), seguido do FloR (78%), o tet(A) (61%), o tet(S) (13%) e o tet(K) (9%). Os xenes tet(B) e FloR detectáronse no 100% dos illados de *L. garvieae* resistentes á oxitetraciclina e ao florfenicol. Ademais, o 83% das *L. garvieae* analizadas eran resistentes a múltiples antimicrobianos, sendo a combinación máis

relevante teta+ tetB (56% das cepas analizadas), tetA+FloR e tetB+ FloR (51%) e tetA+tetB+FloR (48%). En leste mesmo **artigo nº 4** avaliouse tamén o potencial da EM-MALDI-TOF para a identificación de potenciais biomarcadores proteicos de resistencia a antimicrobianos. Este estudo identificou cinco picos proteicos como potenciais marcadores de resistencia á oxitetraciclina e oito picos proteicos como potenciais marcadores de resistencia ao florfenicol presentes nos illados de *L. garvieae*. Este estudo proporciona unha visión sobre a presenza de resistencia a antimicrobianos en cepas de *L. garvieae* illadas en España e constitúe un punto de partida para o establecemento de sistemas de vixilancia da resistencia aos antimicrobianos na acuicultura.

No **Capítulo 4**, desenvolvéronse técnicas de diagnóstico baseadas en PCR específicas para a identificación e/ou detección dos patóxenos *V. salmoninarum*, *S. iniae* e *S. parauberis* nos tecidos de peixes enfermos e/ou en auga.

Para *V. salmoninarum* (**artigo nº 5**) deseñouse un par de cebadores e un protocolo baseado en PCR en tempo real baseado no uso do fluoróforo SYBR Green I, combinado coa análise da curva de melting, para a identificación e cuantificación de *V. salmoninarum* en cultivos bacterianos e tecidos de peixes infectados. Para iso, seleccionouse o xene 16 S rRNA para deseñar un par de cebadores denominados SalF e SalR. A sensibilidade e especificidade deste par de cebadores comparouse con outros deseñados previamente para a PCR convencional. Aínda que ambos pares de cebadores mostraron unha especificidade do 100% utilizando cultivos bacterianos puros ou ADN extraído de bacterias ou tecidos de peixes (Tm = 84,0 ± 0,5 º C), o par de cebadores SalF e SalR (deseñados no **artigo nº 5**) mostraron a maior sensibilidade cun límite de detección de 0,034 x 100 copias de amplicón por ensaio (equivalente a 2 x 10^{-11} ng μl^{-1}, valor Cq de 30,49 ± 1,71). O protocolo de qPCR desenvolto permitiu a detección de *V. salmoninarum* en mostras de peixes non letais e letais con niveis de detección de 0,17 x 10^0 copias do xene en tecidos infectados artificialmente e 0,02 x 10^0 copias do xene en tecidos de peixes infectados experimentalmente con *V. salmoninarum*. Ademais, non se detectaron amplificacións non específicas cando se analizaron tecidos de peixes sans ou de peixes infectados con outros patóxenos.

Para a identificación específica de *S. iniae* (**artigo nº 6**) desenvolveuse e avaliouse un protocolo de PCR cuantitativa baseado nun par de cebadores que amplifican un fragmento de 167 pb do xene que codifica a permeasa de lactato (*lldY*). O xene *lldY* foi seleccionado baseándose nunha análise xenómica comparativa que utilizou 45 secuencias recuperadas da base de datos do xenoma do NCBI. O protocolo mostrou uns niveis de especificidade do 100% con cultivos bacterianos puros ou con ADN extraído de *S. iniae*. A temperatura de melting do produto de amplificación foi de 77,00 ± 0,55 º C. A sensibilidade de detección do protocolo foi de 1,12 x 10^1 copias de produto amplificado por ensaio (equivalente a 2 x 10^{-9} ng μl^{-1}) utilizando ADN bacteriano e de 1,44 x 10^1 copias de xenes en tecidos de peixes infectados con *S. iniae*. O protocolo de qPCR proporciona unha alternativa precisa e sensible para a identificación de *S. iniae*,

así como a súa detección en tecidos de peixes, o que pode ser posto en funcionamento como ferramenta de rutina en laboratorios de microbioloxía.

No **artigo nº 7** descríbese a estrutura prevista para os loci cps implicados na biosíntese da cápsula para os serotipos III, IV e V de *S. parauberis*. Baseándose nas rexións específicas dos serotipos I, II e III dos loci cps, deseñouse un sistema de PCR múltiple para diferenciar os principais serotipos de *S. parauberis* causantes de enfermidade nos peixes. Ademais, descríbese un método de PCR en tempo real e un par de cebadores (cps3K-F e cps3K-R) para identificar cepas de *S. parauberis* pertencentes ao serotipo III en cultivos bacterianos e tecidos de peixes, sen a necesidade de soros obtidos de animais. As análises *in silico* e *in vitro* revelaron que ambos os métodos teñen unha especificidade do 100%. Por unha banda, optimizouse o ensaio de PCR múltiple para a detección de cepas de *S. parauberis* dos subtipos Ia (tamaño do amplicón 213 bp), os subtipos Ib e Ic (ambos con tamaño do amplicón 303 bp), o serotipo II (tamaño do amplicón 403 bp) e o serotipo III (tamaño do amplicón 130- bp) a partir de cultivos bacterianos. Por outra banda, optimizouse o ensaio de PCR en tempo real para a identificación e cuantificación das cepas do serotipo III de *S. parauberis* en cultivos bacterianos e tecidos de peixes (Tm = 73,00 ± 0.33º C). Este ensaio alcanzou unha sensibilidade de 2,67 x 10^2 copias do xene (equivalente a 3,8 x 10^{-9} ng μl^{-1}) utilizando cultivos bacterianos puros de *S. parauberis* do serotipo III e 1,76 x 10^2 copias do xene en tecidos de peixes infectados experimental e naturalmente con *S. parauberis* do serotipo III. A especificidade e sensibilidade dos protocolos descritos neste estudo suxiren que estes métodos poderían utilizarse con fins diagnósticos e/ou epidemiolóxicos nos laboratorios de diagnóstico clínico.

No **Capítulo 5** avaliouse a eficacia e seguridade de vacinas bivalentes para a prevención da vibriose, causada por *Vibrio anguillarum*, e da furunculose atípica, causada por *Aeromonas salmonicida* subsp. *achromogenes*. Para iso, en primeiro lugar, avaliáronse catro vías de infección para *A. salmonicida* subsp. *achromogenes* (inxección intraperitoneal, inxección subcutánea, inmersión e inoculación intragástrica) en rodaballo e linguado (**artigo nº 8**), co fin de establecer un modelo de infección adecuado para o estudo da patoxénese da furunculose atípica e para a avaliación da eficacia de métodos de prevención e control da enfermidade. Neste estudo utilizáronse cepas de *A. salmonicida* subsp. *achromogenes* illadas a partir de rodaballos afectados de furunculose atípica. De todas as vías probadas, a inxección intraperitoneal, subcutánea e por inmersión causaron mortalidade e reproduciron os signos clínicos da furunculose atípica nos peixes infectados experimentalmente. Estes resultados suxiren que as cepas de *A. salmonicida* subsp. *achromogenes* utilizadas non mostran especificidade de hóspede, sendo capaces de infectar tanto aos rodaballos como aos linguados en condicións experimentais alcanzando un 80-100% de mortalidade tras a inoculación intraperitoneal e subcutánea. Confirmouse que *A. salmonicida* subsp. *achromogenes* era o axente causante das mortalidades mediante o cultivo bacteriano e mediante análise de qPCR de mostras de peixes moribundos ou mortos. Con todo, precísanse máis estudos para identificar as vías de entrada e os

modelos de diseminación de *A. salmonicida* subsp. *achromogenes* nos peixes, o que axudará a mellorar o tratamento e as estratexias profilácticas.

Finalmente, no **artigo nº 9** desenvolveuse unha vacina autóxena bivalente fronte a *V. anguillarum* e *A. salmonicida* subsp. *achromogenes* á que se incorporou como adxuvante o composto oleoso Montanide™. A eficacia desta vacina autóxena e da vacina comercial AlphaJect 3000 (Pharmaq AS), administradas mediante inxección intraperitoneal, para previr a furunculose atípica e a vibriose no rodaballo, avaliouse de forma comparativa. Avaliouse en paralelo o efecto da inxección do adxuvante de aceite mineral Montanide™ (ISA 763A VG; Seppic, Francia) e da parafina líquida Eolane 130 (Total Fluids, España). O efecto de ambas as vacinas e dos adxuvantes comprobouse analizando os parámetros sanitarios e a supervivencia dos peixes tras o reto infeccioso con *V. anguillarum* e *A. salmonicida* subsp. *achromogenes*. Os resultados do estudo mostraron que a vacina autóxena conferiu altos niveis de protección e inmunidade duradeira contra ambos patóxenos cunha soa dose. Con todo, os rodaballos inxectados con esta autovacina mostraron efectos secundarios graves, mentres que se observaron menores efectos negativos cando os rodaballos eran inxectados coa vacina AlphaJect 3000 ou cos adxuvantes Montanide ou Eolane. Todos os rodaballos vacinados mostraron títulos de aglutinación de anticorpos notables, superiores aos dos peixes de control, que se mantiveron 160 días despois da vacinación. Este estudo demostrou que a vacina bivalente autóxena induce unha protección duradeira contra a furunculose atípica e a vibriose no rodaballo, tras a administración dunha soa dose, á conta de elevados efectos secundarios nos peixes. Por tanto, o desenvolvemento de novas vacinas debería centrarse nas autovacinas e no uso de adxuvantes de parafina líquida que aumenten a protección con efectos secundarios reducidos ou nulos.

A enfermidade é o resultado dunha perturbación no equilibrio entre o hóspede, o patóxeno e o medio ambiente. Por tanto, as estratexias para previr e controlar as enfermidades bacterianas nas piscifactorías deben considerar todos os aspectos implicados na relación patóxeno-hóspede-ambiente para garantir a protección dos peixes a longo prazo.

Neste traballo de doutoramento estudáronse diferentes aspectos da tipificación e seguimento de patóxenos bacterianos, o diagnóstico e o control das enfermidades producidas polos patóxenos bacterianos *S. parauberis, S. iniae, V. salmoninarum, L. garvieae, A. salmonicida* subsp. *achromogenes* e *V. anguillarum*. Os resultados obtidos mostraron que os métodos serolóxicos, xenotípicos e proteómicos son ferramentas útiles para un diagnóstico e un seguimento rápido e fiable dos patóxenos de peixes *S. parauberis, S. iniae, V. salmoninarum* e *L. garvieae*. Entre todos estes métodos, a EM-MALDI-TOF é a que ten maior poder discriminatorio e é o método máis rápido e menos custoso para a identificación destes patóxenos, así como para a realización de estudos epidemiolóxicos a gran escala. Ademais, a EM-MALDI-TOF e as técnicas baseadas en PCR demostraron unha alta prevalencia de resistencia á oxitetraciclina e ao florfenicol entre as cepas de *L. garvieae* illadas en España. As

técnicas de PCR e EM-MALDI-TOF combinadas coa detección fenotípica da resistencia ofrecen unha alternativa para os sistemas de vixilancia e a xestión rápida da enfermidade, permitindo o desenvolvemento de medidas terapéuticas eficaces contra *L. garvieae* durante un gromo da enfermidade na acuicultura. As técnicas baseadas en PCR en tempo real desenvolvidas neste traballo permitiron a identificación de *V. salmoninarum, S. iniae* e a identificación e tipificación de *S. parauberis* a partir de ADN extraído de cultivos puros, de suspensións bacterianas e de tecidos de peixes infectados. Os protocolos desenvolvidos demostraron ser 100% específicos e ter altos niveis de sensibilidade (de 0,034 a 2,67 x 10^2 copias de amplicón por µl). Todas estas metodoloxías poden utilizarse como ferramenta en estudos de vixilancia epidemiolóxica ou con fins de diagnóstico da estreptococose utilizando como mostras cultivos bacterianos e mostras de tecidos de peixes obtidas por procedementos letais e non letais. Por último, atopouse que as cepas *de A. salmonicida* subsp. *achromogenes* illadas de rodaballos enfermos mostraron pouca ou ningunha especificidade de hóspede, sendo capaces de reproducir os signos clínicos da furunculose atípica de forma similar á da infección natural tras a inoculación por vía intraperitoneal e subcutánea de rodaballos e linguados. Ademais, a vacina autóxena bivalente desenvolta conferiu altos niveis de protección a longo prazo contra a furunculose atípica e a vibriose tras a administración dunha soa dose no rodaballo, á conta de elevados efectos secundarios nos peixes. Isto demostra que son necesarios novos estudos que se centren no desenvolvemento de novas autovacinas e na avaliación doutros adxuvantes que melloren a protección con efectos secundarios reducidos ou nulos. Neste sentido, a parafina líquida Eolane 130 é un candidato prometedor que se podería considerar no desenvolvemento de futuras vacinas.

Resumen

En las últimas décadas, el aumento anual mundial del consumo de pescado ha duplicado el crecimiento demográfico, poniendo de manifiesto que el sector acuícola es uno de los sectores ganaderos con mayor tasa de crecimiento. En la actualidad, los alimentos provenientes del medio acuático representan el 17 % de la producción mundial de carne comestible. No aporta únicamente proteína animal, sino que además contienen micronutrientes biodisponibles y ácidos grasos esenciales que no se encuentran fácilmente en los alimentos terrestres. La acuicultura se ha convertido, por tanto, en un sector fundamental para contribuir a la seguridad alimentaria y nutricional a nivel mundial. En este escenario, se engendró recientemente la estrategia de la Granja a Mesa, que persigue políticas que proporcionen a los ciudadanos alimentos seguros, nutritivos y asequibles y apoyen a los agricultores, ganaderos, acuicultores y pescadores con la sostenibilidad de los alimentos a lo largo de toda su cadena de valor. Esta estrategia de la Granja a la Mesa deberá abarcar cada paso de la cadena de suministro de alimentos, desde la producción hasta el consumo, e impulsar los objetivos de la Economía Circular.

En este contexto, las enfermedades infecciosas en la acuicultura son una amenaza constante para la producción y, por consiguiente, para la seguridad alimentaria. Los brotes de enfermedades infecciosas causan grandes pérdidas económicas debido a la reducción de la productividad, la mala calidad de los productos acuáticos y los costes de los tratamientos. Además, las enfermedades en acuicultura suponen un grave riesgo para la salud humana por la existencia de agentes potencialmente zoonóticos y por el consumo de productos acuícolas infectados.

Las bacterias Gram-negativas son los principales microorganismos patógenos que afectan a los peces. Esto es porque muchos de estos microorganismos cohabitan en el hábitat natural de los organismos acuáticos sin causar enfermedades; sin embargo, hay condiciones que predisponen al desarrollo de la enfermedad, como el bajo nivel de oxígeno disuelto, los problemas de calidad del agua y el estrés en el manejo o los traumatismos. Entre ellas, resaltan las enfermedades causadas por *Aeromonas* sp y *Vibrio* sp, responsables de septicemias hemorrágicas, con enrojecimiento externo y hemorragias en el peritoneo, la pared corporal y las vísceras de los peces enfermos.

Las infecciones por cocos Gram-positivos o "estreptococosis" son también consideradas una de las principales enfermedades emergentes y reemergentes que afectan a los peces. Estas infecciones fueron introducidas en Europa a mediados de los años 80, y se consideran un complejo de enfermedades similares causadas por diferentes géneros y especies capaces de inducir daños en el sistema nervioso central caracterizados por exoftalmia y meningoencefalitis. En estas infecciones, la temperatura del agua se considera un factor predisponente para la aparición de la enfermedad y suelen estar producidas por *Vagococcus* sp., *Lactococcus* sp. y *Streptococcus* sp.

El éxito de la gestión de la salud de los organismos acuáticos comienza con la prevención de las enfermedades más que con el tratamiento. El objetivo principal es

aplicar el enfoque "Una Salud" para lograr una producción acuícola sostenible y segura. Los principios de "Una sola salud" pretenden involucrar a múltiples sectores y disciplinas para lograr resultados beneficiosos para la salud y el bienestar de las personas, los animales y el entorno que comparten. La observación diaria del comportamiento de los peces y el conocimiento integrado de las interacciones entre los patógenos, el huésped y el medio ambiente, permiten detectar problemas de forma temprana cuando se producen, de modo que se puede realizar un diagnóstico y seguimiento antes de que la mayor parte de la población enferma.

La correcta identificación y seguimiento del agente etiológico es uno de los principales factores para una buena gestión de la enfermedad. Históricamente, los diagnósticos de los peces afectados y las identificaciones de los agentes patógenos se realizaban mediante el aislamiento bacteriano y la caracterización fenotípica de los microorganismos aislados. Sin embargo, los diagnósticos basados en estos análisis microbiológicos tardan al menos un día y a menudo no proporcionan una identificación fiable del patógeno. Por ello, cada vez son más necesarias el uso de herramientas analíticas alternativas para la identificación rápida y precisa de las especies bacterianas que son difíciles de discriminar mediante las técnicas clásicas, así como para la discriminación entre cepas estrechamente relacionadas, con el fin de mejorar la gestión de la enfermedad.

El serotipado de los aislados bacterianos se realizaba con fines diagnósticos, epidemiológicos, y para la selección de cepas para el desarrollo de vacunas para prevenir las enfermedades en los peces. Esta técnica generalmente es más rápida, sensible y específica que los métodos de diagnóstico basados en el cultivo de los microorganismos. Sin embargo, el serotipado tiene una capacidad limitada para discriminar entre algunos aislados y, la necesidad de obtener antisueros de los animales constituye una limitación importante.

La identificación y tipificación de microorganismos basada en la amplificación del ADN bacteriano también es un método utilizado habitualmente en la investigación médica y biológica. Los métodos basados en la amplificación del ADN bacteriano proporcionan identificaciones específicas de las especies e información sobre el parentesco genético de las cepas, la fuente de infección y la virulencia de las cepas, así como la distribución geográfica y del huésped de las posibles variantes. Los métodos de tipado basados en patrones de bandas de ADN han demostrado previamente su eficacia en la caracterización de especies bacterianas Gram-positivas. Algunos de estos métodos son la electroforesis en gel de campo pulsado (PFGE), el ADN polimórfico amplificado al azar (RAPD), el ribotipado y la amplificación de secuencias de ADN repetitivas de función desconocida, entre las que se incluyen las secuencias repetitivas palindrómicas extragénicas (secuencias REP) y las secuencias consenso repetitivas intragénicas de enterobacterias (secuencias ERIC), siendo las secuencias REP las más utilizada en el estudio de brotes.

En los últimos años, la espectrometría de masas de desorción/ionización asistida por una matriz con detección de masas por tiempo de vuelo (EM-MALDI-TOF) ha

surgido como un enfoque prometedor para el diagnóstico, la discriminación entre cepas ambientales estrechamente relacionadas o la identificación de microorganismos raros o menos frecuentes que son difíciles de discriminar con las técnicas clásicas. En comparación con otros métodos convencionales o moleculares, la EM-MALDI-TOF es un método de diagnóstico sencillo y de bajo coste, el cual permite obtener resultados en unos pocos minutos tras el cultivo del microorganismo. Además, se trata de un método de diagnóstico no dirigido, de alto rendimiento y que tiene facilidad de automatización. En la EM-MALDI-TOF se genera un espectro de masas del microorganismo, el cual es traducido electrónicamente a una lista de picos expresados como una relación entre su masa/carga y la abundancia relativa de la muestra, y es diferente para cada microorganismo. Este patrón único producido por las proteínas y otras biomoléculas de la muestra analizada se cotejará con el espectro de un microorganismo conocido previamente depositado en una base de datos. Teniendo en cuenta que cada espectro contiene picos de masa únicos para cada género, especie e incluso cepa, este espectro funcionará como una huella dactilar que puede ser cotejada para identificar un microorganismo. Sin embargo, EM-MALDI-TOF también ha demostrado ser valiosa para la caracterización de microorganismos para los que no existen espectros de masas de referencia a través del uso de análisis multivariantes (análisis clúster o análisis de componentes principales) de los datos de masas proteómicas.

Tras la correcta identificación y caracterización de los patógenos presentes en las instalaciones acuícolas, es necesaria una adecuada gestión del brote infeccioso. Para controlar los brotes infecciosos se han utilizado de forma frecuente productos químicos y medicamentos. Sin embargo, estos métodos sirven para ganar tiempo para los peces y permitirles superar las infecciones oportunistas, pero no sustituyen a una cría y seguimiento adecuado. Además, el uso de estos medicamentos tiene repercusiones negativas sobre el medio ambiente y la salud humana; como son la propagación de bacterias resistentes a antimicrobianos y la transferencia de genes de resistencia a patógenos humanos y de animales. En este contexto, las vacunas son consideradas un nuevo enfoque ecológico para prevenir y controlar las enfermedades de los peces, por lo que en algunas zonas del mundo se ha producido una transición del uso de fármacos al uso de vacunas.

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 *Streptococcus*, *Lactococcus*, *Vagococcus*, *Aeromonas* y *Vibrio*. Los objetivos específicos establecidos en la presente tesis fueron:

1. Evaluación de las técnicas moleculares, serológicas y proteómicas para la identificación, tipificación y seguimiento de las bacterias patógenas de los peces.

2. Análisis de perfiles fenotípicos, genotípicos y proteómicos para determinar la susceptibilidad antimicrobiana y la resistencia a múltiples fármacos de las bacterias patógenas de los peces.

3. Desarrollo de métodos moleculares para la identificación y detección cuantitativa de las bacterias patógenas de los peces y su diferenciación de especies estrechamente relacionadas.

4. Estudio de la eficacia y seguridad de nuevas vacunas para la prevención de enfermedades bacterianas en peces.

En el **Capítulo 3**, se describe el potencial de diferentes métodos de diagnóstico para la rápida diferenciación y tipificación de cepas bacterianas aisladas de peces con signos de estreptococosis. Se tipificaron y caracterizaron cepas patógenas de peces pertenecientes a los géneros *Streptococcus*, *Vagococcus* y *Lactococcus* mediante métodos convencionales (métodos bioquímicos y sistemas API), serológicos (aglutinación en portaobjetos y ensayo dot-blot), moleculares (REP y ERIC-PCR), antigénicos (inmunoblot), proteómicos (EM-MALDI-TOF) y quimiotaxonómicos (análisis de ésteres metílicos de ácidos grasos). Además, la potencial virulencia de las cepas bacterianas se evaluó mediate infección experimental de peces.

La identificación y tipificación de cepas de *Streptococcus parauberis* y *Streptococcus iniae* aisladas de peces (**artículo nº 1**) fue realizada de forma comparativa mediante métodos serológicos (aglutinación en portaobjetos y ensayo dot-blot) y moleculares (REP-PCR) y proteómicos (EM-MALDI-TOF). Este estudio demostró que todas las técnicas testadas son útiles como herramientas de diagnóstico, ya que son capaces de diferenciar las cepas de *Streptococcus* según su posición taxonómica. Además, se demostró variabilidad antigénica, proteómica y genética dentro de la especie *S. parauberis*. Por otro lado, las cepas de *S. iniae* mostraron ser antigénica, proteómica y genéticamente homogéneas. Los resultados proteómicos y serológicos permitieron confirmar la existencia de nuevos potenciales serotipos en cepas de *S. parauberis* aisladas de rodaballo en España (definidas como serotipo III) y cepas aisladas de muestras de leche de un animal con mastitis (definida como serotipo IV). Asimismo, se encontraron biomarcadores proteicos específicos de género y/o especie para las cepas de *Streptococcus* analizadas mediante MALDI-TOF-MS. También se observaron perfiles de ADN y patrones proteicos específicos de serotipo y subserotipo para las cepas de *S. parauberis.* Se encontró una buena correlación entre los métodos serológicos, proteómicos y moleculares testados en el **artículo nº 1** para la diferenciación y tipificación de especies de *Streptococcus*.

Para la caracterización de *Vagococcus salmoninarum* (**artículo nº 2**) se utilizaron métodos fenotípicos (métodos bioquímicos, sistemas API y análisis de resistencia a antimicrobianos), serológicos (aglutinación en portaobjetos y ensayos dot-blot), antigénicos (inmunoblot), genéticos (RAPD, ERIC y REP-PCR) y proteómicos (EM-MALDI-TOF). Los resultados demostraron que las cepas de *V. salmoninarum* fueron resistentes a la mayoría de los antimicrobianos probados y sólo el 10% de las cepas fueron sensibles al florfenicol. Los análisis de aglutinación en portaobjetos y ensayos dot-blot pusieron de manifiesto la existencia de una gran homogeneidad antigénica dentro de la especie *V. salmoninarum*, y que no existen reacciones cruzadas con otras especies patógenas de peces causantes de estreptococosis como son *S. parauberis, S.*

iniae, S. agalactiae, L. garvieae o *Carnobacterium maltaromaticum*. Estos resultados fueron apoyados por el análisis electroforético de las proteínas de la superficie celular y por inmunoblot. Además, el análisis genotípico y proteómico también mostró una limitada variabilidad dentro de la especie *V. salmoninarum*, permitiendo establecer patrones y picos de masa específicos de esta especie. Estos resultados indican que los métodos fenotípicos, genómicos y proteómicos permiten diferenciar rápidamente a la especie *V. salmoninarum* de las demás especies causantes de estreptococosis. Sin embargo, la EM-MALDI-TOF permite una tipificación y caracterización rápida y fácil de *V. salmoninarum* sin necesidad de una base de datos específica.

