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Dinámica de la respuesta codificante/no-codificante y su rol en la interacción entre el salmón Atlántico (*Salmo salar*) y *Piscirickettsia salmonis*

Tesis para optar al grado de
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RESUMEN

La Septicemia Rickettsial Salmonidea (SRS) causada por la bacteria Gram negativa *Piscirickettsia salmonis* es considerado como uno de los principales patógenos que afecta la salmonicultura chilena y una de las amenazas más importantes para el desarrollo sustentable del sector. A pesar de su relevancia, el desarrollo de herramientas efectivas para su control ha estado obstaculizado por distintas brechas de conocimiento a nivel molecular tanto de la bacteria como de su hospedero, así como de sus interacciones durante el proceso infectivo. En este contexto, la presente tesis doctoral busca la generación de conocimiento relevante sobre los mecanismos moleculares implicados en la interacción patógeno-hospedero. La información generada permite su aplicación en nuevas herramientas y estrategias terapéuticas para el control de la enfermedad. Como primera etapa del presente estudio, se utilizaron herramientas en genómica funcional para identificar distintos mecanismos moleculares involucrados en la interacción patógeno-hospedero. Interesantemente, los resultados evidenciaron una modulación significativa de transcritos relacionados a la endocitosis mediada por clatrina y homeostasis de hierro durante el proceso infectivo de *P. salmonis*. Paralelamente, se evidenció un aumento en la expresión de genes que favorecen la endocitosis, pero una disminución en la expresión de los genes vinculados a la maduración del endosoma y fagocitosis. Estos procesos biológicos podrían explicar cómo *P. salmonis* se desarrolla en vacuolas citoplasmáticas en su hospedador sin que este sea capaz de lograr la maduración de endosomas para la posterior lisis bacteriana. La regulación de genes que participan en la homeostasis de hierro sugiere que la infección de *P. salmonis* en salmón Atlántico promueve una acumulación de hierro a nivel intracelular, elemento vital para el desarrollo y patogénesis de la bacteria. Esta acumulación podría favorecer la disponibilidad intracelular de hierro para la bacteria, lo que podría contribuir al desarrollo de la enfermedad.

Como segunda etapa del estudio, se exploraron las respuestas moleculares del salmón del Atlántico durante la infección con *P. salmonis* relacionada con RNAs no-codificantes (ncRNAs), incluyendo long non-coding RNAs y micro RNAs (miRNAs). Mediante análisis de co-localización genómica y co-expresión se identificaron lncRNAs putativamente vinculados con la regulación de genes que participan en la endocitosis y homeostasis del hierro. Por otra parte, 99 miRNAs fueron diferencialmente expresados en distintos tejidos de *S. salar* durante la

infección. Mediante predicción de targets, se estableció que estos miRNAs estarían participando en la regulación de distintos genes de inmunidad y particularmente de *hepcidin-1*, gen previamente vinculado a la respuesta de salmónidos frente a *P. salmonis* y a la homeostasis de hierro. Aunque el rol de los ncRNAs en la regulación de procesos biológicos en peces requiere de investigación funcional para dilucidar mecanismos específicos, nuestros resultados sugieren la existencia de un rol activo en la regulación de las repuestas de salmónidos frente a infecciones bacterianas.

Como tercera parte del estudio, se caracterizó la respuesta transcriptómica de *P. salmonis* durante el proceso infectivo en salmón Atlántico mediante Dual RNA-Seq. De esta forma, se determino que la bacteria despliega una amplia batería de genes para favorecer la patogénesis en el salmón. Además de la respuesta canónica asociada con virulencia en patógenos intracelulares, se identificó una respuesta transcriptómica común asociada al metabolismo de aminoácidos entre patógeno y hospedero. Experimentos posteriores demostraron que *P. salmonis* es capaz de explotar distintos tipos de aminoácidos como fuente de energía para favorecer su crecimiento. Ambos resultados sugieren la existencia de una competencia por los nutrientes entre salmón y bacteria, otorgando así nuevos conocimientos sobre la importancia de la inmunidad nutricional y el rol hierro y los aminoácidos durante esta interacción. Finalmente, y con el propósito de identificar cambios genómicos de *P. salmonis* sobre los mecanismos de virulencia, se realizó un experimento de cultivo continuo de la bacteria en medio libre de células. Los resultados evidenciaron cambios genómicos y transcriptómicos en bacterias cultivadas durante 200 pasajes en medio de cultivo libre de células. Estos cambios involucraron la relocalización y regulación de distintos genes del sistema de secreción tipo Dot/Icm, flagelinas, elementos genéticos móviles (MGE) y de captura de hierro (Vibrioferrin). Una posterior infección en líneas celulares de salmón reveló que la bacteria traspasada sistemáticamente en medio liquido sufrió una clara atenuación en la capacidad de generación de efectos citopáticos y lisis en las células expuestas.

En conclusión, los resultados obtenidos nos permiten plantear que mecanismos como la endocitosis mediada por clatrina y la inmunidad nutricional basada en la competencia de hierro y aminoácidos son factores claves de la interacción del salmón con *P. salmonis*. Por otra parte, los ncRNAs emergen como componentes importantes en la regulación de estos procesos. Finalmente, es necesario seguir investigando si la atenuación patogénica mediada por cultivo

continuo en medio libre de células podrá ser usada como base para la generación de una nueva vacuna viva atenuada para el control de SRS, aportando así en un desarrollo más sustentable de la salmonicultura en Chile.

ABSTRACT

The Rickettsial Salmonide Septicemia (SRS) caused by the Gram-negative bacterium *Piscirickettsia salmonis* is considered as one of the main pathogens affecting Chilean salmon aquaculture and one of the most important threats for the sustainable development of the sector. Despite its relevance, the development of effective tools for its control has been hampered by different knowledge gaps at the molecular level of both the bacterium and its host, as well as their interactions during the infective process. In this context, the present doctoral thesis is aimed to generate relevant knowledge about the molecular mechanisms involved in during host-pathogen interaction.

As a first stage of the present study, tools in functional genomics were used to identify different molecular mechanisms involved in the pathogen-host interaction. Interestingly, the results evidenced a significant modulation of transcripts related to the endocytosis mediated by clathrin and iron homeostasis during the infective process of *P. salmonis*. In parallel, there was an increase in the expression of genes that favor endocytosis, but a decrease in the expression of genes linked to endosome maturation and phagocytosis. These biological processes could explain how *P. salmonis* develops in cytoplasmic vacuoles in its host without it being able to achieve endosome maturation for subsequent bacterial lysis. The regulation of genes involved in iron homeostasis suggests that the infection of *P. salmonis* in the Atlantic salmon promotes an accumulation of iron at intracellular level, a vital element for the development and pathogenesis of the bacteria. This accumulation could favor the intracellular availability of iron for the bacteria, which could contribute to the development of the disease.

As a second stage of the study, the molecular responses of Atlantic salmon were explored during infection with *P. salmonis* related to non-coding RNAs (ncRNAs), including long non-coding RNAs and microRNAs (miRNAs). Through genomic co-localization and co-expression analysis, lncRNAs putatively linked to the regulation of genes involved in endocytosis and iron homeostasis were identified. On the other hand, 99 miRNAs were differentially expressed in different tissues of *S. salar* during infection. By predicting targets, it was established that these miRNAs would be involved in the regulation of different immunity genes and particularly of hepcidin-1, a gene previously linked to the salmonid response to *P. salmonis* and to iron homeostasis. Although the role of ncRNAs in the regulation of biological processes in fish

requires functional research to elucidate specific mechanisms, our results suggest the existence of an active role in the regulation of salmonid responses to bacterial infections.

As a third part of the study, the transcriptomic response of *P. salmonis* during the infective process in Atlantic salmon was characterized by Dual RNA-Seq. Thus, it was determined that the bacterium deploys a wide battery of genes to favor the pathogenesis in salmon. In addition to the canonical response associated with virulence in intracellular pathogens, a common transcriptomic response associated with the metabolism of amino acids between pathogen and host was identified. Later experiments showed that *P. salmonis* is capable of exploiting different types of amino acids as an energy source to promote their growth. Both results suggest the existence of competition for nutrients between salmon and bacteria, thus providing new insights into the importance of nutritional immunity and the role of iron and amino acids during this interaction. Finally, and with the purpose of identifying genomic changes of *P. salmonis* on virulence mechanisms, a serial passage experiment of the bacteria was carried out in a cell-free medium. The results showed genomic and transcriptomic changes in bacteria cultured during 200 passages in cell-free culture medium. These changes involved the relocation and regulation of different genes of the Dot/Icm secretion system, flagellins, mobile genetic elements (MGE) and iron capture (Vibrioferrin). A subsequent infection in salmon cell lines revealed that the bacteria systematically transferred in liquid medium underwent a clear attenuation in the ability to generate cytopathic effects and lysis in the exposed cells.

Overall, our results allow us to propose that mechanisms such as clathrin-mediated endocytosis and nutritional immunity based on iron and amino acid competition are key factors in the interaction of salmon with *P. salmonis*. On the other hand, ncRNAs emerge as important components in the regulation of these processes. Finally, it is necessary to continue investigating whether pathogenic attenuation mediated by continuous culture in cell-free medium can be used as a basis for the generation of a new live attenuated vaccine for the control of SRS, thus contributing to a more sustainable development of salmon farming in Chile.

1. INTRODUCCIÓN

1.1 Salmonicultura en Chile: brechas y perspectivas para el desarrollo sustentable de la industria

El sostenido crecimiento de la salmonicultura nacional no ha estado exento de diversas problemáticas económicas, medioambientales y sociales que han comprometido el desarrollo sustentable del sector (Quiñones et al., 2019). Estos problemas están generalmente vinculados a potenciales impactos negativos en los ecosistemas, tales como la pérdida de hábitats, contaminación y escapes de individuos cultivados, entre otras (Maroni, 2000;Tacon et al., 2010;Klinger and Naylor, 2012). Dentro de estas problemáticas, las enfermedades y sus tratamientos constituyen una parte importante de las perdidas económicas para la industria. Por otra parte, el uso intensivo de antibióticos durante el tratamiento de enfermedades bacterianas puede favorecer el desarrollo de bacterias resistentes a los antibióticos, tanto en el medioambiente como en el humano que consuma el producto (Barton and Floysand, 2010;Millanao B et al., 2011). Esto ha conllevado a una tendencia global para la eliminación del uso de antibióticos en la producción de alimentos para el consumo humano. Debido a esto resulta imperiosa la generación de nuevas estrategias para el control de las enfermedades que sean económicamente viables, medioambientalmente responsables y socialmente aceptables. Dentro de las enfermedades que afectan la salmonicultura nacional, la Septicemia Ricketsial Salmonidea (SRS) es una de las más relevantes para la industria. SRS puede llegar a ser responsable de alrededor del 80% de las mortalidades infecciosas en salmónidos, generando un impacto económico estimado de US$ 850 millones de pérdidas anuales (Rozas and Enriquez, 2014). A pesar de la importancia de este patógeno, el desarrollo de herramientas efectivas para su control se ha visto dificultado por distintas brechas de conocimiento asociadas con diversos aspectos propios de la enfermedad.

1.2 Septicemia Rickettsial Salmonídea (SRS), una enfermedad de alto impacto

El primer gran brote de la septicemia rickettsial salmonídea (SRS) en Chile ocurrió durante el 1989 (Bravo and Campos, 1989), causando la muerte de aproximadamente 1,5 millones de salmón Coho (*Oncorhynchus kisutch*) cultivados en el sur de Chile. Las mortalidades asociadas a este evento representaron en algunos casos hasta el 90% de la producción anual, traduciéndose

en pérdidas económicas directas estimadas en US$ 10 millones producto de la mortalidad de salmones aptos para cosecha (Cvitanich et al., 1990). Con el transcurrir de los años, el marcado crecimiento de la salmonicultura en Chile y las condiciones de cultivo han favorecido el número frecuencia de estos brotes. A 30 años del primer brote, SRS es responsable de entre el 60 y 80% del total de las mortalidades producto de enfermedades infecciosas en las principales especies salmónidas cultivadas a nivel nacional. De este modo, el instituto tecnológico del salmón (INTESAL) ha reportado que las pérdidas económicas directas asociadas a SRS fluctúan entre US$ 100–150 millones, las cuales pueden traducirse en US$ 850 millones de pérdida potencial anuales y que representan un 25% de las ganancias totales por concepto de exportación anual (Wilhelm et al., 2006;Rozas and Enriquez, 2014). Estos valores, sumados a los US$ 7,5 millones destinados a tratamientos con antibióticos para el control de SRS (Smith et al., 1999), convierten este enfermedad en una de las principales patologías que afecta la salmonicultura chilena y una de las amenazas más importantes para el desarrollo sustentable del sector.

SRS es definida como una enfermedad sistémica, puesto que afecta múltiples tejidos en peces que desarrollan esta patología. Así, los signos observables incluyen la palidez branquial producto de la profunda anemia que desarrollan, hinchazón abdominal, petequias, hemorragias en la base de las aletas y múltiples ulceras (Cvitanich et al., 1990;Fryer et al., 1990;Branson and Diazmunoz, 1991). Internamente, se puede apreciar inflamación en hígado, riñón y bazo. Sin embargo, uno de los principales signos clínicos durante un cuadro infecciosos SRS corresponde a la aparición de marcas circulares de coloración amarilla en el hígado (Cvitanich et al., 1990;Fryer et al., 1990;Branson and Diazmunoz, 1991).

El patógeno responsable de esta enfermedad es *Piscirickettsia salmonis* una bacteria Gram-negativa, no móvil, pleomórfica, principalmente cocoide con un diámetro de entre 0.5 a 1,5 μm y perteneciente a la subdivisión gamma de las proteobacterias (Fryer and Hedrick, 2003). En relación a su crecimiento, *P. salmonis* se replica primordialmente dentro de vacuolas citoplasmáticas en células del hospedador (Fryer et al., 1990;Fryer et al., 1992), las cuales pueden ser observadas incluso en macrófagos (Rojas et al., 2010;Bakkemo et al., 2011). Por otra parte, se ha demostrado la capacidad de la bacteria para infectar múltiples especies de peces óseos, pudiendo replicarse incluso en células de insectos y anfibios (Birkbeck et al., 2004).

Inicialmente, *P. salmonis* fue considerada como una bacteria intracelular obligatoria, es decir, que solo podía ser cultivada y crecida en células eucariontes (Birkbeck et al., 2004). Esta condición dificultó el estudio de bacteria y por ende muchos aspectos relacionados a su biología básica como virulencia y transmisión permanecieron escasamente estudiadas (Rozas and Enriquez, 2014). La evidencia científica apoyando el carácter intracelular facultativo y la supervivencia extracelular de la bacteria (Gomez et al., 2009;Marshall et al., 2011a) han llevado a un escenario favorable para el desarrollo de nuevos estudios que permitan llenar estos vacíos. Considerando la relevancia de este patógeno para la salmonicultura nacional y la transversalidad de tejidos y especies que puede infectar, *P. salmonis* es un interesante modelo de estudio para esclarecer mecanismos de interacción patógeno-hospedero, a fin de sentar bases para el desarrollo de nuevas estrategias de control y mitigación de esta enfermedad.

1.3 Genómica funcional para el estudio de interacciones patógeno-hospedero

A pesar de la importancia *P. salmonis*, pocos estudios se han destinado a comprender los mecanismos mediante el cual el hospedador responde a la bacteria y a su vez, como *P. salmonis* es capaz de contrarrestar esta respuesta, lograr subsistir y replicarse de manera intracelular. En este contexto, estudios transcriptómicos basados en microarreglos han sido destinados para identificar qué genes son activados y reprimidos durante la infección de *P. salmonis* en salmónidos (Rise et al., 2004;Tacchi et al., 2011;Pulgar et al., 2015). De este modo, se ha determinado que genes relacionados a procesos antioxidantes son inducidos en macrófagos de salmón Atlántico (*Salmo salar*) infectados con *P. salmonis*, mientras tanto, genes asociados con respuesta inmune adaptativa disminuyen sus niveles de expresión en presencia del patógeno (Rise et al., 2004). Estos resultados fueron posteriormente validados en músculo, hígado y riñón cefálico en *S. salar* desafiados con *P. salmonis* (Tacchi et al., 2011). De esta forma, genes relacionados con respuesta oxidativa e inflamatoria aumentaron su expresión, mientras que genes asociados con respuesta inmune adaptativa, vía de señalización por proteína G y apoptosis vieron disminuida su expresión. Si bien estos resultados han aportado al entendimiento de algunos aspectos de la interacción *P. salmonis* y salmón, los estudios transcriptómicos basados en microarreglos presentan algunas limitaciones técnicas. En los últimos años, el desarrollo de tecnologías de secuenciación masiva aplicadas a la secuenciación del mRNAs (RNA-Seq) ha

reemplazado paulatinamente el uso de microarreglos (Shendure, 2008;Ozsolak and Milos, 2011). Además de tener un costo cada vez menor, las tecnologías RNA-Seq ofrecen ventajas comparativas sobre los microarreglos, tales como la posibilidad de identificar nuevos transcritos e isoformas, identificar polimorfismos de un solo nucleótido (SNP) y poseer un rango dinámico más amplio (Fu et al., 2009;Wang et al., 2009b;Sirbu et al., 2012). Sin embargo, una de las mayores ventajas radica en la posibilidad de la identificación de genes diferencialmente expresados sin estar condicionado por la lista de sondas (transcritos) predeterminadas en el microarreglo. Un claro ejemplo de este punto esta asociada al crecimiento exponencial en investigación relacionada con RNAs no codificantes y su rol en la regulación de diversos procesos biológicos.

1.3 RNAs no codificantes y su rol en la interacción patógeno hospedero

Si bien el porcentaje exacto se encuentra aún en discusión, se sabe que una fracción significativa del genoma animal se transcribe en algún en una molécula de RNA (Pertea, 2012). Sin embargo, para algunas especies solo el 2% del genoma codifica para la producción de proteínas, y por ende el transcriptoma está compuesto principalmente por RNAs no-codificantes (ncRNAs) (Collins et al., 2004). Inicialmente, el rol de estos ncRNAs estaba limitado a la producción de proteínas a través del RNA ribosomal (rRNA) y de transferencia (tRNA), sin embargo, una mirada más profunda en el espectro del RNA no-codificante ha permitido la identificación de diferentes clases de ncRNAs funcionales con roles que van más allá de la síntesis de proteínas (Cech and Steitz, 2014). Estos RNAs incluyen microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs) y long non coding RNAs (lncRNAs). Por muchos años, los ncRNAs fueron considerados como subproductos de la maquinaria transcriptómica, sin embargo, evidencia reciente ha sugerido que estos transcritos pueden tener un rol fundamental en la regulación de diversos procesos biológicos sin la necesidad de codificar para una proteína (Mercer et al., 2009;Nie et al., 2012;Kaushik et al., 2013;Cech and Steitz, 2014;Heward and Lindsay, 2014).

Los microRNAs (miRNAs) constituyen al grupo de ncRNAs más estudiados. Corresponden a RNAs de aproximadamente 21 nt que regulan la expresión génica a nivel post-transcripcional mediante la intervención en la estabilidad o en la traducción de mRNA (Chekulaeva and

Filipowicz, 2009). A través de la complementariedad de bases entre el miRNA y el mRNA objetivo, el complejo RISC es dirigido preferentemente al extremo 3' del RNA mensajero. Esta interacción permite el reclutamiento de la maquinaria molecular que promueve la deadenilación y la represión de la transcripción del mRNA objetivo (Fabian et al., 2009;Hafner et al., 2011;Wilson and Doudna, 2013). Esto, le permite a los miRNAs ser un mecanismo de sintonía fina para el silenciamiento de especifico de genes, teniendo así incidencia en la regulación directa o indirecta de prácticamente todos los procesos biológicos que ocurren en una célula. Así, distintas investigaciones han sido destinadas para comprender el rol de los miRNAs en peces (Xia et al., 2011;Xu et al., 2015;Jiang et al., 2016). Particularmente en salmónidos, se ha descrito que algunos miRNAs se expresan diferencialmente dependiendo del estado de maduración sexual (Farlora et al., 2015) y en la respuesta inmune contra virus (Bela-ong et al., 2015;Schyth et al., 2015). Sin embargo, el rol de los miRNAs durante la respuesta inmune contra bacterias permanece aún poco estudiado.