En el **artículo nº 3** se describe la caracterización fenotípica (ensayos bioquímicos y API), molecular (RAPD y REP-PCR), antigénica (Inmunoblot), proteómica (EM-MALDI-TOF), quimiotaxonómica (análisis de ésteres metílicos de ácidos grasos) y de virulencia de aislados de *L. garvieae*. En este estudio se analizaron de forma comparativa cepas de *L. garvieae* aisladas a partir del año 2015 de truchas arco iris cultivadas, que habían sido inmunizadas con la vacuna Ictiovac LG (Hipra, España) o con la autovacuna combinada contra *L. garvieae* y *Yersinia ruckeri* (Acuipharma Aquaculture Health solutions, España), con cepas de *L. garvieae* aisladas de trucha arco iris cultivadas en diferentes granjas de España durante el periodo 2002 a 2014. Este estudio permitió detectar cierta heterogeneidad fenotípica dentro de las cepas de *L. garvieae*. Esta heterogeneidad fue confirmada mediante técnicas de genotipado y proteómicas, las cuales permitieron separar las cepas de *L. garvieae* aisladas de humanos y bovinos de las cepas de *L. garvieae* aisladas del medio acuático. Por otra parte, los métodos de tipificación por PCR convencional mostraron que ninguna de los aislados de *L. garvieae* analizados pertenecía al serotipo II y no portaban el clúster de genes de la cápsula descrito en el genoma o en plásmidos de cepas japonesas de *L. garvieae*. En el **artículo nº3**, se identificaron diferentes patrones clonales en *L. garvieae* mediante RAPD y REP-PCR (siete perfiles) y MALDI-TOF (seis patrones), los cuales estaban estrechamente relacionados con la fuente de aislamiento de las cepas de *L. garvieae*, pero no con la virulencia de estas. Además, se detectaron dos variantes antigénicas de la proteína (W1 y W2), que difieren en reactividad en el rango de 35 a 70 KDa, utilizando antisueros de conejo y de trucha arco iris. Ambos antisueros detectaron dos bandas proteicas altamente inmunogénicas de 30 y 45 KDa. Estos resultados podrían contribuir a una mejor comprensión de la patogénesis de la lactococosis y ayudar a la selección de antígenos para el desarrollo de vacunas eficaces.

En el **artículo nº 4** se realizó un estudio sobre la susceptibilidad a los antimicrobianos y la resistencia a múltiples fármacos (MDR) de *L. garvieae* aisladas de trucha arco iris, y se determinó la correlación entre el fenotipo de resistencia, el genotipo y el perfil proteómico. Los resultados del ensayo de susceptibilidad antimicrobiana demostraron la resistencia de los aislados al trimetoprima-sulfametoxazol y a la flumequina (100% de resistencia), a la enrofloxacina (67%), a la oxitetraciclina (44%), a la amoxicilina (23%), al cloranfenicol (18%) y al florfenicol (13%). La detección mediante PCR identificó varios genes de resistencia a los antimicrobianos (ARG), siendo el tet(B) el de mayor prevalencia (85%), seguido del

FloR (78%), el tet (A) (61%), el tet(S) (13%) y el Tet(K) (9%). Los genes tet(B) y FloR se detectaron en el 100% de los aislados de *L. garvieae* resistentes a la oxitetraciclina y al florfenicol. Además, el 83% de las *L. garvieae* analizadas eran resistentes a múltiples antimicrobianos, siendo la combinación más relevante tetA+tetB (56% de las cepas analizadas), tetA+FloR y tetB+ FloR (51%) y teA+tetB+FloR (48%). En este mismo **artículo nº 4** se evaluó también el potencial de la MALDI-TOF-MS para la identificación de potenciales biomarcadores proteicos de resistencia a antimicrobianos. Este estudio identificó cinco picos proteicos como potenciales marcadores de resistencia a la oxitetraciclina y ocho picos proteicos como potenciales marcadores de resistencia al florfenicol presentes en los aislados de *L. garvieae*. Este estudio proporciona una visión sobre la presencia de resistencia a antimicrobianos en cepas de *L. garvieae* aisladas en España y constituye un punto de partida para el establecimiento de sistemas de vigilancia de la resistencia a los antimicrobianos en la acuicultura.

En el **Capítulo 4**, se desarrollaron técnicas de diagnóstico basadas en PCR específicas para la identificación y/o detección de los patógenos *V. salmoninarum*, *S. iniae* y *S. parauberis* en los tejidos de peces enfermos y/o en agua.

Para *V. salmoninarum* (**artículo nº 5**) se diseñó un par de cebadores y un protocolo basado en PCR en tiempo real basado en el uso del fluoróforo SYBR Green I, combinado con el análisis de la curva de melting, para la identificación y cuantificación de *V. salmoninarum* en cultivos bacterianos y tejidos de peces infectados. Para ello, se seleccionó el gen 16S rRNA para diseñar un par de cebadores denominados SalF y SalR. La sensibilidad y especificidad de este par de cebadores se comparó con otros diseñados previamente para la PCR convencional. Aunque ambos pares de cebadores mostraron una especificidad del 100% utilizando cultivos bacterianos puros o ADN extraído de bacterias o tejidos de peces (Tm = 84,0 ± 0,5 ºC), el par de cebadores SalF y SalR (diseñados en el **artículo nº 5**) mostraron la mayor sensibilidad con un límite de detección de 0,034 x 10^0 copias de amplicón por ensayo (equivalente a 2 x 10^{-11} ng μl^{-1}, valor Cq de 30,49 ± 1,71). El protocolo de qPCR desarrollado permitió la detección de *V. salmoninarum* en muestras de peces no letales y letales con niveles de detección de 0,17 x 10^0 copias del gen en tejidos infectados artificialmente y 0,02 x 10^0 en tejidos de peces infectados experimentalmente con *V. salmoninarum*. Además, no se detectaron amplificaciones inespecíficas cuando se analizaron tejidos de peces sanos o de peces infectados con otros patógenos.

Para la identificación específica de *S. iniae* (**artículo nº 6**) se desarrolló y evaluó un protocolo de PCR cuantitativa basado en un par de cebadores que amplifican un fragmento de 167 pb del gen que codifica la permeasa de lactato (*lldY*). El gen *lldY* fue seleccionado basándose en un análisis genómico comparativo que utilizó 45 secuencias recuperadas de la base de datos del genoma del NCBI. El protocolo mostró unos niveles de especificidad del 100% con cultivos bacterianos puros o con ADN extraído *de S. iniae.* La temperatura de melting del producto de amplificación fue de 77,00 ± 0,55 ºC. La sensibilidad de detección del protocolo fue de 1,12 x 10^1 copias de producto amplificado por ensayo (equivalente a 2 x 10^{-9} ng/µl) utilizando ADN bacteriano y de

1,44 x 10^1 copias de genes en tejidos de peces infectados con *S. iniae*. El protocolo de qPCR proporciona una alternativa precisa y sensible para la identificación de *S. iniae* y su detección en tejidos de peces que puede implementarse como herramienta de rutina en los laboratorios microbiológicos.

En el **artículo nº 7** se describe la estructura prevista para los loci cps implicados en la biosíntesis de la cápsula para los serotipos III, IV y V de *S. parauberis*. Basándose en las regiones específicas de los serotipos I, II y III de los loci cps, se diseñó un sistema de PCR múltiple para diferenciar los principales serotipos de *S. parauberis* causantes de enfermedad en los peces. Además, se describe un método de PCR en tiempo real y un par de cebadores (cps3K-F y cps3K-R) para identificar cepas de *S. parauberis* pertenecientes al serotipo III en cultivos bacterianos y tejidos de peces, sin la necesidad de sueros obtenidos de animales. Los análisis *in silico* e *in vitro* revelaron que ambos métodos tienen una especificidad del 100%. Por un lado, se optimizó el ensayo de PCR múltiple para la detección de cepas de *S. parauberis* de los subtipos Ia (tamaño del amplicón 213-bp), los subtipos Ib e Ic (ambos con tamaño del amplicón 303-bp), el serotipo II (tamaño del amplicón 403-bp) y el serotipo III (tamaño del amplicón 130-bp) a partir de cultivos bacterianos. Por otro lado, se optimizó el ensayo de PCR en tiempo real para la identificación y cuantificación de las cepas del serotipo III de *S. parauberis* en cultivos bacterianos y tejidos de peces (Tm = 73,00 ± 0.33 ºC). Este ensayo alcanzó una sensibilidad de 2,67 x 10^2 copias del gen (equivalente a 3,8 x 10^{-9} ng/µl) utilizando cultivos bacterianos puros de *S. parauberis* del serotipo III y 1,76 x 10^2 copias del gen en tejidos de peces infectados experimental y naturalmente con *S. parauberis* del serotipo III. La especificidad y sensibilidad de los protocolos descritos en este estudio sugieren que estos métodos podrían utilizarse con fines diagnósticos y/o epidemiológicos en los laboratorios de diagnóstico clínico.

En el **Capítulo 5** se evaluó la eficacia y seguridad de vacunas bivalentes para la prevención de la vibriosis, causada por *Vibrio anguillarum*, y de la forunculosis atípica, causada por *Aeromonas salmonicida* subsp. *achromogenes*. Para ello, en primer lugar, se evaluaron cuatro rutas de infección para *A. salmonicida* subsp. *achromogenes* (inyección intraperitoneal, inyección subcutánea, inmersión e inoculación intragástrica) en rodaballo y lenguado (**artículo nº 8**), con el fin de establecer un modelo de infección adecuado para el estudio de la patogénesis de la forunculosis atípica y para la evaluación de la eficacia de métodos de prevención y control de la enfermedad. En este estudio se utilizaron cepas de *A. salmonicida* subsp. *achromogenes* aisladas a partir de rodaballos afectados de forunculosis atípica. De todas las vías probadas, la inyección intraperitoneal (i.p.), subcutánea y por inmersión causaron mortalidad y reprodujeron los signos clínicos de la forunculosis atípica en los peces infectados experimentalmente. Estos resultados sugieren que las cepas de *A. salmonicida* subsp. *achromogenes* utilizadas no muestran especificidad de hospedador, siendo capaces de infectar tanto a los rodaballos como a los lenguados en condiciones experimentales alcanzando un 80-100% de mortalidad tras la inoculación intraperitoneal y subcutánea. Se confirmó que *A. salmonicida* subsp. *achromogenes* era el agente causante de las mortalidades mediante el cultivo bacteriano y mediante análisis de qPCR de muestras de peces moribundos o

muertos. Sin embargo, se necesitan más estudios para identificar la ruta de entrada y la diseminación de *A. salmonicida* subsp. *achromogenes* en los peces, lo que ayudará a mejorar el tratamiento y las estrategias profilácticas.

Finalmente, en el **artículo nº 9** se desarrolló una vacuna autógena bivalente frente a *V. anguillarum* y *A. salmonicida* subsp. *achromogenes* a la que se incorporó como adyuvante el compuesto oleoso Montanide™. La eficacia de esta vacuna autógena y de la vacuna comercial AlphaJect 3000 (Pharmaq AS), administradas mediante inyección intraperitoneal, para prevenir la forunculosis atípica y la vibriosis en el rodaballo, se evaluó de forma comparativa. Se evaluó en paralelo el efecto de la inyección. del adyuvante de aceite mineral MontanideTM (ISA 763A VG; Seppic, Francia) y de la parafina líquida Eolane 130 (Total Fluids, España). El efecto de ambas vacunas y de los adyuvantes se comprobó analizando los parámetros sanitarios y la supervivencia de los peces tras el reto infeccioso con *V. anguillarum* y *A. salmonicida* subsp. *achromogenes*. Los resultados del estudio mostraron que la vacuna autógena confirió altos niveles de protección e inmunidad duradera contra ambos patógenos con una sola dosis. Sin embargo, los rodaballos inyectados con esta autovacuna mostraron efectos secundarios graves, mientras que se observaron menores efectos negativos cuando los rodaballos eran inyectados con la vacuna AlphaJect 3000 o con los adyuvantes Montanide o Eolane. Todos los rodaballos vacunados mostraron títulos de aglutinación de anticuerpos notables, superiores a los de los peces de control, que se mantuvieron 160 días después de la vacunación. Este estudio ha demostrado que la vacuna bivalente autógena induce una protección duradera contra la forunculosis atípica y la vibriosis en el rodaballo, tras la administración de una sola dosis, a costa de elevados efectos secundarios en los peces. Por consiguiente, el desarrollo de nuevas vacunas debería centrarse en las autovacunas y en el uso de adyuvantes de parafina líquida que aumenten la protección con efectos secundarios reducidos o nulos.

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 de la tipificación y seguimiento de patógenos bacterianos, el diagnóstico y el control de las enfermedades producidas por los patógenos bacterianos S. *parauberis, S. iniae, V. salmoninarum, L. garvieae, A. salmonicida* subsp. *achromogenes* y *V. anguillarum*. Los resultados obtenidos mostraron que los métodos serológicos, genotípicos y proteómicos son herramientas útiles para un diagnóstico y un seguimiento rápido y fiable de los patógenos de peces *S. parauberis, S. iniae, V. salmoninarum* y *L. garvieae*. Entre todos estos métodos, el EM-MALDI-TOF es el que tiene mayor poder discriminatorio y es el más rápido y menos costoso para la identificación de estos patógenos y para la realización de estudios epidemiológicos a gran escala. Además, la EM-MALDI-TOF y las técnicas basadas en PCR demostraron una alta prevalencia de resistencia a la

oxitetraciclina y al florfenicol entre las cepas de *L. garvieae* aisladas en España. Las técnicas de PCR y EM-MALDI-TOF combinadas con la detección fenotípica de la resistencia ofrecen una alternativa para los sistemas de vigilancia y la gestión rápida de la enfermedad, permitiendo el desarrollo de medidas terapéuticas eficaces contra *L. garvieae* durante un brote de la enfermedad en la acuicultura. Las técnicas basadas en PCR en tiempo real desarrolladas en este trabajo permitieron la identificación de *V. salmoninarum*, *S. iniae* y la identificación y tipificación de *S. parauberis* a partir de ADN extraído de cultivos puros, de suspensiones bacterianas y de tejidos de peces infectados. Los protocolos desarrollados demostraron ser 100% específicos y tener altos niveles de sensibilidad (de 0,034 a 2,67 x 10^2 copias de amplicón por µl). Todas estas metodologías pueden utilizarse como herramienta en estudios de vigilancia epidemiológica o con fines de diagnóstico de la estreptococosis utilizando como muestras cultivos bacterianos y muestras de tejidos de peces obtenidas por procedimientos letales y no letales. Por último, se encontró que las cepas de *A. salmonicida* subsp. *achromogenes* aisladas de rodaballos enfermos mostraron poca o ninguna especificidad de hospedador, siendo capaces de reproducir los signos clínicos de la forunculosis atípica de forma similar a la de la infección natural tras la inoculación por vía intraperitoneal y subcutánea de rodaballos y lenguados. Además, la vacuna autógena bivalente desarrollada confirió altos niveles de protección a largo plazo contra la forunculosis atípica y la vibriosis tras la administración de una sola dosis en el rodaballo, a costa de elevados efectos secundarios en los peces. Esto demuestra que son necesarios nuevos estudios que se centren en el desarrollo de nuevas autovacunas y en la evaluación de otros adyuvantes que mejoren la protección con efectos secundarios reducidos o nulos. En este sentido, la parafina líquida Eolane 130 es un candidato prometedor que podría considerarse en el desarrollo de futuras vacunas.

Abstract

In recent decades, the annual global increase in fish consumption has doubled population growth, making the aquaculture sector one of the fastest growing livestock sectors. Today, aquatic feed accounts for 17% of the world's edible meat production. It not only provides animal protein, but also contains bioavailable micronutrients and essential fatty acids that are not readily available in terrestrial feeds. Aquaculture has therefore become a key sector for contributing to global food and nutrition security. In this scenario, the Farm to Fork strategy was recently engendered, which pursues policies that provide citizens with safe, nutritious, and affordable food and support farmers, livestock keepers, aqua-culturists, and fishermen with food sustainability along their entire value chain. This Farm to Fork strategy should cover every step of the food supply chain, from production to consumption, and drive the objectives of the Circular Economy.

In this context, infectious diseases in aquaculture are a constant threat to production and therefore to food safety. Infectious disease outbreaks cause large economic losses due to reduced productivity, poor quality of aquatic products and treatment costs. In addition, diseases in aquaculture pose a serious risk to human health due to the existence of potentially zoonotic agents and the consumption of infected aquaculture products.

Gram-negative bacteria are the main pathogenic microorganisms affecting fish. This is because many of these microorganisms cohabit in the natural habitat of aquatic organisms without causing disease; however, there are conditions that predispose to the development of disease, such as low dissolved oxygen, water quality problems and stress in handling or trauma. These include diseases caused by *Aeromonas* sp. and *Vibrio* sp., which are responsible for hemorrhagic septicemias, with external reddening and hemorrhages in the peritoneum, body wall and viscera of diseased fish.

Gram-positive cocci infections or "streptococcosis" are also considered one of the major emerging and re-emerging diseases affecting fish. These infections were introduced in Europe in the mid-1980s and are considered a complex of similar diseases caused by different genera and species capable of inducing central nervous system damage characterized by exophthalmia and meningoencephalitis. In these infections, water temperature is considered a predisposing factor for disease onset and are usually caused by *Vagococcus* sp., *Lactococcus* sp. and *Streptococcus* sp.

Successful management of the health of aquatic organisms begins with disease prevention rather than treatment. The main objective is to apply the "One Health" approach to achieve sustainable and safe aquaculture production. The One Health principles aim to involve multiple sectors and disciplines to achieve beneficial outcomes for the health and welfare of people, animals, and the environment. Daily observation of fish behavior and integrated knowledge of the interactions between pathogens, host and environment allow problems to be detected early when they occur, so that diagnosis and monitoring can be carried out before most of the population becomes ill.

Correct identification and monitoring of the etiological agent is one of the main factors for good disease management. Historically, diagnoses of affected fish and identifications of pathogens were made by bacterial isolation and phenotypic characterization of the isolated microorganisms. However, diagnoses based on these microbiological analyses take at least one day and often do not provide a reliable identification of the pathogen. Therefore, the use of alternative analytical tools for rapid and accurate identification of bacterial species, that are difficult to discriminate by classical techniques, as well as for discrimination between closely related strains, is increasingly necessary to improve disease management.

Serotyping of bacterial isolates was performed for diagnostic and epidemiological purposes, and for the selection of strains for the development of vaccines to prevent fish diseases. This technique is generally faster, more sensitive, and more specific than diagnostic methods based on culture of the microorganisms. However, serotyping has a limited ability to discriminate between some isolates and the need to obtain antisera from animals is a major limitation.

Identification and typing of microorganisms based on amplification of bacterial DNA is also a commonly used method in medical and biological research. Methods based on bacterial DNA amplification provide species-specific identifications and information on genetic relatedness of strains, source of infection and virulence of strains, as well as geographic and host distribution of possible variants. Typing methods based on DNA banding patterns have previously proven effective in the characterization of Gram-positive bacterial species. Some of these methods are pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), ribotyping and amplification of repetitive DNA sequences of unknown function, including extragenic palindromic repetitive sequences (REP sequences) and enterobacterial intragenic repetitive consensus sequences (ERIC sequences), with REP sequences being the most used in the study of outbreaks.

In recent years, matrix-assisted desorption/ionization mass spectrometry with time-of-flight mass detection (MALDI-TOF-MS) has emerged as a promising approach for diagnosis, discrimination between closely related environmental strains or identification of rare or less frequent microorganisms that are difficult to discriminate with classical techniques. Compared to other conventional or molecular methods, MALDI-TOF-MS is a simple and low-cost diagnostic method, which allows results to be obtained within a few minutes after culture of the microorganism. Moreover, it is a non-targeted, high-throughput diagnostic method that is easy to automate. In MALDI-TOF, a mass spectrum of the microorganism is generated, which is electronically translated into a list of peaks expressed as a ratio between their mass/charge and the relative abundance of the sample and is different for each micro-organism. This unique pattern produced by the proteins and other biomolecules in the analyzed sample shall be compared with the spectrum of a known microorganism previously deposited in a database. Since each spectrum contains mass peaks unique to each genus, species and even strain, this spectrum will function as a fingerprint that can be matched to identify a microorganism.

However, MALDI-TOF-MS has also proven valuable for the characterization of microorganisms for which no reference mass spectra exist using multivariate analysis (cluster analysis or principal component analysis) of the proteomic mass data.

After correct identification and characterization of the pathogens present in aquaculture facilities, proper management of the infectious outbreak is necessary. Chemicals and drugs have often been used to control infectious outbreaks. However, these methods serve to buy time for the fish and allow them to overcome opportunistic infections but are no substitute for proper husbandry and monitoring. Furthermore, the use of these drugs has negative repercussions on the environment and human health, such as the spread of antimicrobial resistant bacteria and the transfer of resistance genes to human and animal pathogens. In this context, vaccines are seen as a new environmentally friendly approach to prevent and control fish diseases, and in some areas of the world there has been a transition from the use of drugs to the use of vaccines.

In view of the above, this PhD thesis has focused on solving some of the main problems related to diseases caused by bacterial pathogens of the genera *Streptococcus*, *Lactococcus*, *Vagococcus*, *Aeromonas* and *Vibrio*. The specific objectives established in this thesis were:

1. Evaluation of molecular, serological, and proteomic techniques for the identification, typing and monitoring of fish pathogenic bacteria.

2. Phenotypic, genotypic, and proteomic profiling analysis to determine antimicrobial susceptibility and multidrug resistance of fish pathogenic bacteria.

3. Development of molecular methods for the identification and quantitative detection of fish pathogenic bacteria and their differentiation from closely related species.

4. Study of the efficacy and safety of new vaccines for the prevention of bacterial diseases in fish.

In **Chapter 3**, the potential of different diagnostic methods for rapid differentiation and typing of bacterial strains isolated from fish showing signs of streptococcosis is described. Pathogenic fish strains belonging to the genera *Streptococcus*, *Vagococcus* and *Lactococcus* were typed and characterized by conventional (biochemical methods and API systems), serological (slide agglutination and dot-blot assay), molecular (REP and ERIC-PCR), antigenic (immunoblot), proteomic (MALDI-TOF-MS) and chemotaxonomic (fatty acid methyl ester analysis) methods. In addition, the virulence potential of the bacterial strains was assessed by experimental infection of fish.

Identification and typing of *Streptococcus parauberis* and *Streptococcus iniae* strains isolated from fish (**article nº 1**) was carried out comparatively by serological (slide agglutination and dot-blot assay) and molecular (REP-PCR) and proteomic (MALDI-TOF-MS) methods. This study demonstrated that all tested techniques are useful as diagnostic tools, as they can differentiate *Streptococcus* strains according to

their taxonomic position. Furthermore, antigenic, proteomic, and genetic variability within the *S. parauberis* species was demonstrated. On the other hand, *S. iniae* strains were shown to be proteomic, antigenically, and genetically homogeneous. Proteomic and serological results confirmed the existence of new potential serotypes in *S. parauberis* strains isolated from turbot in Spain (defined as serotype III) and strains isolated from milk samples of an animal with mastitis (defined as serotype IV). Furthermore, genus and/or species-specific protein biomarkers were found for the *Streptococcus* strains analyzed by MALDI-TOF-MS. Serotype- and subserotype-specific DNA profiles and protein patterns were also observed for *S. parauberis* strains. A good correlation was found between the serological, proteomic, and molecular methods tested in **article nº 1** for differentiation and typing of *Streptococcus* species.

Phenotypic (biochemical methods, API systems and antimicrobial resistance analysis), serological (slide agglutination and dot-blot assays), antigenic (immunoblot), genetic (RAPD, ERIC, and REP-PCR) and proteomic (MALDI-TOF-MS) methods were used for the characterization of *Vagococcus salmoninarum* (**article nº 2**). The results showed that *V. salmoninarum* strains were resistant to most of the antimicrobials tested and only 10% of the strains were sensitive to florfenicol. Slide agglutination and dot-blot analyses showed a high antigenic homogeneity within the *V. salmoninarum* species and no cross-reactivity with other pathogenic fish species causing streptococcosis such as *S. parauberis*, *S. iniae*, *S. agalactiae*, *L. garvieae* or *Carnobacterium maltaromaticum*. These results were supported by electrophoretic analysis of cell surface proteins and by immunoblot. In addition, genotypic and proteomic analysis also showed limited variability within the *V. salmoninarum* species, allowing the establishment of species-specific patterns and mass peaks. These results indicate that phenotypic, genomic, and proteomic methods allow rapid differentiation of *V. salmoninarum* species from other streptococcosis-causing species. However, MALDI-TOF-MS allows rapid and easy typing and characterization of *V. salmoninarum* without the need for a specific database.

Article nº 3 describes the phenotypic (biochemical and API assays), molecular (RAPD and REP-PCR), antigenic (immunoblot), proteomic (MALDI-TOF-MS), chemotaxonomic (fatty acid methyl ester analysis) and virulence characterization of *L. garvieae* isolates. In this study, *L. garvieae* isolates isolated from 2015 onwards from farmed rainbow trout, which had been immunized with the Ictiovac LG vaccine (Hipra, Spain) or with the combined autovaccine against *L. garvieae* and *Yersinia ruckeri* (Acuipharma Aquaculture Health solutions, Spain), were comparatively analyzed with *L. garvieae* isolates from rainbow trout farmed in different farms in Spain during the period 2002 to 2014. This study allowed detecting some phenotypic heterogeneity within *L. garvieae* strains. This heterogeneity was confirmed by genotyping and proteomic techniques, which allowed separating *L. garvieae* strains isolated from humans and cattle from *L. garvieae* strains isolated from the aquatic environment. Moreover, conventional PCR typing methods showed that none of the *L. garvieae* isolates tested belonged to serotype II and did not carry the capsule gene cluster described in the genome or plasmids of Japanese *L. garvieae* strains. In **article nº 3**,

different clonal patterns in *L. garvieae* were identified by RAPD and REP-PCR (seven profiles) and MALDI-TOF-MS (six patterns), which were closely related to the source of isolation of the *L. garvieae* strains, but not to the virulence of the *L. garvieae* strains. In addition, two antigenic variants of the protein (W1 and W2), differing in reactivity in the range of 35 to 70 KDa, were detected using rabbit and rainbow trout antisera. Both antisera detected two highly immunogenic protein bands of 30 and 45 KDa. These results could contribute to a better understanding of the pathogenesis of lactococcosis and help in the selection of antigens for the development of effective vaccines.

A study on antimicrobial susceptibility and multidrug resistance (MDR) of *L. garvieae* isolated from rainbow trout was conducted in **article nº 4**, and the correlation between resistance phenotype, genotype and proteomic profile was determined. Antimicrobial susceptibility test results demonstrated resistance of isolates to trimethoprim-sulfamethoxazole and flumequine (100% resistance), enrofloxacin (67%), oxytetracycline (44%), amoxicillin (23%), chloramphenicol (18%) and florfenicol (13%). PCR screening identified several antimicrobial resistance genes (ARGs), with tet(B) being the most prevalent (85%), followed by FloR (78%), tet(A) (61%), tet(S) (13%) and tet(K) (9%). The tet(B) and FloR genes were detected in 100% of *L. garvieae* isolates resistant to oxytetracycline and florfenicol. Furthermore, 83% of the *L. garvieae* tested were resistant to multiple antimicrobials, the most relevant combination being tetA+tetB (56% of the strains tested), tetA+FloR and tetB+ FloR (51%) and teA+tetB+FloR (48%). The potential of MALDI-TOF-MS for the identification of potential protein biomarkers of antimicrobial resistance was also evaluated in the same **article nº 4**. This study identified five protein peaks as potential oxytetracycline resistance markers and eight protein peaks as potential florfenicol resistance biomarkers present in *L. garvieae* isolates. This study provides insight into the occurrence of antimicrobial resistance in *L. garvieae* isolates from Spain and constitutes a starting point for the establishment of antimicrobial resistance surveillance systems in aquaculture.

In **Chapter 4**, specific PCR-based diagnostic techniques were developed for the identification and/or detection of the pathogens *V. salmoninarum*, *S. iniae* and *S. parauberis* in diseased fish tissues and/or water.

For *V. salmoninarum* (**article nº 5**), a primer pair and a real-time PCR-based protocol based on the use of the SYBR Green I fluorophore, combined with melting curve analysis, were designed for the identification and quantification of *V. salm*oninarum in bacterial cultures and infected fish tissues. For this purpose, the 16S rRNA gene was selected to design a primer pair called SalF and SalR. The sensitivity and specificity of this primer pair was compared with previously designed primers for conventional PCR. Although both primer pairs showed 100% specificity using pure bacterial cultures or DNA extracted from bacteria or fish tissues (Tm = 84.0 ± 0.5 ºC), the SalF and SalR primer pair (designed in **article nº 5**) showed the highest sensitivity with a detection limit of 0.034 x 10^0 amplicon copies per assay (equivalent to 2 x 10^{-11} ng µl-1, Cq value of 30.49 ± 1.71). The developed qPCR protocol allowed the detection

of *V. salmoninarum* in non-lethal and lethal fish samples with detection levels of 0.17 x 10^0 copies of the gene in artificially infected tissues and 0.02 x 100 in tissues from fish experimentally infected with *V. salmoninarum*. In addition, no non-specific amplifications were detected when tissues from healthy fish or fish infected with other pathogens were analyzed.

For the specific identification of *S. iniae* (**article nº 6**), a quantitative PCR protocol based on a primer pair amplifying a 167 bp fragment of the gene encoding lactate permease (*lldY*) was developed and evaluated. The *lldY* gene was selected based on a comparative genomic analysis using 45 sequences retrieved from the NCBI genome database. The protocol showed 100% specificity levels with pure bacterial cultures or with DNA extracted from *S. iniae*. The melting temperature of the amplification product was 77.00 ± 0.55 ºC. The detection sensitivity of the protocol was 1.12 x 10^1 copies of amplified product per assay (equivalent to 2 x 10^{-9} ng/µl) using bacterial DNA and 1.44 x 10^1 gene copies in fish tissues infected with *S. iniae*. The qPCR protocol provides an accurate and sensitive alternative for *S. iniae* identification and detection in fish tissues that can be implemented as a routine tool in microbiological laboratories.

The predicted structure of the cps loci involved in capsule biosynthesis for serotypes III, IV and V of *S. parauberis* is described in **article nº 7**. Based on the serotype-specific regions I, II and III of the cps loci, a multiplex PCR system was designed to differentiate the main disease-causing serotypes of *S. parauberis* in fish. Furthermore, a real-time PCR method and primer pair (cps3K-F and cps3K-R) are described to identify *S. parauberis* strains belonging to serotype III in bacterial cultures and fish tissues, without the need for animal sera. *In silico* and *in vitro* analyses revealed that both methods have 100% specificity. On the one hand, the multiplex PCR assay was optimized for the detection of *S. parauberis* strains of subtypes Ia (amplicon size 213-bp), subtypes Ib and Ic (both with amplicon size 303-bp), serotype II (amplicon size 403-bp) and serotype III (amplicon size 130-bp) from bacterial cultures. Furthermore, a real-time PCR assay was optimized for the identification and quantification of *S. parauberis* serotype III strains in bacterial cultures and fish tissues (Tm = 73.00 ± 0.33 ºC). This assay achieved a sensitivity of 2.67 x 10^2 gene copies (equivalent to 3.8 x 10^{-9} ng/µl) using pure bacterial cultures of *S. parauberis* serotype III and 1.76 x 10^2 gene copies in fish tissues experimentally and naturally infected with *S. parauberis* serotype III. The specificity and sensitivity of the protocols described in this study suggest that these methods could be used for diagnostic and/or epidemiological purposes in clinical diagnostic laboratories.

Chapter 5 evaluated the efficacy and safety of bivalent vaccines for the prevention of vibriosis, caused by *Vibrio anguillarum*, and atypical furunculosis, caused by *Aeromonas salmonicida* subsp *achromogenes*. Therefore, four routes of infection for *A. salmonicida* subsp. *achromogenes* (intraperitoneal injection, subcutaneous injection, immersion and intragastric inoculation) were evaluated in turbot and sole (**article nº 8**), to establish a suitable infection model for the study of the pathogenesis of atypical furunculosis and for the evaluation of the efficacy of methods for the prevention and

control of the disease. This study used strains of *A. salmonicida* subsp. *achromogenes* isolated from turbot affected by atypical furunculosis. Of all routes tested, intraperitoneal, subcutaneous and immersion injection caused mortality and reproduced the clinical signs of atypical furunculosis in experimentally infected fish. These results suggest that the strains of *A. salmonicida* subsp. *achromogenes* used do not show host specificity, being able to infect both turbot and sole under experimental conditions reaching 80-100% mortality after intraperitoneal and subcutaneous inoculation. *A. salmonicida* subsp. *achromogenes* was confirmed as the causative agent of mortalities by bacterial culture and by qPCR analysis of samples from moribund or dead fish. However, further studies are needed to identify the route of entry and dissemination of *A. salmonicida* subsp. *achromogenes* in fish, which will help to improve treatment and prophylactic strategies.

Finally, in **article nº 9**, a bivalent autogenous vaccine against *V. anguillarum* and *A. salmonicida* subsp. *achromogenes* was developed with the oily compound Montanide™ as an adjuvant. The efficacy of this autogenous vaccine and the commercial vaccine AlphaJect 3000 (Pharmaq AS), administered by intraperitoneal injection, to prevent atypical furunculosis and vibriosis in turbot was evaluated comparatively. The effect of the injection of the mineral oil adjuvant Montanide™ (ISA 763A VG; Seppic, France) and the liquid paraffin Eolane 130 (Total Fluids, Spain) was evaluated in parallel. The effect of both vaccines and adjuvants was tested by analyzing health parameters and fish survival after infectious challenge with *V. anguillarum* and *A. salmonicida* subsp. *achromogenes*. The results of the study showed that the autogenous vaccine conferred high levels of protection and long-lasting immunity against both pathogens with a single dose. However, turbot injected with this autovaccine showed severe side effects, whereas fewer negative effects were observed when turbot were injected with the AlphaJect 3000 vaccine or with the adjuvants Montanide or Eolane. All vaccinated turbot showed remarkable antibody agglutination titers, higher than those of control fish, which were maintained 160 days after vaccination. This study has shown that the autogenous bivalent vaccine induces long-lasting protection against atypical furunculosis and vibriosis in turbot, after administration of a single dose, at the cost of high side effects in the fish. Therefore, the development of new vaccines should focus on autovaccines and the use of liquid paraffin adjuvants that increase protection with reduced or no side effects.

Disease is the result of a disturbance in the balance between host, pathogen, and environment. Therefore, strategies to prevent and control bacterial diseases in fish farms must consider all aspects involved in the pathogen-host-environment relationship to ensure long-term protection of fish.

In this PhD work, different aspects of bacterial pathogen typing, and monitoring, diagnosis and control of diseases caused by the bacterial pathogens *S. parauberis*, *S. iniae*, *V. salmoninarum*, *L. garvieae*, *A. salmonicida* subsp. *achromogenes* and *V. anguillarum* have been studied. The results obtained showed that serological, genotypic, and proteomic methods are useful tools for rapid and reliable diagnosis and monitoring

of the fish pathogens *S. parauberis*, *S. iniae*, *V. salmoninarum* and *L. garvieae*. Among all these methods, MALDI-TOF-MS has the highest discriminatory power and is the fastest and least expensive method for the identification of these pathogens and for large-scale epidemiological studies. In addition, MALDI-TOF-MS and PCR-based techniques demonstrated a high prevalence of resistance to oxytetracycline and florfenicol among *L. garvieae* strains isolated in Spain. PCR and MALDI-TOF-MS techniques combined with phenotypic detection of resistance offer an alternative for surveillance systems and rapid disease management, allowing the development of effective therapeutic measures against *L. garvieae* during a disease outbreak in aquaculture. The real-time PCR-based techniques developed in this work allowed the identification of *V. salmoninarum*, *S. iniae* and the identification and typing of *S. parauberis* from DNA extracted from pure cultures, bacterial suspensions, and infected fish tissues. The developed protocols proved to be 100% specific and to have high levels of sensitivity (from 0.034 to 2.67 x 10^2 amplicon copies per µl). All these methodologies can be used as a tool in epidemiological surveillance studies or for streptococcosis diagnostic purposes using bacterial cultures and fish tissue samples obtained by lethal and non-lethal procedures as samples. Finally, it was found that strains of *A. salmonicida* subsp. *achromogenes* isolated from diseased turbot showed little or no host specificity, being able to reproduce the clinical signs of atypical furunculosis in a manner like that of natural infection after intraperitoneal and subcutaneous inoculation of turbot and sole. Furthermore, the bivalent autogenous vaccine developed conferred high levels of long-term protection against atypical furunculosis and vibriosis after single dose administration in turbot, at the cost of high side effects in fish. This demonstrates the need for further studies focusing on the development of new autovaccines and the evaluation of other adjuvants that improve protection with reduced or no side effects. In this respect, the liquid paraffin Eolane 130 is a promising candidate that could be considered in the development of future vaccines.

CHAPTER 1. General introduction

In recent decades, aquaculture has become the fastest growing livestock sector. The ever-increasing population and the resulting demand for fish as a source of animal protein are the main reasons for the growth of the aquaculture industry. However, this increase in production implies the use of intensive farming methods, where animals are reared at high stocking densities, often resulting in disease.

Disease outbreaks cause high economic losses due to reduced productivity, poor quality of aquatic products or the cost of treatment. Thus, the estimated annual economic loss worldwide in the aquaculture industry due to disease amounts to billions of US dollars (Pridgeon and Klesius, 2012). In addition, diseases in aquaculture are a risk to human health from the consumption of infected aquaculture products.

The main pathogens responsible for diseases in the aquaculture industry are bacteria, viruses, fungi, and parasites (Figure 1). Bacteria are the major cause of mortality in aquaculture due to their high adaptive capacity and their ability to survive in the aquatic environment independently of their hosts (Pridgeon and Klesius, 2012).

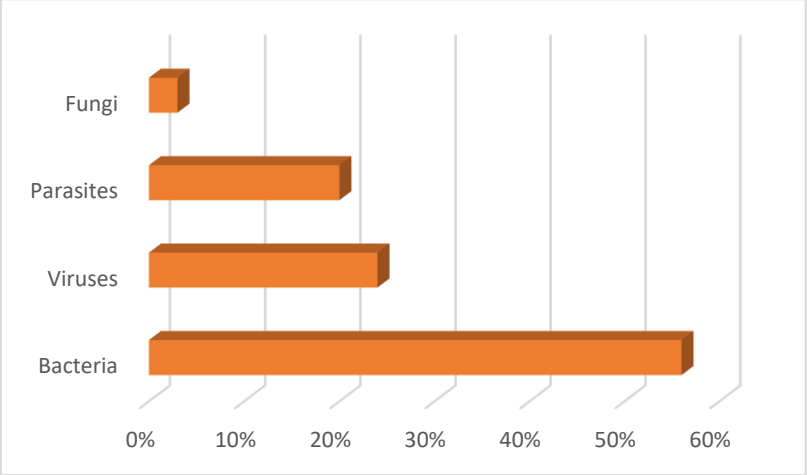

Figure 1. Percentage of infectious disease outbreaks caused by major causative agents in aquaculture. Image prepared by the author.

Successful fish health management in aquaculture begins with disease prevention rather than treatment. Prevention of fish diseases is achieved through good management of water quality, nutrition, and sanitation. Fish are in constant contact with opportunistic pathogens, including bacteria. Low water quality, poor nutrition or suppression of the immune system generally associated with stressful conditions allow these opportunistic pathogens to develop diseases in fish. Drugs used to treat these diseases provide a means to buy time for the fish and allow them to overcome opportunistic infections but are not a substitute for proper husbandry.

Daily observation of fish behaviour and integrated knowledge of the interactions between pathogens, host, and environment (Sitjà-Bobadilla and Oidtmann, 2017) (Figure 2) allows early detection of problems when they occur, so that a diagnosis can be made before most of the population becomes sick.

The integrated fish health management model provides that when a pathogen causes an infectious outbreak, the steps to be taken are as follows:

1. **Knowledge** of the pathogen(s) causing the outbreak (life cycle, host invasion strategies, natural enemies, vectors, etc.), as well as the host and environmental risk factors that favour the spread of the pathogen and its impact on a fish population. This information comes from the experience of fish farmers, scientific studies, and literature reviews.
2. **Prevention**, which involves the development, evaluation of feasibility, cost-effectiveness, and application of the best preventive strategies for each pathogen.
3. **Disease surveillance**, including detection of the pathogen, surveillance of host performance and the possible potential impact on the environment.
4. **Control**, which involves the development and application of intervention strategies when prevention is not enough to stop the disease.
5. **Re-evaluation and planning** in view of the results obtained from the various strategies.

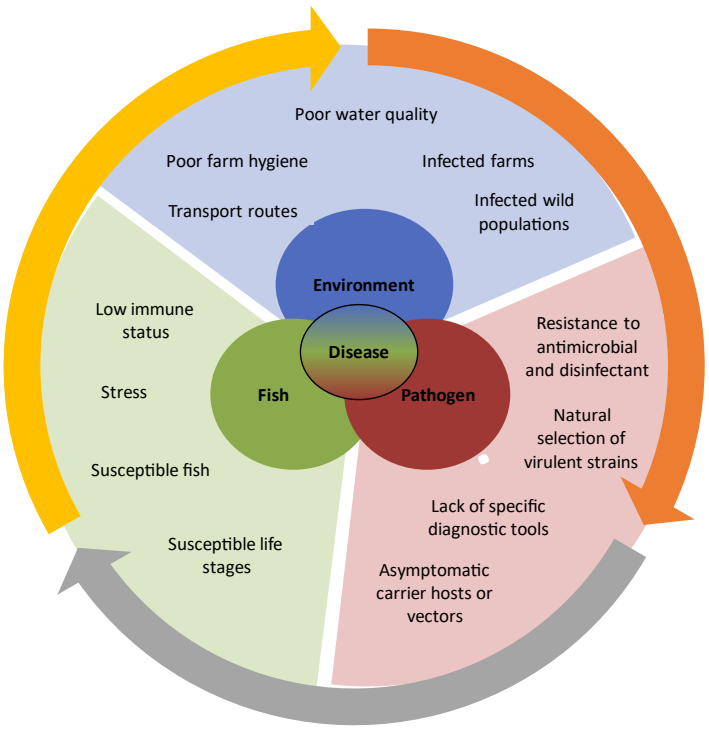

Figure 2. Venn diagram showing the interaction between pathogens, fish and environment as the factors involved in the occurrence of fish diseases. Image prepared by the author.

Therefore, the main objective of this PhD research work will focus on the integrated study of some of the main pathogenic bacterial species responsible for diseases in farmed fish. The economic impact of these pathogenic bacteria on the aquaculture industry has increased the interest in epidemiological studies or evaluation of the phenotypic, molecular, serological, and chemotaxonomic characteristics of these pathogens. The design of new, faster, and more effective diagnostic methods, or new sustainable and ecological prevention mechanisms has also become a priority. A summary of the pathogens responsible for diseases in freshwater and marine fish studied in this work is presented below.

1.1.1. Gram-positive bacteria

Gram-positive cocci infections of fish or 'streptococcosis' were introduced in Europe in the mid - 1980s. These infections are regarded as a complex of similar diseases caused by different genera and species capable of inducing damage to the central nervous system characterized by exophthalmia and meningoencephalitis. Water temperature is considered a predisposing factor for the onset of the disease (Ghittino et al., 2003; Buller, 2014; Austin and Austin, 2016; Yardimci et al., 2016). Diseases occurring at water temperatures below 12 ºC, termed cool water streptococcosis, usually are produced by *Carnobacterium maltaromaticum* (previous *Carnobacterium piscicola*), *Lactococcus piscium*, and *Vagococcus salmoninarum*. While diseases that occur at water temperatures above 15 ºC, warm-water streptococcosis, are produced by *Streptococcus iniae, Streptococcus parauberis, Streptococcus agalactiae* (synonym of *S. difficilis*), and *Lactococcus garvieae,* (Ghittino et al., 2003; Mata et al., 2004b; Ruiz-Zarzuela et al., 2005; Didinen et al., 2011; Yardimci et al., 2016).

1.1.1.1. The family *Streptococcaceae*

The family *Streptococcaceae* comprises three genera, of which only the genera *Streptococcus* and *Lactococcus* have reported species pathogenic for fish. Originally, members of the *Streptococcaceae* family were classified into four broad categories that were nominated by Sherman as pyogenic, viridans, lactic and enterococci (Facklam, 2002; Buller, 2014). The pyogenic group includes β-haemolytic strains of *Streptococcus*, which are classified into Lancefield groups according to the carbohydrate's composition of bacterial antigen on their wall. However, there are many species belonging to this group of which his Lancefield group is unknown. The viridans group are non-haemolytic bacteria or show a green colouration around the colony grown in blood agar (α-haemolysis), do not tolerate high pH, salt, or temperatures at or below 10 ºC. The Lactic group includes bacteria of the genus *Lactococcus*, while the enterococci group currently includes bacteria of the genus *Enterococcus* (Buller, 2014).

The bacteria of this family show an ecological distribution wide and diverse; because they can adapt and survive to a wide range of conditions. This ability allows them to infect and survive in both animal and human hosts, turning them into potentially

zoonotic organisms. Table 1 represents the differential characteristics of streptococcal species pathogenic to fish. Among these fish streptococci, *S. iniae*, *S. parauberis* and *L. garvieae* can be regarded as the main aetiological agents causing diseases in aquaculture.

Table 1. Differential characteristics of streptococcal species pathogenic to fish.

Character	*L. garvieae*	*S. iniae*	*V. salmoninarum*	*L. piscium*	*S. parauberis*	*S. agalactiae*
Morphology	ovoid-shaped coccus	coccus	ovoid-shaped coccus	ovoid-shaped coccus	Coccus	Coccus
Motility	-	-	-	-	-	-
Haemolysis	α	ß	α	-	α	-
Catalase	-	-	-	-	-	-
ADH	+	+	-	+	+	+
VP	+	-	-	+	+	+
Amylase	-	-	+	+	-	V
Growth at:						
45ºC	+	-	-	-	-	-
pH 9.6	+	-	-	+	-	-
40% bile salts	+	-	+	-	-	-
6.5% NaCL	+	-	-	-	-	-
Acid production:						
Mannitol	V	+	-	+	+	-
Galactose	+	-	-	-	-	-
Tagatose	V	-	+	-	-	-

ADH, arginine dehidrolase; +, positive reaction; -, negative reaction; V, variable

1.1.1.1.1. *Streptococcus iniae*

Streptococcus iniae is a Gram-positive bacterium that was first isolated from freshwater dolphins (*Inia geoffrensis*) (Pier and Madin, 1976). This pathogen was named *Streptococcus shiloi* in isolates from tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) from Israel, until it was discovered that it was the same bacteria as *S. iniae* by genetic and phenotypic tests (Eldar et al., 1995). *S. iniae* is a non-motile coccus (0.3-0.5 µm), aerobic and facultative aerobic, encapsulated, β-haemolytic on blood agar. The optimal temperature for growth of *S. iniae* isolates is in the range 35-40ºC and at pH range of 6-7 (Perera et al., 1994). Growth does not occur at 10 or 45 °C, at 6.5% NaCl or 10% and 40% bile broth (Buller, 2014; Austin and Austin, 2016). This species is not currently assigned to any Lancefield group (Agnew and Barnes, 2007). This bacterium grows well on several common media such as Brain–Heart Infusion Agar (BHIA), Trypticase-Soy Agar (TSA), Blood Agar (BA) and Nutrient Agar (NA).

S. iniae is a zoonotic bacterium of marine and freshwater fish that causes outbreaks of warm-water streptococcosis in aquatic species (Agnew and Barnes, 2007; Austin and Austin, 2016) and invasive disease in humans (Agnew and Barnes, 2007; Baiano and Barnes, 2009). In fish, *S. iniae*-infection produces external clinical signs such as exophthalmia, diffuse or petechial skin haemorrhages predominantly in the cephalic and caudal region, ascites and splenomegaly (Figure 3) (Buller, 2014; Austin and Austin, 2016). The economic impact associated to *S. iniae* infections on aquaculture industry has been estimated in 1997 at US$ 10 million in the USA and US$ 100 million worldwide (Shoemaker et al., 2001).

The importance of this bacterium transcends the veterinary field. *S. iniae* was noted as an emerging zoonotic disease transmitted by food-producing animals at the International Conference on Emerging Infectious Diseases in 2000 (Agnew and Barnes, 2007). Human infections with *S. iniae* have been sporadic at all times, but new cases continue to be reported in 2009 (Koh et al., 2009) . The number of human infected with *S. iniae* is likely to be higher than reported due to failures in detection and identification methods (Agnew and Barnes, 2007; Baiano and Barnes, 2009). The clinical presentation includes bacterial cellulitis, septicaemia, endocarditis, arthritis, meningitis, osteomyelitis, fever and abdominal distension, and pneumonia (Lau et al., 2003). The risk factors associated with *S. iniae* infection include Asian ethnic origin (85 % of human infected are Asian descent), old age and pre-existing medical conditions like diabetes mellitus (Sun et al., 2007).

Figure 3. Clinical signs produced by streptococcosis caused by experimental infection of *S. iniae* in rainbow trout. Image prepared by the author.

S. iniae is phenotypically heterogeneous, which difficult its identification based on the analysis of some morphological and physiological characteristics. Moreover, *S. iniae* is not listed in many commercial or clinical databases; so many *S. iniae* strains are assigned a low level of compatibility or "unidentified" (Facklam et al., 2005; Lau et al., 2006). Recently, new technologies have been developed based on serological methods (Dodson et al., 1999; Nho et al., 2009), polymerase chain reaction (PCR) (Berridge et al., 1998; Goh et al., 1998; Zlotkin et al., 1998b; Mata et al., 2004a; Zhou et al., 2011) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Kim et al., 2017) to carry out reliable identifications or typing of that microorganism. Serological studies demonstrated the existence of antigenic heterogeneity in Japanese isolates of *S. iniae* with two serotypes described. These serotypes were classified according to the presence/absence of capsular polysaccharide antigens in KG^+ (capsulated phenotype) and KG^- (non-capsulated phenotype) (Bachrach et al., 2001; Kanai et al., 2006). Agglutination tests showed that (i) antisera against encapsulated isolates reacted with non-encapsulated isolates and (ii) antisera against non-encapsulated isolates did not react with any encapsulated isolates (Kanai et al., 2006). In addition, the presence of the capsule was related to the pathogenicity of this bacterium, being virulent only the isolates of *S. iniae* with capsule (Kanai et al., 2006). Molecular characterization of *S. iniae* using Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) (Bachrach et al., 2001), Pulse-Field Gel Electrophoresis (PFGE) (Lau et al., 2003; Facklam et al., 2005) and ribotyping (Eldar et al., 1997) have also demonstrated genetic heterogeneity within this species.

1.1.1.1.2. *Streptococcus parauberis*

Streptococcus parauberis (formerly *S. uberis* genotype II) is a Gram-positive short-rod (cocci-bacilli)-shaped bacterium, facultative anaerobic, non-motile, encapsulated and α-haemolytic on blood agar. Catalase and oxidase are negative. The optimal temperature for growth of *S. parauberis* isolates is in the range 25-37 ºC (Garrido López et al., 2016). Growth does not occur at 4 or 45ºC or at 4.5 % NaCl (Domenech et al., 1996; Austin and Austin, 2016). Of the phylogenetic groups of genus

Streptococcus, *S. parauberis* belongs to the pyogenic group and Lancefield E serogroup (Facklam, 2002). This bacterium grows well on several common media such as Brain–Heart Infusion Agar (BHIA), Trypticase-Soy Agar (TSA), Blood Agar (BA), Tryptone-Soy Yeast Extract Agar (TSYEA) or Agar Brucella (Austin and Austin, 2016; Garrido López et al., 2016).