Otro grupo de RNAs no-codificantes con un creciente interés en la comunidad científica corresponde a los lncRNAs. Estos, se diferencian con otros tipos de ncRNAs principalmente por la longitud de su secuencia (> 200 nt). Al igual que un RNA mensajero (mRNA), los lncRNAs se transcriben por la acción de una RNA polimeraza II, poseen una caperuza y una cola poly-A, sin embargo, no contienen información para la síntesis de proteínas (Mercer et al., 2009;Moran et al., 2012). La caracterización molecular de lncRNAs en diferentes especies modelos ha llevado al descubrimiento de que estos transcritos pueden participar en la regulación de la expresión de proteínas. Aunque los mecanismos moleculares de esta regulación están aún son discutidos, se ha sugerido que estarían cumpliendo un rol clave en el remodelamiento de la cromatina, el control de la transcripción y el procesamiento post-transcripcional (Mercer et al., 2009). Esto ha permitido asociar a los lncRNAs en la regulación de múltiples procesos biológicos, tales como reproducción, desarrollo, metabolismo y respuesta inmune (Imamura and Akimitsu, 2014;Taylor et al., 2015;Zhao and Lin, 2015). Debido a que los lncRNAs corresponden al tipo de ncRNA más recientemente estudiado, poco se conoce del rol de los lncRNAs en la regulación de la respuesta inmune en peces y como estos RNAs no codificantes podrían estar involucrados en la interacción patógeno-hospedero. En este contexto, estudios transcriptomicos basados en RNA-seq podrían complementar el estado actual de conocimiento

en relación con los mecanismos de respuesta del salmón frente a una infección de *P. salmonis* y como estos mecanismos podrían ser regulados por RNAs no codificantes.

1.4 Dual RNA-seq: una mirada desde el patógeno durante el proceso de infección.

Con relación a *P. salmonis*, distintas aproximaciones han sido destinadas para comprender las bases moleculares que le confieren su amplio potencial infectivo. Así, se ha logrado evidenciar la presencia de sistemas de secreción Dot/Icm tipo 4B en *P. salmonis* el cual corresponde a un importante mecanismo de virulencia en bacterias y que es responsable de la supervivencia intracelular y replicación en otros patógenos Gram-negativos (Gomez et al., 2013b). Por otra parte, también se ha reportado evidencia que sugiere la secreción de exotoxinas de carácter proteico por parte de *P. salmonis*, las cuales son capaces de producir efectos citopáticos en líneas celulares de embriones de salmón (Rojas et al., 2013). Recientemente, se han caracterizado vesículas de membrana externa (OMV) secretadas por *P. salmonis*, las cuales al ser purificadas son capaces de generar efectos citopáticos en células de salmónidos (Oliver et al., 2016). Por otra parte, se ha descrito que la interacción de *P. salmonis* con proteínas como clatrina y actina resultan fundamentales para la internalización y el desarrollo del proceso infectivo en células de salmones (Ramirez et al., 2015). A pesar de que se han realizado avances en comprender como *P. salmonis* es capaz de generar patogenicidad en su hospedador y como este responde ante la presencia del patógeno, todas estas aproximaciones han sido abordadas desde la perspectiva del patógeno o del hospedador. No obstante, el desarrollo de una enfermedad implica una constante interacción entre ambos componentes y que no corresponden a fenómenos aislados, sino más bien paralelos.

En este escenario una nueva aplicación basada en secuenciación masiva ha sido desarrollada para lograr sobreponerse a esta problemática. Esta aproximación se denomina Dual RNA-seq y se basa en la secuenciación paralela del transcriptomas del patógeno y hospedador a fin de identificar nuevas formas de interacción entre ambos agentes (Westermann et al., 2012). Inicialmente, este enfoque se desarrolló para el estudio de infecciones virales, fúngicas y parasitarias, donde los RNAs mensajeros de patógeno y hospedador comparten características estructurales que facilitan su análisis simultaneo (Tiemey et al., 2012;Strong et al., 2013;Choi et al., 2014;Pittman et al., 2014) y apenas se usada en modelos bacterianos (Westermann et al.,

2012). Sin embargo, la mejora en las tecnologías de secuenciación y el desarrollo de nuevos métodos de captura/depleción ARN ofrecen una oportunidad prometedora para expandir también este enfoque a las infecciones bacterianas (Westermann et al., 2016).

Aunque la mayoría de los enfoques duales de RNA-seq aplicados en infecciones bacterianas han sido exploratorios, algunos de ellos han revelado nuevos mecanismos de interacción hospedero-patógeno. Por ejemplo, mediante Dual RNA-seq se logró descubrir una posible estrategia empleada por *Chlamydia trachomatis* para la infección in vitro de células epiteliales humanas basada en una adquisición temprana de hierro y una estrategia de depleción inmune del hospedador (Humphrys et al., 2013). Además, el análisis simultaneo del transcriptoma de la bacteria Gram-negativa *Haemophilus influenza* durante la infección de las células epiteliales de la mucosa reveló la importancia de la respuesta oxidativa del huésped y los mecanismos empleados por las bacterias para superar este ambiente adverso (Baddal et al., 2015). Además, se utilizó Dual RNA-seq para caracterizar el papel regulador de small RNAs (sRNAs) en la infección por *Salmonella enterica*. Los investigadores identificaron sRNAs bacterianos involucrado en la regulación de los genes huésped y patogénicos, revelando nuevos roles de estos transcritos bacterianos durante la patogénesis (Westermann et al., 2016). Así, el estudio simultaneo de transcriptomas de huésped y patógeno mediante Dual RNA-seq se ha convertido en una poderosa herramienta para desentrañar aspectos clave durante el proceso de infección. En el presente estudio, aplicamos un enfoque dual de RNA-Seq para revelar nuevos aspectos del proceso infeccioso de la bacteria intracelular *Piscirickettsia salmonis* durante la infección en el salmón del Atlántico (*Salmo salar*).

1.5 Atenuación por pasajes de virulencia bacteriana: hacia el desarrollo de nuevas herramientas de control de *P. salmonis*

Las bacterias son organismos altamente dinámicos que pueden sobrevivir y adaptarse a una amplia variedad de entornos. En condiciones de laboratorio, estos microorganismos se propagan rutinariamente en diferentes medios de cultivo. Si estas condiciones son lo suficientemente persistentes, las bacterias eventualmente se adaptarán (domesticarán) a este entorno no natural (Eydallin et al., 2014;Kram et al., 2017). Junto con ser organismos de fácil manejo y cortos tiempos de generación, las bacterias se han utilizado como modelos de estudio para abordar

aspectos clave sobre la adaptación y la evolución en respuesta a entornos difíciles. La adaptación al medio se ha explorado principalmente en *Escherichia coli*, donde esta bacteria se ha ha expuesto durante multiples pasajes a distintas condiciones adversas, incluido el estrés por nutrientes, medios ácidos o en presencia de antibiótico (Herring et al., 2006;Conrad et al., 2009;Toprak et al., 2012;Barrick and Lenski, 2013;Wiser et al., 2013;Spagnolo et al., 2016). Estos estudios evidenciaron que *E. coli* experimenta a diferentes modificaciones genotípicas y fenotípicas que les proporcionan una ventaja comparativa para sobrevivir o explotar completamente las condiciones en las que se cultivan. Por ejemplo, a través de la secuenciación del genoma completo, se identificaron 13 mutaciones de novo en *E. coli* cultivadas en un medio de crecimiento a base de glicerol durante un período de 44 días (Herring et al., 2006). Aquí, los autores demuestran que estas modificaciones espontáneas confirieron ventajas comparativas por sobre la población nativa. Incluso durante un corto período de adaptación (2-3 días) *E. coli* puede mostrar cambios fenotípicos como respuesta de domesticación (Eydallin et al., 2014). Sin embargo, cada adaptación a un nuevo entorno trae consigo un costo.

Para los agentes patógenos, se ha reportado que la adaptación a un medio mediante el pasaje serial de la bacteria mejora el rendimiento en nuevas condiciones de cultivo, pero conlleva el desarrollo de una disminución de la virulencia. Esto ha sido estudiado ampliamente en virus, donde los pasajes en serie se han utilizado como un enfoque clásico para el desarrollo de vacunas vivas atenuadas contra infecciones virales (Hanley, 2011). Por ejemplo, se han desarrollado vacunas contra el virus del sarampión, la fiebre amarilla y el virus de la varicela, entre otros basados en el pasaje en serie del virus en un huésped no natural (Hanley, 2011). También se ha reportado una reducción de la virulencia después de la adaptación de los medios en bacterias. Se ha informado una reducción en la patogenesis de *Staphylococcus aureus* después de 6 semanas de pasaje en serie, probablemente asociado con mutaciones puntuales en la región de codificación del gen *agrC* (Somerville et al., 2002). Otros ejemplos clásicos de atenuación patogénica a través de pasajes en serie incluyen el desarrollo de vacunas vivas atenuadas contra infecciones por *Salmonella typhi* y *Mycobacterium bovis* (Germanier et al., 1975;Toida, 2000). La mayoría de las veces estas adaptaciones se centran en la aparición de mutaciones puntuales aleatorias que modificarán un número restringido de proteínas, lo que confiere a los mutantes una ventaja comparativa en comparación con las poblaciones de tipo salvaje. Con miras al

desarrollo de una vacuna viva atenuada contra *P. salmonis*, el presente trabajó buscó determinar si el pasaje serial de la bacteria en un medio de cultivo artificial libre de células generará un impacto en la patogenesis de la bacteria, y de ocurrir, cuales serían las bases moleculares de esta atenuación más allá de mutuaciones puntuales.

2. HIPÓTESIS

3.1 Hipótesis 1

Mediante la utilización de genómica funcional es posible identificar mecanismos moleculares claves de la interacción entre *Piscirickettsia salmonis* y el salmón Atlántico (*Salmo salar*), así como los elementos no codificantes que participan en la regulación de estos procesos.

3.2 Hipótesis 2

La propagación de *P. salmonis* a largo plazo en un medio de cultivo libre de células genera modificaciones genómicas/transcriptómicas en respuesta a la adaptación al medio, atenuando la patogenicidad de la bacteria en células de salmones.

3. OBJETIVOS

3.1 Objetivo general

Determinar los mecanismos transcriptómicos codificantes y no-codificantes que rigen la interacción entre *P. salmonis* y el salmón Atlántico, y evaluar el impacto de la modificación de estos procesos en la patogenicidad de la bacteria.

3.2 Objetivos específicos

1. Caracterizar la respuesta transcriptómica codificante y no codificante del salmón Atlántico durante una infección con *P. salmonis.*

2. Caracterizar la respuesta transcriptómica codificante de *P. salmonis* durante la infección en salmón Atlántico.

3. Evaluar las modificaciones genómicas/transcriptómicas de *P. salmonis* adaptadas a un cultivo continuo en medio libre de células y evaluar sus efectos en la virulencia.

4. MATERIALES Y MÉTODOS

4.1 Caracterización de la respuesta transcriptómica codificante y no codificante del salmón Atlántico durante una infección con *P. salmonis.*

La siguiente metodología describirá los pasos realizados para el desarrollo del objetivo especifico 1. En resumen, utilizando herramientas de secuenciación masiva aplicada a mRNAs (RNA-seq) se buscó la identificación de los genes diferencialmente expresados en salmones infectados con *P. salmonis*. Estos genes fueron clasificados posteriormente en funciones moleculares a fin de determinar los procesos bilógicos involucrados en la respuesta del salmón a la bacteria. Utilizando estos mismos datos se procedió a la caracterización de lncRNAs diferencialmente expresados y mediante co-localización y co-expresión se busco determinar la existencia de posibles lncRNAs que pudiesen estar vinculados a la regulación de estos procesos. Finalmente, utilizando herramientas de secuenciación de small RNAs, se buscó la caracterización de miRNAs diferencialmente expresados en salmón expuestos a la bacteria. Identificados los miRNAs diferencialmente expresados, se procedió a la predicción de targets *in silico* a fin de determinar si estos miRNAs podrían estar regulando procesos claves de la interacción salmón-bacteria. El detalle de la metodología empleada para este objetivo se explica a continuación.

4.1.1 Diseño experimental

Salmones Atlánticos no vacunados (154.7 ± 13.5 g) se obtuvieron de un centro de cultivo ubicada en Puerto Montt, Chile y se transfirieron a la Estación de Biología Marina de la Universidad de Concepción (Dichato, Chile). Aquí, individuos seleccionados al azar, fueron monitoreados para descartar la presencia de diferentes patógenos comúnmente presentes en salmones. Después de la cuarentena, los individuos se dividieron aleatoriamente en 2 líneas de recirculación independientes de agua marina, cada una con cinco tanques de 370 litros. Por cada línea, cuatro estanques fueron destinados para muestreo y el uno para registro de mortalidad. Por cada estanque, 50 individuos fueron mantenidos por un periodo de aclimatación de 14 días previo al desafío. Después de este período, cada individuo fue anestesiado e inyectado intraperitonealmente con 0,2 ml de *P. salmonis* (cepa EM-90) con una concentración de $1x10^6$

bacterias por dosis (grupo desafío) o con 0,2 ml de PBS (grupo control). De ambos grupos, se colectó hígado, riñón, cerebro y bazo de 7 individuos a los 0, 3, 7, 14 y 30 días posteriores a la infección (ppp). Cada tejido fue almacenado en RNA-Later (Ambion, EE. UU.) a -80º C hasta el momento de su procesamiento. La mortalidad fue registrada diariamente y confirmada como resultado de SRS clínica y molecularmente. Todos los procedimientos involucrando animales se llevaron a cabo según las pautas aprobadas por el Comité de Ética de la Universidad de Concepción.

4.1.2 Estrategia de secuenciación de mensajeros (RNA-seq)

RNA total fue aislado desde 30 mg de tejido colectado previamente utilizando el kit RiboPure ™ (Ambion, EE.UU.), incluyendo un tratamiento con DNAse I de acuerdo con las instrucciones del fabricante para evitar Contaminación con ADN genómico. De esta manera, se realizaron extracciones de cerebro, bazo y riñón cefálico para los puntos 0, 7, 14 y 30 dias post inyección (dpi). La integridad del RNA obtenido fue estimada usando el 2200 TapeStation (Agilent Technologies, EE. UU.) y el screentape R6K. Las muestras con valores de integridad de RNA (RIN) mayores a 8 fueron consideradas válidas y utilizadas para posteriores análisis. Un pool de RNA por cada punto y tejido fue preparado utilizando 5 individuos y utilizado para la preparación de librerías de secuenciación Illumina utilizando el kit Truseq ™ v2 (Illumina, EE. UU.) De acuerdo con las instrucciones del fabricante. La calidad de las librerías obtenidas fue evaluada con relación a su distribución de tamaño, según lo estimado por la 2200 TapeStation (Agilent Technologies) utilizando el screentape D1K (Agilent Technologies). Cada librería fue cuantificada mediante qPCR utilizando el kit de cuantificación de librerías Illumina/Universal (Kappa, EE. UU.) De acuerdo con las instrucciones del fabricante. Dos réplicas biológicas fueron secuenciadas en la plataforma Miseq (Illumina) con 2 x 250 pair end reeds en el Laboratorio de Biotecnología y Genómica Acuática del Centro Interdisciplinario de Investigación en Acuicultura (INCAR), Chile.

4.1.3 Determinación de genes diferencialmente expresados

Los datos de secuenciación masiva obtenidos fueron analizados utilizando el software CLC Genomics Workbench v9.0 (CLC bio, Dinamarca). La data cruda (Raw Data) obtenida fue

filtrada por calidad y trimmiada en base a la secuencia de adaptadores utilizada durante la preparación de las librerías. Los reads restantes fueron ensamblados *de novo* utilizando el algoritmo incluido en CLC y considerando los siguientes parametros: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, y minimum contig length = 250. Los contigs resultantes fueron mapeados y ajustados utilizando la versión más reciente del genoma de *S. salar* (GenBank: GCA_000233375.4) (Lien et al., 2016), considerando los siguientes parámetros: mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, y similarity fraction = 0.8. Los contigs mapeados se usaron como secuencia de referencia para el análisis de expresión utilizando la herramienta CLC bio RNA-Seq y considerando los siguientes parámetros: minimum read length fraction = 0.9, minimum read similarity fraction = 0.9 y unspecific read match limit = 10. Los valores de expresión fueron estimados como transcritos por millón de reads (TPM). Los valores de expresión originales se normalizaron con el método de escalamiento, utilizando la media como valor de normalización y la media de la mediana como referencia. El análisis estadístico basado en la proporción se utilizó para evaluar expresiones diferenciales. Para esto, se determinó la distribución binomial (Baggerly et al., 2003), y los valores de p se ajustaron utilizando la corrección de la tasa de descubrimiento falso (FDR) (Benjamini and Hochberg, 1995). Aquellos genes con un valor absoluto de fold change mayor a 4 y con un p-value menor a 0,05 en relación con el grupo control fueron considerados como diferencialmente expresados. Una vez identificados los genes diferencialmente expresados, estos fueron anotados y clasificados en distintas rutas metabólicas utilizando el servidor de anotación automática KEGG (Moriya et al., 2007), y el servidor de anotación de enriquecimiento GO (https://david.ncifcrf.gov) (Huang et al., 2009b;a).

4.1.4 Validación por qPCR y estimación de carga bacteriana

Para corroborar los valores de expresión obtenidos mediante RNA-seq, se realizaron diferentes validaciones de RT-qPCR. Brevemente, cada RT-qPCR se realizó en StepOnePlus ™ (Applied Biosystems, Life Technologies, EE. UU.) Utilizando el mastermix de qPCR Maxima® SYBR Green / ROX (Thermo Scientific, EE. UU.) El ciclo de amplificación consistió en un paso a 95 ºC durante 10 min, 40 ciclos a 95 ° C durante 30 s, 60 ° C durante 30 s, y 72 ° C durante 30 s. Los análisis fueron realizados utilizando tres replicas técnicas y biológicas por carda gen

evaluado. Los valores de expresión se estimaron a través del método de ΔΔ Ct comparativo utilizando como gen normalizador el factor de elongación 1-alfa del salmón. Cada expresión se midió en al menos tres individuos, y se estimaron diferencias significativas entre las condiciones con la prueba t de Student ($p < 0.05$). Por su parte, la carga bacteriana de *P. salmonis* en los tejidos fue determinada utilizando la sonda TaqMan tal como se describió previamente (Corbeil et al., 2003).

4.1.5 Caracterización de lncRNAs.

Dado el poco grado de conservación de los lncRNAs, la caracterización estos transcritos se basó en la identificación y descarte de transcritos que evidencien algún potencial de codificación para proteínas. Brevemente, mediante BLASTx los contigs ensamblados se compararon con la base de datos de proteínas no redundantes de NCBI y UNIPROT, considerando los siguientes parámetros: word size = 3, gap cost existence = 11, extension = 1, y una matriz BLOSUM62. Contigs con un E value $< 1 \times 10{-}5$ fueron considerados como transcritos anotados. Para descartar la presencia de nuevos transcritos codificantes entre las secuencias restantes, se aplicaron tres filtros más: existencia de marcos de lectura abiertos (ORF), presencia de dominios de proteínas conservados (http://www.ncbi.nlm.nih.gov) y el potencial de codificación. Este úñltimo estimado utilizando el Coding Potential Assesment tool (CPAT) (Wang et al., 2013). Transcritos con ORFs > 200, con dominios de proteínas conservados dentro de cualquiera de sus 6 marcos de lectura, o exhibiendo potencial de codificación fueron descartados. Los contigs restantes se clasificaron como lncRNAs putativos y se compararon con lncRNAs reportados previamente para *S. salar* (Boltaña et al., 2016) y mapeados contra el genoma *de S. salar* para la obtención de la lista final de lncRNAs caracterizados para el salmón.

4.1.6 Determinación *in silico* de interacción lncRNAs genes codificantes

A fin de determinar si los lncRNAs caracterizados podrían tener un rol en la regulación de la respuesta transcriptómica codificante, se realizó un análisis *in silico* de co-localizacion genómica y co-expressón génica. Para esto, primero se determinó el numero de lncRNAs diferencialmente expresados entre los grupos tal como se describió previamente (Sección 4.1.3). Una vez obtenidos los lncRNAs y los mRNAs diferencialmente expresados estos fueron

mapeados en el genoma de *S. salar* según se describió previamente (Sección 4.1.5). De cada gen diferencialmente expresado se extrajeron los lncRNAs ubicados en una ventana génica de 10 kb (co-localización). Finalmente, se estimo el coeficiente de correlación de Pearson entre los valores de cada gen y los lncRNAs co-localizados en la ventana de 10kb previamente seleccionada (co-expresión). Esta correlación fue estimada utilizando y graficada utilizando la librería de R Corrplot (https://cran.r-project.org/).

4.1.7 Estrategia de secuenciación miRNAs.

Los tejidos del bazo y riñón, provenientes del diseño experimental descrito previamente (Sección 4.1.2), fueron usados para la extracción de RNA total y secuenciación de small RNAs (miRNA-Seq). El tiempo utilizado fue el día 14 post infección en el grupo infectado con P. salmonis (+Psal) y el grupo control (-Psal). El RNA total fue extraído y verificado tal como se describe en la sección 4.1.2. Las librerías fueron preparadas utilizando el kit TruSeq Small RNA Kit (Illumina, San Diego, CA, EE. UU) según las instrucciones del fabricante y secuenciadas en triplicado utilizando la plataforma MiSeq (Illumina) utilizando cartridge de corrida de 50 ciclos en el Laboratorio de Biotecnología y Genómica Acuática del Centro Interdisciplinario de Investigación en Acuicultura (INCAR), Chile.

4.1.8 Caracterización de miRNAs y selección de diferencialmente expresados

Los reads sin procesar (Raw data) fueron analizados como se describe en la sección 4.1.3. Los reads filtrados fueron posteriormente mapeados contra distintas bases de datos de RNAs no codificantes incluyendo NCBI (http://www.ncbi.nlm.nih.gov/), RFam (http://rfam.janelia.org/), y Repbase (http://www.girinst.org/repbase/) para remover cualquier read perteneciente a mRNAs, rRNA, tRNA y snoRNA. Los reads además fueron mapeados contra la última versión del genoma del salmón Atlántico. Cualquier read con un match perfecto con cualquiera de estas bases de datos o con una región codificante del salmón fueron descartados de análisis posteriores. Finalmente, se realizo un “extract and count” a fin de obtener una lista de secuencias small RNAs única, la cual fue posteriormente anotada utilizando la base de datos de miRNA miRBase 21 (Griffiths-Jones et al., 2006;Kozomara and Griffiths-Jones, 2014a). La

determinación de los miRNAs diferencialmente expresados fue realizada como fue descrito previamente en la sección 4.1.3 usando la lista de miRNAs anotados mediante miRBase.

4.1.9 Predicción de targets de miRNAs y análisis de Gene Ontology (GO).

Una vez identificados los miRNAs diferencialmente expresados de procedió a determinar *in silico* los más posibles targets para cada secuencia. Para esto se usaron los softwares PITA (Kertesz et al., 2007) y miRanda (John et al., 2004). Las comparaciones se realizaron utilizando los miRNAs diferencialmente expresados y las regiones 3' UTR de los mRNAs diferencialmente expresados identificados en la sección 4.1.3. Los parámetros utilizados para PITA y miRanda fueron: open gap penalty = -9, extended gap penalty = - 4, score threshold = 140, energy threshold = 1 kcal/mol, y scaling parameter = 4. Aquellas interacciones miRNA-mRNA con una energía libre de Gibbs estimada menor a -12 fueron consideradas como probables interacciones. Finalmente, se realizó un análisis de enriquecimiento GO considerando las interacciones miRNA-mRNA probable a fin de determinar las rutas probables reguladas por los miRNAs diferencialmente expresados. Los resultados fueron graficados utilizando la plataforma REVIGO (Supek et al., 2011) y el software R.

4.2 Caracterización la respuesta transcriptómica codificante de *P. salmonis* durante la infección en salmón Atlántico

A continuación, se describen los métodos utilizados para la realización del segundo objetivo especifico. Brevemente, se utilizo la aproximación Dual RNA-seq para estudiar de manera paralela el transcriptoma del salmón y de la bacteria durante un proceso de infección. Mediante esta aproximación se logró identificar distintas rutas reguladas durante la patogénesis. Sin embargo, se evidenció una respuesta transcriptómica común entre patógeno y hospedador asociada al metabolismo de aminoácidos. Debido a esto, se caracterizó un conjunto de genes asociados a la biosíntesis y degradación de aminoácidos en *P. salmonis* y se comparó con otras bacterias patógenas. Posteriormente, se realizaron cultivos experimentales con distintos niveles de suplementación de aminoácidos a fin de determinar la importancia de estos nutrientes para el crecimiento de la bacteria. A continuación, se describe en detalle los procedimientos realizados para el cumplimiento de este objetivo.

4.2.1 Estrategia de secuenciación

El Dual RNA-seq fue realizado utilizando muestras de riñón cefálico y bazo a 3, 7 y 14 días post infección obtenidas del diseño experimental descrito en la sección 4.1.1. A diferencia de las extracciones de RNA realizadas para los ensayos previos, la preparación del RNA fue realizada mediante depleción de RNA ribosomal en vez de captura de mensajeros mediante cola Poly-A. Esto fue realizado utilizando el kit Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, EE. UU.). El RNA resultante conteniendo transcritos tanto de la bacteria como del hospedador fueron usados para preparar librerías de secuenciación masiva utilizando el kit TrueSeq RNA sample preparation kit (Illumina, San Diego, CA, EE. UU.). Cada librería fue secuenciada en duplicado utilizando la plataforma HiSeq a paired-en reads de 100bp utilizando el servicio de secuenciación de Macrogen (Corea).

4.2.2 Dual RNA-seq y análisis de expresión diferencial.

Los reads sin procesar fueron filtrados tal como se describió en la sección 4.1.3. Una vez filtrados, utilizando el genoma de *S. salar* y de *P. salmonis*, se discriminó bioinformáticamente los reads pertenecientes tanto al salmón como a la bacteria. Para esto los reads fueron mapeados a ambos genomas utilizando los siguientes parámetros: mismatch cost of 2, insertion/deletion cost of 3 y similarity/length fraction of 0.8. Los reads mapeados a ambos genomas fueron extraidos y utilizados para la realización de RNA-seqs individuales tanto para *S. salar* como para *P. salmonis*. De esta manera, se determinaron los genes diferencialmente expresados para ambas especies utilizando la metodología descrita en la sección 4.1.3. Los valores de expresión provenientes de los 3 días post infección fueron utilizado como línea base para la determinación de expresión diferencial de genes en P. salmonis. Esto debido a que en los controles (-Psal) no existen reads bacterianos sobre los cuales hacer la comparación.

4.2.3 Anotación funcional y validación por qPCR.

Los genes diferencialmente expresados tanto para la bacteria como para el salmón fueron anotados en diferentes rutas moleculares, tal como fue descrito en la sección 4.1.3. De esta forma, se buscaron rutas moleculares comunes diferencialmente reguladas entre la respuesta de *P. salmonis* y el salmón Atlántico. Una lista de genes diferencialmente expresados tanto para el

hospedador como para la bacteria fueron utilizados para la validación de los resultados de expresión génica obtenidos mediante RNA-seq. Estas qPCRs fueron realizadas tal como se describe en la sección 4.1.4. En el caso de P. salmonis, el gen 16S fue elegido como normalizador de la expresión génica. Las diferencias significativas entre 7 y 14 días post infección con respecto a 3 días post infección se estimaron con la prueba t de Student ($p < 0,05$).

4.2.4 Rol del metabolismo de aminoácidos en la interacción patógeno-hospedero.

El análisis Dual RNA-seq reveló un gran número de genes expresados diferencialmente asociados con el metabolismo de los aminoácidos durante el proceso de infección. Debido a esto, se exploró la importancia de los aminoácidos en el metabolismo de *P. salmonis*. Primero se comparó el repertorio de genes correspondientes al metabolismo de aminoácidos en *P. salmonis* en comparación con otro patógeno que afecta la salmonicultura (*Aeromonas salmonicida*) y una bacteria filogenéticamente cercana a *P. salmonis* (*Francisella tularensis*). De esta forma, se compararon el número de genes anotados en los genomas de cada incluyendo las rutas KEGG del metabolismo de la histidina (00340), la degradación de la valina, leucina e isoleucina / biosíntesis (00280 y 00290), el metabolismo de la arginina y la prolina (00330), la biosíntesis / degradación de la lisina 00300 y 00310), metabolismo de cisteína y metionina (00270), metabolismo de glicina, serina y treonina (00260), fenilalanina, tirosina y biosíntesis de triptófano (00400) y metabolismo de alanina, aspartato y glutamato (00250). Además, se realizaron cultivos líquidos de *P. salmonis* suplementados con distintos tipos de aminoácidos. Se preparó un medio basal (BM) con Eugon (30,4 g/ l) suplementado con $FeCl_3$ (2 mM) (control negativo), un medio completo (CM) preparado con Eugon (30,4 g / l) suplementado con $FeCl_3$ (2 mM) y Casaminoácido (1%) (Control positivo). Además, se prepararon medios experimentales suplementados con 1% de valina, leucina e isoleucina. Todos los cultivos se realizaron por triplicado y se mantuvieron a 20 ºC con una agitación constante de 100 rpm. El crecimiento bacteriano se controló diariamente a través del cambio en la densidad óptica a una absorbancia de 600 nm. Se llevó a cabo un t-test para identificar diferencias estadísticamente significativas ($p < 0,01$) entre los tratamientos.

4.3 Evaluación de las modificaciones genómicas/transcriptómicas de *P. salmonis* adaptadas a un cultivo continuo en medio libre de células y evaluar cambios en su virulencia.

A continuación, se presenta la metodología utilizada para el desarrollo del objetivo especifico 3. En resumen, se realizó un cultivo serial de *P. salmonis* en un medio libre de células por un periodo aproximado de 2 años (200 pasajes). Combinando short y long reads secuencing, se identificaron los cambios genómicos y transcriptómicos que sufrió la bacteria durante este proceso de cultivo. Los resultados evidenciaron cambios en distintos genes vinculados a patogenicidad. Debido a esto, se realizó una infección in vitro de células de riñón cefálico de salmón (SHK-1) y se comparó la capacidad de producir efectos citopáticos y lisis en la bacteria original (P0) y la cultivada continuamente en medio libré de células (P200)

4.3.1 Cultivo continuo de *P. salmonis* en medio libre de células

Medio de cultivo Eugon fue seleccionado para el experimento de cultivo serial de *P. salmonis*. Este medio se preparó disolviendo 30,4 g de caldo Eugon (BactoTM) en 1 litro de agua destilada autoclavada (121 ° C durante 20 minutos). Una vez disuelto, el se autoclavó nuevamente y se dejó enfriar a temperatura ambiente. En este punto, el medio fue suplementado con 1% (m/v) de Casaminoácidos (BactoTM) y con $FeCl_3$ (MERCK) a una concentración final de 2 mM. Tanto los casaminoácidos como el $FeCl_3$ agregados asépticamenteal medio mediante la filtración a de una membrana de 0,22um. Para el pasaje 0 P0, células CHSE-214 se cultivaron en matraces T75 a 20 ºC con 20 ml de DMEM (HyClone) suplementado con 10% de suero bovino fetal inactivado por calor (Biological Industries) y 1% de aminoácidos no esenciales (HyClone). Cuando los cultivos celulares alcanzaron una confluencia del 70%, los matraces se inocularon con 10^6 *P. salmonis*. Los cultivos se incubaron a 20ºC hasta la aparición de efectos citopáticos (CE) producto de la infección bacteriana en alrededor del 80% de las células CHSE-214. En este punto, las células fueron colectadas y el sobrenadante se obtuvo para su posterior centrifugación a fin de sedimentar los desechos celulares de CHSE-214. Se obtuvo el sobrenadante que contenía *P. salmonis*, el cual se consideró como pasaje P0. Se utilizó 1 ml de bacteria P0 para inocular un tubo falcon de 15 ml que contenía 2 ml de cultivo en caldo Eugon. Los cultivos se mantuvieron a 20º C con agitación constante de 100 rpm. Cuando el cultivo de

bacterias alcanzó la fase exponencial (A_{600} 0,3-0,4), se transfirieron 300 µl a un nuevo falcon de 15 ml que contenía 2 ml frescos de caldo Eugon (P1). Esto se consideró como un pasaje y el proceso se repitió 200 veces (hasta P200) cada vez que el cultivo alcanzó la fase exponencial. Dada la lenta tasa de crecimiento de *P. salmonis*, la fase exponencial se logró cada 3 a 4 días, por lo que fue necesario mantener un cultivo continuo de la bacteria durante aproximadamente 2 años. Muestras de DNA y RNA fueron colectadas desde los cultivos P0, P1 y P200.

4.3.2 Secuenciación de genomas P0 y P200

Se realizó la secuenciación del genoma completo para comparar la dinámica del genoma entre las bacterias originales (P0) y después de 200 pasajes (P200). Para esto, se aisló el DNA total de los cultivos P0 y P200 utilizando el kit DNeasy Blood & Tissue Kits (Qiagen) de acuerdo con las instrucciones del fabricante. La integridad del DNA aislado se confirmó a través de un gel de agarosa al 1% y la pureza se evaluó a través de la relación de absorbancia 260/280 y 260/230 estimada mediante el espectrofotómetro NanoDrop 1000 (Thermo Scientific). Las muestras de DNA sin degradación y con relaciones de absorbancia superiores a 1,8 se enviaron a Macrogen Inc. (Corea) para la secuenciación del genoma completo utilizando PacBio Single Molecule Real Time (SMRT). Los reads obtenidos fueron filtrados por calidad y tamaño y ensamblados *de novo* utilizando el flujo de trabajo descrito mediante la plataforma HGAP3. Los genomas y plásmidos obtenidos tanto para P0 como para P200 se anotaron con el servidor RAST (http://rast.nmpdr.org/rast.cgi) (Overbeek et al., 2014) utilizando como referencia los genomas de *P. salmonis* (NCBI ID: 1238) y parámetros por defecto. Para identificar el reordenamiento genómico, se detectaron bloques de sintenidad entre los genomas P0 y P200 y los plásmidos. Para este propósito, se usó la herramienta Synteny Block ExpLoration (Sibelia) (http://bioinf.spbau.ru/sibelia) para identificar regiones compartidas entre ambos genomas. Los resultados se visualizaron luego utilizando el software Circos versión 0.69-6 (http://circos.ca/) (Krzywinski et al., 2009).

4.3.3 Secuenciación de transcriptomas en distintos pasajes

Se realizaron análisis de RNA-seq para identificar genes expresados diferencialmente entre las bacterias P0, P1 y P200. La extracción de RNA total, control de calidad, preparación de librerías

y secuenciación se realizó según lo descrito en la sección 4.1.2. La indentificacion de genes de *P. salmonis* diferencialmente expresados entre los pasajes se llevó a cabo según lo descrito en la sección 4.1.3.

4.3.4 Explorando la atenuación por pasajes de *P. salmonis.*

Se realizó un experimento *in vitro* para evaluar el efecto del cultivo serial de *P. salmonis* en un medio libre de células sobre un cultivo celular de salmónidos. Para esto, se usó una línea celular proveniente de riñón cefálico de salmón (SHK-1), las cuales se cultivaron en frascos T75 a 20 ºC con 20 ml de medio Leibovitz L-15 (HyClone) suplementado con 10% de suero bovino fetal inactivado por calor (Biological Industries) y 1% de Aminoácidos no esenciales (HyClone). Cuando los cultivos celulares alcanzaron una confluencia del 70%, los frascos se inocularon con 10^6 *P. salmonis* provenientes de pasaje P0 o P200. Los matraces se incubaron durante 14 días a 20 ºC y se registraron efectos citopáticos después de este período.

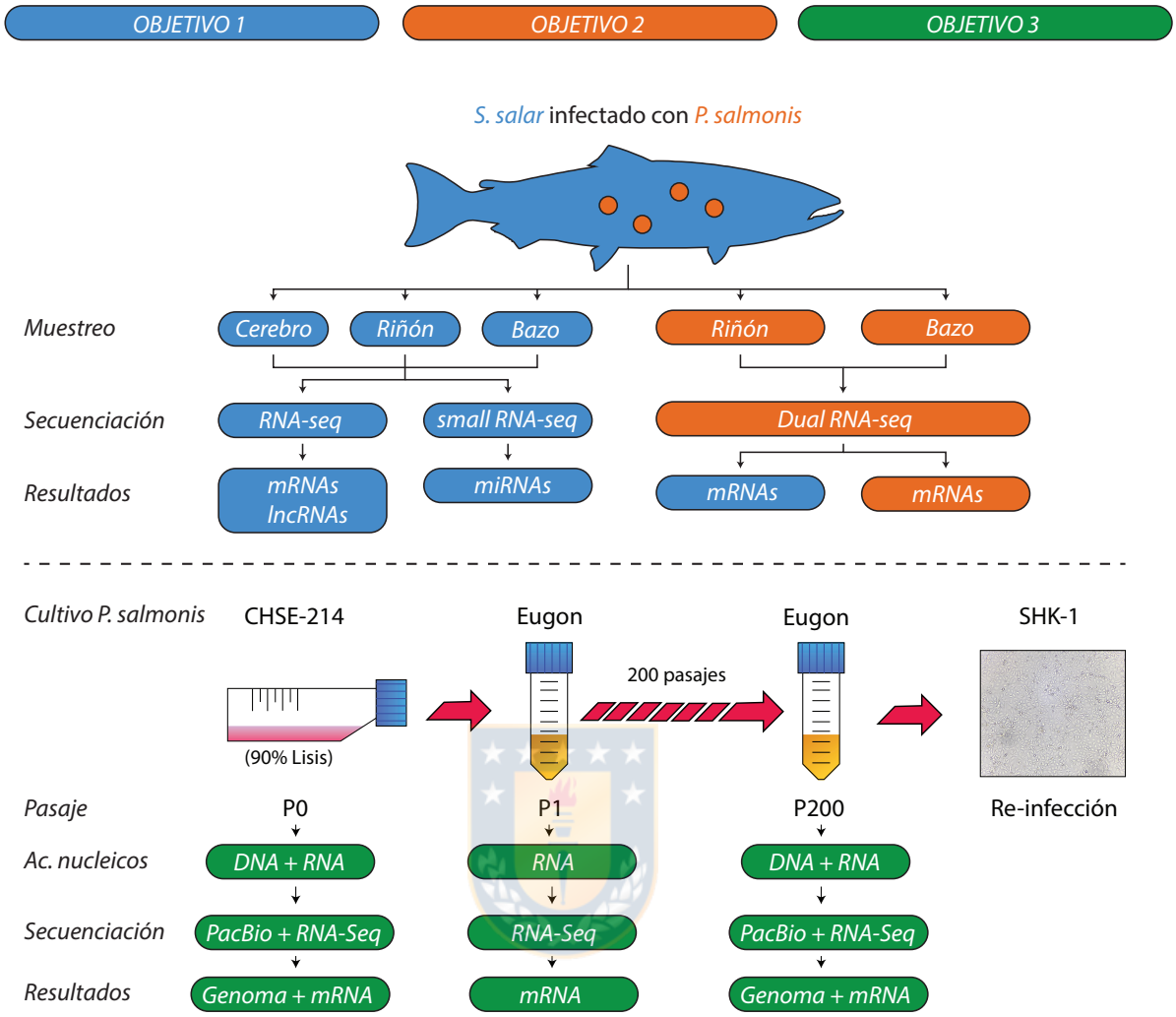

Figura 1. Esquema general de la metodología utilizada.