S. parauberis was initially described as the aetiological agent of bovine mastitis (Williams and Collins, 1990; Khan et al., 2003). *S. parauberis* as fish pathogen was originally recognised in farmed turbot (*Scophthalmus maximus*) from five sites in northern Spain during 1993 and 1994 (Domenech et al., 1996; Romalde et al., 1999). Since then, it has become a major disease in the aquaculture industries of multitude of countries, with particular importance in Northeast Asia (Korea, Japan and China) (Aoki et al., 1990; Baeck et al., 2006). The *S. parauberis*-infection produces external clinical signs such as darkening of skin, weight loss, unilateral or bilateral exophthalmia, haemorrhagic septicaemia, and meningitis with abnormal swimming. Internally, lesions include ascites, paleness and congestion of the liver, spleen and kidney (Figure 4) (Domenech et al., 1996; Woo & Park, 2013). The evidence suggests a major impact of the disease causing mortalities over 50% over a period of 3-7 days and chronic infections with few deaths each day over a period of several weeks (Woo and Park, 2013). In addition to a wide variety of aquatic (Austin and Austin, 2016) and terrestrial animals (McDonald et al., 2005), *S. parauberis* has also been isolated from food (Koort et al., 2006; Fernández-No et al., 2012; Böhme et al., 2013) and, recently, from humans (Zaman et al., 2016).

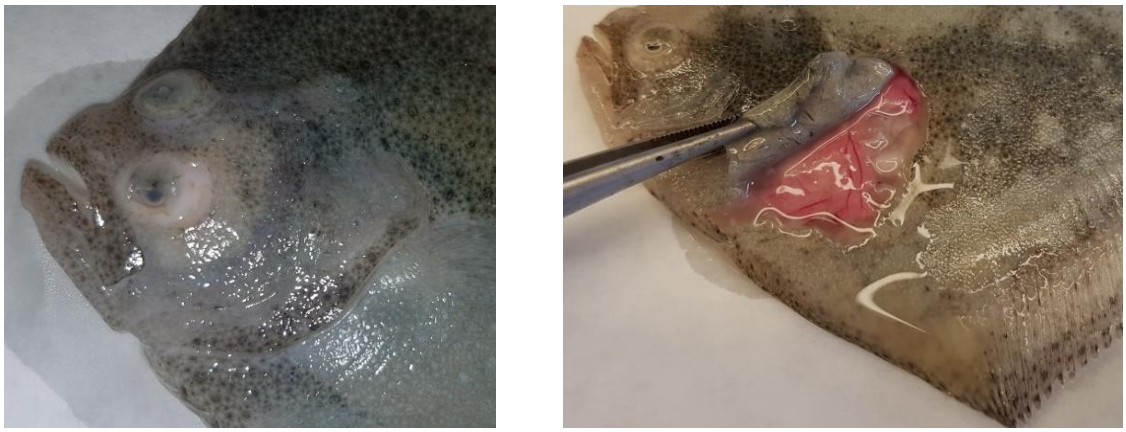

Figure 4. Clinical signs produced by streptococcosis caused by *S. parauberis* in turbot. Image prepared by the author.

The phenotypic heterogeneity of *S. parauberis* (Nho et al., 2009) and the high genetic proximity of this species to other bacteria of the same family (e.g. *S. uberis*, *S. iniae* or *L. garvieae*) difficult the identification based on the analysis of its morphological or physiological characteristics (Nho et al., 2009; Austin and Austin, 2016; Zaman et al., 2016). To overcome these problems, several molecular methods

based on PCR have been developed for the identification (Hassan et al., 2001; Mata et al., 2004b; Nguyen et al., 2014) and characterization (Romalde et al., 1999) of this pathogen. However, the molecular method most effectively reported in the identification of α-haemolytic streptococci, including *S. parauberis*, is MALDI-TOF mass spectrometry (Davies et al., 2012; Fernández-No et al., 2012; Kim et al., 2015; Zaman et al., 2016).

Serological studies have also been used to identify and differentiate *S. parauberis* species. These studies showed that this species is serologically indistinguishable from *S. uberis* (Jayarao et al., 1991) and antigenically different from *S. iniae* (Nho et al., 2009). Slide agglutination test using rabbit anti-*Streptococcus parauberis* sera has been successfully applied for identification and serotype differentiation of *S. parauberis* isolates (Tu et al., 2015a). In this sense, agglutination assays carried out by Kanai et al. (2015) indicated that Japanese flounder *S. parauberis* isolates can be divided into five serological groups (serotypes Ia, Ib and Ic, serotype II and nontypeable strains), according to the presence/absence of capsular polysaccharide antigens or structural variation in the capsular polysaccharides. The *S. parauberis* strains isolated in Spain showed serological homogeneity (Toranzo et al., 1995). The differentiation of serotypes within a bacterial species is beneficial for the development of prevention methods or for the selection of the most appropriate treatment. In this regard, it should be noted that all *S. parauberis* strains belonging to serotype II were found to be resistant to tetracycline because they harbour a Tet(S) gene in an 11 kbp plasmid (Meng et al., 2009).

Molecular characterization of *S. parauberis* has demonstrated genetic heterogeneity in Japanese flounder *S. parauberis* isolates by PFGE method, which revealed the relationship between pulsotypes and serotypes (Kanai et al., 2015), and genetic homogeneity in Spanish *S. parauberis* isolates by RAPD and ribotyping (Romalde et al., 1999).

1.1.1.1.3. *Lactococcus garvieae*

Lactococcus garvieae is a Gram-positive bacterium that was first isolated as a causative agent of bovine mastitis. It was previously described as *Streptococcus garvieae* (Collins et al., 1983) and further reclassified to the new genus *Lactococcus* (Schleifer et al., 1985). *L. garvieae* is a non-motile anaerobic facultative ovoid coccus (1.4 x 0.7 µm) that usually produces α-hemolysis on blood agar. General culture media such as brain-heart infusion agar (BHIA), trypticase-soy agar (TSA), blood agar (BA), trypticase-soy broth (TSB) and bile-esculin agar (BEA) are routinely used for *in vitro* growth of this bacterium at a temperature between 4°C and 45°C, with the optimum temperature for growth being 37°C. Growth is possible at pH 4.5 and 9.6 and with 4% sodium chloride (NaCl). Some strains can also grow weakly at a NaCl concentration of 6.5% (Kusuda et al., 1991; Gibello et al., 2016; Austin and Austin, 2016; Meyburgh et al., 2017). Negative results for Lancefield groups A, B, C, D, E, F, G, H, K, L, M, N and O are shown for *L. garvieae* strains (Kusuda et al., 1991).

Lactococcus garvieae is the causative agent of lactococcosis in fish, a type of warm- water streptococcosis. Lactococcosis is a particularly devastating pathology in freshwater salmonids and other marine fish species. Outbreaks of this disease cause severe economic losses due to high mortality rates (50-80% of total production), declining growth rates and clinical signs that impede commercialization of these fish (Vendrell et al., 2006; Buller, 2014; Austin and Austin, 2016). Lactococcosis has been reported to affect fish of all sizes, from juveniles of 5g to adults over 1 kg (Chang et al., 2002; Buller, 2014). The clinical signs of this disease are hyperacute septicaemia and haemorrhagic septicaemia, anorexia, lethargy, erratic swimming, exophthalmia (uni or bilateral) (Figure 5). It is also very common to observe fish with swollen abdomen and anal prolapse (Domenech et al., 1993; Eldar et al., 1999; Vendrell et al., 2006; Gibello et al., 2016; Meyburgh et al., 2017).

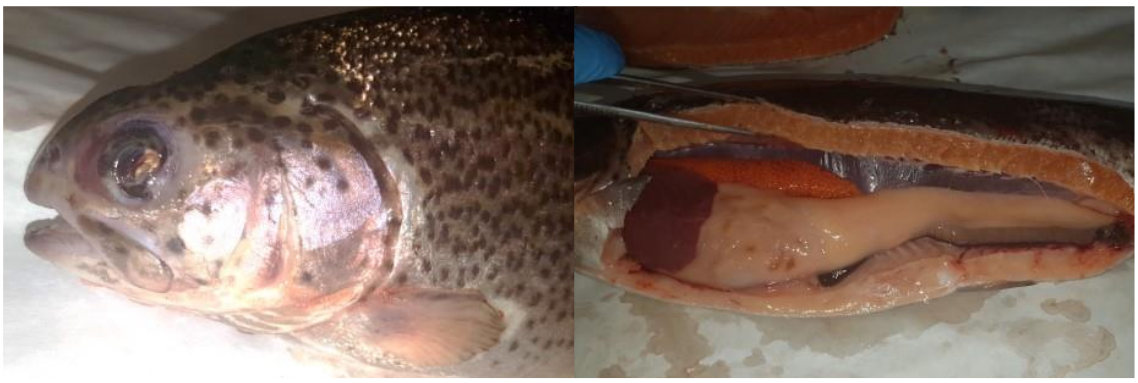

Figure 5. Clinical signs produced by lactococcosis caused by *L. garvieae* in rainbow trout. Image prepared by the author.

The ability of *L. garvieae* to adapt and survive in many environmental conditions is likely related to its wide distribution. In fact, *L. garvieae* has been also isolated from different terrestrial animals (Kawanishi et al., 2006; Tejedor et al., 2011), from river and sewage waters (Aguado-Urda et al., 2010) and from different food and feedstuffs (Olstorpe et al., 2008). An increasing number of human infections due to *L. garvieae* has been reported in recent years, with handling and ingestion of raw fish notified as a source or risk factor in the majority of clinical cases (Gibello et al., 2016), resulting in the status of an emerging zoonotic pathogen (López-Campos et al., 2015; Meyburgh et al., 2017).

Phenotypic studies of *L. garvieae* (Vela et al., 2000) have identified up to 13 biotypes based on the acidification of some sugars and the presence of the enzymes pyroglutamic arylamidase and N-acetyl-β-glucosaminidase. However, this categorisation of biotypes is now being questioned because further research indicates that *L. garvieae* strains are homogeneous, regardless of geographical location or aquatic hosts (Buller, 2014). Based on these studies, phenotypic characteristics have been used

for the correct identification of *L. garvieae* (Buller, 2014; Gibello et al., 2016; Austin and Austin, 2016). Nevertheless, diagnosis based on bacterial isolation and phenotypic characterisation takes at least one week and may provide an unreliable identification of this pathogen (Buller, 2014; Austin and Austin, 2016). Therefore, correct identification of *L. garvieae* is obtained through 16S rRNA gene sequencing, PCR-based techniques (Zlotkin et al., 1998a; Mata et al., 2004b; Jung et al., 2010; Odamaki et al., 2011; Dang et al., 2012) or MALDI-TOF mass spectrometry (Heras Cañas et al., 2015; Assis et al., 2017; Tandel et al., 2017).

Agglutination tests using *L. garvieae* strains isolated from fish have demonstrated the existence of two serotypes, one Japanese and one European, associated with variation of the capsular antigen (KG-) and a third serotype that include non-capsulated isolates (KG+) from both geographic regions (Barnes and Ellis, 2004). Further studies (Fukuda et al., 2015; Oinaka et al., 2015) using Japanese isolates have established the existence of two serotypes; serotype I that includes KG- and KG+ phenotypes and serotype II that grouped strains isolated from *Seriola* spp. that did not agglutinate with anti-KG- serum. These antigenic variations seem to be related to the virulence of this species in fish. The capsulated strains (KG^- type) of *L. garvieae* were more virulent than those not capsulated (KG^+ type) in yellowtail, with LD_{50} values lower than 10^2 bacteria per fish (Kitao, 1996).

At the genetic level, major variations were found associated with the host or geographic origin using molecular genomic methods such as RAPD, PFGE and ribotyping (Vela et al., 2000; Ravelo et al., 2003; Tejedor et al., 2011). In recent years, combining phenotypic and molecular data, two subspecies have been proposed within the species *L. garvieae*: *Lactococcus garvieae* subsp. *garvieae* and *Lactococcus garvieae* subsp. *bovis* (Varsha and Nampoothiri, 2016a).

1.1.1.2. The family *Enterococcaceae*

The family *Enterococcaceae* comprises seven genera, of which the genera *Enterococcus, Vagococcus* and *Catellicoccus* have reported species pathogenic for fish. The organisms of this family can be found in diverse environments, and many are colonizers of humans. In general, the *Enterococcaceae* are fastidious, and consequently, they thrive in environments where various nutritional needs are provided, usually by other living or dead organisms.

1.1.1.2.1. *Vagococcus salmoninarum*

Vagococcus salmoninarum is a Gram-positive bacterium isolated for the first time from an adult rainbow trout (*Oncorhynchus mykiss*) in USA in 1968 (Wallbanks et al., 1990). *V. salmoninarum* is a non-motile anaerobic facultative coccus that produces α-haemolysis on blood agar. Growth occurs at 10-37ºC but is variable for growth at 45ºC and in 6.5% NaCl. All strains are catalase-negative and variable reactions were found in Voges-Proskauer reaction (Buller, 2014).

Vagococcosis, also called cold-water streptococcosis, is an emerging disease in the European trout industry caused by the pathogen *V. salmoninarum*. This bacterium has been isolated from diseased subadults or adults of rainbow trout in Spain (Ruiz-Zarzuela et al., 2005), juvenile and adult rainbow trout in France (Michel et al., 1997), and rainbow trout broodstock and those over 50 g in Italy (Ghittino et al., 2004; Salogni et al., 2007) and Turkey (Didinen et al., 2011; Tanrikul et al., 2014). It has also been isolated from rainbow trout, brown trout (*Salmo trutta*) and Atlantic salmon (*Salmo salar*) in Australia and Norway (Schmidtke and Carson, 1994). Streptococcosis caused by this bacterium has a typically chronic course, with mortality rates up to 50%, and affects either subadult or adult fish (>150-200 g) (Michel et al., 1997; Ruiz-Zarzuela et al., 2005; Didinen et al., 2011; Austin and Austin, 2016). Disease outbreaks are associated with water temperatures of 10-12ºC and post-spawning stress (Ghittino et al., 2004; Salogni et al., 2007). Clinical signs include external and internal haemorrhages, exophthalmia, and accumulation of ascitic fluid in the body cavity, paleness and enlargement of internal organs (Figure 6) (Buller, 2014; Austin and Austin, 2016).

Although the vagococcosis is considered an emerging pathology in fish, its diagnosis remains difficult. This is because, although high phenotypic homogeneity has been reported within this species (Schmidtke and Carson, 1994; Michel et al., 1997; Tanrikul et al., 2014), the phenotypic characteristics of all Gram-positive cocci responsible for streptococcosis in fish are highly similar (Buller, 2014; Austin and Austin, 2016). To date, there are only two molecular methods based on PCR for identification of *V. salmoninarum* (Ruiz-Zarzuela et al., 2005; Standish et al., 2020).

Figure 6. Clinical signs produced by Vagococcosis caused by *V. salmoninarum* in rainbow trout. Image prepared by the author.

1.1.2. Gram-negative bacteria

Gram-negative bacteria are the main pathogenic microorganisms affecting fish. Many of these microorganisms are cohabiting in the natural habitat of aquatic organisms without causing disease; however, there are conditions that predispose to the development of the disease, such as low level of dissolved oxygen, water quality problems, and stress in management or trauma. Gram-negative bacteria are characterized by the presence of a unique cell wall component termed lipopolysaccharide (LPS), which is associated with substantial diseases in humans and marine organisms (Anwar and Choi, 2014).

Among diseases caused by the Gram-negative bacteria there are some that are characterized by haemorrhagic septicaemias, with external redness and haemorrhages in the peritoneum, body wall and viscera of the diseased fish. Ulcerative lesions are common as the disease progresses, and mortality can be significant if stress is not controlled. In these cases, the most common bacterial isolates in affected fish include *Aeromonas* and *Vibrio* (Austin and Austin, 2016).

There is another group of Gram-negative filamentous bacteria reported as the main cause of disease in seawater fish (mainly the genus *Tenacibaculum*) and freshwater fish (mainly the genus *Flavobacterium*) (Austin and Austin, 2016).

1.1.1.3. The family *Aeromonadaceae*

The family *Aeromonadaceae* comprises the genera *Aeromonas*, *Tolumonas*, *Oceanimonas*, *Oceanisphaera*, and *Zobellella*. Bacteria included in this family are strict aerobes or facultative anaerobes typically associated with aquatic environments. In the first edition of *Bergey's Manual of Systematic Bacteriology*, the genus *Aeromonas* was assigned to the eubacterial family *Vibrionaceae* based primarily on phenotypic characters. However, subsequent DNA hybridization studies evidenced the significant evolutionary distance between both taxonomic groups, which are now grouped into distinct families (Rosenberg et al., 2013). The genus *Aeromonas* is the main genus of this family responsible for fish diseases.

1.1.1.3.1. *Aeromonas salmonicida*

Aeromonas salmonicida is a Gram-negative short to oval rod-shaped bacterium. This bacterium is a facultative anaerobe, non-motile, non-spore forming and non-acid fast. The temperature growth range is 6 to 34.5 ºC, with the optimum growth in the range of 20 to 22ºC. It shows a similarly broad pH growth range of pH 5.3 to 9.0.

A. salmonicida is the oldest known fish pathogen and is currently endemic almost worldwide in both fresh and marine waters. *A. salmonicida* has a broad host range, affecting salmonid species (Austin and Austin, 2007), and a variety of non-salmonid fish, including sablefish (*Anoplopoma fimbria*), lumpfish (*Cyclopterus lumpus*), cunner (*Tautogolabrus adspersus*), turbot (*Scophthalmus maximus*), Senegalese sole (*Solea senegalensis*), Atlantic cod (*Gadus morhua*), halibut (*Hippoglossus hippoglossus*),

lamprey (*Petromyzon marinus*), carp (*Cyprinus carpio*), sea bass (*Dicentrarchus labrax*) and European eel (*Anguilla anguilla*) (Austin and Austin, 2016; Fernández-Álvarez et al., 2016). Five subspecies of *A. salmonicida* affecting cultured and wild fish have been described: *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *smithia*, *achromogenes*, *masoucida* and *pectinolytica*. *A. salmonicida* subsp. *salmonicida* is the main causative agent of furunculosis, also called "typical furunculosis", characterized by the presence of the typical furuncles or ulcers in the skin, exophthalmia, haemorrhages, septicaemia (Figure 7) and acute mortality in susceptible fish (Wiklund and Dalsgaard, 1998; Toranzo et al., 2005; Austin and Austin, 2016). Growth of the bacteria may be accompanied by the release of a brown pigment when cultured in the presence of the amino acids, tyrosine, or phenylalanine. The production of this pigment is often used in presumptive diagnosis of infection by *A. salmonicida* subspecies *salmonicida*. The remaining subspecies of *A. salmonicida* are referred to as "atypical strains" which produce "atypical furunculosis", characterized by the presence of dermal ulcerations and external pathology with or without subsequent septicaemia (Wiklund and Dalsgaard, 1998). The clinical signs of atypical furunculosis are indistinguishable from those associated to typical furunculosis or other diseases produced by motile *Aeromonas* species (Austin and Austin, 2016).

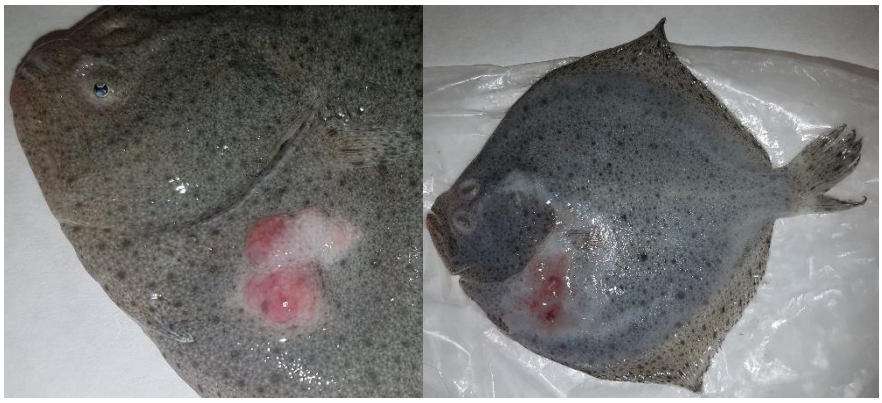

Figure 7. Clinical signs produced by furunculosis caused by *A. salmonicida* subsp. *salmonicida* in turbot. Image prepared by the author.

A. salmonicida subsp. *salmonicida* has long been described as a very homogeneous species on a biochemical and molecular level (Menanteau-Ledouble et al., 2016). Hirvelä-Koski et al. (1994) used 14 different biochemical tests to biochemically characterize 71 isolates of *A. salmonicida* subsp. *salmonicida*, and obtained equal results for all strains in 12 of the biochemical tests used. This same homology has also been reported at the genomic level, using RAPD analyses performed by Miyata et al. (1995), Inglis (1996) and by O'hIci et al. (2000) and ribotyping (Nielsen et al., 1994). The genetic homology of some of these isolates was checked by PFGE analysis, giving very similar results for isolates of *A. salmonicida* subsp. *salmonicida* studied (O'Hici et al., 2000).

The biochemical characteristics of the subspecies of *A. salmonicida* are well known and may be useful in differentiating this species from other fish pathogens or in differentiation between subspecies (Austin and Austin, 2016). Some differential biochemical characteristics of these subspecies are summarized in table 2. However, phenotypic characterization can give imprecise results, especially in the case of complex genera such as *Aeromonas*. Therefore, the use of biochemical and genetic methods in parallel is necessary for a correct identification of the species (Beaz-Hidalgo and Figueras, 2012). The most used genetic methods for the identification of *A. salmonicida* strains are based on the amplification of genomic DNA using the PCR method, such as multiplex-PCR (Altinok et al., 2008), multiplex PCR coupled to a DNA microarray (González et al., 2004), conventional PCR (Hiney et al., 1994; Beaz-Hidalgo et al., 2008; Beaz-Hidalgo and Figueras, 2012; Buller, 2014) or real-time PCR (Fernández-Álvarez, González, & Santos, 2016).

Recently, other authors (Benagli et al., 2012; Donohue, Smallwood, Pfaller, Rodgers, & Shoemaker, 2006; Fernández-Álvarez, Gijón, Álvarez, & Santos, 2016b) have reported that specific peak masses obtained from the MALDI-TOF MS analysis of intact cells could be used as specific biomarkers for the identification and differentiation between *Aeromonas* species and subspecies.

Table 2. Characteristics for differentiation between subspecies of *Aeromonas salmonicida* and atypical isolates. Table elaborated from Wiklund and Dalsgaard, 1994.

Subspecies or group	Pigment production	Oxidase reaction	Gas from glucose	Acid from sucrose	Indole production	Degradation of aesculin	Sensitivity to amplicillin	Sensitivity to cephalothin
A. salmonicida	+(-)	+(-)	+	-(+)	-	+	S	S
A. achromogenes	-(+)	+	-	+	+	-	R	R
A. masoucida	-	+	+	+	+	+	ND	ND
A. nova	-/+	+	-	+/-	+(-)	-/+	R/S	R/S
A. smithia	-(+)	+	ND	+(-)	-	-	S(R)	ND
Phenon 13	+(-)	+	ND	+	-	-	R	ND
Slow growing	-	-/+	-	+/-	-(+)	+(-)	S	S(R)
Atypical isolates	+/-	+	-	+	-/+	-(+)	R(S)	R(S)

+: positive reaction; -, negative reaction>; (), reaction of a few strains; S, sensitive; R, resistant; ND, no data available.

The appearance and development of diseases in fish is the result of the interaction between the pathogen, the host, and the environment. In addition, the complexity and dynamism of the aquatic environment makes it difficult to control these diseases. Therefore, multidisciplinary studies in which a potentially pathogenic microorganism is detected and characterised at an early stage are particularly important for prevention and management of disease in this type of environment. Aspects of host fish biology and a better understanding of the environmental factors affecting such cultures, will also allow the application of appropriate measures to prevent and management the disease that limits fish production (Austin, 2017; Toranzo et al., 2005). This monitoring of diseases in the form of surveillance and rapid diagnosis is important to allow appropriate measures to be taken when pathogens are detected and diseases are diagnosed, before they become a major problem for the farmer (Adams and Thompson, 2011).

Several conventional, serological, molecular, and chemotaxonomic microbiology techniques have been developed over the years. Some of these techniques are widely used to carry out epidemiological studies, detection, and identification of bacterial pathogens in aquaculture.

1.2.1. Conventional or phenotypic methods

Conventional or phenotypic methods are based on the characterization of the biochemical, physiological, and metabolic properties of bacteria, which must be previously grown in pure culture (culture-dependent) (Buller, 2014; Austin and Austin, 2016; Austin, 2017). Several biochemical kits are commercially available which are not specifically designed for use with aquatic pathogens, but several authors have found them useful after minor adaptations. There are several manual, semi-automated and automated systems available for bacterial identification including the API 20E, API ZYM, API 20NE, API 50CH, Vitek or Biolog (Popovic et al., 2007). These commercial kits are routinely used in bacterial diagnostic laboratories, as they are easy to use, fast and cost-effective; however the reliability of it for use with fish pathogens needs to be carefully evaluated (Austin, 2011, 2017).

Currently, phenotypic methods are widely used for the diagnosis of bacterial diseases. However, these methods have major constraints that make them a bad choice when quick and reliable diagnoses are required. Some of the main disadvantages of these methods for the identification of aquatic pathogens are:

- Limited precision because they are unable to differentiate closely related bacterial species (e.g., *Aeromonas salmonicida* from *A. hydrophila* or *Streptococcus parauberis* from *S. uberis*) or subtypes within the same species (e.g. *A. salmonicida* subsp *salmonicida* from *A. salmonicida* subsp *achromogenes*). The possibility therefore exists of erroneous or ambiguous results.

- Misidentifications or non-identifications using commercial kits and the manufacturer's probabilistic databases have been reported because of the absence of aquatic pathogens in these databases.
- They are time-consuming and labour intensive.
- They are not enabling discrimination of several bacterial species important in aquaculture.