5. RESULTADOS

CAPÍTULO I (H1, OE1). Novel insights into the response of Atlantic salmon (*Salmo salar*) to *Piscirickettsia salmonis*: Interplay of coding genes and lncRNAs during bacterial infection

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Abstract

Despite the high prevalence and impact to Chilean salmon aquaculture of the intracellular bacterium *Piscirickettsia salmonis*, the molecular underpinnings of host-pathogen interactions remain unclear. Here, the interplay of coding and non-coding transcripts has been proposed as a key mechanism of immune response regulation. Therefore, the aim of this study was to characterize the coding and non-coding transcriptional response of Atlantic salmon (*Salmo salar*) during *P. salmonis* infection. For this, RNA-Seq was conducted in brain, spleen, and head kidney samples, revealing different transcriptional modulations according to bacterial load. Additionally, while most of the regulated genes annotated for diverse biological processes during infection, a common response associated with clathrin-mediated endocytosis and iron homeostasis was present in all tissues. Interestingly, while endocytosis-promoting factors and clathrin inductions were upregulated, endocytic receptors were mainly downregulated. Furthermore, the regulation of genes related to iron homeostasis suggested an intracellular accumulation of iron, a process in which heme biosynthesis/degradation pathways might play an important role. Regarding the non-coding response, 918 putative long non-coding RNAs were identified, where 425 were newly characterized for *S. salar*. Finally, co-localization and co-expression analyses revealed a strong correlation between the modulations of long non-coding RNAs and genes associated with endocytosis and iron homeostasis. These results represent the first characterization for long non-coding RNA regulation during bacterial infection in a bony fish, with findings suggesting a correlation between the coding and non-coding transcriptional response.

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Novel insights into the response of Atlantic salmon (*Salmo salar*) to *Piscirickettsia salmonis*: Interplay of coding genes and lncRNAs during bacterial infection

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ABSTRACT

Despite the high prevalence and impact to Chilean salmon aquaculture of the intracellular bacterium *Piscirickettsia salmonis*, the molecular underpinnings of host-pathogen interactions remain unclear. Herein, the interplay of coding and non-coding transcripts has been proposed as a key mechanism involved in immune response. Therefore, the aim of this study was to evidence how coding and non-coding transcripts are modulated during the infection process of Atlantic salmon with *P. salmonis*. For this, RNA-seq was conducted in brain, spleen, and head kidney samples, revealing different transcriptional profiles according to bacterial load. Additionally, while most of the regulated genes annotated for diverse biological processes during infection, a common response associated with clathrin-mediated endocytosis and iron homeostasis was present in all tissues. Interestingly, while endocytosis-promoting factors and clathrin inductions were upregulated, endocytic receptors were mainly down-regulated. Furthermore, the regulation of genes related to iron homeostasis suggested an intracellular accumulation of iron, a process in which heme biosynthesis/degradation pathways might play an important role. Regarding the non-coding response, 918 putative long non-coding RNAs were identified, where 425 were newly characterized for *S. salar*. Finally, co-localization and co-expression analyses revealed a strong correlation between the modulations of long non-coding RNAs and genes associated with endocytosis and iron homeostasis. These results represent the first comprehensive study of putative interplaying mechanisms of coding and non-coding RNAs during bacterial infection in salmonids.

1. Introduction

Salmonid Rickettsial Septicemia (SRS) is a severe systemic disease associated with major economic losses worldwide in aquaculture [1]. Since the first report of SRS in Chile at the end of the 1980s [2], this disease has rapidly become the principal infectious disease in and the main threat to sustainable salmonid aquaculture in this country [3]. The etiological agent of SRS is *Piscirickettsia salmonis*, a Gram-negative, non-motile, generally cocoid and aerobic bacterium belonging to the gamma subdivision of Proteobacrteria [4]. Initially, *P. salmonis* was described as an obligate intracellular bacterium that, consequently, could only be cultivated in eukaryotic cell lines [5]. This hampered *P. salmonis* culture studies, translating into a poor understanding of many aspects, including the basic biology of transmission, virulence, and host responses [3]. Nevertheless, evidence supporting a facultative intracellular nature of *P. salmonis* [6,7] and the development of cell-free culture media [8] favorably supported performing novel studies to fill knowledge gaps. Although genetic selection is a proposed management strategy for *P. salmonis*, a negative genetic correlation has been reported between harvest weight and *P. salmonis* resistance in *Oncorhynchus kisutch* [9]. Therefore, novel and effective SRS treatments are needed.

Developing new disease mitigation strategies requires understanding host-pathogen interaction mechanisms beyond pathogen virulence and the host response. However, few studies have aimed to clarify the molecular response mechanisms of salmonids to *P. salmonis* infections. Regarding this, microarray-based approaches

Abbreviations: Abcg2, ATP-binding cassette sub-family G member 2; ARF6, ADP-ribosylation factor 6b; dpi, days post-injection; ISAv, infectious salmon anemia virus infection; lncRNAs, long non-coding RNAs; SRS, salmonid rickettsial septicemia.

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coupled with qPCR validations have identified the most regulated genes during the infective process of *P. salmonis* [10–12]. In these reports, antioxidant genes were positively regulated in Atlantic salmon macrophages infected with *P. salmonis*. In turn, genes associated with adaptive immune responses were strongly downregulated in infected hematopoietic kidney [10]. Similar results were obtained via transcriptomic analyses of *Salmo salar* livers, head kidneys, and muscles following a *P. salmonis* challenge [11]. Specifically, oxidative and inflammatory response genes were upregulated while genes associated with the adaptive immune response, G protein signaling pathway, and apoptotic process were downregulated. Consequently, the reported transcriptome modulation suggests a putative mechanism employed by *P. salmonis* to evade host defenses. Furthermore, transcriptional response in head kidney of *S. salar* with distinct levels of susceptibility to the bacteria was assessed [12]. The results suggested the importance of the iron-deprivation defense system based on the iron content, bacterial load and the regulation of genes associated to cellular iron depletion [12]. Although these studies have contributed to better understanding of host response to *P. salmonis*, there are still key questions unresolved beyond the microarray analysis.

RNA-seq offers some comparative advantages to other large scale transcriptomic technologies, such as a large dynamic range that permits biological isoforms to differentiate transcription profiles; the detection of genetic variants; and the detection of unexpected transcripts involved in several biological processes [13]. Among them, it has been proposed that a large portion of the transcriptome is dedicated to the production of non-coding transcripts, which can functionally act as RNAs [14]. Here, long non-coding RNAs (lncRNAs), which are non-coding RNAs longer than 200 nucleotides have emerged with key roles in the regulation of protein coding genes [15]. Recently, it has been demonstrated that lncRNAs might play a key role in the activation and repression of the immune system, the regulation of the inflammatory response and even the regulation of T cell differentiation and migration [16–19]. Despite evidence for the various regulatory roles of lncRNAs, few studies have characterized lncRNAs in bony fish [20–24]. Here, recently our research group reported strong regulated of lncRNAs in *S. salar* following infectious salmon anemia virus infection (ISAv), suggesting putative roles in response to viral infection [24]. However, no additional information is available regarding lncRNA functions during intracellular bacterial infections in salmonids.

Therefore, the aim of this study was to reveal the coding and non-coding transcriptional responses of *S. salar* during *P. salmonis* infection. The results evidenced strong clathrin-mediated endocytosis pathway regulation, suggesting a putative mechanism of immune evasion by *P. salmonis*. In turn, *S. salar* defended against bacterial infection with a complex transcriptomic response, which included a strong regulation of the heme biosynthesis/degradation pathway and of iron transporter proteins such as hepcidin and haptoglobin, among others. Finally, there was a significant transcriptional correlation between highly overregulated genes and adjacent lncRNAs during infection.

2. Materials and methods

2.1. Experimental design

Unvaccinated Atlantic salmon (*S. salar*; n = 200, ≈158.3 ± 35.4 g) were acquired from a commercial farm (Puerto Montt, Chile) and maintained in six tanks (500 L) at Aquagestion facilities (Puerto Montt, Chile). Individuals underwent random sanitary exams to discard the presence of *P. salmonis* (SRS), the infectious pancreatic necrosis virus, and ISAv. After six weeks of acclimation, individuals were anesthetized with benzocaine (Sigma; 20% w/v, 50 mg/L) and intraperitoneally injected with 0.2 mL of *P. salmonis* (LF89 strain) at 1×10^4 PFU/mL (challenged group) or with PBS (control group). After injection, four tanks were used for sampling, and the remaining tanks were used to record mortality. At 0, 7, 14, and 30 days post-injection (dpi), 15 total individuals per sampling point were sacrificed with an anesthetic overdoses and brain, spleen and head kidney were isolated. These tissues were selected due their role in immunity [25] and also because spleen and head kidney are one of the most affected tissues during the infection with *P. salmonis* [3]. On the other hand, brain is also affected and even this tissue is used for the isolation of *P. salmonis* from infected fish [26,27]. All tissues were stored in the RNAlater® solution (Ambion, USA) at −80 °C until RNA isolation. Mortalities were molecularly and clinically confirmed as resulting from *P. salmonis* infection (data not shown).

2.2. RNA isolation and sequencing

RNA isolation and sequencing were performed as previously described [28]. Briefly, total RNA was isolated from sampled fish using brain, spleen, and head kidney tissues (30 mg each) and the RiboPure™ Kit (Ambion) and treated with DNAse I, RNAse-free (Thermofisher) according to the manufacturer's instructions to avoid gDNA contamination. RNA integrity was estimated by the 2200 TapeStation (Agilent Technologies, USA) using R6K screen tape. Samples with RIN values > 8 were used in further analyses. RNA pools were constructed using combined values from five different individuals for each tissue (brain, spleen, and head kidney) and sampling point (0, 7, 14, and 30 dpi). Illumina sequencing libraries were constructed for each RNA pool using the Truseq™ RNA Sample Preparation Kit v2 (Illumina, USA) according to the manufacturer's instructions. The quality of constructed libraries was assessed based on length distribution, as estimated by the 2200 TapeStation (Agilent Technologies) using D1K screen tape (Agilent Technologies). Libraries with a mean length >300 bp were used for sequencing, and libraries were qPCR quantified using the Library Quantification Kit Illumina/Universal (Kappa, USA) according to the manufacturer's instructions. Two biological replicates were sequenced on the Miseq platform (Illumina) with 2 × 250 paired-end reads in six different runs at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), Chile.

2.3. Differential expression and pathway analyses

Sequencing data were analyzed with the CLC Genomics Workbench software v9.0 (CLC bio, Denmark). Illumina adapters/index were trimmed, and a quality-based filter was applied to raw sequencing reads. Trimmed reads were *de novo* assembled using the CLC bio algorithm and considering a mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, and a minimum contig length = 250. Resulting contigs were mapped and adjusted using the most recent version of the *S. salar* genome (GenBank: http://www.ncbi.nlm.nih.gov/assembly/GCA_000233375.4) [29], considering a mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, and a similarity fraction = 0.8. The mapped contigs were used as reference sequence for transcriptomic analysis using the CLC bio RNA-Seq tool and considering a minimum read length fraction = 0.9, minimum read similarity fraction = 0.9, and unspecific read match limit = 10. Expression values were calculated as reads per kilobase of exon model per million mapped reads. Original expression values were normalized with the scaling method, using the mean as a normalization value and the median mean as a

reference. Proportion-based statistical analysis was used to assess differential expressions. For this, Beta-binomial distribution was determined [30], and p-values were adjusted using false discovery rate correction [31]. Expression values were compared against respective control groups, and contigs with |fold change| > 4 and corrected p-values < 0.01 were considered differentially expressed. Finally, the KEGG Automatic Annotation Server [32], GO Enrichment Annotation Server (https://david.ncifcrf.gov) [33,34], and the bidirectional best-hit assignment method were used to annotate the differentially expressed genes among different signaling pathways.

2.4. RT-qPCR validation and bacterial load

To corroborate the expression values obtained by RNA-seq, different RT-qPCR validations were performed. The evaluated genes and applied primers are listed in Supplementary Table S1. Briefly, each RT-qPCR was conducted in StepOnePlus™ (Applied Biosystems, Life Technologies, USA) using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) and an amplification cycle of 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Analyses considered three biological and technical replicates for each time point and tissue. Expression values were estimated through the comparative ΔCt method using *elongation factor 1 alpha* as an internal control, per previous validation as a reference gene during *P. salmonis* infections [11,12]. Each expression was measured in at least three individuals, and significant differences between conditions were estimated with the Student's *t*-test ($p < 0.05$). Comparisons between the fold-changes obtained with RNA-seq and RT-qPCR revealed a correlation of $R^2 > 0.8$ (Supplemental Fig. S1). Furthermore, bacterial load was estimated through the TaqMan assay, as previously described for *P. salmonis* [35] and using RNA pools for each tissue and sampling time.

2.5. Identification of lncRNAs and correlation analysis

A step-wise approach, based on a previously described pipeline [24], was used to identify lncRNAs. Briefly, to discard protein coding transcripts, assembled contigs were blasted against the non-redundant BLASTx protein database and UNIPROT protein database, considering a word size = 3, gap cost existence = 11, extension = 1, and a BLOSUM62 matrix. An E value = $1 \times 10-5$ threshold was used to identify unannotated sequences. To discard the presence of novel coding transcripts among the remaining sequences, three more filters were applied – the presence of open reading frames, conserved protein domains (http://www.ncbi.nlm.nih.gov), and coding potential, as estimated by the coding potential assessment tool [36]. Transcripts with interior open reading frames >200, conserved protein domains within any of the six translation frames, or coding potential were removed. The remaining contigs were classified as putative lncRNAs and mapped against previously reported lncRNAs for *S. salar* [24] considering a mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, and a similarity fraction = 0.8. Expression values for lncRNAs were estimated through RNA-Seq, as detailed above. The Pearson correlation coefficient between lncRNAs and coding genes was estimated and plotted with the Corrplot library in R (https://cran.r-project.org/).

3. Results

3.1. Infection and transcriptional responses

The first mortality due to *P. salmonis* infection was recorded 11 dpi. Thereafter, survival rates remained in declined, with a maximum mortality of 70% (Fig. 1A). Together with the clinical observations (data not shown), this result confirmed the pathogenicity of the bacterial strain used as the challenge. Furthermore, estimated bacterial loads were highest in the spleen and head-kidney at 14 dpi and at 30 dpi in the brain (Fig. 1B).

To assess the transcriptional response of Atlantic salmon during *P. salmonis* infection, whole transcriptome sequencing was carried out in infected and uninfected individuals. For this, total RNA was isolated and sequenced from brain, spleen, and head kidney samples. After quality control and adapter/index trimming, each library contained an equal number of reads (≈53 million clean reads). The remaining reads were assembled and mapped against the Atlantic salmon genome (GenBank: GCA_000233375.4), resulting in 225,576 high quality contigs that were used as reference sequences for further transcriptional analysis. Global expression patterns for each tissue and time were estimated, and different transcriptional modulations were found.

While the spleen and head kidney had a similar temporal distribution of differentially expressed transcripts (fold-change > 4, p-values < 0.01), as compared to the control, the brain evidenced late activation of the transcriptional response (Fig. 1C). There were 146 and 56 differentially expressed contigs in the brain 7 and 14 dpi, respectively, but the highest number of differential transcripts (i.e. 2093) in this tissue was obtained 30 dpi. In turn, at 7, 14, and 30 dpi, the spleen and head kidney respectively evidenced (371, 515, and 506) and (478, 527, and 1267) differential transcripts. Regarding modulation intensity, the head kidney registered the greatest number of regulated contigs (fold-changes > 20; Fig. 1C).

3.2. Annotation and pathway analyses

To establish the most regulated genes and molecular pathways during bacterial infection, both GO and KEGG annotations were applied considering the differentially expressed contigs. In the brain, the most represented GO terms were associated with vesicle-mediated transport, nerve impulse transmission, and the neurological system process, while the most related KEGG pathways included the glutamatergic synapse and synaptic vesicle cycle (Fig. 2). In the spleen, the most regulated biological processes were associated with the immune response, proteolysis, and a response to wounding, while the most related KEGG pathways included carbon metabolism and cytokine-cytokine receptor interactions (Fig. 2). In the head kidney, differentially expressed genes were distributed between protein localization and the homeostatic and oxidation-reduction processes (Fig. 2). Furthermore, a common response associated with endocytosis and the ion homeostasis regulation was found in all tissues. The terms "homeostatic process" and "cellular ion homeostasis" were represented in the spleen, while the head kidney presented the terms "response to metal ions" and "homeostatic processes" (Fig. 2 and Supplemental Fig. S2).

Due to the common regulation of endocytosis and iron homeostasis, bioinformatics analyses focused on the main molecular components involved in these pathways. Based on the KEGG annotation, the classical pathway of endocytosis was mapped (Fig. 3). Using *Danio rerio* as a reference, all genes comprising the endocytosis pathway were identified, with the exception of the charged *Multivesicular body protein* 5 (*CHMP5*) and the *ESCRT-II complex subunit VPS22* (*VPS22*). Additionally, matching the expression levels in each tissue revealed an upregulation of both clathrin-mediated and -independent endocytosis stimulating factors, such as of the *ADP-ribosylation factor 6b (ARF6)*, *phosphatidylinositol-4-phosphate 5-kinase-like 1 (PIP5k)*, *phospholipase D1a (PLD)*, and *v-src avian sarcoma viral oncogene homolog (SRC)*. However, several endocytic receptors and respective adaptor proteins

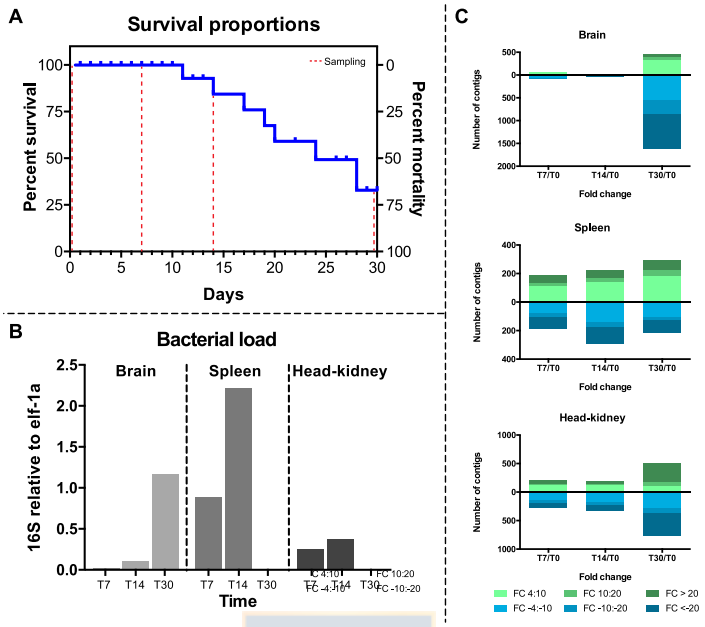

Fig. 1. **(A)** Survival curve after intraperitoneal injection with *P. salmonis*. Blue line represents the survival/mortality rate (%), and the dashed red line represents sampling days (0, 7, 14, and 30 days post-injection). **(B)** Relative amount of *P. salmonis* load at each sampling point in the brain, spleen, and head kidney. **(C)** Transcriptional modulation of the differentially expressed transcripts for each tissue (fold change > 4, p-values < 0.01). Distinct colors represent a different range of fold change (FC) value for each time point as compared to the respective controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were mainly downregulated after bacterial infection, with the exceptions of *Clathrin* and *Spartin*. Furthermore, downstream genes associated with the endocytosis signaling pathway and endosome maturation showed no significant variations among tissues. The downregulation of key genes was also found in the phagosome pathway, where phagocytosis-promoting receptors and genes related to phagosome maturation were less expressed in the brain and head kidney as compared to the spleen (Fig. 4).