The limitations of conventional methods in terms of sensitivity and specificity led to the development and use of molecular techniques.

1.2.2. Molecular methods

Molecular methods for bacterial diagnosis and typing are mainly based on the amplification and subsequent analysis of nucleic acids (DNA or RNA). Since their appearance in the 1980s, these methods have emerged as complementary, alternative, or even reference procedures to phenotypic or serological methods for the detection, identification, and epidemiological analysis. In recent decades, molecular methods have emerged as critical tools in diagnostic laboratories for routine detection and fingerprinting, as well as to assist in animal and human health surveillance, allowing rapid implementation of infection prevention and control practices. Molecular methods are applied to detect pathogens in a diverse range of environmental samples including water, soil, food, and aquatic pathogens. These methods are very useful for the detection of microorganisms that are difficult to grow, exist in a dormant state or are involved in a zoonosis (Morshed et al., 2007; Buller, 2014; Austin and Austin, 2016).

In aquaculture, the first molecular methods used for diagnosis and bacterial typing were based on hybridization of target DNA using specific, pre-marked, single-stranded DNA probes. However, these methods required a high amount of starting DNA, resulting in low detection sensitivity. To overcome these problems, the Polymerase Chain Reaction (PCR) technique was developed in 1985 (Saiki et al., 1985), capable of directly amplifying bacterial DNA. Since its development, this technique has become the most widely used technique in molecular biology in recent years and has been adapted to a wide range of fields, including DNA cloning, gene expression and the diagnosis and epidemiology of infectious diseases, among others (Altinok and Kurt, 2003; Morshed et al., 2007; Adams and Thompson, 2011; Buller, 2014).

1.2.2.1. PCR-based diagnostic methods

Methods based on the amplification of bacterial DNA are also known as methods based on the PCR technique. The PCR method has been widely used in aquaculture welfare through the detection and identification of bacterial fish pathogens and discrimination between non-pathogenic and pathogenic strains (Morshed et al., 2007; Buller, 2014). These tests have advantages over conventional diagnostic methods, such as sensitivity, specificity, or elimination of the need for bacterial isolation, dramatically reducing the time required for reporting results and decision making. In addition, PCR can be carried out using non-destructive samples as mucus, blood, or fish

faeces. All these advantages have an impact on improved management of aquaculture diseases and more effective preventive and control measures, even with early or mild bacterial infections (Adams and Thompson, 2011).

Conventional PCR may be enough to detect or identify a bacterial pathogen and, in many cases, the identification of PCR products by sequencing is the "*gold standard*" method to finally identify the pathogen. Traditionally, the most widely used bacterial genetic sequence for these purposes has been the 16S rRNA gene (Zlotkin et al., 1998a, 1998b; Hassan et al., 2001; Ruiz-Zarzuela et al., 2005; Torres-Corral et al., 2019). This is because the 16S rRNA is a highly conserved gene or *housekeeping* marker. In recent years, many studies have focused on the search for new target genes for differentiation of closely related species (Goh et al., 1998; Mata et al., 2004a; Nguyen et al., 2014), differentiation of serovars (Kannika et al., 2017; Ohbayashi et al., 2017; Rochat et al., 2017; Shoemaker et al., 2017) or genotypes (Olsen et al., 2017), for the detection of antibiotic resistance genes (Walther et al., 2008; Park et al., 2009; Raissy and Shahrani, 2015) or virulence genes (Allen and Neely, 2011; Salighehzadeh et al., 2020).

In aquaculture, many other variations of this technique are also used, including two-step or nested PCR, multiplex PCR (m-PCR), reverse transcriptase PCR (RT-PCR), and real time PCR that differs in the levels of sensitivity (Adams and Thompson, 2011; Buller, 2014) (Figure 8). The real time PCR has several competitive advantages over conventional PCR, such as high sensitivity and accuracy, the possibility of quantification and reduced potential for contamination due to the closed tube system used during amplification and post-amplification analysis (Adams and Thompson, 2011).

In this regard, real-time PCR has been developed for *S. parauberis* bacteria using as target the DNA gyrase subunit B (*gyrB*) gene (Nguyen et al., 2014), for *V. salmoninarum* using as target the phenylalanyl-tRNA synthetase (*pheS*) gene (Standish et al., 2020), for *L. garvieae* using as target the 16S rRNA gene (Jung et al., 2010) and for *A. salmonicida* using the surface array protein (*VapA*) gene (Keeling et al., 2013) and the gene that code for a serine/threonine protein kinase of the type III secretion system (*aopO* gene) (Fernández-Álvarez, González, et al., 2016).

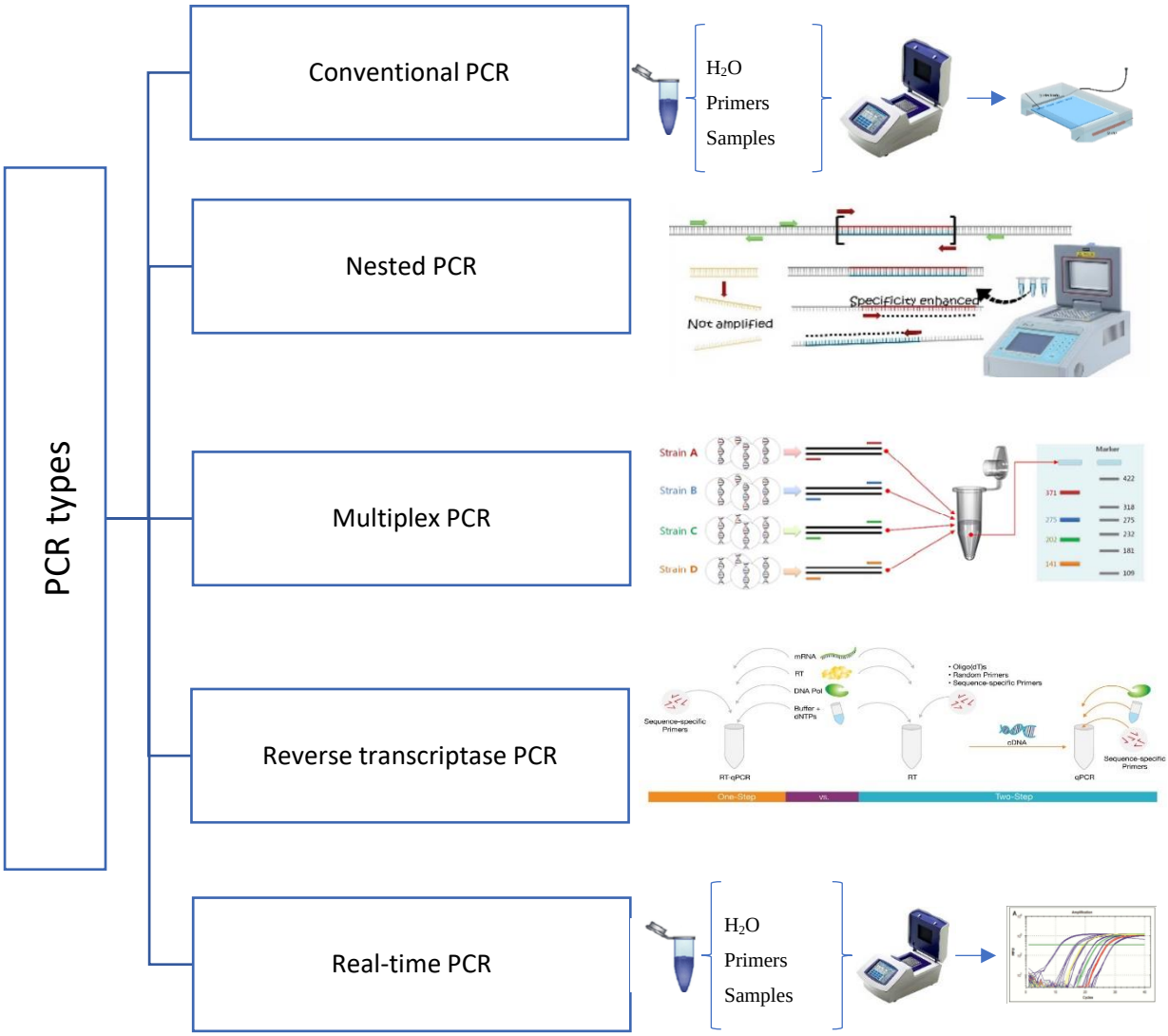

Figure 8. PCR-based diagnostic methods used in aquaculture. Image prepared by the author.

1.2.2.2. Genotypic methods

Microbial genotypic characterization methods can be divided into two broad categories: (1) pattern or fingerprint-based and (2) sequence-based techniques.

Fingerprint-based techniques typically use a systematic method to produce a series of fragments of an organism's chromosomal DNA. These fragments are separated into agarose gels by size to generate a profile, or fingerprint, that is unique to that organism and its close relatives (Emerson et al., 2008). The differences in these fingerprints between organisms are often interpreted as genetic distances. Differences in genetic fingerprints should reflect variations or polymorphisms in DNA sequences rather than

artefacts, so when the profiles of two organisms match, they can be considered very closely related, usually at the strain or species level (Adams and Thompson, 2011; Buller, 2014). Various DNA fingerprinting techniques are currently available for microbiological typing such as PCR-RFLP, ribotyping, random polymorphic DNA amplification (RAPD), repetitive extragenic palindromic (REP)-PCR or enterobacterial repetitive intergenic consensus (ERIC)-PCR.

Ideal requirements for fingerprint techniques are speed and low costs, not requiring prior investment in terms of sequence analysis, primer synthesis or DNA probe characterisation, as well as providing a good level of strain discrimination. For all these reasons, the most widely used DNA amplification-based typing techniques in recent years have been the RAPD-PCR method, based on the use of arbitrarily small sequence primers that hybridise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures, and the repetitive sequence amplification methods, REP-PCR and ERIC-PCR, based on the use of primers that amplify repeated sequences of high homology and a high degree of evolutionary conservation in the bacterial genome (Versalovic et al., 1991; Emerson et al., 2008; Buller, 2014). In this sense, RAPD, REP-PCR and ERIC-PCR techniques have been widely used for the genotype analysis of some of the main fish pathogens such as *S. parauberis* (Romalde et al., 1999), *S. iniae* (Dodson et al., 1999; Bachrach et al., 2001; Keirstead et al., 2014; Kim et al., 2014), *S. agalactiae* (Amal et al., 2013; Kayansamruaj et al., 2014), *V. salmoninarum* (Tanrikul et al., 2014), *L. garvieae* (Ravelo et al., 2003; Varsha and Nampoothiri, 2016a), *A. salmonicida* (Beaz-Hidalgo et al., 2008) and *V. anguillarum* (Vaseeharan et al., 2008).

In bacteriology, the sequence-based technique can be based on the analysis of the sequence of a specific part of bacterial DNA, usually associated with a specific gene, or on the analysis of the entire bacterial genome. These analyses are based on the comparison of target DNA sequences or genomes with extensive genomic databases. The degree of similarity, or coincidence, between the two sequences is a measure of how closely the two organisms are related to each other. Several computer algorithms have been created to compare multiple sequences with each other and build a phylogenetic tree based on the results (Emerson et al., 2008; Austin and Austin, 2016).

The most widely used sequence for both bacterial identification and bacterial typing has been 16S rRNA gene. However, there are bacteria with high similarity in the 16S rRNA gene, for which a more thorough analysis using the multilocus sequence typing (MLST) technique is required. In the MLST studies, partial sequences from 6 to 10 genes coding for proteins with conserved functions (housekeeping genes) are concatenated to establish a single long sequence that can be compared and used to generate phylogenetic trees. This technique has proved useful for epidemiological studies on the bacteria *L. garvieae* (Reguera-Brito et al., 2016), *S. agalactiae* (Jones et al., 2003), *Aeromonas* spp. (Martino et al., 2011; Wamala et al., 2018) or *V. anguillarum* (Steinum et al., 2016).

All these techniques have proven to be useful for many of the bacterial pathogens affecting fish and, in recent years, they have become particularly important to compare

complete bacterial genomes, to determine the average number of identical nucleotides between two genomes as well as to determine antibiotic resistance genes or virulence genes.

1.2.3. Immunological methods

Immunological tests in fish have been widely used for diagnosis of fish diseases, antigenic characterisation of strains for developing vaccines, demonstration of vaccine efficacy, understanding virulence mechanisms of the organism, monitoring the presence or absence of infection or to estimate the prevalence of infection. In the OIE *Manual of Diagnostic Tests for Aquatic Animals* (OIE, 2021) are described some serological tests for diagnosis of bacterial and viral diseases of fish. Immunological tests are based on highly specific and sensitive binding reaction between antigens and antibodies. Development of immunodiagnostic methods had revolutionized aquaculture diagnostics as they are more sensitive and specific than traditional approaches and can be used at the farm level without the aid of instruments. Also, these methods can detect non-culturable micro-organisms. However, the sensitivity and specificity of these techniques lies in the correct antibody selection, specifically polyclonal or monoclonal antibodies (Austin, 2017).

Polyclonal antibodies contain heterogeneous mixed immunoglobulin molecules that can recognize multiple epitopes in a single antigen. The antibodies are usually obtained from the blood of rabbits or small mammals that have been previously immunised with antigens. Polyclonal antibodies are most used for the diagnosis of diseases in aquaculture and the determination of serotypes of bacterial strains. Polyclonal antibodies are used more frequently in aquaculture because their production is cheaper, less time-consuming, and easier to process than monoclonal antibodies. However, the use of polyclonal antibodies has some limitations, such as cross-reactivity, and the inability to discriminate antigen at the epitope level. These limitations could be overcome using monoclonal antibodies but obtaining them requires high technology and qualified personnel. Today there are several commercial monoclonal and polyclonal antibodies against various fish pathogens (González and Santos, 2009; Adams and Thompson, 2011).

The immunological methods most frequently used in the diagnosis of fish pathogens are direct and indirect immunofluorescence (FAT and IFAT), immunohistochemistry (IHC), enzyme-linked immunosorbent assay (ELISA), immunoblot and dot blot, and agglutination techniques (Ooyama et al., 2002a; Kang et al., 2004; Klesius et al., 2006; Adams and Thompson, 2011). FAT and IFAT allow the detection of pathogens in cultured samples of infected fish or directly in infected tissue sections. The success of these techniques lies in their simplicity, sensitivity, and rapidity. However, for FAT and IFAT, fluorescent or confocal microscopes are required to visualize the results, as well as qualified personnel (González and Santos, 2009). IHC allows the detection of pathogens in paraffin-embedded tissue samples after incubation with a pathogen-specific antibody. This is an easy-to-perform method, which has the advantage of allowing co-localisation of bacterial antigens and a lesion, increasing

diagnostic accuracy, and understanding of pathogenesis (González and Santos, 2009; Fernández-Álvarez and Santos, 2018). However, IHC is less sensitive than IFAT. ELISA technique is based on the use of antibodies that react with the pathogen on a plate and a colour change is observed when the sample is positive. It is frequently used to measure the antigens of the pathogen or host antibody response. This technique has the advantage of high throughput and can be automated and provides quantitative values. This assay is particularly useful for detecting and quantifying pathogens during clinical disease but is less useful for subclinical infections due to the sensitivity limits of the assay and may give false negative results or display cross-reactivity with related antigens (Fernández-Álvarez and Santos, 2018). Finally, agglutination and blotting methods are commonly applied for screening, identification and serotyping of fish tissue samples and bacterial culture samples.

1.2.3.1. Agglutination tests

Agglutination tests are based on the generation of a macroscopically visible precipitate due to the formation of large antigen-antibody complexes, usually after 1-2 minutes incubation (Figure 9). This precipitate is formed due to a specific antibody-antigen bond. Agglutination tests can be performed on plates or slides (qualitative antibody detection) (Figure 9a), or on microtitre plates or tubes (quantitative antibody detection) (Figure 9b) (Austin, 2017).

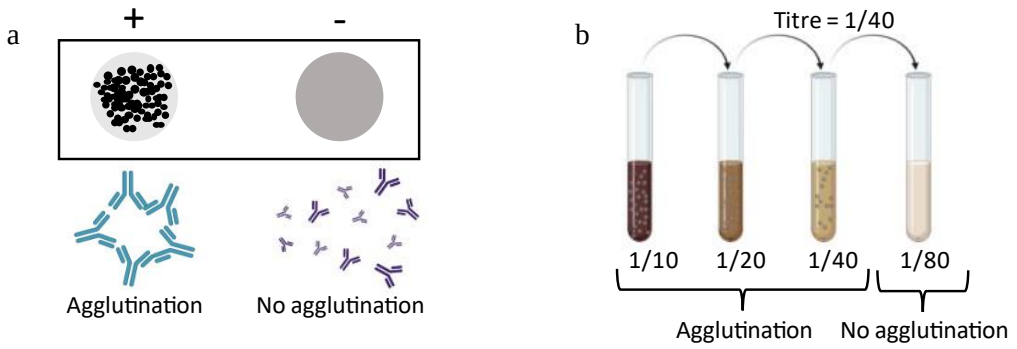

Figure 9. Conventional serological methods. Agglutination test uses the principles of precipitation occurring by formation of large antigen-antibody clumps visible with the naked eye or under microscope; (a) slide agglutination test; (b) tube agglutination test. Image prepared by the author.

These tests can be used for bacteria identification or typing. In bacterial agglutination, dead or inactivated bacteria suspensions are used as antigens. The sites to which antibodies bind are cell wall components or flagella. For agglutination tests, a defined antigen suspension is incubated for 1-2 minutes with an equal volume of antiserum. Each test must be accompanied by a negative and a positive serum control, as well as negative control of the strains to be tested to rule out problems of autoagglutination. Agglutination assays are also used to detect previous contact with the

pathogen. However, it should be noted that negative reactions can be found in an acute phase of infection (Austin, 2017).

Agglutination tests have been successfully used for the differentiation of different bacterial fish pathogens (Buller 2014; Austin and Austin, 2016). However, almost all researchers report that these methods can be ambiguous and difficult to perform, because many institutes and laboratories do not have the anti-serum required to perform the typing (Kanai et al., 2006, 2015). To solve these problems, agglutination kits are commercially available for the rapid preliminary screening of *A. salmonicida* and *V. anguillarum* (BIONORTM MONO-AQUA Test system, Bionor, Spain) (Gonzalez et al., 2004; González and Santos, 2009).

1.2.3.2. Blot methods

Dot blot or immunoblot methods are characterized by the fact that the antigen-antibody reaction occurs on a carrier membrane. In these techniques, the antigens are transferred and fixed to a carrier membrane, usually made of nitrocellulose. The detection of the antigen-antibody binding is based on an enzymatic colour reaction.

The membranes with the fixed antigens are incubated with specific antibodies. Subsequently, the detection of specific antibodies bound to the antigen is performed through enzyme-labelled secondary antibodies. These enzyme-labelled secondary antibodies are responsible of forming visible bands on the carrier membrane as a result of a dye reaction (Austin, 2017).

Dot blot technique is commonly applied for the screening, identification and serotyping of samples of fish tissue and bacterial culture samples. Dot-blot has been applied for identification and serotyping of *L. garvieae* (Eyngor et al., 2004) or *V. anguillarum* (Santos et al., 1995).

On the other hand, the immunoblot technique in aquaculture is mainly used for the identification of pathogens that are difficult to identify based on their proteomic profile, the identification of certain proteins, virulence-related studies and the detection of immunogenetic proteins (Buller, 2014; Austin, 2017). Inmunoblot has been applied for identification and serotyping of *L. garvieae* (Shin et al., 2007; Yesiltas et al., 2019), *S.parauberis* and *S. iniae* (Toranzo et al., 1995; Shin et al., 2006; Nho et al., 2009) or *V. anguillarum* (Santos et al., 1995) *Aeromonas salmonicida* (Fernández-Álvarez et al., 2016a).

Blot techniques provide the advantage of being rapid and sensitive diagnostic methods, as well as being able to specifically detect several immunogenic proteins in a single assay. The major drawbacks of these techniques are that they may require qualified personnel or may give erroneous results because of cross-reactivity between antigens, particularly using polyclonal antibodies.

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

Proteomic and chemo-taxonomic studies have improved comprehension in the microbial world, helping to explore aspects beyond genomics. Applications of studies of proteins and other bacterial components have involved understanding the adaptations of bacteria to the lethal actions of antibiotics, mechanisms of bacterial virulence or the pathogenesis of infectious diseases. These studies may therefore allow the development of new diagnostic and therapeutic strategies in the fight against some of the most lethal bacteria affecting aquaculture.

1.2.4.1. MALDI-TOF

Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) has emerged as a promising approach for diagnosis, discrimination between closely related environmental strains or identification of rare or less frequent microorganisms that are difficult to discriminate with classical techniques (Kim et al., 2015, 2017; Topić Popović et al., 2017; Fernández-Álvarez et al., 2018a). A mass spectrum is a ratio of the ions present in a sample, expressed as a ratio of their mass/charge (m/z) and the relative abundance (intensity) in the sample. The main advantage of MALDI-TOF-MS over other types of mass spectrometry is the analysis of macromolecules with minimal fragmentation. This feature provides an excellent option for microbiology laboratories.

MALDI-TOF methodology typically analyses fingerprint spectra in the range of 2,000 to 20,000 Da, which reflects ribosomal and other housekeeping and structural proteins that are abundant in the bacterial cell and relatively independent of growth state and external stimuli (Welker, 2011; Fernández-Álvarez and Santos, 2018). The sample molecules are ionised in the resulting hot column of ablated gases and are funnelled in a TOF mass spectrometer which records the mass-to-charge (m/z) ratio of the ions. This is achieved by measuring the time it takes for the ions to pass through a known length under the acceleration of an electric field of known strength. The resulting mass spectrum is produced from the pattern (i.e. the position and relative intensity) of the detected m/z peaks, generating a different profile for a sample (Cho et al., 2015; Patel, 2015). This unique pattern produced by the proteins and other biomolecules of the sample analysed will be matched with the spectrum of a known microorganism previously deposited in a database. Considering that each spectrum contains unique mass peaks for each genus, species and even strain, this spectrum will function as a fingerprint that can be matched to identify a microorganism (Cho et al., 2015; Topić Popović et al., 2017).

This technique has been commonly used in microbiological laboratories around the world to identify bacteria, viruses and/or fungi due to its speed, ease of execution and precision. Over the past years, significant efforts have been made to build protein databases for the identification and study of the phyloproteomics of fish bacterial pathogens that are closely related such as *Vibrio* (Erler et al., 2015), *Aeromonas*

(Fernández-Álvarez, Gijón, et al., 2016b; Topić Popović et al., 2017), *Photobacterium* (Pérez-Sancho et al., 2016), *Tenacibaculum* (Bridel et al., 2020; Fernández-Álvarez et al., 2018; Fernández-Álvarez, Torres-Corral, Saltos-Rosero, & Santos, 2017), *Streptococcus* (Kim et al., 2015, 2017) and *Flavobacterium* (Fernández-Álvarez et al., 2018b; Pérez-Sancho et al., 2017). However, MALDI-TOF has also proven valuable for the characterisation of microorganisms for which no reference mass spectra exist, using cluster analysis and principal component analysis of proteomic mass data (Böhme et al., 2011; AlMasoud et al., 2014; Starostin et al., 2015; Fernández-Álvarez and Santos, 2018).

The MALDI-TOF technique's success in recent years is due to several advantages that have made it popular, such as being a non-directed, high-performance method with ease and speed of sample preparation and data acquisition, ease of automation and low sample cost. However, the initial purchase of a MALDI-TOF instrument is relatively expensive (Patel, 2015; Topić Popović et al., 2017).

1.2.4.2. SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) of proteins has been widely used in the last decade for the analysis of bacteria, both for comparative purposes and for the study of their protein biochemistry at the molecular level. The most popular technique is the discontinuous SDS-PAGE technique, first described by Laemmli (Laemmli, 1970), as a method for the separation of proteins into complex mixtures and the determination of their molecular weights. In this technique the protein samples are treated with SDS and a reducing agent, causing the denaturation and negative charge of the proteins, which makes them electrically mobile. Therefore, in the SDS-PAGE the proteins are migrated through a polyacrylamide gel that allows the separation and analysis of the proteins. These proteins will be separated according to their molecular weight.

The SDS-PAGE method has advantages over other taxonomic techniques such as high resolution and good reproducibility, as well as serving as a preliminary step to other techniques such as immunoblot or MALDI-TOF-MS. The SDS-PAGE technique has been widely used in aquaculture to detect and estimate the molecular weight of antigenic bacterial proteins (Toranzo et al., 1995; Van Gelderen et al., 2010), to identify protein antigens possibly associated with virulence or host immunity (Sudheesh et al., 2007), to compare the proteomic profile of different microorganisms for strain identification or typing (Bandín et al., 1993; Nho et al., 2009; Fernández-Álvarez et al., 2016a), as well as to detect variations in the expression or structure of proteins in the cell surface (Santos et al., 1995; Ma et al., 2019b).

1.2.4.3. Analysis of methyl esters of fatty acids (FAME)

The analysis of labelled fatty acids by an automated gas chromatograph (GC) has been found useful to identify nematodes (Sekora et al., 2009), fungi and bacteria (Kunitsky, 2006). In this process, fatty acids are hydrolysed from

phospholipids, triacylglycerols, sterols and other lipid structures. A methyl group is then added to the carboxyl group of fatty acids which forms a methyl ester that acts as a label for the GC, which then reads and identifies those fatty acids. This system was called FAME analysis, as it uses methyl esters of fatty acids (Sasser, 2001; Kunitsky, 2006; Sekora et al., 2009).

The identification of fatty acids with this system is done with retention time measurements, which is the time it takes for a specific fatty acid to pass through the GC column (Sherlock® Analysis Software, MIDI Systems, Inc.). These measurements will be compared with a database of fatty acid retention times of a known sample. The percentage of each fatty acid is calculated by the response rate (measured in the electrical response mV) produced when it passes through the detector at the end of the column over the total response of the sample (Sekora et al., 2009). These measurements will be compared with a database of fatty acid of a known sample.

The MIDI- FAME technique has been successfully applied in the study of the taxonomy (Osterhout et al., 1991; Shoemaker et al., 2005; Huang, 2013; Varsha and Nampoothiri, 2016b) and for the epidemiological typing (Piñeiro-Vidal et al., 2008) of many bacterial species of interest in aquaculture. This is because this technique represents a fast, accurate and economical solution for the identification of a wide range of microbial species.

APPROACHES FOR PREVENTION AND CONTROL OF BACTERIAL FISH DISEASES

Pathogens are one of the main limiting factors in aquaculture production. Poorly managed or handled, these pathogens can develop to unmanageable proportions and contribute to large economic losses. Chemicals and drugs have been used indiscriminately in aquaculture to control pathogens and increase production. However, the indiscriminate use of antibiotics increased the existence of antimicrobial-resistant bacteria as well as drug residues in aquaculture products, becoming one of the major problems that motivate scientist to search for other safe and effective methods (Subramani and Michael, 2017; Soliman et al., 2019).

The integration of management solutions started decades ago as a holistic way to combat infectious outbreaks in aquaculture without relying on chemicals or drugs. The main objective is to apply the One Health approach to achieve sustainable aquaculture production (Stentiford et al., 2020). The One Health principles aim to engage multiple sectors and disciplines to achieve beneficial outcomes for the health and welfare of people, animals, and their shared environment (Figure 10). One Health is an approach increasingly recognised as a means to address the global health challenges that involve the collaboration of multiple stakeholders, such as wider industry, government, producers, scientists, and the public (Stentiford et al., 2020). The One Health measures of success (SM) covering environment, organism, and human health for the development of sustainable aquaculture are shown in Figure 10.

From a human health perspective, food derived from aquaculture must be nutritious and not contaminated with harmful microorganisms or contaminants. Diseases in aquaculture often require treatment with veterinary drugs such as disinfectants or antibiotics. Disinfection is used to prevent the entry or exit of pathogens and their spread in aquaculture facilities. The main methods of disinfection used in aquaculture include chemical and physical treatment or a combination of both. The chemicals most frequently used for this purpose are chloramine T, formalin, hypochlorite (sodium or calcium), iodophors, ozonation, quaternary ammonium compounds, ethyl alcohol, hydrogen peroxide, peracetic acid and formaldehyde. Of these, hydrogen peroxide and peracetic acid stand out as the safest disinfectants since they decompose easily in oxygen and water without harming the environment or the host. On the other hand, the most frequently used physical disinfection is based on the use of UV irradiation, drying or heat treatment (OIE, 2021)

Historically, antimicrobials in aquaculture have been used therapeutically, prophylactically or metaphylactically (Romero et al., 2012; Okocha et al., 2018). However, under Regulation (EU) 2019/6 on veterinary medicinal products, applicable from January 28, 2022, antimicrobials for prophylactic and metaphylactic use can only be used in exceptional cases. According to this regulation, antimicrobials can be used in prophylaxis on an animal or a limited number of animals at high risk of infectious disease with potentially serious consequences. In metaphylactic use, the antimicrobials can be used after the diagnosis of an infectious disease in cases with high risk of spreading an infectious disease in the treated group of animals and no suitable alternatives are available.

Figure 10. One Health measures of success (SM) covering environment, organism, and human health for sustainable aquaculture development. Image prepared by the author.

Currently, all medicines legally used in food animal species, including aquaculture species, must be approved by government agencies responsible for veterinary medicine, such as the Food and Drug administration (FDA) of the United States or the Spanish Agency of Medicines and Medical Devices (SAMMD). These regulatory agencies may establish standards for antibiotic use, including permissible routes of administration, dosage forms, withdrawal times, tolerance and use by species, including dose rates and limitations. Thus, the FDA authorizes the use of oxytetracycline, florfenicol and sulfadimethoxine/ormetprim in aquaculture, while the SAMMD allows the use of flumequine, oxytetracycline and florfenicol for bathing and oral hygiene.

Where there is a no suitable veterinary medicine authorised in your territory for the specific condition in the animal being treated, to avoid unacceptable suffering to the animal, the exceptional prescription regime, commonly known as the prescription “cascade", could be used. The cascade is a risk-based decision tree. Prescribing decisions in accordance with the cascade must be made on a case-by-case basis (“UK National Ecosystem Assessment,” 2021).

In aquaculture, the most common way of administering antimicrobials is to mix them with specially formulated feed, considering the number and weight of the animals to be treated. However, there are other routes of administration, such as immersing the fish in water containing antimicrobials or administering them by injection (Okocha et al., 2018; Santos and Ramos, 2018). Most of these delivery methods involve selective pressure on exposed environments (usually water), either by direct application of the antimicrobials in this environment or through fish feed and faeces containing antimicrobials and unabsorbed antimicrobial metabolites (Okocha et al., 2018; Santos & Ramos, 2018; Subramani & Michael, 2017; Watts, Schreier, Lanska, & Hale, 2017). Therefore, the responsible veterinarian should use different antimicrobials over time to avoid the development of resistance. This management tool is known as a "rotation program" and aims to preserve long-term efficacy by minimizing the selective pressure that increases the level of resistance. The emergence of bacterial resistance poses a high risk to public health due to the development and spread of bacteria and antimicrobial resistance genes (ARGs). Figure 11 shows a schematic of the drivers of ARGs in relation to antimicrobial use in aquaculture.

Bacteria may become resistant to antibiotics because of inherent or intrinsic resistance, where the species is not normally susceptible to a particular drug, or acquired resistance, where bacterial strains or species susceptible to a particular drug may be transformed into resistant strains or species because of transfer of the genes responsible for antibiotic resistance (Romero et al., 2012; Watts et al., 2017). For acquired resistance, bacteria use mostly mobile elements, such as plasmids and transposable elements, to access a large set of ARG that move from a bacterial cell through horizontal gene transfer (HGT) and can then spread through bacterial populations (Watts et al., 2017).

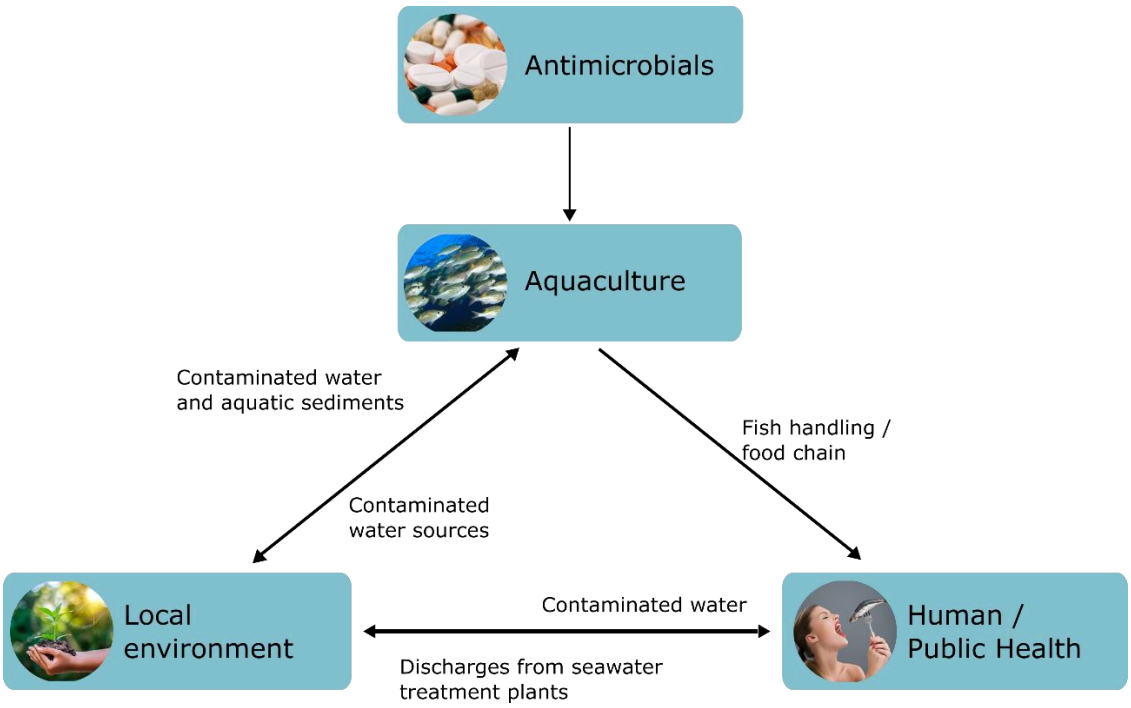

Figure 11. Schematic view of the drivers of ARGs in relation to antimicrobial use in aquaculture. Image prepared by the author.

Recently, numerous globally distributed ARGs have been reported to be detected in aquatic sediments, such as the sulphonamide resistance genes *sul 1* and *sul 2*, the tetracycline resistance genes *tetB*, *tetC*, *tetM*, *tetO* and *tetW*, the quinolone resistance gene *qnrA*, the aminoglycoside resistance gene *aadA* and the β-lactamase resistance genes bla_{TEM} , bla_{SHV} , $bla_{CTX\text{-}M}$ and bla_{NDM} (Yang et al., 2013; Czekalski et al., 2014; Chen et al., 2015). Tetracycline resistance genes have also been identified in marine sediments (Yang et al., 2013). Many ARGs have also been reported in bacteria causing disease in aquaculture, such as *A. salmonicida*, *Aeromonas hydrophila*, *Edwardsiella tarda*, *Edwarsiella ictaluri*, *Yersinia ruckeri*, *Photobacterium damselae* subsp. *piscicida*, *Flavobacterium psychrophilum*, *S. iniae*, *S. parauberis*, *S. agalactiae* and *L. garvieae* and *Vibrio anguillarum*. These ARGs may be shared with human pathogens, and some appear to have originated from fish pathogens or aquatic sediments.

Prevention of infectious diseases can be achieved through a combination of measures such as biosecurity, hygiene, feeding, establishment of husbandry conditions that ensure animal welfare, implementation of surveillance systems and immunization protocols through vaccination. Vaccination has been an essential tool over time in preventing many infectious diseases. Vaccination plays an important role in One Health approach in the protection of animal and public health, as part of integrated health programs against infectious diseases. Vaccination is also an important part of effective and early response strategies to prevent the spread of emerging infectious diseases. In addition, vaccination can reduce the need for antibiotic use, contributing to the fight against the emergence of resistance (Adams and Thompson, 2011; Subramani and Michael, 2017).

Vaccination is the most appropriate method of controlling fish diseases, contributing to the environmental, social, and economic sustainability of global aquaculture. Vaccines are non-pathogenic preparations of the pathogen that induce an adaptative immune response in the host, enabling it to recognize and destroy the pathogen when it encounters it later. However, vaccination is too expensive for widespread use by fish producers and since it is extremely difficult to develop multiple strain vaccines, most vaccines are only effective against one type of pathogen (Austin and Austin, 2016).

Since the first reported use of fish vaccines for disease prevention in the 1940s, several vaccines have been developed to reduce the impact of some bacterial and viral diseases in fish. Vaccines are considered an environmentally friendly approach to disease prevention compared to antibiotics, so in some areas of the world there has been a transition from the use of antibiotics to the use of vaccination (Ma et al., 2019a).

The use of vaccines by the aquaculture industry has grown rapidly in recent years, both regarding the number of fish species and the number of microbial diseases addressed (Håstein et al., 2005; Evensen, 2009). More than 26 licensed fish vaccines are available worldwide for use in various fish species to date (Table 3). Many of these vaccines use conventional production methods consisting mainly of formalin-killed bacteria, although some live attenuated or subunit protein vaccines (formulated with adjuvants) have also been commercialised (Ma et al., 2019a).

Currently it is recognised that many of these dead or inactivated vaccines can induce weak immunogenicity because of poor activation of cellular immunity in fish species and therefore may require the use of adjuvants or multiple booster doses to induce protective immunity. Modern technology has therefore focused on the development of vaccines using specific pathogenic components. Vaccines developed using these modern approaches may include subunit or recombinant DNA vaccines containing new antigens produced by various expression systems. In general, these advances are promising; however, the reality has been somewhat limited in the case of aquaculture due in part to the challenges of the aquatic environment and the practical application of mass vaccination due to the nature of fish farming (Subramani and Michael, 2017; Ma et al., 2019a).

Vaccination by injection is the most popular method of administering vaccines, especially with the introduction of automated vaccination machines to help vaccinate fish in mass. However, injection vaccination can cause local reactions at the injection site and is not feasible to use with larvae because it is difficult and labour intensive. This is a major drawback since most fish mortalities occur at larval stages (Håstein et al., 2005; Subramani and Michael, 2017).

To overcome these problems, oral administration in feed or immersion methods are available. During vaccination by immersion the fish are kept in the vaccine solution. Fish can receive the vaccine through their mucosal surfaces present in gills and skin and, produce both systemic and mucosal antibodies that lead to protection against the

disease. Immersion vaccination is ideal for immunising larvae and large numbers of fish without stressing them. Immunisation by immersion, however, only confers protection for a limited period and therefore requires subsequent repeated administration of antigens (Subramani and Michael, 2017; Ma et al., 2019a). On the other hand, oral vaccination is the most affordable method, especially in large-scale intensive farming. In this method, bacterial suspensions are mixed directly with the feed and given to the fish. As there are no handling procedures or stress factors, it can be used routinely to immunise the entire fish population, even many times. The disadvantage of oral immunisation is that there are ambiguous results regarding the effectiveness and duration of protection. Thus, oral immunisation is mostly used to give regular booster shots for vaccines that were administered by other means (Subramani and Michael, 2017).

Table 3. Overview of the main licensed fish vaccines that have been used in global aquaculture. Table elaborated from Ma, Bruce, Jones, et al., 2019

Disease	Pathogen	Major Fish Host	Vaccine type	Antigens/Targets	Administration method	Country/Region
Enteric redmouth disease (ERM)	*Yersinia ruckeri*	Salmonids	Inactivated	Inactivated *Y. ruckeri*	IMM or oral	USA, Canada, Europe
Vibriosis	*Vibrio anguillarum* *Vibrio ordalii* *Vibrio salmonicida*	Salmonids, ayu, grouper, sea bass, sea bream, yellowtail, cod, halibut	Inactivated	Inactivated *Vibrio* spp.	IP or IMM	USA, Canada, Japan, Europe, Australia
Enteric septicemia of catfish (ESC)	*Edwardsiella ictalurii*	Catfish	Inactivated	Inactivated *E. ictaluri*	IP	Vietnam
Columnaris disease	*Flavobacterium columnare*	All freshwater finfish species, bream, bass, turbot, salmon	Attenuated	Attenuated *F. columnare*	IMM	USA
Furunculosis	*Aeromonas salmonicida* subsp. *salmonicida*	Salmonids	Inactivated	Inactivated *A. salmonicida* spp.	IP or IMM	USA, Canada, Chile, Europe, Australia
Wound Disease	*Moritella viscosa*	Salmonids	Inactivated	Inactivated *M. viscosa*	IP	Spain
Streptococcosis	*Streptococcus agalactiae*	Tilapia	Inactivated	Inactivated *S. agalactiae*	IP	Taiwan, Province of China, Japan, Brazil, Indonesia
Streptococciosis	*Streptococcus iniae*	Tilapia	Inactivated	Inactivated *S. iniae*	IP or IM	Taiwan, Province of China, Japan, Brazil, Indonesia
Pasteurellosis	*Photobacterium damselae*	Seabream	Inactivated	Inactivated *P. damselae* subsp.	IM	Spain

				piscicida		
Pasteurellosis	*Pasteurella piscicida*	Sea bass, sea bream, sole	Inactivated	Inactivated *P. piscicida*	IMM	USA, Europe, Taiwan, Japan
Tenacibaculosis	*Tenacibaculum maritimum*	Turbot	Inactivated	Inactivated *T. maritimum*	IP	Spain
Motile *Aeromonas* septicemia (MAS)	*Aeromonas* spp.	Striped catfish and salmonids	Inactivated	Inactivated Aermonas spp.	IP	Vietnam
Bacterial Kidney Disease (BKD)	*Renibacterium salmoninarum*	Salmonids	Avirulent live culture	Arthrobacter davidanieli	IP	Canada, Chile, USA
Salmonid rickettsial septicemia	*Piscirickettsia salmonis*	Salmonids	Inactivated	Inactivated *P. salmonis*	IP	Chile
Lactococcosis	*Lactococcus garvieae*	Rainbow trout, amberjack, yellowtail	Inactivated	Inactivated *L. garvieae*	IP	Spain

IMM: Immersion inmunization; IP: intraperitoneal injection inmunization

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CHAPTER 2. General objectives

Bacterial diseases in aquaculture are responsible for large economic losses and represent a risk to human health in terms of food safety. Pathogenic bacteria can be introduced to aquaculture at every stage of production. As part of a One Health approach, it is crucial for farmers to understand the occurrence, ecology and dynamics of the etiological agents present along the aquaculture chain. Therefore, new molecular, serological, and proteomic methods for rapid and accurate identification of these etiological agents are essential, as well as monitoring them. In addition, the routine use of antimicrobials in the treatment of diseased fish has contributed to the emergence of drug-resistant bacteria. The resistant bacteria could enter the human food chain via direct contact with humans or through contaminated aquaculture products. New vaccines have been developed to prevent and control bacterial disease outbreaks because of this worrying situation. This PhD work has focused on the One Health concept to manage the current concern of emerging and re-emerging bacterial diseases of fish produced by *Streptococcus* sp., *Lactococcus* sp., *Vagococcus* sp., *Aeromonas* sp. and *Vibrio* sp. The specific objectives of this work were:

1. Evaluation of molecular, serological, and proteomic techniques for the identification, typing, and monitoring of fish pathogenic bacteria.

2. Phenotypic, genotypic, and proteomic profiling analysis to determine antimicrobial susceptibility and multidrug resistance of fish pathogenic bacteria.

3. Development of molecular methods for the identification and quantitative detection of fish pathogenic bacteria and their differentiation from closely related species

4. Study of the efficacy and safety of new vaccines for the prevention of bacterial diseases in fish.

The following reference strains (Table 1) obtained from culture collections and clinical strains of *Streptococcus* spp., *Lactococcus garvieae*, *Vagococcus salmoninarum*, *Aeromonas salmonicida* subsp. *achromogenes* and *Vibrio anguillarum* isolated from clinical specimens (Table 2, 3, 4 and 5) were used to carry out these objectives:

Table 1. Reference strains used in the present work.

Strain	Species	Origin
CECT 7363	*Streptococcus iniae*	*Inia geoffrensis*
NCDO 2020	*Streptococcus parauberis*	Cattle
NCIMB 703043	*S. parauberis*	*Scopthalmus maximus*
DSM 16828	*Streptococcus agalactiae*	*Oreochromis niloticus*
CECT 183	*S. agalactiae*	Fish
CECT 958	*Streptococcus suis*	Pig
CECT 479	*Streptococcus mutans*	Carious dentine
KCCM 40105	*S. mutans*	Carious dentine
CECT 994	*Streptococcus uberis*	Unknown
CECT 985	*Streptococcus pyogenes*	Scarlet fever
CECT 5810	*Vagococcus salmoninarum*	*Oncorhynchus mykiss*
NCDO 2155	*Lactococcus garvieae*	Bovine mastitis
CECT 5274	*L. garvieae*	*Seriola quinqueradiata*
CECT 4493	*Lactococcus piscium*	*Oncorhynchus mykiss*
CECT 185	*Lactococcus lactis* subsp. *lactis*	Food
KCTC 3769	*L. lactis* subsp. *lactis*	Human
ATCC 35586	*Carnobacterium maltaromaticum*	*Salmo clarkii*
CECT 4016	*Carnobacterium divergens*	Minced beef
CECT 934	*Listeria monocytogenes*	Brain of sheep
ATCC 33209	*Renibacterium salmoninarum*	*Oncorhynchus tshawytscha*
ATCC 33658	*Aeromonas salmonicida* subsp. *salmonicida*	*Salmo salar*
NCIMB 2261	*A. salmonicida* subsp. salmonicida	*S. salar*
CECT 895	*A. salmonicida* subsp. *achromogenes*	*Salmo trutta*
CECT 896	*A. salmonicida* subsp. *masoucida*	*Oncorhynchus masou*
CECT 4330	*Aeromonas hydrophila*	Water from an eel farm
CECT 7443	*Aeromonas piscicola*	*Salmo salar*
NCIMB 2034	*Edwarsiella tarda*	Human faeces
CECT 849	*E. tarda*	Human faeces
CECT 99	*Escherichia coli*	Unknown
NCIMB 2154	*Tenacibaculum maritimum*	*Pagrus major*
NCIMB 14598	*Tenacibaculum dicentrarchi*	*Dicentrarchus labrax*
DSM 18841	*Tenacibaculum gallaicum*	Seawater turbot tank
CECT 7292	*Tenacibaculum soleae*	*Solea senegalensis*
ATCC 43306	*Vibrio anguillarum*	*Gadus morhua*
NCIMB 1900	*Vibrio Pelagius*	Seawater
NCIMB 13384	*Flavobacterium psychrophilum*	*O. mykiss*
ATCC 35035	*Flavobacterium branchiophilum*	*Oncorhynchy masou*
CECT 7844	*Flavobacterium plurextorum*	Eggs of rainbow trout
CECT 955	*Yersinia ruckeri*	*Salmo gairdneri*
CECT 108	*Pseudomonas aeruginosa*	Blood

Table 2. Clinical strains used in the present work.

Strain	Species	Origin (year of isolation), country

Streptococcus parauberis strains		
SK466/1	*S. parauberis*	*Scopthalmus* maximus (2001), Spain
Sp01.1	*S. parauberis*	*S. maximus* (2001), Spain
SK466/2	*S. parauberis*	*S. maximus* (2002), Spain
SK272/04	*S. parauberis*	*S. maximus* (2004), Spain
SK451/04	*S. parauberis*	*S. maximus* (2004), Spain
SK415/04	*S. parauberis*	*S. maximus* (2004), Spain
SK644/04	*S. parauberis*	*S. maximus* (2004), Spain
SK17/05	*S. parauberis*	*S. maximus* (2005), Spain
SK145/05	*S. parauberis*	*S. maximus* (2005), Spain
SK537/10	*S. parauberis*	*S. maximus* (2010), Spain
NUF1003[a]	*S. parauberis*	*Paralichthys olivaceus*, Japan
NUF1032[a]	*S. parauberis*	*P. olivaceus*, Japan
KRS02083[a]	*S. parauberis*	*P. olivaceus*, Japan
2007-1[a]	*S. parauberis*	*P. olivaceus*, Japan
NUF1095[a]	*S. parauberis*	*P. olivaceus*, Japan
NUF1071[a]	*S. parauberis*	*P. olivaceus*, Japan
Sp15.1	*S. parauberis*	*P. olivaceus* (2015), Spain
Sp15.2	*S. parauberis*	*P. olivaceus* (2015), Spain

Streptococcus iniae strains		
Si314	*S. iniae*	Marine fish (2010), Spain
Si315	*S. iniae*	Marine fish (2010), Spain
Si317	*S. iniae*	Marine fish (2010), Spain
Si318	*S. iniae*	Marine fish (2010), Spain
Si322	*S. iniae*	Marine fish (2010), Spain
Si**323**	*S. iniae*	Marine fish (2010), Spain
Si324	*S. iniae*	Marine fish (2010), Spain
Si**325**	*S. iniae*	Marine fish (2010), Spain
Si335	*S. iniae*	Marine fish (2010), Spain
Si336	*S. iniae*	Marine fish (2010), Spain
Si337	*S. iniae*	Marine fish (2010), Spain
Si341	*S. iniae*	Marine fish (2010), Spain
Si349	*S. iniae*	Marine fish (2010), Spain
Si350	*S. iniae*	Marine fish (2010), Spain
Si351	*S. iniae*	Marine fish (2010), Spain
Si356	*S. iniae*	Marine fish (2010), Spain
Si363	*S. iniae*	Marine fish (2010), Spain
Si367	*S. iniae*	Marine fish (2010), Spain
Si372	*S. iniae*	Marine fish (2010), Spain
Si404	*S. iniae*	Marine fish (2010), Spain
Si408	*S. iniae*	Marine fish (2010), Spain
Si415/10	*S. iniae*	Marine fish (2010), Spain
Si420	*S. iniae*	Marine fish (2010), Spain
Si429	*S. iniae*	Marine fish (2010), Spain
Si444	*S. iniae*	Marine fish (2010), Spain
Si503	*S. iniae*	Marine fish (2010), Spain
Si528	*S. iniae*	Marine fish (2010), Spain