Considering the high quantity of regulated ion homeostasis genes and the reported importance of iron deprivation during *P. salmonis* infection [12], the transcriptional regulation of iron homeostasis, as mainly occurs via the heme biosynthesis/degradation pathway [37], was also evaluated (Fig. 5). Genes related to the extracellular export of heme, such as the Feline leukemia virus subgroup C receptor-related protein (*FLVCR*) and the ATP-binding cassette sub-family G member 2 (*Abcg2*) [38], evidenced significantly lower expression values in the brain and head kidney during *P. salmonis* infection. An inverse response was found for the *Haptoglobin* and *Hepcidin* genes, in which expression significantly increased (Fig. 5). These results were later validated through RT-qPCR, with *Hepcidin* and *Haptoglobin* strongly upregulated in particularly the spleen and head kidney (Fig. 6). The *transferrin receptor* (*TR*) and *ferritin middle subunit* (*FM*) were also significantly induced 14 dpi in the head kidney and spleen, respectively.

3.3. Discovery and transcriptome correlation of lncRNAs

Owing to the growing evidence supporting the regulation of lncRNAs in different biological processes [18,19,24,39,40], a search was conducted for these transcripts in the obtained sequencing data. For this, a bioinformatics pipeline for lncRNA discovery was implemented (Supplemental Fig. S3). Contigs (225 K total) previously generated and validated against the most recent Atlantic salmon genome were used as the input, and different strict filters were applied. BLASTx annotation removed 39 K contigs with E-values lower than 1×10^{-5}. The remaining contigs were filtered based on coverage to minimize the presence of assembly artifacts, resulting in 2256 contigs. Finally, the coding potential of these remaining transcripts was evaluated through (I) the presence of open reading frames > 200 nt, (II) coding potential, as estimated through the coding potential assessment tool, and (III) the presence of conserved domains on the six different translation frames. As a result, 918 transcripts were classified as putative lncRNAs for Atlantic salmon.

This information was used to evaluate the transcriptional modulation of protein-coding transcripts and lncRNAs. A similar distribution between coding and lncRNA transcripts was evidenced in tissues infected by *P. salmonis*, with the head kidney registering the largest quantity of differentially expressed transcripts (fold-change > 4, p-values < 0.01) and the spleen the least. However, the percentage of transcripts shared between all tissues was slightly larger for lncRNA transcripts (Fig. 7A). Regarding transcriptional modulation intensity, a similar distribution of fold-change values was obtained between coding and non-coding transcripts (Fig. 7B). These results suggest that lncRNAs might be involved in the transcriptomic response to bacterial infection or act as modulators of immune-related genes. To complement these results, a comparative analysis was performed between previously reported *S. salar* lncRNAs [24] and those presently obtained. Analysis results reveled that near half of the lncRNAs (i.e. 423) were similar to those

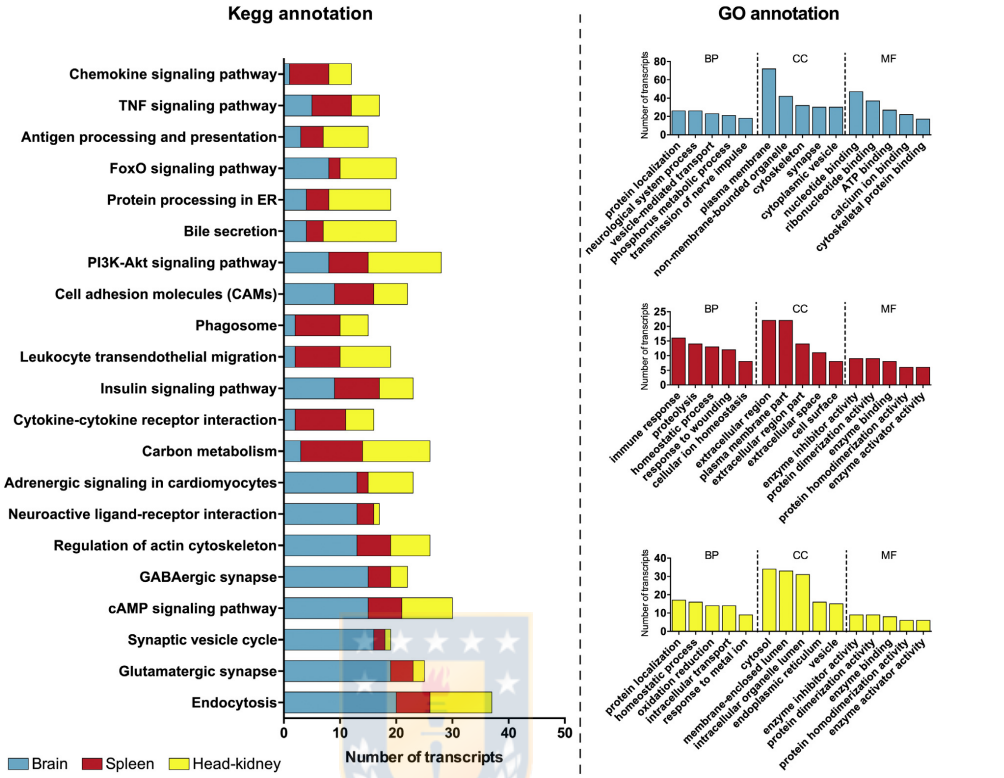

Fig. 2. KEGG and GO annotation distributions for differentially expressed transcripts among different pathways; biological processes (BP), cellular components (CC) and molecular functions (MF). The brain, spleen, and head kidney are represented in light blue, red, and yellow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

previously reported, whereas 425 corresponded to newly described lncRNAs (Supplemental Fig. S4).

To identify the Atlantic salmon lncRNAs that could play pivotal roles during *P. salmonis* infection, co-localization and co-expression analyses were carried out. The candidate genes *Clathrin*, *Hepcidin*, and *haptoglobin* were highly and positively regulated. Subsequently, the genomic localization of each these genes was extracted considering 100,000 bp both up- and downstream, and putative lncRNAs were mapped against the genomic region. Through this, 15, 11, and 15 different lncRNAs were mapped near *clathrin*, *hepcidin-1*, and *haptoglobin*, respectively (Fig. 8). Further transcriptional and correlation analyses between these genes and the respective lncRNAs evidenced different transcriptional relationships. Meanwhile, significant Pearson correlations were found between gene expressions and proximal lncRNAs for *clathrin* and *hepcidin-1*, while a less, but still significant correlation was obtained for *haptoglobin*. The most significant correlation for *clathrin* was with *Ss_lncRNA_2892*, which was negatively correlated in all tissues. In turn, the most significant correlation for *hepcidin-1* was with *Ss_lncRNA_2892*, which was positively correlated in all tissues (Fig. 8).

4. Discussion

The facultative intracellular bacterium *P. salmonis* has become the principal infectious disease and the main threat to sustainable salmonid aquaculture in Chile. However, despite the importance of this pathogen, several biological aspects of transmission, virulence, and host response (i.e. resistance/susceptibility) remain poorly understood [3]. Nevertheless, large-scale transcriptomic studies have been used in recent years to assess the molecular responses of salmonids to several infectious diseases [11,12,28,41–43]. Related to this, the aim of the present study was to explore the transcriptional response of *S. salar* when challenged with *P. salmonis*, with focus given to the interplay between protein coding and non-coding gene transcripts during infection.

The obtained results evidenced tissue-specific transcriptional patterns over time. While a similar quantity of up- and down-regulated genes were observed in the head kidney and spleen, the brain activated a late transcriptional response. Considering that maximum bacterial load in the brain was reached 30 dpi, a strong relationship could exist between bacterial load and transcriptional response in this tissue. This response type can be explained by considering that intraperitoneal injections were employed as the bacterial entry rout. This methodology normally first affects tissues close to the injection site [44]. Additionally, although *P. salmonis* can be isolated from salmonid brains [26,27], *P. salmonis* infections most predominantly affect the liver, spleen, and kidney [3,45]. Therefore, a strong and early modulation of the transcriptional

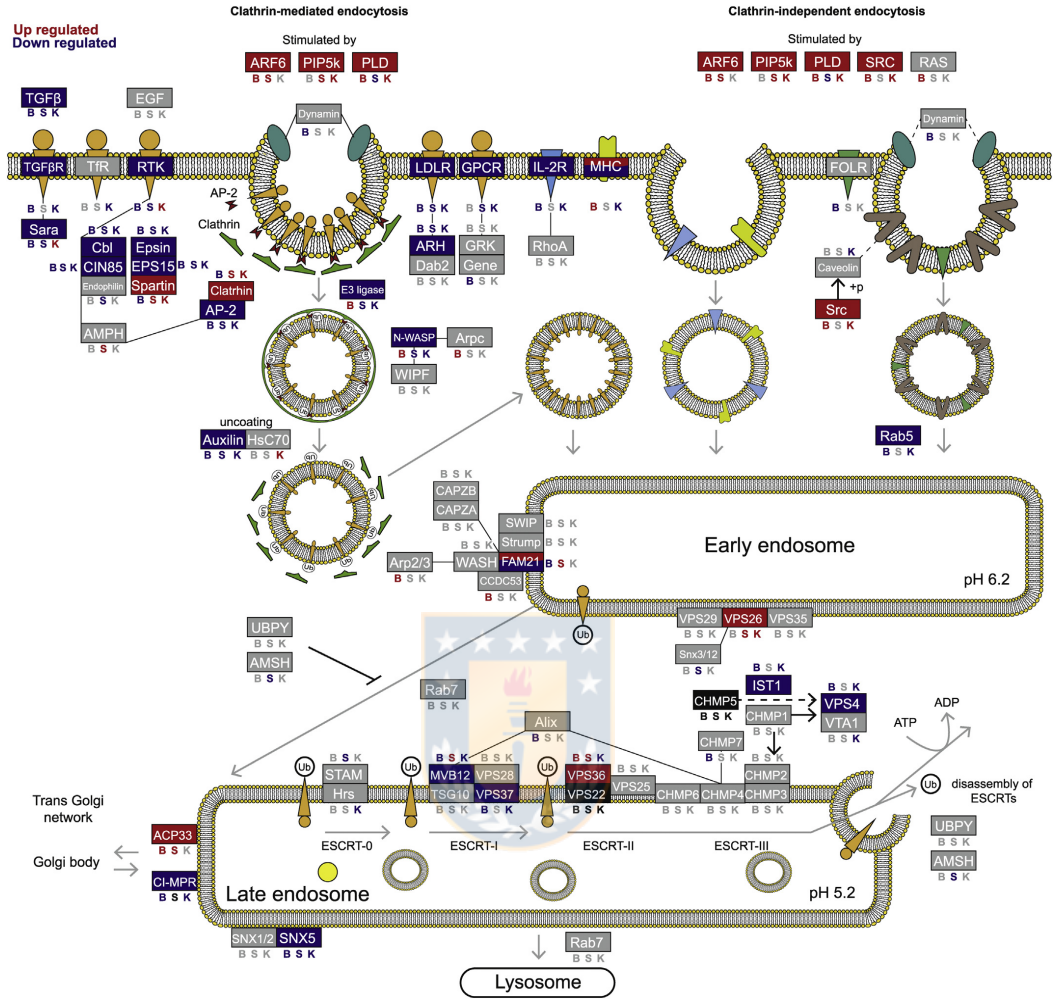

Fig. 3. Endocytosis pathway based on KEGG annotation, where each box represents a gene within the pathway. A list of gene abbreviations can be found in Pathway Entry (http://www.genome.jp/kegg-bin/show_pathway?dre04144). The letters below each gene represents the brain (B), spleen (S), and head kidney (H). The color of each letter represents the regulation of the gene, where blue indicates gene downregulation, red indicates gene upregulation, and grey indicates that the gene had no significant difference (fold change > 4, p-values < 0.01), all genes as compared to the control. Finally, the color of the box for each gene represents the predominant modulation of the gene among all tissues, where blue is predominant downregulation, red upregulation, and grey no significant regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

machinery in these tissues is expected.

The gene annotation of differential expressions during *P. salmonis* infection revealed different pathways associated with molecular responses in each tissue. In the brain, regulated processes were mainly associated with nerve impulse transmissions, such as the glutamatergic synapse and synaptic vesicle cycle. Previous evidence in mammals suggests that *Streptococcus pneumonia* can disrupt glutamate regulation, leading to synaptic damage in the brain [46]. Although barely understood in teleosts, *S. salar* brains infected with rickettsial-like organism can show granulomatous meningitis, parenchymal perivascular, and mononuclear hypercellularity [47]. Therefore, a strong transcriptional regulation of synapsis-associated genes could alter normal brain functions that, in turn, could be associated with the erratic swimming behavior of salmonids affected by SRS [27].

Although different transcriptional patterns were evidenced among analyzed tissues, a common response associated with oxidation-reduction processes, endocytosis and ion responses were present within brain, spleen and head kidney. Among these, oxidation-reduction processes are known important components

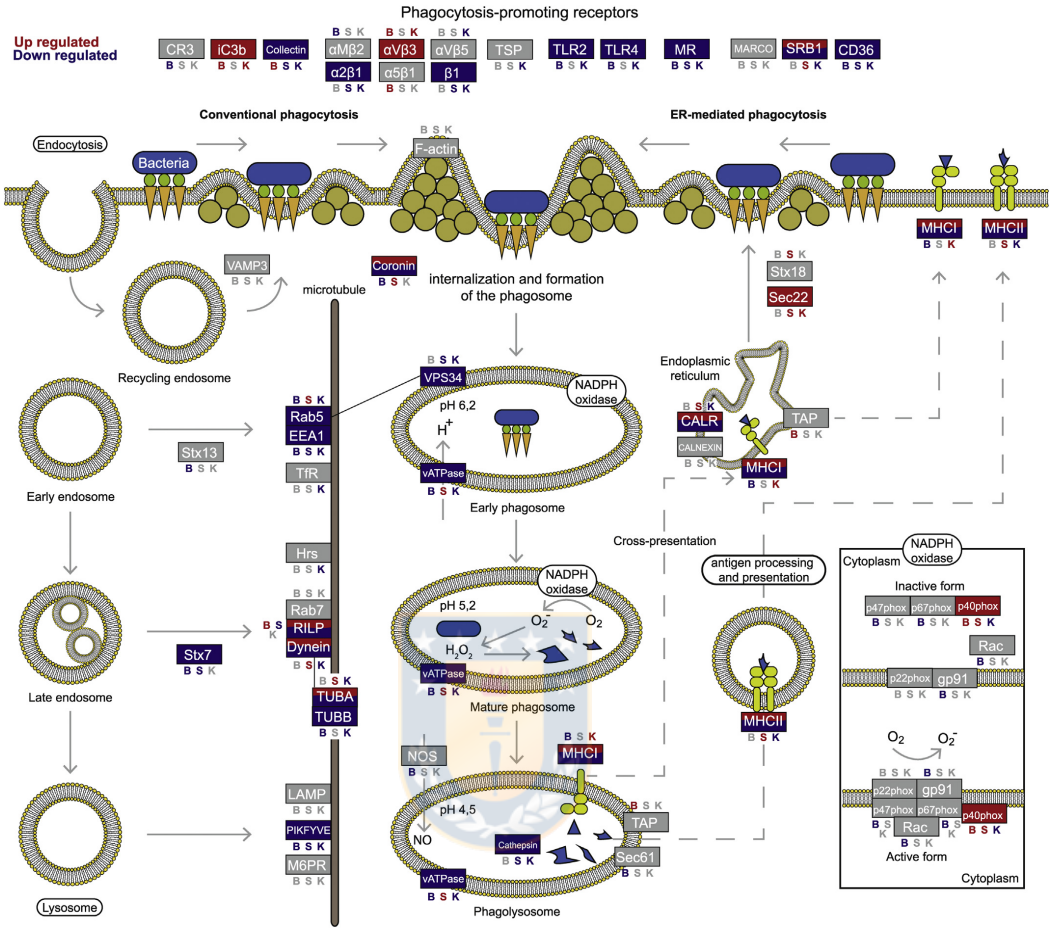

Fig. 4. Phagosome pathway based on KEGG annotation, where each box represents a gene within the pathway. A list of gene abbreviations can be found in Pathway Entry (http://www.genome.jp/kegg-bin/show_pathway?dre04145). The letters below each gene represent the brain (B), spleen (S), and head kidney (H). The color of each letter represents the regulation of the gene, where blue indicates gene downregulation, red indicates gene upregulation, and grey indicates that the gene had no significant difference (fold change > 4, p-values < 0.01), all genes as compared to the control. Finally, the color of the box for each gene represents the predominant modulation of the gene among all tissues, where blue is predominant downregulation, red upregulation, and grey no significant regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the Atlantic salmon innate immune response against *P. salmonis* in the liver, head kidney and muscle [11]. In addition endocytosis likely serves as the main entry mechanism for many intracellular bacteria [48]. One of the most common cellular signs of *P. salmonis* infection is the presence of cytoplasmic vacuoles in which the bacteria live and replicate [2,49,50], thus evidencing the use of host-mediated endocytosis machinery for cell entry. The currently presented findings suggest that different endocytosis-promoting factors are induced, such as *ARF6*. Similarly, the obligate intracellular bacterium *Chlamydia* induces endogenous *ARF6* activation, thereby facilitating bacterial endocytosis through actin remodeling [51]. This cytoskeleton regulation can be also observed in *P. salmonis* infections, where the interaction with endogenous *actin* and *clathrin* has been suggested as a key step for the bacterial internalization [52]. Interestingly, *Clatrhin* was also significantly up-regulated in infected individuals, suggesting the importance of clathrin-mediated endocytosis for the bacterial internalization. In contrast, several endocytosis receptors and their respective adaptors proteins were repressed during challenge. Consistently, downregulation of receptors and cell signaling genes has been previously evidenced during *P. salmonis* infections in *Salmo salar* [10,11] and considered as a result of a potential mechanism whereby the bacteria can affect the expression of host genes to evade the immune response.