Si Li-bar	*S. iniae*	Marine fish (2018), Spain
	***Streptococcus uberis* strains**	
196 (H)	*S. uberis*	Unknown, Spain
	***Streptococcus agalactiae* strains**	
SaCSp08.18.4	*S. agalactiae*	Marine fish (2018), Spain
SaCSp08.18.1	*S. agalactiae*	Marine fish (2018), Spain
SaCSp08.18.9	*S. agalactiae*	Marine fish (2018), Spain
	***Vagococcus salmoninarum* strains**	
MM2339/92[b]	*V. salmoninarum*	*Oncorhynchus mykiss* (1992), France
JIP 24/98[b]	*V. salmoninarum*	*O. mykiss* (1998), France
JIP 30/99[b]	*V. salmoninarum*	*O. mykiss* (1999), France
JIP 20/00[b]	*V. salmoninarum*	*Carassius carassius* (2000), France
JIP 27/01	*V. salmoninarum*	*O. mykiss* (2001), Spain
SK379/05	*V. salmoninarum*	*O. mykiss* (2001), Spain
SK389/05	*V. salmoninarum*	*O. mykiss* (2005), Spain
A/362[c]	*V. salmoninarum*	*O. mykiss*, Spain
SK241/09	*V. salmoninarum*	*O. mykiss* (2009), Spain
SK23/11	*V. salmoninarum*	*O. mykiss* (2011), Spain
SK42/11	*V. salmoninarum*	*O. mykiss* (2011), Spain
OJOS7	*V. salmoninarum*	*O. mykiss* (2012), Spain
OJOS9	*V. salmoninarum*	*O. mykiss* (2012), Spain
SK104/12	*V. salmoninarum*	*O. mykiss* (2012), Spain
SK147/12	*V. salmoninarum*	*O. mykiss* (2012), Spain
SK154/12	*V. salmoninarum*	*O. mykiss* (2012), Spain
SK246/12	*V. salmoninarum*	*O. mykiss* (2012), Spain
SK85/17	*V. salmoninarum*	*O. mykiss* (2017), Spain
	***Lactococcus garvieae* strains**	
JIP 31/90[b]	*L. garvieae*	*Oreochromis niloticus* (1990), France
JIP 27/99[b]	*L. garvieae*	*Oncorhynchus mykiss* (1999), France
Lg02.1	*L. garvieae*	*O. mykiss* (2002), Spain
Lg05.1	*L. garvieae*	*O. mykiss* (2005), Spain
Lg06.1	*L. garvieae*	*O. mykiss* (2006), Spain
Lg08.1	*L. garvieae*	*O. mykiss* (2008), Spain
Lg09.1	*L. garvieae*	*O. mykiss* (2009), Spain
Lg11.1	*L. garvieae*	*O. mykiss* (2011), Spain
Lg11.2	*L. garvieae*	*O. mykiss* (2011), Spain
Lg14.1	*L. garvieae*	*O. mykiss* (2014), Spain
Lg14.2	*L. garvieae*	*O. mykiss* (2014), Spain
Lg14.3	*L. garvieae*	*O. mykiss* (2014), Spain
Lg14.4	*L. garvieae*	*O. mykiss* (2014), Spain
Lg15.1	*L. garvieae*	*O. mykiss* (2015), Spain
Lg15.2	*L. garvieae*	*O. mykiss* (2015), Spain
Lg15.3	*L. garvieae*	*O. mykiss* (2015), Spain
Lg15.4	*L. garvieae*	*O. mykiss* (2015), Spain

Lg15.5	*L. garvieae*	*O. mykiss* (2015), Spain
Lg6.15	*L. garvieae*	*O. mykiss* (2015), Spain
Lg16.1	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.2	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.3	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.4	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.5	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.6	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.7	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.8	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.9	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.10	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.11	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.12	*L. garvieae*	*O. mykiss* (2016), Spain
Lg16.13	*L. garvieae*	*O. mykiss* (2016), Spain
Lg17.1	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.2	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.3	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.4	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.5	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.6	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.7	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.8	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.9	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.10	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.11	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.12	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.13	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.14	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.15	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.16	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.17	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.18	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.19	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.20	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.21	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.22	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.23	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.24	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.25	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.26	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.27	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.28	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.29	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.30	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.31	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.32	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.33	*L. garvieae*	*O. mykiss* (2017), Spain
Lg17.34	*L. garvieae*	*O. mykiss* (2017), Spain

Lg18.1	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.2	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.3	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.4	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.5	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.6	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.7	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.8	*L. garvieae*	*O. mykiss* (2018), Spain
Lg18.9	*L. garvieae*	*O. mykiss* (2018), Spain
Lg7[d]	*L. garvieae*	*O. mykiss*, Turkey
1684[e]	*L. garvieae*	*Anguilla Anguilla*
H-14[e]	*L. garvieae*	Bird faeces
16007529-B[e]	*L. garvieae*	Bloom algal
MAM-77[e]	*L. garvieae*	Bovine mastitis
Lg1[d]	*L. garvieae*	*O. mykiss* Turkey
Lg11	*L. garvieae*	*O. mykiss*
CLFP-33[e]	*L. garvieae*	*O. mykiss* Spain
Lg6[d]	*L. garvieae*	*O. mykiss* Turkey
Lg5[d]	*L. garvieae*	*O. mykiss* Turkey
Lg4[d]	*L. garvieae*	*O. mykiss* Turkey
Lg3[d]	*L. garvieae*	*O. mykiss* Turkey
Lg2[d]	*L. garvieae*	*O. mykiss* Turkey
Lg8[d]	*L. garvieae*	*O. mykiss* Turkey
Lg9[d]	*L. garvieae*	*O. mykiss* Spain
Lg10	*L. garvieae*	*O. mykiss* Spain
1481/03[e]	*L. garvieae*	Repiratory process pig
156(H)[e]	*L. garvieae*	Urinary tract infection
138(H)[e]	*L. garvieae*	Urinary tract infection
BA06/02133[e]	*L. garvieae*	Water
DP-1[e]	*L. garvieae*	Repiratory process pig
***Aeromonas salmonicida* subsp. *achromogenes* strains**		
R0.16.01.01	*A. salmonicida* subsp. *achromogenes*	*Scopthalmus* maximus (2001), Spain
Asa-21.09.01	*A. salmonicida* subsp. *achromogenes*	*S.* maximus (2001), Spain
Asa-16.01.02	*A. salmonicida* subsp. *achromogenes*	*S.* maximus (2002), Spain
RO16.02.02	*A. salmonicida* subsp. *achromogenes*	*S.* maximus (2002), Spain
***Vibrio anguillarum* strains**		
R0.12.04.01	*V. anguillarum*	*S.* maximus (2001), Spain
RO15.11.2	*V. anguillarum*	*S.* maximus (2002), Spain

a, strains provided by Dr. Kinya Kanai; b, strains provided by Dr. Jean-Francois Bernardet, c, strains provided by Dr. Imanol Ruiz-Zarzuela; d, strains provided by Dr. Behire Işıl Didinen and e, strains provided by Dr. Alicia Gibello.

CHAPTER 3. Evaluation of molecular, serological and proteomic techniques for the identification and tracking of pathogenic fish bacteria

Article nº 1: Torres-Corral, Y., Fernández-Álvarez, C., Santos, Y. (2019). Proteomic and molecular fingerprinting for identification and tracking of fish pathogenic *Streptococcus*. Aquaculture, 498, 322-334. ISSN: 0044-8486. Doi: 10.1016/j.aquaculture.2018.08.041.

Title: Proteomic and molecular fingerprinting for identification and tracking of fish pathogenic *Streptococcus*.

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

Affiliation: Departamento de Microbiología y Parasitología, Edificio CIBUS Facultad de Biología and Instituto de Investigación y Análisis Alimentario, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

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Article nº 2: Torres-Corral, Y., Santos, Y. (2019). Identification and typing of *Vagococcus salmoninarum* using genomic and proteomic techniques. Journal of fish diseases, 42 (4), 597-612. ISSN: 0140-7775. DOI: 10.1111/jfd.12967.

Title: Identification and typing of *Vagococcus salmoninarum* using genomic and proteomic techniques.

Authors: Yolanda Torres-Corral, Ysabel Santos

Affiliation: Departamento de Microbiología y Parasitología, Instituto de Análisis Químico y Biológico (IIAQBUS), Universidad de Santiago de Compostela, Santiago de Compostela, Spain.

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Torres-Corral and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Article nº 3: Torres-Corral, Y., Santos, Y. (2021). Clonality of *Lactococcus garvieae* isolated from rainbow trout cultured in Spain: a molecular, immunological, and proteomic approach. Aquaculture, 545, 737190. ISSN: 0044-8486. Doi: 10.1016/j.aquaculture.2021.737190

Title: Clonality of *Lactococcus garvieae* isolated from rainbow trout cultured in Spain: a molecular, immunological, and proteomic approach.

Authors: Yolanda Torres-Corral, Ysabel Santos

Affiliation: Departamento de Microbiología y Parasitología, Instituto de Análisis Químico y Biológico (IIAQBUS), Universidad de Santiago de Compostela, Santiago de Compostela, Spain.

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Article nº 4: Torres-Corral, Y., Santos, Y. (2022). Predicting antimicrobial resistance of *Lactococcus garvieae*: PCR detection of resistance genes versus MALDI-TOF protein profiling. Aquaculture, 553, 738098. ISSN: 0044-8486. Doi: 10.1016/j.aquaculture.2022.738098

Title: Predicting antimicrobial resistance of *Lactococcus garvieae*: PCR detection of resistance genes versus MALDI-TOF protein profiling.

Authors: Yolanda Torres-Corral, Ysabel Santos

Affiliation: Departamento de Microbiología y Parasitología, Instituto de Análisis Químico y Biológico (IIAQBUS), Universidad de Santiago de Compostela, Santiago de Compostela, Spain.

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Santos. The first draft of the manuscript was written by Yolanda Torres-Corral and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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CHAPTER 4. Development of molecular methods for the identification and quantification of bacterial fish pathogens and its differentiation from closely related species.

Article nº 5: Torres-Corral, Y., Fernández-Álvarez, C., Santos, Y. (2019). High-throughput identification and quantification of *Vagococcus salmoninarum* by SYBR Green I-based real-time PCR combined with melting curve analysis. Journal of fish diseases, 42(10), 1359-1368. ISSN: 0140-7775. Doi: 10.1111/jfd.13053.

Title: High-throughput identification and quantification of *Vagococcus salmoninarum* by SYBR Green I-based real-time PCR combined with melting curve analysis.

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

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Article nº 6: Torres-Corral, Y., Santos, Y. (2021). Development of a real-time PCR assay for detection and quantification of *Streptococcus iniae* using the lactate permease gene. Journal of fish diseases, 44(1), 53-61. ISSN: 0140-7775. Doi: 10.1111/jfd.13267

Title: Development of a real-time PCR assay for detection and quantification of *Streptococcus iniae* using the lactate permease gene.

Authors: Yolanda Torres-Corral, Ysabel Santos

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Article nº 7: Torres-Corral, Y., Santos, Y. (2020). Comparative genomics of *Streptococcus parauberis*: new targets for molecular diagnosis of serotype III. Applied microbiology and biotechnology, 104(14), 6211-6222. ISSN: 0175-7598. Doi: 10.1007/s00253-020-10683-z

Title: Comparative genomics of *Streptococcus parauberis*: new targets for molecular diagnosis of serotype III.

Authors: Yolanda Torres-Corral, Ysabel Santos

Affiliation: Departamento de Microbiología y Parasitología, Instituto de Análisis Químico y Biológico (IIAQBUS), Universidad de Santiago de Compostela, Santiago de Compostela, Spain.

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CHAPTER 5. Efficacy and safety of new vaccines for the prevention of bacterial diseases in fish

Article nº 8: Torres-Corral, Y., González-Barreiro, O., Riaza, A., Santos, Y. (2022). Establishment of different challenge models for *Aeromonas salmonicida* subsp. *achromogenes* in turbot and sole. Aquaculture, 555, 738261. ISSN: 0044-8486. Doi: 10.1016/j.aquaculture.2022.738261

Title: Establishment of different challenge models for *Aeromonas salmonicida* subsp. *achromogenes* in turbot and sole.

Authors: Yolanda Torres-Corral, Óscar González-Barreiro, Ana Riaza, Ysabel Santos

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Article nº 8: Torres-Corral, Y. Girons, A., González-Barreiro, O. , Seoane, R. , Riaza, A. and Santos, Y. (2021). Effect of bivalent vaccines against *Vibrio anguillarum* and *Aeromonas salmonicida* subspecie *achromogenes* on health and survival of turbot. Vaccines, 9(8), 906. Doi: 10.3390/vaccines9080906

Title: Effect of bivalent vaccines against *Vibrio anguillarum* and *Aeromonas salmonicida* subspecie *achromogenes* on health and survival of turbot.

Authors: Yolanda Torres-Corral, Albert Girons, Óscar González-Barreiro, Rafael Seoane, Ana Riaza, Ysabel Santos

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CHAPTER 6. General discussion

Aquaculture is the fastest growing food production sector in the world. In recent years, the trend in aquaculture is to increase the intensification and commercialisation of aquaculture production. This trend has led to an increase in diseases and problems caused by viruses, bacteria, fungi, parasites, and other emerging and undiagnosed pathogens. Diseases in aquaculture are the main constraint to the farming of aquatic species, limiting the economic and social development of aquaculture in many countries. Production costs increase with disease outbreaks due to lost investment in dead production, treatment costs and reduced growth during convalescence (Toranzo et al., 2005; Buller, 2014; Austin and Austin, 2016). In addition, as aquaculture expands and new species are cultured, new diseases emerge that adversely affect both wild and farmed fish. The rate and the extent of occurrence can be reduced by implementing biosecurity programmes aimed at mitigating the risk factor for disease emergence (Murray and Peeler, 2005). To address these problems, the universal "One Health" approach, a model to promote synergy between the disciplines of human, animal, and environmental health sciences, should be applied and expanded.

The general aims of the present research were: 1) the evaluation of molecular, serological and proteomic techniques for the identification and monitoring of fish pathogenic bacteria (Chapter III), 2) the phenotypic, genotypic, and proteomic profiling analysis to determine antimicrobial susceptibility and multidrug resistance of fish pathogenic bacteria (Chapter III), 3) the development of molecular diagnostic techniques that are more sensitive and specific and less laborious than the conventional methods, for the identification and/or quantification of pathogenic bacteria in the tissues of diseased fish (Chapter IV) and 3) the evaluation of the efficacy and safety

of new vaccines for the prevention of bacterial diseases in fish (Chapter V).

The first part of this work (Chapter III) described the potential of different diagnostic methods for rapid differentiation and typing of strains isolated from fish with signs of streptococcosis. Streptococcosis is considered a complex of similar diseases caused by different genera and species capable of inducing central nervous system damage characterised by exophthalmia and meningoencephalitis (Austin & Austin, 2016; Buller, 2014; Ghittino et al., 2003; Yardimci et al., 2016). Therefore, in Chapter III of this PhD work, fish pathogenic bacterial strains belonging to the genera *Streptococcus* (article nº 1), *Vagococcus* (article nº 2) and *Lactococcus* (article nº 3) were characterised and typed using molecular, serological, and proteomic techniques. These studies are essential to understand the epidemiological factors related to the bacteria (host, distribution, virulence) that help to define optimal disease management and prevention strategies (Austin & Austin, 2016; Buller, 2014; Toranzo et al., 2005) using a coordinated and multi-sectoral approach, such as One Health.

Conventional methods based on bacterial isolation and phenotypic characterization are the most used methods in clinical laboratories to identify the causative agent of a disease; however, these methods take at least a day and often do not provide reliable identification of the pathogen (Buller, 2014; Austin and Austin, 2016). Serotyping of bacterial isolates is performed for diagnostic, epidemiological purposes and for the development and implementation of effective management strategies (Buller, 2014; Shin et al., 2009), although it can provide unreliable identifications in species with high antigenic heterogeneity and has the major limitation of the need to obtain

antisera from animals. Therefore, new diagnostic and typing methods based on bacterial DNA amplification have been developed (Hassan et al., 2001; Jung et al., 2010; Mata et al., 2004; Nguyen et al., 2016; Tu et al., 2015). In Chapter III, rapid and low-cost molecular tools (RAPD, REP-PCR and ERIC-PCR) have been used to type Gram-positive strains isolated from fish with clinical signs of streptococcosis.

This chapter also tested the capability of matrix-assisted laser desorption ionisation-assisted laser desorption ionisation mass spectrometry (MALDI-TOF-MS) for the identification and typing of streptococcosis-causing bacteria in fish in two approaches (i) using the commercial database Bruker Biotyper (V. 3.1) to compare proteomic profiles and (ii) using multivariate statistical analyse. MALDI-TOF-MS is a technique that has emerged in recent years as a promising technology for rapid, accurate and cost-effective identification of microorganisms isolated in clinical laboratories, food processing and many diverse settings (Suarez et al., 2013; Erler et al., 2015; Zhu et al., 2015; Assis et al., 2017; Topić Popović et al., 2017).

Article nº 1 derived from this study described the discriminatory power of serological (dot-blot and agglutination tests), genetic (REP-PCR), and proteomic (MALDI-TOF-MS) methods for the differentiation of *S. parauberis*, *S. iniae*, *S. agalactiae* y *S. uberis* pathogenic for fish, epidemiological studies, and strain typing. All these methods showed concordant results, being able to differentiate *Streptococcus* strains according to their taxonomic position. Furthermore, this work demonstrated that strains of *S. parauberis* are antigenically, proteomically and genetically heterogeneous. Kanai et al (Kanai et al., 2009, 2015) reported the existence of serotype I (with three subtypes, Ia, Ib and Ic), serotype II and non-typeable

strains within *S. parauberis* isolated from Japanese flounder. However, in the present work (article nº 1) we reported the existence of new potential serotypes in strains of *S. parauberis* isolated from turbot (defined as serotype III) and in the reference strain NCDO 2020 isolated from a mastitis milk sample (defined as serotype IV). *S. parauberis* serotype III was confirmed by MALDI-TOF-MS, REP-PCR, and multiplex PCR techniques. REP-PCR revealed a specific molecular profile for each *S. parauberis* serotype (serotypes I, II, III and non-typeable), providing a useful and low-cost tool to predict *S. parauberis* serotype. Moreover, MALDI-TOF-MS using the Biotyper V. 3.1. database identified 61% *of S. parauberis* strains to genus level. This low success rate is probably due to a lack of proper or sufficient spectral signals of these bacteria in this reference database. Subsequent statistical analyses performed in this work allowed the detection of a *Streptococcus* genus-specific biomarker at *m/z* 4451.60, presumably assigned to the large subunit (50S) ribosomal protein L-36 by sequence comparison. In addition, *S. parauberis*-specific biomarkers at *m/z* 4752.78 and 9501.23 were detected and assigned to a phage protein and a phosphotransferase system (PTS) protein, respectively. Peak mass patterns associated with *S. parauberis* serotype and subtypes were also reported in this study.

The *S. iniae* strains tested (article nº 1) showed high antigenic and molecular homogeneity using agglutination and REP-PCR tests, respectively. The high homogeneity found within this species and the ability of these techniques to group *Streptococcus* strains according to their taxonomic position indicate that these tests could be used for rapid and accurate identification of *S. iniae* strains. MALDI-TOF-MS and Biotyper V. 3.1. database allowed the identification at genus level of

65.5% of the *S. iniae* strains. Species-specific biomarkers for *S. iniae* were also detected at *m/z* 4135.82, 6353.12 and 8270.44 and were mainly assigned to ribosomal subunit proteins. Several studies have reported the usefulness of mass peaks derived from ribosomal proteins or housekeeping proteins for the identification of bacterial species, as well as for differentiation at the strain or clonal complex level (Fernández-Álvarez et al., 2018; Fiedoruk et al., 2016).

The strains of *V. salmoninarum* (article nº 2) tested in the present PhD work, showed a high biochemical and serological homogeneity. This homogeneity is useful to differentiate *V. salmoninarum* strains from other strains of Gram-positive cocci pathogenic to fish. However, an immunoblot analysis allowed the detection of cross-reactivity with some cellular proteins of reference strains of *S. parauberis* and *L. garvieae*. The lack of correlation between serological test results and immunoblot analysis of cell surface proteins has also been described in *Enterococcus* strains (Toranzo et al., 1995) and could be attributed to the fact that common protein antigens are found in low amounts or are not fully exposed to antibodies, being detected only when highly sensitive immunological techniques are used, as suggested by Toranzo et al. (1995). These results could allow the establishment of antigenic, immunological, and epidemiological relationships between species or strains and provide data for the formulation of effective vaccines. At genetic level, results of genomic fingerprinting obtained with RAPD, ERIC-PCR and REP-PCR typing techniques also allowed the correct identification of *V. salmoninarum* and its differentiation from the other Gram-positive bacteria analysed. Furthermore, all these techniques demonstrated the existence of genetic heterogeneity within *V. salmoninarum* species, and the

existence of RAPD, ERIC and REP-PCR profiles apparently related to host species. MALDI-TOF-MS and Biotyper V 3.1 database did not allow the identification of any strain of *V. salmoninarum* since this database does not contain any proteomic profile of this bacterium. To overcome this problem, multivariate statistical analyses were applied to the proteomic data obtained by MALDI-TOF-MS for the detection of species-specific biomarker of *V. salmoninarum* and to group the strains according to their taxonomic position. *V. salmoninarum*-specific biomarkers were reported at *m/z* 4521.41, 4845.06, 5291.74, 6229.97, 6965.50, 9043.02, 9686.66 and 10579.43. Host-specific biomarkers were also detected for *V. salmoninarum* isolated from crucian carp (*m/z* 6649.48, 7944.65) and from rainbow trout (*m/z* 11547.75). These peaks could not be presumptively assigned to proteins based on their sequence comparison due to the non-existence of this bacterial genus in UniProt database. The multivariate statistical analyses applied in this work to the proteomic data obtained by MALDI-TOF-MS can be applied quickly and easily in all microbiological diagnostic laboratories without the need for a specific database.

On the other hand, to clarify the cause of the increasing isolation of *L. garvieae* strains from vaccinated trout reared in Spain, a characterisation of *L. garvieae* strains isolated during the years 2002 to 2018 has been carried out (article nº 3). The analysis of morphological, biochemical, and physiological characteristics of *L. garvieae* strains showed some heterogeneity within the species. This phenotypic variability has been previously reported in other studies (Buller, 2014; Gibello et al., 2016) and may result in misidentifications of *L. garvieae*. Therefore, different molecular techniques have been described for the identification and typing of *L. garvieae* strains such as

classical ribotyping (Eldar et al., 1999), pulsed field gel electrophoresis (PFGE) (Vela et al., 2000), RAPD (Ravelo et al., 2003), REP-PCR (Schmidtke and Carson, 2003; Varsha and Nampoothiri, 2016) and DNA sequencing (Reguera-Brito et al., 2016). Among them, RAPD and REP-PCR were selected in article nº 3 for typing *L. garvieae* strains because of their ability to differentiate bacteria at the strain level quickly and at low cost. Both genotyping techniques demonstrated throughout this studey the existence of genetic heterogeneity within this species with the appearance of clonal genomic profiles that appear to be related to the source of isolation of *L. garvieae* strains. The results found were consistent with previous studies that detected different genetic clones in *L. garvieae* strains isolated from mammals and fish (Kawanishi et al., 2006; Vela et al., 2000). Proteomic characterisation using MALDI-TOF-MS has allowed to group the strains analysed in article nº 3 according to their taxonomic position, clearly separating *L. garvieae* from other Gram-positive strains analysed. *L. garvieae*-specific biomarkers were detected at *m/z* 4717.04 and 9431.98, which were identified in the UniProt database as a DNA-binding protein and a small ribosomal protein subunit (30S), respectively. Multivariate analyses applied to these data also grouped the *L. garvieae* strains into clusters that appear to be related to the source of isolation. These results agreed with those obtained by genomic analyses that also separate strains of *L. garvieae* isolated from humans and cattle from strains of *L. garvieae* isolated from the aquatic environment. In this work, an analysis of cell surface proteins was also carried out to assess whether possible antigenic and/or capsular variation could be responsible for the existing problem with vaccines developed against lactococcosis. This study of cell surface proteins by SDS-PAGE and Immunoblot using rabbit antiserum revealed the existence

of two antigenic variants named W1 and W2 that differ in protein antigen reactivity in the range of 35 to 70 KDa. The W1 antigenic variant, which showed the lowest immunoreactivity, was presented by Spanish and French strains isolated from rainbow trout between 1999 and 2018, as well as by strains isolated from Nile tilapia and the reference strain CECT5274; while the W2 antigenic variant, which showed the highest immunoreactivity was presented by Spanish and Turkish strains isolated from rainbow trout between 2015 and 2018. The immunoreactivity of rainbow trout antiserum was weaker than that observed with rabbit antiserum with only two highly immunogenic protein bands (30 and 45 KDa) being detected by both antisera. The two antigenic variants W1 and W2 were also detected using rainbow trout antiserum. The occurrence of these antigenic variants could be due to selective pressure induced by the mass vaccination as indicated by others (Bachrach et al., 2001; Telford, 2008).

The overall results of article nº 3 of the present work revealed a large variability within *L. garvieae* at both genetic and proteomic levels. This heterogeneity also represents a therapeutic challenge for humans and animals. Therefore, antimicrobial resistance profiles of *L. garvieae* strains isolated from rainbow trout cultured in Spain were investigated in article nº 4, combining phenotypic (zone diameter in disc diffusion tests), molecular (detection of ARGs by PCR), and proteomic (protein biomarkers by MALDI-TOF-MS) methods. The results of the disc diffusion antimicrobial susceptibility testing performed in this work showed that the *L. garvieae* strains tested were resistant to trimethoprim-sulfamethoxazole and flumequine (100% resistance), enrofloxacin (67%), oxytetracycline (44%), amoxicillin (23%), chloramphenicol (18%) and florfenicol

(13%). Among the antimicrobial agents approved for use in aquaculture by the FDA and SAMMD, the most effective *in vitro* against *L. garvieae* was florfenicol. The literature also reported *L. garvieae* strains phenotypically resistant to trimethoprim-sulfamethoxazole, flumequine, erythromycin, kanamycin, penicillin, enrofloxacin, ciprofloxacin, chloramphenicol, oxytetracycline and ampicillin (Ravelo et al., 2001; Diler et al., 2002; Walther et al., 2008; Austin and Austin, 2016; Teker et al., 2019). Article nº 4 also investigated the use of PCR-based molecular methods for ARG detection to improve the current antimicrobial resistance surveillance system as proposed by the World Health Organisation (WHO) (WHO, 2019). These methods provide faster results than phenotypic methods and can be used for clinical management, for surveillance and for monitoring resistance mechanisms (WHO, 2019). This study showed that almost all tested *L. garvieae* strains isolated from fish (78/81 strains) had one or more ARGs, with *tet(B)* being the most prevalent (85%), followed by *FloR* (78%) and *tet(A)* (61%). These rates seem to reflect an increase in the use of tetracyclines and phenicol for the treatment of lactococcosis in intensive fish farming. The WHO cautions that there is not always a correlation between molecular and phenotypic testing results for antimicrobial resistance, however in this study the *tet(B)* and *FloR* genes were detected in 100% of *L. garvieae* isolates resistant to oxytetracycline and florfenicol. The *tet(A)* and *tet(B)* genes encode for tetracycline-specific efflux pumps, while the FloR gene encodes for a phenol-specific efflux pump (Grossman, 2016). The *tet* genes have been most frequently described in negative bacteria, however a high presence of these genes has been described in strains of the genus *Lactococcus* (Raissy and Shahrani, 2015; Ture and Boran, 2015; Shahi and Mallik, 2020) This may be caused to the

rapid spread of tetracycline-resistant determinants among bacterial populations due to the localisation of tetracycline genes in mobile elements such as plasmids, transposons or gene cassettes (13, 17). Moreover, 83% of the *L. garvieae* tested showed the presence of multiple antimicrobial resistance genes, the most relevant combination being *tetA+tetB* (56% of the strains tested), *tetA+FloR* and *tetB+ FloR* (51%) and *teA+tetB+FloR* (48%).