Several transcripts associated with ion regulation and homeostasis were also significantly regulated in the brain, spleen, and head kidney. Among ions, iron is vital for almost all living organisms, especially bacteria [53–55]. Accordingly, one of the first lines

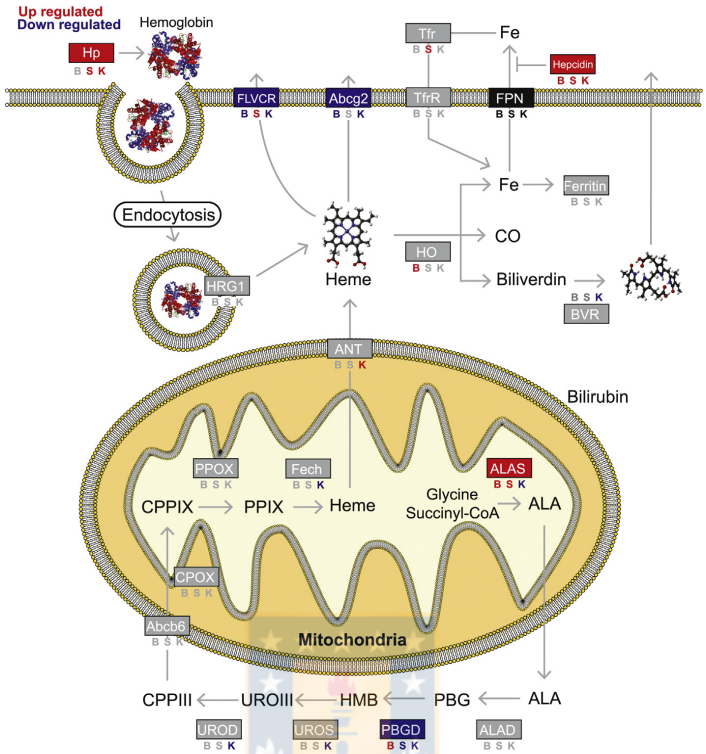

Fig. 5. Heme metabolism pathway based on Larsen et al. (2012), where each box represents a gene within the pathway. The letters below each gene represent the brain (B), spleen (S), and head kidney (H). The color of each letter represents the regulation of the gene, where blue indicates gene downregulation, red indicates gene upregulation, and grey indicates that the gene had no significant difference (fold change > 4, p-values < 0.01), all genes as compared to the control. Finally, the color of the box for each gene represents the predominant modulation of the gene among all tissues, where blue is predominant downregulation, red upregulation, and grey no significant regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of host defense against pathogens is iron sequestration, or the limiting of iron availability [55,56]. This nutritional immunity could be a key difference between *P. salmonis* susceptible and resistant Atlantic salmon families [12], where resistant families can decrease the cellular iron content. The currently assessed *S. salar* individuals showed more *P. salmonis*-susceptible expressions. Specifically, genes associated with the intracellular accumulation of iron, such as *haptoglobin*, *hepcidin-1*, *transferrin receptor*, and *Ferritin middle chain*, were significantly induced while genes associated with extracellular iron export through the heme group, such as the *feline leukemia virus receptor* and *ABCg2*, were mainly repressed in the assessed tissues. Although haptoglobin is not directly involved in iron uptake, it binds free hemoglobin in bony fish [57], thus preventing oxidative activity and, primarily, preventing pathogen iron acquisition within free hemoglobin [58]. In turn, while *hepcidin* has been extensively studied as an antimicrobial peptide in bony fish [59–64], its role in iron homeostasis is not well understood. However, experimental iron overloads can induce *hepcidin* expression in the sea bass (*Dicentrarchus labrax*) [65], marine medaka (*Oryzias melastigma*) [66], and rainbow trout (*Oncorhynchus mykiss*) [67]. Therefore, hepcidin might play an iron homeostasis role similar to that in mammals.

To gain a better understanding about key gene regulations during *P. salmonis* infection, lncRNA transcriptional responses were explored. An ever-increasing number of reports suggest that lncRNAs function in regulating multiple biological processes [68], consequently leading to increased investigative interests. Although mammals have been mainly used for the genomic characterization of lncRNAs [40,69], cumulative evidence supports the presence of lncRNA in bony fish [21–24]. Furthermore, lncRNAs might play an important role during the immune response of Atlantic salmon to ISAv [24].

The present study conducted a comparative analysis between the lncRNAs identified during ISAv, and *P. salmonis* infections revealed a divergence in ≈50% of transcripts. This finding suggests that lncRNAs can displays pathogen-specific profiles during the infection process and their transcription activity could be associated to specific immune-related genes. Although there is no functional evidence to date that supports the specific role of lncRNA during the fish immune response, others well-studied types of non-coding transcripts, such as microRNAs have been reported as primary modulators of the immune response [70].

Like microRNAs, the modulation of lncRNAs can impact the expression of protein-coding genes. While microRNAs are mainly associated with post-transcriptional gene silencing, lncRNAs have a wider spectrum of regulation and can be involved at an epigenetic

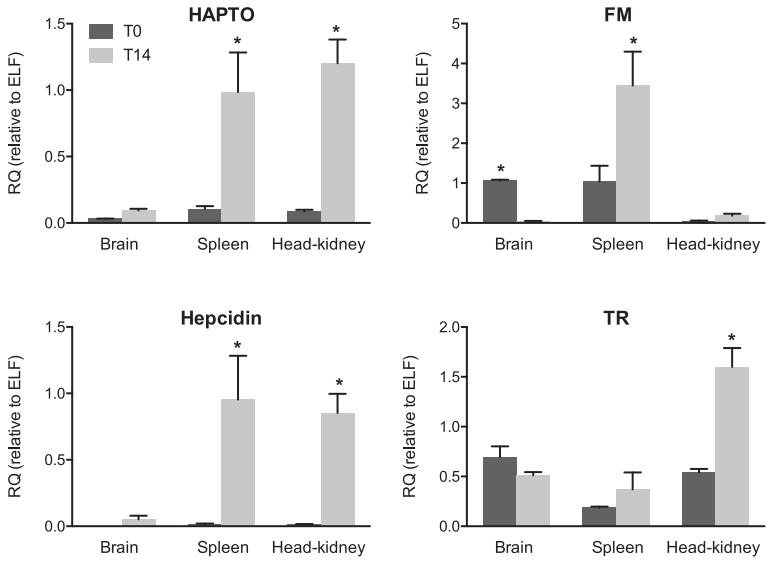

Fig. 6. RT-qPCR validation of genes associated with the response to *P. salmonis*. Relative quantification, normalized to *ELF-1α*, of haptoglobin (HAPTO), ferritin middle subunit (FM), hepcidin-1, and the transferrin receptor (TR). Significance was confirmed through the Student's *t*-test (*$p < 0.05$).

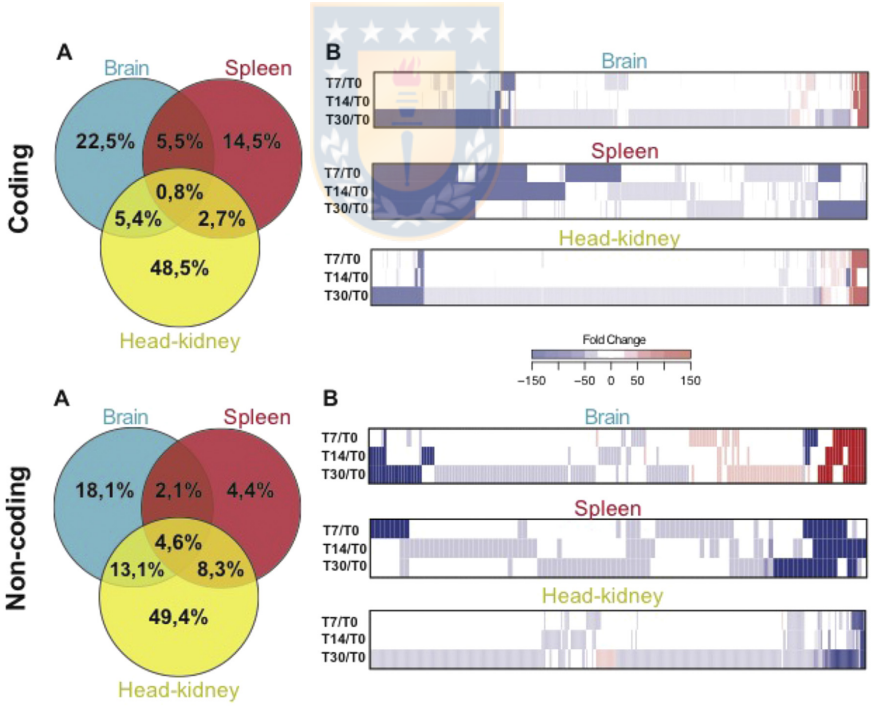

Fig. 7. Transcriptome profiles of protein-coding genes and lncRNAs between tissues infected with *P. salmonis*. **(A)** Venn diagram of differentially expressed transcripts for each tissue (fold change > 4, p-values < 0.01). (B) Heat-map of fold-changes for the differentially expressed genes in each tissue and at each time point (T0 to T30) as compared to the control group.

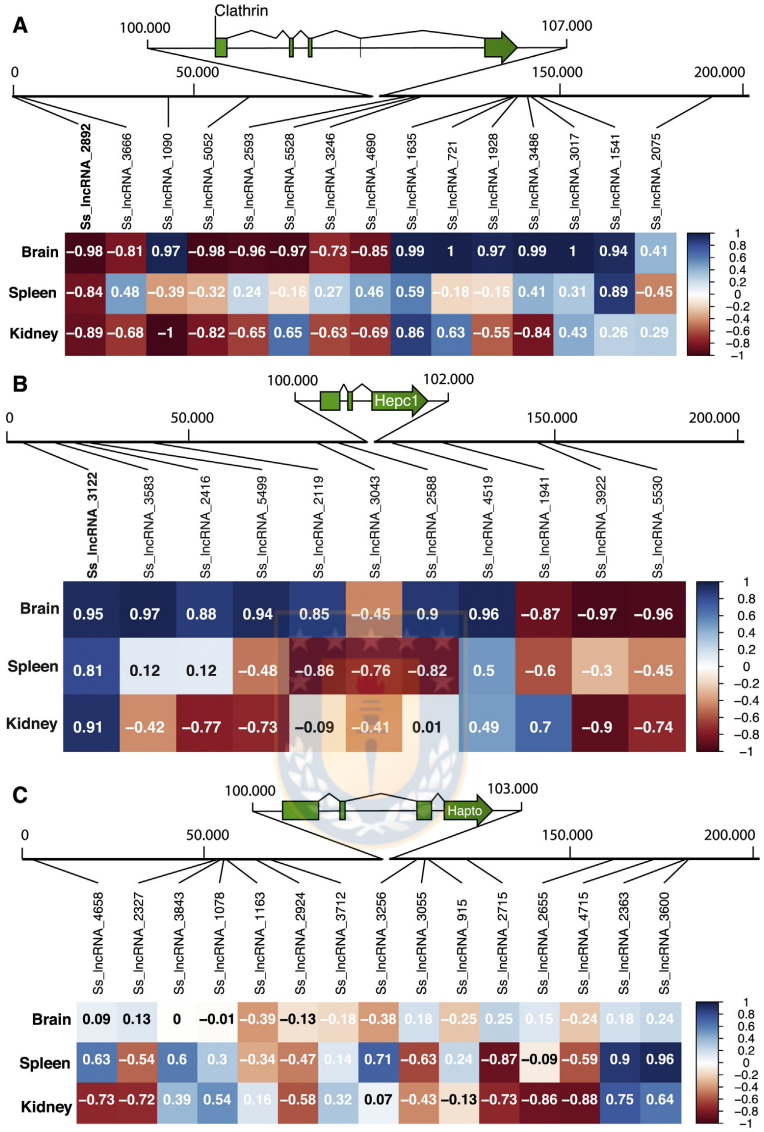

Fig. 8. Genomic localizations of highly modulated lncRNAs and genes during *P. salmonis* infection. Pearson correlation analyses were applied to evidence co-expressions between clathrin, hepcidin-1 (Hepc1), and haptoglobin (Hapto) genes and neighboring lncRNAs. Color scale represents the correlation values.

level [15]. Therefore, lncRNAs might act as *cis* regulators for protein coding genes [71]. Applied co-localization and co-expression approaches have been used to estimate the functional correlations between coding and non-coding transcripts. To link the coding/non-coding response, focus was given to the transcriptional correlations between *clathrin*, *hepcidin*, and *haptoglobin* and the proximal lncRNAs. The genomic context of each gene evidenced the presence of multiple lncRNAs adjacent to target genes, where two lncRNAs were significantly downregulated and maintained in correlation with the respective genes. While this correlation was negative between *Clathrin* and Ss_lncRNA_2892, a positive correlation was estimated between *Hepcidin* and SS_lncRNA_3122 within all tissues. MicroRNAs are mostly associated with gene silencing and, therefore, a negative correlation with the target gene

is expected [72]. In contrast, lncRNAs have a broader spectrum of regulation [73], and even a single lncRNA can mediate both the activation and repression of biological processes [18]. In support of this, the lncRNAs of the present study showed greater versatility in gene regulation than microRNAs, and, consequently, both negative and positive transcriptional correlations between lncRNAs and target genes can be expected. However, considering that correlation does not necessary imply causation, further functional analyses are still required to fully demonstrate the regulation of lncRNAs in the transcriptional response of Atlantic salmon to *Piscirickettsia salmonis.*

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2016.11.001.

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CAPÍTULO II (H1,OE1). MicroRNA-based transcriptomic responses of Atlantic salmon during infection by the intracellular bacterium *Piscirickettsia salmonis*

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Abstract

MicroRNAs (miRNAs) are small non-coding RNAs that have emerged as key regulators in diverse biological processes across taxa. However, despite the importance of these transcripts, little is known about their role during the immune response in salmonids. Because of this, we use deep sequencing technologies to explore the microRNA-based transcriptomic response of the Atlantic salmon (*Salmo salar*) to the intracellular bacteria *Piscirickettsia salmonis*, one of the main threats to salmon aquaculture in Chile. Hence, 594 different miRNAs were identified from head kidney and spleen transcriptomic data. Among them, miRNA families mir-181, mir-143 and mir-21 were the most abundant in control groups, while after stimulation with *P. salmonis,* mir-21, mir-181 and mir-30 were the most predominant families, respectively. Furthermore, transcriptional analysis revealed 84 and 25 differentially expressed miRNAs in head kidney and spleen respectively, with an overlapping response of 10 miRNAs between the analyzed tissues. Target prediction, coupled with GO enrichment analysis, revealed that the possible targets of the most regulated miRNAs were genes involved in the immune response, such as cortisol metabolism, chemokine-mediated signaling pathway and neutrophil chemotaxis genes. Among these, predicted putative target genes such as *C-C motif chemokine 19-like*, *stromal cell-derived factor 1-like*, *myxovirus resistance protein 2* and *hepcidin-1* were identified. Overall, our results suggest that miRNA expression in co-modulation with transcription activity of target genes is related to putative roles of non-coding RNAs in the immune response of Atlantic salmon against intracellular bacterial pathogens.

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MicroRNA-based transcriptomic responses of Atlantic salmon during infection by the intracellular bacterium *Piscirickettsia salmonis*

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that have emerged as key regulators in diverse biological processes across taxa. However, despite the importance of these transcripts, little is known about their role during the immune response in salmonids. Because of this, we use deep sequencing technologies to explore the microRNA-based transcriptomic response of the Atlantic salmon (*Salmo salar*) to the intracellular bacteria *Piscirickettsia salmonis*, one of the main threats to salmon aquaculture in Chile. Hence, 594 different miRNAs were identified from head kidney and spleen transcriptomic data. Among them, miRNA families mir-181, mir-143 and mir-21 were the most abundant in control groups, while after infection with *P. salmonis*, mir-21, mir-181 and mir-30 were the most predominant families. Furthermore, transcriptional analysis revealed 84 and 25 differentially expressed miRNAs in head kidney and spleen respectively, with an overlapping response of 10 miRNAs between the analyzed tissues. Target prediction, coupled with GO enrichment analysis, revealed that the possible targets of the most regulated miRNAs were genes involved in the immune response, such as cortisol metabolism, chemokine-mediated signaling pathway and neutrophil chemotaxis genes. Among these, predicted putative target genes such as *C-C motif chemokine 19-like*, *stromal cell-derived factor 1-like*, *myxovirus resistance protein 2* and *hepcidin-1* were identified. Overall, our results suggest that miRNA expression in co-modulation with transcription activity of target genes is related to putative roles of non-coding RNAs in the immune response of Atlantic salmon against intracellular bacterial pathogens.

1. Introduction

MicroRNAs (miRNAs) encompass a class of small (~21 nucleotide) evolutionarily conserved and single-stranded non-coding RNAs that regulate gene expression at the post-transcriptional level (Chekulaeva and Filipowicz, 2009). Through base pairing, miRNAs are mainly directed to the 3′ untranslated regions (3′ UTR) of messenger RNAs (mRNAs), triggering the recruitment of the molecular machinery that promotes either the translational repression, cleavage or deadenylation of target mRNA (Hafner et al., 2011). Because of this, miRNAs have emerged as a key mechanism of post-transcriptional gene silencing, being involved in the regulation of diverse biological processes like reproduction, growth, and immune response, among others (Bartel, 2004). The high degree of sequence conservation, coupled with the implementation of high-throughput sequencing technologies, has led to the discovery and characterization of multiple miRNAs across taxa (Berezikov, 2011). Since the first discovery of miRNAs in *Caenorhabditis elegans* in 1993 (Lee et al., 1993), thousands of miRNAs have been identified in vertebrates (Kozomara and Griffiths-Jones, 2014). It has even been predicted that around 60% of mammalian mRNAs present conserved miRNA binding sites (Friedman et al., 2009). While research into miRNAs in mammals and plants has made substantial progress, the regulatory role of miRNAs in lower vertebrates, such as teleost fish, remains poorly understood (Bizuayehu and Babiak, 2014).

From the first report of miRNAs in zebrafish (Lim et al., 2003), different approaches have aimed to clarify the role of miRNAs as fine-tuning regulators of different biological processes in teleosts. Among them, it has been proposed that miRNAs constitute key

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switches for the activation or inhibition of the immune response (Andreassen and Hoyheim, 2017). Recently, large scale transcriptomic studies, coupled with *in silico* target prediction, have identified differentially expressed miRNAs in response to viral (Guo et al., 2015; Han et al., 2016; Liu et al., 2016; Najib et al., 2016; Wu et al., 2015; Zhang et al., 2014) and bacterial (Gong et al., 2015; Ordas et al., 2013; Wang et al., 2016; Yuhong et al., 2016; Zhao et al., 2016) infections in several species. In relation to salmonids, it was recently reported that mir-462 and mir-731 in rainbow trout *Oncorhynchus mykiss* may be involved in IFN-mediated defense against the viral hemorrhagic septicemia virus (VHSV), even becoming part of the protective effect elicited by rhabdovirus G-based DNA vaccines (Bela-ong et al., 2015). Likewise, it has been proposed that two miRNAs, miR-462 and miR-731, described only in teleost fish, play roles in the immune response to viral pathogens in *O. mykiss* (Schyth et al., 2015). Although, these transcripts share homology with miRNAs involved in cell cycle control in humans, genomic organization and target prediction evidence that teleost variants might involve different mechanisms than human orthologues with respect to their regulation and functional roles (Schyth et al., 2015). Despite the potential of miRNAs as fine-tuning regulators of the immune response in teleosts, the role of miRNAs in the response to bacterial infection in salmonids remains unexplored.

Piscirickettsia salmonis is a facultative intracellular gram negative bacterium that causes the salmonid rickettsial septicemia (SRS), a severe systemic disease associated with major mortalities in salmonid aquaculture (Mauel and Miller, 2002). Due to the negative impact and prevalence of this bacterium, different approaches have sought to understand the molecular basis underpinning salmonid responses to this pathogen. Among these, it has recently been proposed that non-coding RNAs (ncRNAs) might be involved in the regulation of the immune response of Atlantic salmon to *P. salmonis* (Valenzuela-Miranda and Gallardo-Escarate, 2016), making them interesting targets to better understand host-pathogen interactions beyond protein coding genes. In this context, the aim of this study was to reveal the miRNA-based transcriptomic response of the Atlantic salmon to *P. salmonis*, providing novel insights into the role of ncRNAs as potential modulators of the immune response in salmonids during bacterial infections.

2. Materials and methods

2.1. Experimental design

Individuals and tissues used in this study were obtained from a previously reported challenge for Atlantic salmon infected with *P. salmonis* (Valenzuela-Miranda and Gallardo-Escarate, 2016). Briefly, around 200 unvaccinated *Salmo salar* (158.3 ± 35.4 g) from a commercial farm (Puerto Montt, Chile) were maintained in six 500-L tanks at Aquagestion facilities (Puerto Montt, Chile). For six weeks individuals were acclimatized and subjected to random sanitary examinations to rule out the presence of different pathogens, including *P. salmonis*, infectious pancreatic necrosis virus (IPNv) and the infectious salmon anemia virus (ISAV). After this period, individuals were anesthetized and intraperitoneally (IP) injected with 0.2 mL of *P. salmonis* (LF89 strain) at 1×10^4 PFU/mL (challenged group) or with PBS (control group). After IP injection, two tanks were used to record mortality and the remaining four were used for tissue sampling. During sampling, the development of SRS and the presence of *P. salmonis* within sampled individuals was first clinically confirmed and later verified through qPCR (Corbeil et al., 2003). As the highest bacterial load among infected individuals was reached at 14 days post-injection (DPI) (Fig. S1), this period was selected to sample spleen and head kidney from 15 randomly selected individuals. The tissues were isolated and stored in RNA-later solution (Ambion, USA) at −80 °C until small RNA isolation.

2.2. Small RNA isolation and high-throughput sequencing

RNA isolation and small RNA sequencing were performed based on previously reported protocols (Farlora et al., 2015; Gallardo-Escárate et al., 2017). Thus, total RNA was isolated from around 30 mg of each tissue using the Trizol™ Kit (Ambion®), according to the manufacturer's instructions. Quality and purity of the isolated RNA were evaluated using the NanoDrop 1000 Spectrophotometer (Thermo Scientific), while the RNA integrity number (RIN) was evaluated through the 2200 TapeStation (Agilent technologies, USA) using the R6K screen tape following the manufacturer's instructions. Samples with RIN >8 and A260/280 > 1.8 were used for high throughput sequencing library construction using the TruSeq Small RNA Kit (Illumina®, San Diego, CA, USA), according to the manufacturer's instructions. Sequencing libraries were constructed with an RNA pool of 5 individuals for each tissue (head kidney and spleen) and for uninfected (-Psal) and infected (+Psal) individuals. Finally, three biological replicates were sequenced for each condition (-Psal/+Psal) in the MiSeq (Illumina) platform using different sequencing runs of 41 cycles at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Chile.

2.3. Discovery and characterization of the miRNA repertory in Salmo salar

After index/adapter removal, raw reads were filtered and quality trimmed using the CLC Genomics Workbench Software (Version 9.1, CLC Bio, Denmark). Thus, read with a quality score <0.05 on the Phred scale, with a short length, or with three or more ambiguous nucleotides were removed. All reads were matched against different coding and non-coding databases, including the NCBI (http://www.ncbi.nlm.nih.gov/), RFam (http://rfam.janelia.org/), and Repbase databases (http://www.girinst.org/repbase/), to remove mRNA, rRNA, tRNA and snoRNA reads remaining in sequencing data. The small RNA reads were mapped against the last version of the *S. salar* genome (https://www.ncbi.nlm.nih.gov/genome/369) (Lien et al., 2016). Any read perfectly matching a database or coding sequence was removed from further analysis. Finally, identical sequences were grouped and counted prior to annotation against miRBase 21 (Griffiths-Jones et al., 2006; Kozomara and Griffiths-Jones, 2014) using CLC Genomics Workbench Software and considering default conditions.

2.4. Identification of differentially regulated miRNAs

RNA-seq analysis were applied to identify the most regulated miRNAs in *S. salar* after exposure to *P. salmonis*. Cleaned and filtered reads were matched against the annotated miRNAs using the CLC Genomics Workbench Software RNA-seq algorithm. The considered mapping settings included a length fraction = 0.9, a similarity fraction = 0.9 and a mismatch, insertion and deletion cost of 2, 3 and 3 respectively. Expression values were calculated as transcripts per million (TPM) and normalized by totals per million reads. To determine statistically significant differences, a proportion-based statistical analysis was conducted using the Baggerley's test (Baggerly et al., 2003) and adjusting p-values by false discovery rate (FDR) correction. MiRNAs with a fold change >4 regarding control group and with p-values < 0.01 were selected as differentially expressed miRNAs. Finally, heat-maps were constructed by plotting the $\log_2$ of the normalized TPM values and were hierarchically clustered estimating Manhattan distances with an average linkage criterion.

2.5. RT-qPCR validations

To validate RNA-seq results, RT-qPCR were conducted using cDNAs synthetized from previously isolated RNA with miScript II RT Kit (Qiagen Inc., USA) according manufacturer's instructions. Thus three differentially expressed miRNAs were randomly selected for qPCR validations, including the Ss-miR-455-5p which has been previously validated as housekeeping miRNA (Johansen and Andreassen, 2014; Valenzuela-Munoz et al., 2017). All RT-qPCRs were conducted in three biological and technical replicates using the miScript SYBR Green PCR Kit (Qiagen, USA) and the StepOnePlus™ (Applied Biosystems®, Life Technologies, USA) with amplification program of 95 °C for 10 min, 40 cycles at 95 °C for 15s and 60 °C for 1 min. Thus, relative miRNA expression was estimated using

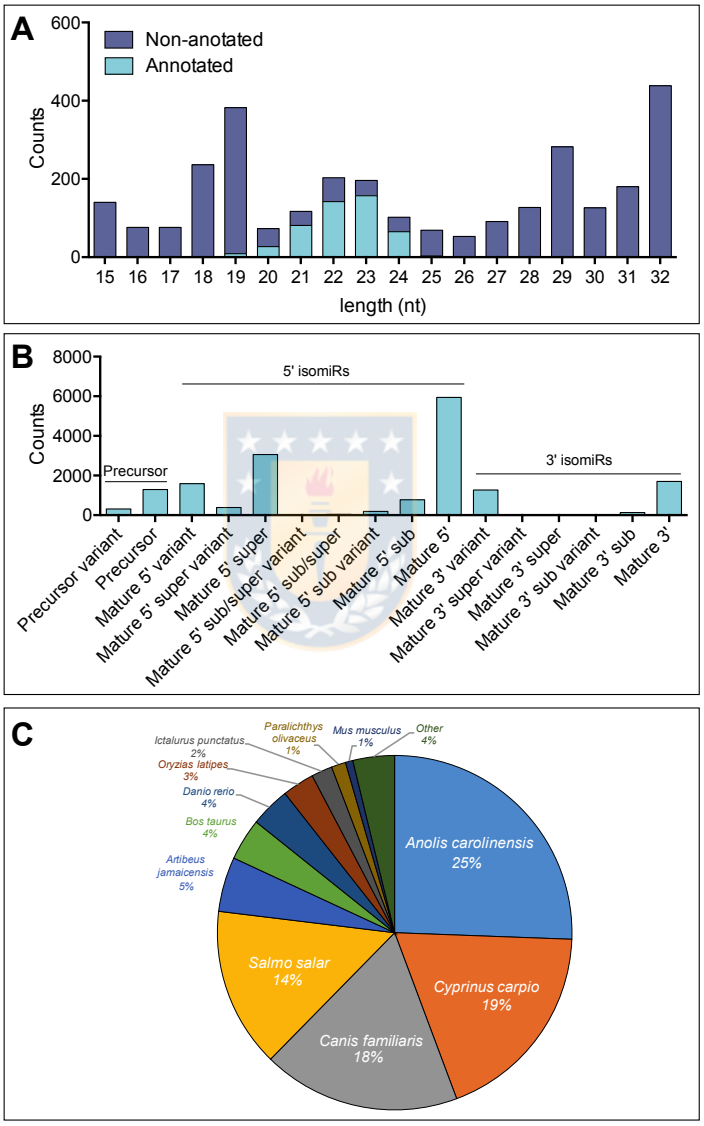

Fig. 1. A) Length distribution of annotated (light blue) and non-annotated (blue) miRNAs. B) Classification and distribution of the different isomiRs among annotated miRNAs. C) Species-specific distribution for annotated miRNAs regarding previously reported sequences in the miRBase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the comparative ΔΔCt method (Livak and Schmittgen, 2001). Finally, a Student's t-test was used to identify statistically significant differences ($p < 0.05$) using the JMP v9 software (SAS Institute Inc., USA).

2.6. miRNA target prediction and GO enrichment analysis

PITA (Kertesz et al., 2007) and miRanda (John et al., 2004) software were used to target genes of the differentially expressed miRNAs. For this purpose, 3′ UTR regions were identified from the complete list of annotated genes in the lasts version of the Atlantic salmon genome (GenBank: http://www.ncbi.nlm.nih.gov/assembly/GCA_000233375.4). An additional target prediction analysis was performed by considering 3′UTR of all mRNAs that we previously identified as differentially expressed during *P. salmonis* infection in *S. salar* (Valenzuela-Miranda and Gallardo-Escarate, 2016). For target prediction, the default parameters used for PITA, and miRanda were an open gap penalty = −9, extended gap penalty = - 4, score threshold = 140, energy threshold = 1 kcal/mol, and scaling parameter = 4. The only genes considered as targets were those predicted by both programs and with ΔG > 12. GO enrichment analysis (Huang da et al., 2009) identified the most representative biological processes among potential miRNA targets. The results were plotted using the REVIGO platform (Supek et al., 2011) and R software.

3. Results

3.1. Identification and characterization of miRNAs in S. salar

We analyzed small RNA sequencing of Atlantic salmon spleen and head kidney infected by *P. salmonis* to gain a better understanding of the role of miRNAs during teleost immune response to intracellular bacterial infection. The first step was to identify and characterize the miRNA repertory for the species in our sequencing data. Some 594 miRNAs were annotated from a total of 3060 unique sequences in the miRNA database (Table S1). The largest number of annotated miRNAs were sequence between 21 and 22 nucleotides (nt), while for the non-annotated transcripts, the highest counts of sequences were in 32 and 19 nt (Fig. 1A). We found different types of miRNA isoforms (isomiRs) in terms of sequences and length. The most abundant classes were the mature 5′ isomiRs, 5′ super variant and 3' isomiRs (Fig. 1B). The species-specific analyses for annotated miRNAs evidenced that only 14% belong to previously reported sequences in the miRBase in *S. salar*, while the rest are associated with other teleost species, including *Cyprinus carpio* (24%), *Danio rerio* (4%), *Oryzias latipes* (3%), *Ictalurus punctatus* (2%) and *Paralichthys olivaceus* (1%) (Fig. 1C). Among identified miRNAs, a comparison between previously reported miRNA repertory for *S. salar* (Andreassen et al., 2013; Bekaert et al., 2013) evidenced that 27 putative novel miRNAs were identified for this salmon species (Fig. S2; Table S1).

MiRNAs are often classified in different groups according to sequence conservation and/or structural criteria. These groups are called families and can gather multiple mature miRNAs. To explore how the diversity of families is altered by the presence of *P. salmonis*, we compared the miRNA family distribution in the absence (-Psal) and presence (+Psal) of the bacteria. The results evidence a shift in the diversity of families under both conditions, the miRNA families mir-181, mir-143 and mir-21 being the most abundant in control groups, while mir-21, mir-181 and mir-30 were the most abundant after stimulation with *P. salmonis* (Fig. 2).

3.2. Identification of differentially regulated miRNAs

Differential expression analysis was performed in order to identify statistically significant differences in miRNA profiling following bacterial infection. Here, the previously observed miRNA family distribution was partially modified by considering only differentially expressed transcripts. While mir-27, mir-215 and mir-1388 were the most abundant families in downregulated miRNA clusters in head kidney, mir-30, mir-21 and mir-462 were the most abundant in upregulated clusters (Fig. 3). The differential expression analysis in spleen revealed that the largest number of downregulated miRNAs were associated with mir-143, mir-181 and mir-21 families, while the most upregulated belonged to mir-21, mir-181 and mir-30 families (Fig. 3). Some 74 miRNAs were differentially expressed exclusively in head kidney and 15 in spleen, while 10 miRNAs were shared between both tissues (Fig. 4). Among these, the top up and downregulated miRNAs in head kidney included mir-21_25, mir-181a-1, mir-143_3, while mir-21a-1, mir-181a-1, mir-21_23 and mir-21_15 were the most regulated in the spleen (Fig. 4). Moreover, the transcriptional responses overlapping between the two types of tissue included mir-30e_4, mir-462b-6, mir-143_24 and mir-143_29, among others. While the transcriptional modulations of most miRNAs in response to *P. salmonis* were the same in head kidney and spleen, some miRNAs like mir-10d-1 and mir-456 had distinct expression patterns (Fig. 4). Finally, to validate the identification of differentially regulated miRNAs through RNA-seq, three differentially expressed miRNAs were randomly selected for qPCR validations. Thus, qPCR was conducted in the Ss-mir-10b (differentially expressed in spleen) and the Ss-mir-181A (differentially expressed in head kidney and spleen) and in Ss-miR-455-5p

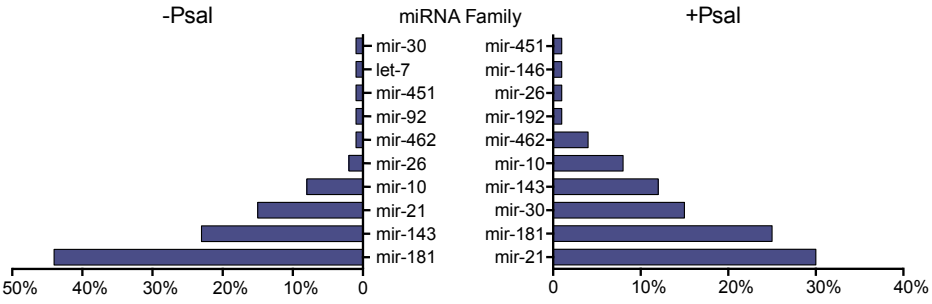

Fig. 2. Diversity of miRNAs families identified in the sequencing data during the absence (-Psal) and presence (+Psal) of the bacteria in *Salmo salar.*

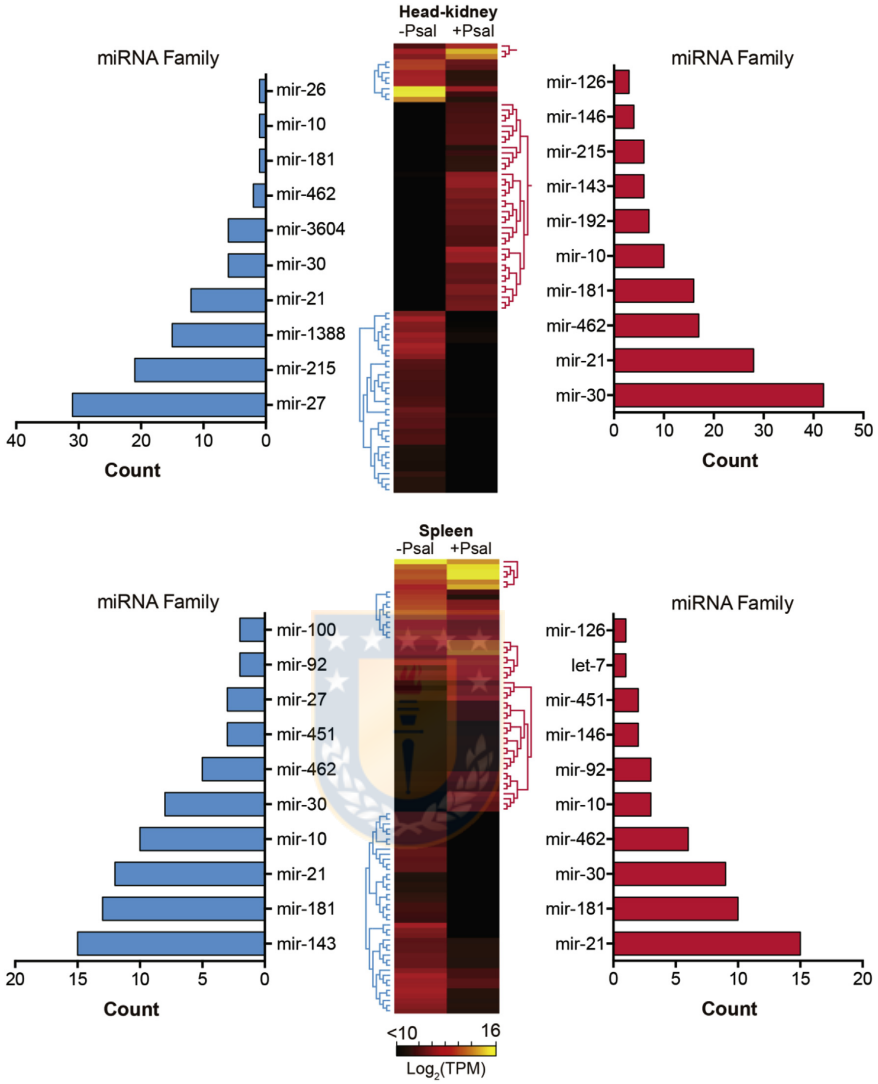

Fig. 3. Differential expression analysis of annotated miRNAs in control (-Psal) and infected (+Psal) individuals. Heat-maps were constructed by plotting the $\log_2$ of the normalized TPM values and were hierarchically clustered estimating Manhattan distances with an average linkage criterion. Fold change >4 and corrected p-values < 0.01 were used as selection criteria to identify differentially expressed miRNAs.

as reference gene. The results showed a direct correlation between the expression values obtained by qPCR and RNA-seq, being differentially expressed in both analysis (Fig. 5).

3.3. Target prediction and pathway analysis

In silico target prediction was applied to identify genes that can be regulated by differentially expressed miRNAs. The first comparison was used to identify the targets in the 3' UTRs of all annotated genes in the lasts version of the Atlantic salmon genome. GO enrichment analysis for genes presenting putative miRNA binding sites revealed 3 main clusters of biological processes, including those related to morphogenesis, transport and signal transduction. Among them, the most enriched terms were annotated to negative regulation of intracellular signal transduction, skeletal system morphogenesis, vesicle-mediated transport and small GTPase mediated signal transduction, among others (Fig. 6A). However, the analysis using differentially expressed genes and

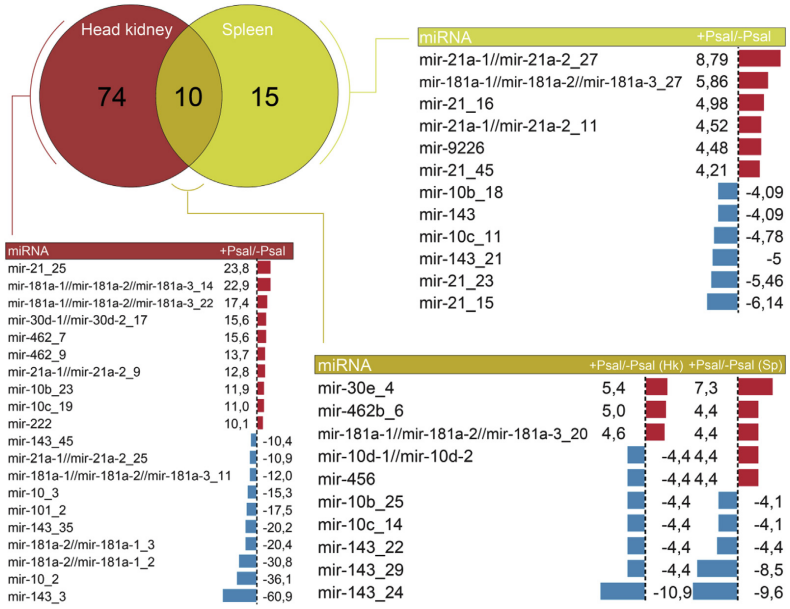

Fig. 4. Venn diagram showing the distribution of differentially regulated miRNAs in head-kidney and spleen. The top up and down regulated miRNAs for each tissue are also showed.

miRNAs evidenced that the most probable targets of these miRNAs are associated with different immune system processes, such as chemokine-mediated signaling pathway, cortisol metabolism, neutrophil chemotaxis and the immune system development (Fig. 6B). A deeper examination revealed that these genes include *Hepcidin-1*, *myxovirus resistance protein 2*, *Stroma cell-derived factor 1-like* and the *C-C motif chemokine 19-like* which, beyond the significant ΔG values, evidence a contrasting modulation following bacterial infection (Fig. 7).