MALDI-TOF-MS has proven to be an efficient and rapid method not only for the identification (article nº 3), but also for the identification of putative protein biomarkers of resistance to oxytetracycline (m/z 4062.27, 4798.95, 6386.64, 9179.42 and 10368.39). 27, 4798.95, 6386.64, 9179.42 and 10368.39) and florfenicol (m/z 2364.22, 3142.72, 3264.40, 4798.95, 6787.64, 7512.63, 9179.42 and 10368.39) (article nº 4) of *L. garvieae* isolates. Some of the protein biomarkers of oxytetracycline resistance were identified in the UniProt database as chorismate synthase and adenine methyltransferase. These results are consistent with previous studies (Hansen et al., 2012; Atkinson et al., 2013; Lupien et al., 2015; Grossman, 2016) that concluded that methyltransferases may be responsible for some of the mechanisms of resistance to different classes of antimicrobials of clinical and veterinary importance. While some of the biomarkers of resistance to florfenicol were identified as putative leader peptide, garvicin KS peptide B or adenine methyltransferase. Leader peptides increase resistance to antimicrobials such as tetracycline, erythromycin, chloramphenicol and the flavonoid genistein, which are substrates of the major drug efflux pump SmeAB (Melior et al., 2020). The 23S rRNA methyltransferases confer resistance to phenicols and four groups of structurally unrelated

antimicrobials (lincosamides, oxazolidinones, pleuromutilinones and streptograminones) (Atkinson et al., 2013; Adesoji and Call, 2020). These results seem to indicate that, although antimicrobial efflux pumps are the most widely described mechanism for florfenicol and oxytetracycline, they are not the only resistance mechanisms present in *L. garvieae* strains isolated from fish. This study provides a starting point for the establishment of antimicrobial resistance surveillance systems in aquaculture based on PCR and MALDI-TOF-MS.

In general, serological methods (slide agglutination and dot-blot), genotyping methods (RAPD, REP-PCR and ERIC-PCR) and MALDI-TOF-MS analyses have proven to be rapid and reliable diagnostic tools capable to distinguish strains of the genera *Streptococcus*, *Vagococcus* and *Lactococcus* pathogenic to fish. However, the accuracy and rapidity of MALDI-TOF-MS make it the most promising tool for rapid identification and typing of bacterial fish pathogens, being able to detect putative protein biomarkers of serotype and antimicrobial resistance. Thus, the MALDI-TOF-MS protocols described in chapter III of this PhD work represent a simple, cost-effective, and time-saving detection system with high throughput capability, which does not require specific mass spectrometry expertise and could be integrated into the workflow of clinical laboratories for the diagnosis and monitoring of streptococcosis.

To determine the potential risk to economically relevant fish cultures of the microorganisms characterized in Chapter III, pathogenicity tests were also performed. The results obtained demonstrated that only strains of *S. parauberis* of serotype III and of subserotypes Ia and Ic tested were virulent for turbot (article nº 1). Strains of serotype III seems to be the most virulent serotype for turbot, causing acute mortality with a LD_{50}

of 2.82 x10^3 CFU per fish. Strains of subserotypes Ia and Ic (isolated from Japanese flounder), showed a lower degree of virulence, causing a chronic mortality pattern, which may indicate some degree of host specificity. The lack of mortalities observed in rainbow trout experimentally infected with strains of *S. parauberis* representatives of serotypes I (subtypes Ia, Ib and Ic), II and III support this hypothesis. Further pathogenicity studies with a higher number of strains from different sources should be carried out for a better understanding of host specificity, which is one of the key factors governing diversity and geographic distribution of pathogenic microorganisms. The virulence of eight strains of *L. garvieae* isolated from diseased rainbow trout in Spain between 2011 and 2017 was also evaluated using juvenile rainbow trout (article nº 3). Regardless to of their genetic and proteomic clonal type, the eight strains of *L. garvieae* tested in the article nº 3 were virulent for rainbow trout, causing 80 - 100 % fish mortality with doses ranging from 10^6 to 10^9 CFU per fish (Table 1). Therefore, no correlation could be established between degree of virulence for fish and the clonal group to which *L. garvieae* strains were assigned.

Traditionally, diagnosis in clinical laboratories is based on the isolation of the pathogen and its identification by conventional microbiological methods or commercial phenotypic identification systems. However, the high similarity of the phenotypic characteristics of Gram-positive cocci responsible for streptococcosis in fish can lead to misidentification, especially when rapid phenotypic systems or automated devices are used (Al-Harbi, 2011; Buller, 2014; Austin and Austin, 2016). New technologies based on serological methods (Dodson et al., 1999; Nho et al., 2009), polymerase chain reaction (PCR) (Berridge et al., 1998; Goh et

al., 1998; Zlotkin et al., 1998b; Mata et al., 2004; Ruiz-Zarzuela et al., 2005; Zhou et al., 2011) or MALDI-TOF-MS (Kim et al., 2015, 2017) have emerged to solve these problems. Nevertheless, these methods are laborious, require use of animals or no provide quantitative analysis in clinical samples. To develop species- or serotype-specific molecular techniques not requiring prior isolation of the micro-organism from the diseased fish, Chapter IV focused on the design of new diagnostic techniques for the detection of *V. salmoninarum* (Article nº 4), *S. iniae* (Article nº 5) and strains of *S. parauberis* of serotype III (Article nº 6).

Real-time or quantitative PCR (qPCR) is a technique that has been widely used for the detection and simultaneous quantification of some of the pathogens responsible for streptococcosis in fish, such as *L. garvieae* (Jung et al., 2010), *S. parauberis* (Nguyen et al., 2014) or *S. agalactiae* (Sebastião et al., 2015), using either a fluorescent dye that associates with the DNA molecule by interacting with the minor groves of the DNA or a modified DNA oligonucleotide (DNA probe) that is fluorescent when hybridized with the complementary DNA. Real-time PCR with the intercalating dye SYBR Green I combined with melting curve analysis (MCA) is a reliable and cost-effective technique that allows highly specific and sensitive detection and analysis of PCR products in a single tube in "real time", which reduces post-processing steps and delays, and minimizes potential experimental error (Winder et al., 2011).

In article nº 4, the 16S rRNA gene of *V. salmoninarum* was evaluated as a target molecule for the development of a specific and sensitive real-time PCR protocol. In this study, the alignment and comparison of the variable regions of the 16S rRNA gene sequences of 15 type strains of six different genera

(*Vagococcus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Carnobacterium* and *Flavobacterium*) appeared to be enough for the design of specific primers for *V. salmoninarum*. A comparative analysis using the primers pSal-1 and pSal-2, described by Ruiz-Zarzuela et al. (2005), and the primers SalF and SalR, designed in this study, showed that both primer pairs were 100% specific, allowing the specific identification of all *V. salmoninarum* strains tested and the detection of the bacterium in lethal and non-lethal fish samples. Both pairs of primers and the qPCR protocol developed clearly differentiated *V. salmoninarum* from other fish pathogens responsible for streptococcosis, such as *S. parauberis*, *S. iniae*, *S. agalactiae*, *L. garvieae*, *L. piscium*, *C. maltaromaticum*, or other unrelated bacterial pathogens, even in tissues of diseased fish. The SalF and SalR primers, which amplify a 543 bp region of the 16S rRNA gene, showed the best sensitivity when using DNA (detection level of 0.034 x 10^0 copies of the gene) and tissue samples of infected fish (detection levels of 0.02 x 10^0 copies of the gene). The high sensitivity of these primers may be because, in theory, the larger amplification products should integrate more SYBR Green molecules. Therefore, as the size of the amplicon increases, a higher level of fluorescence would be expected, which compensates for the lower levels of amplicons produced (Debode et al., 2017). In this sense, the SalF and SalR primers and qPCR based on SYBR green I, combined with the melting curve analysis designed in this study, are the most effective qPCR protocol for the detection and quantification of the fish pathogen *V. salmoninarum*.

The housekeeping gene 16S rRNA is the most widely used in bacteria as target gene for species-specific PCR assays. However, the design of specific primers for *S. iniae* can be

difficult, due to high levels of similarity in the 16S rRNA gene with *S. agalactiae* (Mata et al., 2004) or *S. parauberis* (Nguyen et al., 2014). Therefore, in the article nº5 a comparative genomic approach was used for the selection of the specific lactate permease-encoding gene (GenBank accession nº Y07622.1) as a target for the design of *S. iniae*- specific primers (SilldP-F and SilldP-R) and qPCR-MCA protocol. The qPCR method developed showed 100 % specificity for *S. iniae*. In contrast to other conventional PCR protocols (Zlotkin et al., 1998b; Mata et al., 2004; Nawawi et al., 2009), this assay clearly differentiates *S. iniae* from a large collection of non-target pathogenic bacteria. The qPCR method developed showed a high sensitivity when using DNA (detection levels of 1.04 x 10^1 copies of the gene) and tissue samples of infected fish (detection levels of 1.44 $\times 10^1$ copies of the gene). The detection limit of this qPCR assay was higher than that described in conventional PCR for *S. iniae* using as target the 16S-23S intergenic spacer region, which presented a detection limit of 20 pg of DNA (Zhou et al., 2011), or the single-copy gene *lctO*, which presented a detection limit of 25 pg DNA (Mata et al., 2004). Therefore, the detection protocol described in article nº 5 could be applied as a routine diagnostic tool for *S. iniae* in microbiological diagnostic laboratories.

The recent determination of complete genomic sequences of a wide variety of bacteria has allowed the development of new molecular methods based on PCR useful for both diagnosis and bacterial typing. In recent years, different PCR-based bacterial typing protocols have emerged as an alternative for the use of antisera in pathogenic fish species, such as *S. agalactiae* (Demczuk et al., 2017; Kannika et al., 2017; Shoemaker et al., 2017), *F. psychrophylum* (Rochat et al., 2017), *S. parauberis*

(Tu et al., 2015a) and *L. garvieae* (Ohbayashi et al., 2017). In the article nº6, whole genomes of 15 strains of *S. parauberis* isolated from different hosts and geographical regions were compared to determine the genetic structure of the locus *cps*, which comprises genes responsible for polysaccharide capsule synthesis, from strains of serotype III of *S. parauberis* described previously in Chapter III. This study (article nº6) identified three variable regions (named III, IV and V) within the loci *cps*, different from those described by Tu et al (2015b) for serotypes I and II. These variable regions could represent serotype-specific regions of three different serotypes (serotypes III, IV and V) of *S. parauberis*. *In silico* comparative analysis of a partial sequence of the *cps3K* gene from the variable region III obtained from three *S. parauberis* strains of serotype III showed that the *cps3K* gene is specific to serotype III strains. In addition, the comparative genomic studies carried out in article nº6 suggest that isolates from turbot in Spain and striped bass in the United States, whose genomes are deposited in the NCBI database, could be included into the serotype III of *S. parauberis*. Based on these results, the *cps3K* gene was selected as a target for the design of specific primers for the detection and quantification of strains of *S. parauberis* serotype III, thus reducing the possibility of false positive results. Primers designed in this study were used to optimize: (a) a multiplex PCR protocol capable of differentiating the main *S. parauberis* serotypes that cause disease in fish (serotypes I, II and III) and, (b) a qPCR protocol capable of identifying and quantifying *S. parauberis* strains belonging to serotype III from bacterial cultures and fish tissues.

Optimized multiplex PCR assay in article nº 6 showed a high correspondence between the results observed in the

conventional serotyping (Chapter III; article nº1) using antisera and the molecular serotyping with multiplex PCR performed in this study (article nº 6). Similar results were found in previous studies (Tu et al., 2015a), where they performed a molecular serotyping of strains of *S. parauberis* belonging to serotypes I and II using multiplex PCR. On the other hand, the qPCR method developed in this study showed 100 % specificity for *S. parauberis* serotype III, with no amplifications observed using other serotypes of *S. parauberis* or unrelated bacteria. The qPCR method displayed a great sensitivity when using DNA (detection levels of 2.67 x 10^2 copies of the gene) and tissue samples of infected fish (detection levels of 1.76 × 10^2 gene copies of the gene). These results revealed that the PCR protocols developed in the article nº 6 could be a useful tool in epidemiological surveillance studies or for diagnostic purposes using cultured bacteria and tissues from lethal and non-lethal fish as samples. However, further studies using many isolates from different fish species and other animals in different geographical areas would be necessary to help determine the serological diversity of *S. parauberis* and to develop new diagnostic tools.

Effective management for the prevention of infectious outbreaks in aquaculture goes hand in hand with the integration of all available preventive and curative strategies to minimize the impact of pathogens in the production chain and minimize the impact on the environment. These strategies include the assessment of water quality inside and outside the fish farming plant, contact with suppliers of disease-free fish and eggs, the use of pathogen-free feed, monitoring for the presence of pathogens in tanks, the use of chemotherapy and immunostimulants, or the vaccination of fish. However, the

development of safe and effective vaccines requires, as a first step, studying the complex interaction between fish, infectious agents, and the environment. Therefore, Chapter nº V evaluated the efficacy and safety of new vaccines for the prevention of bacterial diseases in fish. For this purpose, different challenge models for *Aeromonas salmonicida* subsp. *achromogenes* in turbot and sole were established. Experimental infections in fish are an essential mechanism to study the pathogenesis of infectious diseases, to characterize the role of host-associated factors or pathogenic microorganisms, or to develop new effective drugs or vaccines (Faílde et al., 2013). However, few studies have focused on the infection pathways of atypical *A. salmonicida* such as *A. salmonicida* subsp. *achromogenes*. In the article nº 8, *A. salmonicida* subsp. *achromogenes* was inoculated by prolonged bathing (1 h) of the fish in a concentrated suspension of this bacterium. Abrasions of the skin were simulated to simulate a natural infection, and the results of this test suggest that the skin could be a portal for entry of *A. salmonicida* subsp. *achromogenes*. Fifteen days after infection, 73% and 7% mortality were observed in Senegalese sole and turbot infected by immersion with a strain of *A. salmonicida* subsp. *achromogenes*. However, 100% survival was observed in both turbot and sole inoculated by bathing with another strain of *A. salmonicida* subsp. *achromogenes*. Non-mortality results using the bathing infection pathway have been reported for *A. salmonicida* subsp. *achromogenes* in a previous study (Björnsdóttir et al., 2005) and for other fish-pathogenic bacteria such as *Pasteurella piscicida* (Kawahara et al., 1989) or *Enterococcus* strains (Romalde et al., 1996). The gastrointestinal tract has been postulated to be another common portal of entry for many bacterial fish pathogens, such as *Enterorococcus* (Romalde et al., 1996), *Photobacterium damselae* subsp

piscicida (Kawahara et al., 1989), *Vibrio anguillarum* (Horne and Baxendale, 1983; Oisson et al., 1996; Frans et al., 2011) or *Yersinia ruckeri* (Abdel-Latif et al., 2014). For *A. salmonicida*, transmission through the gastrointestinal tract via infected feed has also been investigated, but the results of various authors are contradictory (Hiney et al., 1994; Hirvelä-koski, 2005; Kim et al., 2013; Lodemel et al., 2001; Ringø and Olsen, 1999). In article nº 8, turbot and sole experimentally infected with *A. salmonicida* subsp. *achromogenes* by inoculation of infected food into the gastrointestinal tract also did not develop mortality and/or clinical signs of furunculosis. These results suggest that the route of entry of *A. salmonicida* subsp. *achromogenes* into fish is no through the gastrointestinal tract. Further studies on the pathogenesis of *A. salmonicida* subsp. *achromogenes* are needed to clarify the mechanisms of transmission of this bacterium to fish and to develop effective prevention and control measures against atypical furunculosis. In this sense the real time PCR protocol developed in the present study could be useful for rapid and sensitive detection of the bacterium.

On the other hand, article nº 8 showed that intraperitoneal and subcutaneous injections can reproduce atypical furunculosis in turbot and sole and could be used for future studies on the pathogenesis of disease or challenge studies to improve treatment and the development of prophylactic measures. Clinical signs of natural furunculosis were observed in turbot and sole inoculated intraperitoneally (ip) and subcutaneously (sc) with *A. salmonicida* subsp. *achromogenes*, according to the macroscopic lesions described (Wiklund and Dalsgaard, 1998; Björnsdóttir et al., 2005). The strains of *A. salmonicida* subsp. *achromogenes* used in this study showed no host specificity, being capable of infecting turbot and sole in experimental

conditions reaching 80-100% of mortality after ip and sc inoculation. In both fish an inflammatory response was observed at the site of inoculation, causing in the early stages of the disease a slight swelling of the region near the inoculation site. Since the disease progressed, skin haemorrhages and boils were observed in the regions close to the inoculation site, with the presence of ulcerations. *A. salmonicida* subsp. *achromogenes w*as confirmed as the causative agent of mortalities by culture and qPCR analysis of kidney and spleen from moribund or dead fish. The detection of *A. salmonicida* subsp *achromogenes* by PCR in the kidney and spleen, organs involved in the defence against pathogens, of diseased fish suggests that the high mortality rate observed in fish inoculated with sc and ip could be due to their compromised defence mechanisms. The results obtained in article nº 8 were the basis to test the effect of bivalent vaccines against *A. salmonicida* subsp *achromogenes* and *Vibrio anguillarum* on health and survival of turbot (article nº 9).

Article nº 9 tested the efficacy of oil-based commercial (AlphaJect 3000, Pharmaq AS) and autogenous vaccines against atypical furunculosis and vibriosis in farmed turbot. The effect of i.p injection of mineral oil adjuvant Montanide™ (ISA 763A VG; Seppic, France) and the liquid paraffin Eolane 130 (Total Fluids, Spain) was evaluated in parallel.

The impact of vaccines and adjuvants on health parameters, and fish survival after experimental challenge was checked. In this study, the autogenous vaccine was prepared by mixing pure inactivated cultures of *A. salmonicida* subsp. *achromogenes* and *V. anguillarum* (serotype O2α) and, non-mineral oil adjuvant Montanide™ (ISA 763A VG; Seppic, France). Vaccine efficacy can be improved by using adjuvants together with the selected

antigens. However, vaccine formulations with mineral oil adjuvants have been associated with side effects, such as growth impairment (Durbin et al., 1999; Melingen and Wergeland, 2002; Menanteau-Ledouble et al., 2016), pigmentation, inflammation, and granulomatous lesion formation (Midtlyng, 1997; Durbin et al., 1999; Koppang et al., 2005) and fibrous adhesions in internal organs (Koppang et al., 2005; Berg et al., 2007; Coscelli et al., 2015; Menanteau-Ledouble et al., 2016). Article nº 9 describes the presence of inflammation, granulomas, and organ adhesion in turbot immunised with autogenous and commercial vaccine or injected with Montanide™ adjuvant after 45 days of treatment. These alterations could be caused by the deposition of adjuvants on or between organs, which infiltrate, forming a vaccine cell mass that facilitates the formation of adhesions (Aucouturier et al., 2001; Noia et al., 2014; Jaafar et al., 2015). In addition, microscopic liver lesions were also observed in fish injected with oil adjuvants and adjuvanted vaccines, such as mild to moderate necrosis and granulomatous structures attached to the serosa and occasionally to the exocrine pancreas. Local necrosis or granulomatous lesions in liver tissue are some of the histological alterations previously observed in salmonids injected with oil-adjuvanted vaccines (Mutoloki et al., 2008; Noia et al., 2014).

The efficacy and duration of immunity of the commercial and autogenous vaccine (article nº 9) was tested by infectious challenge test with *V. anguillarum* and *A. salmonicida* subsp. *achromogenes* and determination of Relative Percentage of Survival (RPS). After 45 days post-treatment, the commercial and autogenous vaccine induced complete protection against *V. anguillarum* (RPS values of 100 %). While the autogenous vaccine (RPS values of 83 %) conferred better levels of

protection against *A. salmonicida* subsp. *achromogenes* than the commercial vaccine (RPS values of 50 %). The high levels of protection of the autogenous vaccine could be due to the presence of specific antigens, which are important in the stimulation of the immune system of this fish species. Similar results have been found with vaccines against typical and atypical furunculosis (Santos et al., 2005; Gudmundsdóttir and Björnsdóttir, 2007). After 160 days post-treatment, moderate protection values against *V. anguillarum* were obtained using the commercial vaccine (RPS=52) and the autogenous vaccines (RPS= 67%). In contrast, significantly high levels of protection against *A. salmonicida* subsp. *achromogenes* were observed in turbot vaccinated with AlphaJect 3000 (RPS of 77 %) or autogenous vaccines (RPS=100 %). These results demonstrated that both vaccines (autogenous and commercial) appear to offer significant protection against natural infections with *V. anguillarum* and *A. salmonicida* subsp. *achromogenes*. However, the autogenous vaccine developed in this study and tested in turbot showed the best short- and long-term protection against atypical furunculosis caused by *A. salmonicida* subsp. *achromogenes* and short-term protection against vibriosis caused by *V. anguillarum*. This vaccine causes severe adverse effects in fish, which precludes the possibility of a second vaccination. This is probably due to the nature of the adjuvant used in the vaccine formulation (Montanide™ ISA 763A VG), which allows a continuous release of antigen with prolonged protections at the cost of high side effects in fish. On the other hand, the AlphaJect 3000 vaccine, marketed for salmon farming, and tested on turbot in this study, offers only high levels of short-term protection against vibriosis caused by *V. anguillarum*. The side effects caused by this vaccine were less than those caused by the autogenous vaccine, probably due to

the nature of its adjuvant (liquid paraffin). In fact, fish injected with the adjuvant Eolane 130 showed only mild or minor adhesions. Thus, it would be interesting to test a vaccine formulated with specific antigens characteristic of turbot isolates supplemented with a liquid paraffin adjuvant such as Eolane 130.

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CHAPTER 7. Conclusions

1. Serological and genotypic methods, and MALDI-TOF mass spectrometry have proven to be rapid and reliable diagnostic tools capable of differentiating the main pathogens responsible for streptococcosis in fish such as *Streptococcus parauberis*, *Streptococcus iniae*, *Vagococcus salmoninarum*, and *Lactococcus garvieae*. Among all these methods, MALDI-TOF-MS has the highest discriminatory power and is the fastest and least expensive method for the identification of these pathogens and for conducting large-scale epidemiological studies. MALDI-TOF-MS combined with conventional microbiological methods offers a potential alternative for surveillance systems and rapid disease management in fish, allowing the development of effective therapeutic measures against streptococcosis during a disease outbreak in aquaculture.

2. PCR assays designed for the identification and quantification of *V. salmoninarum*, *S. iniae* and *S. parauberis* belonging to serotype III were shown to be 100% specific and with high levels of sensitivity (from 0.034×10^0 to 2.67×10^2 amplicon copies per µl). These protocols can be used as a tool in epidemiological surveillance studies or for streptococcosis diagnostic purposes using bacterial cultures and lethal and non-lethal fish tissues as samples.

3. PCR and proteomic based methods demonstrated a high prevalence of resistance to oxytetracycline and florfenicol among *L. garvieae* strains isolated in Spain. PCR and MALDI-TOF-MS techniques combined with phenotypic detection of resistance offer an alternative for surveillance systems and rapid disease management, allowing the

development of effective therapeutic measures against *L. garvieae* during a disease outbreak in aquaculture.

4. Strains of *A. salmonicida* subsp. *achromogenes* isolated from diseased turbot showed little or no host specificity, being able to reproduce the clinical signs of atypical furunculosis in a similar way to that of natural infection following intraperitoneal and subcutaneous inoculation of turbot and sole. The developed bivalent autogenous oil-based vaccine conferred high levels of long-term protection against atypical furunculosis and vibriosis after administration of a single dose in turbot, at the cost of high side effects in fish. Further studies should therefore focus on the development of autovaccines and the evaluation of other adjuvants that improve protection with reduced or no side effects. In this respect, the liquid paraffin Eolane 130 is a promising candidate that could be considered in the development of future vaccines.

Appendix 1

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Appendix 2
PATENTE SOLICITADA

1. **Composición inmunogénica para la prevención de la tenacibaculosis marina causada por *Tenacibaculum maritimum* y *Tenacibaculum soleae* en peces, procedimiento de obtención y uso**

   Patente solicitada
   Número de solicitud: P202131002
   Inventores: **Yolanda Torres Corral**; Ysabel Santos Rodríguez
   Institución: Universidad de Santiago de Compostela
   Priority: 26/10/2021

The inability of the extractive fishing industry to meet the growing demand for aquatic products has prompted the rapid growth of aquaculture, a trend that has persisted over the last ten years. However, bacterial infections, which cause significant economic losses and pose a risk to human health, are one of the main constraints facing this industry. Therefore, in this PhD work, new methods for the identification and typing of pathogenic bacteria based on the study of their genome and proteome have been developed and tested as an alternative to conventional diagnostic methods. In addition, the efficacy and safety of new vaccines as a prophylactic method for the prevention of bacterial diseases in fish has been studied.