4. Discussion

In recent years, host-pathogen molecular interactions during infection have mainly been studied in terms of how the protein coding genes are modulated by the host's immune response. However, cumulative evidence suggesting the importance of ncRNAs as fine-tuning modulators of the transcriptional response has expanded this approach. Here, miRNAs and lncRNAs are identified as key components in host-pathogen interaction in teleosts (Bela-ong et al., 2015; Boltana et al., 2016; Gong et al., 2015; Guo et al., 2015; Han et al., 2016; Liu et al., 2016; Najib et al., 2016; Ordas et al., 2013; Schyth et al., 2015; Tarifeno-Saldivia et al., 2017; Valenzuela-Miranda and Gallardo-Escarate, 2016; Wang et al., 2016; Wu et al., 2015; Yuhong et al., 2016; Zhang et al., 2014; Zhao et al., 2016). However, the role of miRNAs in the immune response of salmonids to bacterial infection remains unexplored. In this context, using high-throughput small RNA sequencing we focused our attention on the miRNA transcriptional response of Atlantic salmon to the intracellular bacterium *P. salmonis*.

Based on the last release of the miRNA database (miRBase), 594 miRNAs were characterized in our data. These miRNAs were classified in different families that are traditionally categorized based on sequence and/or structural criteria (Ding et al., 2011; Kozomara and Griffiths-Jones, 2014). Our results evidence that the presence of the bacteria promotes a shift in the diversity of families in the transcriptome, indicating a strong transcriptional modulation of different miRNAs, as reported for different teleost species following bacterial infection (Gong et al., 2015; Sha et al., 2014; Wang et al., 2016; Xu et al., 2016; Yuhong et al., 2016; Zhao et al., 2016). in particular, the most diverse families under control condition included mir-181, mir-143 and mir-21, while mir-21, mir-181 and mir-30 were the most abundant families in the presence of the pathogen. Members of these miRNA families have been associated with immune response in different teleost species (Andreassen and Hoyheim, 2017). Among them, miRNA-181 has been proposed as an evolutionary conserved miRNA with roles in the immune response of teleosts and higher vertebrates (Forster et al., 2015; Zhou et al., 2011).

The differential expression analysis evidenced a subset of miRNAs modulated in (Andreassen et al., 2013) head kidney and spleen following *P. salmonis* infection. Here, 84 and 25 miRNAs were identified in head kidney and spleen respectively, with an overlapping response of 10 miRNAs. While some miRNAs fulfill common housekeeping roles in different tissues, it has been also reported that organ specification in teleosts involves miRNA-based regulation (Bizuayehu and Babiak, 2014; Juanchich et al., 2016; Wienholds et al., 2005). A recent characterization of rainbow trout miRNA transcriptomic response in multiple tissues revealed that a large portion of these transcripts are expressed in a tissue-specific manner (Juanchich et al., 2016), in a manner similar to what is reported in higher vertebrates (Clop et al., 2006). The differences we report in the miRNA transcriptomic repertory following *P. salmonis*

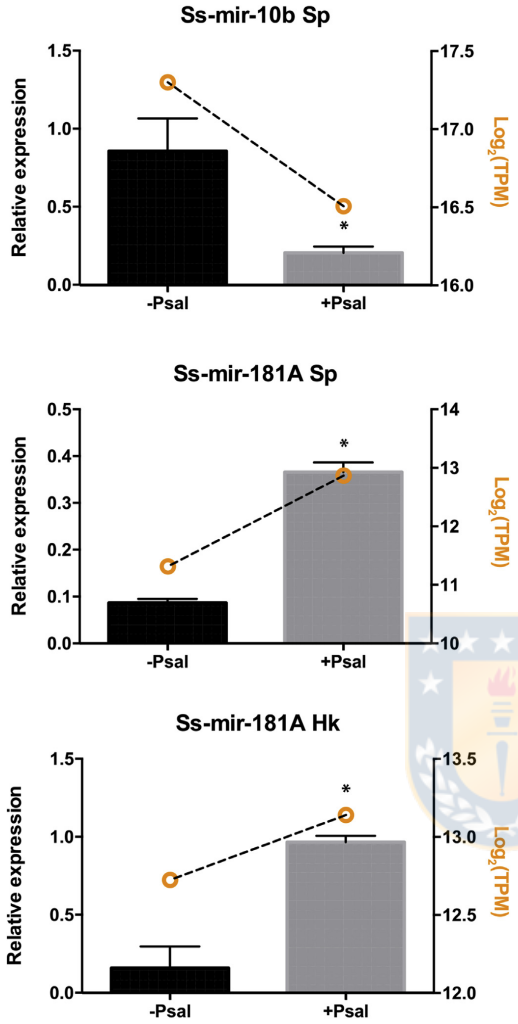

Fig. 5. RT-qPCR validations for three different differentially expressed miRNAs. Relative miRNA expression was estimated using the comparative ΔΔCt method using as reference Ss-miR-455-5p. * indicates statistically significant differences ($p < 0.05$).

infection could be associated with regulating the tissue-specific coding response of the Atlantic salmon to the bacteria (Valenzuela-Miranda and Gallardo-Escarate, 2016). Overexpression of mir-21 has been characterized as key switch in inflammatory response, mainly associated with resolution of inflammation and in negatively regulating the pro-inflammatory response in macrophages (Sheedy, 2015). It has been argued these functions are also present in teleosts, but mainly associated with the response to viral infections (Andreassen and Hoyheim, 2017). Our results show that different mir-21 isoforms are induced during bacterial infection, and may be fulfilling similar roles in the immune response to those demonstrated in higher vertebrates.

Interestingly, mir-21 has been also associated with immune evasion strategies employed by some pathogens. Like *P. salmonis*, mycobacteria are intracellular bacteria that persist and replicate in host macrophages (Ernst, 1998; McCarthy et al., 2008). It has been shown that mycobacterial species induce mir-21 in order to target and regulate multiple pathways required for pathogen infection, including the suppression of IL-12 production, which impairs anti-mycobacterial T cell responses (Liu et al., 2012; Wu et al., 2012). Temporal and specific blocking of mir-21 has been proposed as an ideal strategy for vaccine development in response to this type of pathogen (Sheedy, 2015). Therefore, further research into the role of the mir-21 family in salmonids would deepen our understanding of the molecular mechanism behind the immune evasion of *P. salmonis* to infect host macrophages and replicate itself within them.

In silico target prediction was applied to the complete set of *S. salar* genes currently annotated in its genome to identify the genes regulated by the most modulated miRNAs. The results showed a wide range of biological processes that these miRNAs may regulate, including tissue morphogenesis, transport and signal transduction. Similar results were obtained in the genome-wide miRNA target prediction for rainbow trout, where signal transduction was one of the most overrepresented in terms of being regulated by miRNAs (Mennigen and Zhang, 2016). Taken together, this evidence supports the view that salmonids miRNAs fulfill a similar role in the regulation of cellular signaling as it does described for higher vertebrates, which is accomplished by altering the responsiveness of cells to signaling molecules (Inui et al., 2010). Overall, the predicted regulation of salmon miRNAs over a wide landscape of biological processes concurs with the idea that ncRNAs are a versatile mechanism of post-transcriptional gene silencing (Pratt and MacRae, 2009).

Considering the high degree of plasticity in targets evidenced by salmon miRNAs, we filtered the analysis by comparing only differentially expressed genes and miRNAs after *P. salmonis* infection. These was done by comparing the 3′UTR of all mRNAs that we have previously identified as differentially expressed during *P. salmonis* infection in *S. salar* (Valenzuela-Miranda and Gallardo-Escarate, 2016). Our results indicate that the main targets are genes associated with different immune system processes like the chemokine-mediated signaling pathway, cortisol metabolism, neutrophil chemotaxis and immune system development, which play pivotal roles in the immune response of teleost to bacterial infection (Bird and Tafalla, 2015; Philip and Vijayan, 2015; Uribe et al., 2011). A deeper look into predicted miRNA-gene interactions revealed that some possible target genes of these miRNAs included *C-C motif chemokine 19-like*, *stromal cell-derived factor 1-like*, *myxovirus resistance protein 2* and *hepcidin-1*. In particular, *hepcidin-1* is a small peptide that has shown antimicrobial activity against different bacteria, including *P. salmonis* (Alvarez et al., 2014). Overexpression of *Hepcidin-1* in different Atlantic salmon tissue has been observed in response to *P. salmonis* infection, where lncRNAs may be involved in this regulation (Valenzuela-Miranda and Gallardo-Escarate, 2016). In addition to high ΔG values obtained for miRNA-gene interaction, the expression patterns were mainly complementary between miRNAs and genes in both head kidney and spleen. Although these results support the idea that *Salmo salar* miRNAs are involved in the regulation of different genes triggered by *P. salmonis* infection, further functional studies are still required to fully understand the regulatory role of miRNAs in the salmonid immune system.

5. Conclusion

The attention of researchers has been caught by the emerging role of non-coding RNAs as regulatory elements of diverse

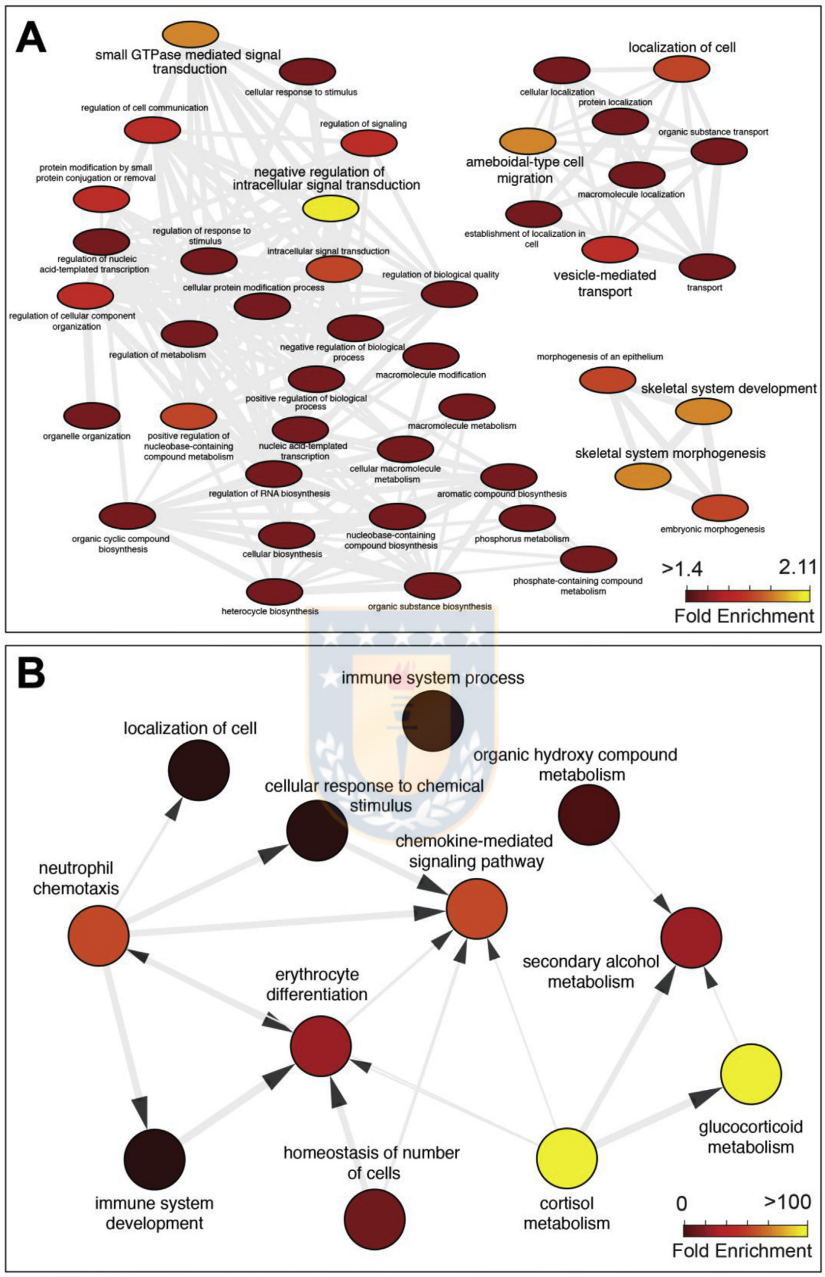

Fig. 6. GO enrichment analysis of the different *in silico* predicted target genes for the differentially expressed miRNAs. A) Genome wide target identification. B) Against *Salmo salar* differentially expressed genes following bacterial infection (Valenzuela-Miranda and Gallardo-Escarate, 2016).

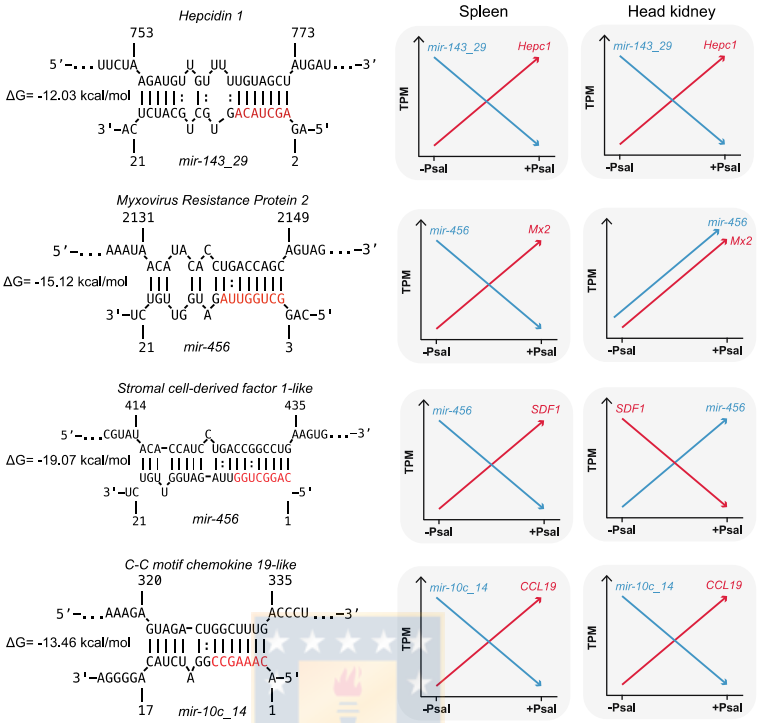

Fig. 7. miRNA-gene target prediction for *Hepcidin-1, Myxovirus Resistance Protein 2, Stromal cell-derived factor 1-like* and *C-C motif chemokine 19-like*. Seed region is represented in red and predicted ΔG values are also reported. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biological processes, including their role. In this context, our results provide novel insights into the role of miRNAs in the immune response of salmonids to intracellular bacteria. This will benefit our understanding of the regulation of molecular mechanisms underlying the immune response to one of the most persistent pathogens in Chilean aquaculture.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2017.08.016.

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CAPÍTULO III (H1,OE2). Dual RNA-Seq uncovers metabolic amino acids dependency of the intracellular bacterium *Piscirickettsia salmonis* infecting Atlantic salmon

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Abstract

High-throughput sequencing technologies have offered the possibility to understand the complexity of the transcriptomic responses of an organism during a wide variety of biological scenarios, such as the case of pathogenic infections. Recently, the simultaneous sequencing of both pathogen and host transcriptomes (Dual RNA-seq) during the infection has become a promising approach to uncover the complexity of the host-pathogen interactions. In this study, through a double rRNA depletion and RNA sequencing protocols, we simultaneously analyzed the transcriptome of the intracellular bacterium *Piscirickettsia salmonis* and its host the Atlantic salmon (*Salmo salar*) during the course of the infection. Beyond canonical host immune-related response and pathogen virulent factors, both bacteria and host displayed a large number of genes associated with metabolism and particularly related with the amino acid metabolism. Notably, genome-wide comparison among *P. salmonis* genomes and different fish pathogens genomes revealed a lack of the biosynthetic pathway for several amino acids such as valine, leucine and isoleucine. To support this finding, *in vitro* experiments evidenced that when these amino acids are restricted the bacterial growth dynamics is significantly affected. However, this condition is phenotypically reversed when the amino acids are supplemented in the bacterial growth medium. Based on our results, a metabolic dependency of *P. salmonis* on *S. salar* amino acids is suggested, which could imply novel mechanisms of pathogenesis based on the capacity to uptake nutrients from the host. Overall, dual transcriptome sequencing leads to the understanding of host-pathogen interactions from a different perspective, beyond biological processes related to immunity.

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Dual RNA-Seq Uncovers Metabolic Amino Acids Dependency of the Intracellular Bacterium *Piscirickettsia salmonis* Infecting Atlantic Salmon

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High-throughput sequencing technologies have offered the possibility to understand the complexity of the transcriptomic responses of an organism during a wide variety of biological scenarios, such as the case of pathogenic infections. Recently, the simultaneous sequencing of both pathogen and host transcriptomes (dual RNA-seq) during the infection has become a promising approach to uncover the complexity of the host–pathogen interactions. In this study, through a double rRNA depletion and RNA sequencing protocols, we simultaneously analyzed the transcriptome of the intracellular bacterium *Piscirickettsia salmonis* and its host the Atlantic salmon (*Salmo salar*) during the course of the infection. Beyond canonical host immune-related response and pathogen virulent factors, both bacteria and host displayed a large number of genes associated with metabolism and particularly related with the amino acid metabolism. Notably, genome-wide comparison among *P. salmonis* genomes and different fish pathogens genomes revealed a lack of the biosynthetic pathway for several amino acids such as valine, leucine, and isoleucine. To support this finding, *in vitro* experiments evidenced that when these amino acids are restricted the bacterial growth dynamics is significantly affected. However, this condition is phenotypically reversed when the amino acids are supplemented in the bacterial growth medium. Based on our results, a metabolic dependency of *P. salmonis* on *S. salar* amino acids is suggested, which could imply novel mechanisms of pathogenesis based on the capacity to uptake nutrients from the host. Overall, dual transcriptome sequencing leads to the understanding of host–pathogen interactions from a different perspective, beyond biological processes related to immunity.

Keywords: dual RNA-seq, *Piscirickettsia salmonis*, Atlantic salmon, nutritional immunity, metabolic dependency, amino acids

INTRODUCTION

High-throughput sequencing technologies applied to transcriptomic studies (RNA-Seq) have offered the possibility to understand complex molecular responses under different biological scenarios. Among them, a pathogenic infection entails a deep transcriptomic remodeling of the host to promote the pathogenic clearance; in turn, pathogens display the expression of different

genes to grant their survival and replicate within the host. In this context, the simultaneous analysis of host and pathogen transcriptomes (dual RNA-Seq) during their interaction can reveal novel aspects of the infective process (Westermann et al., 2017). Initially, this approach was limited to viral, fungal and parasitic infections, where the pathogen resembles host transcripts (Tierney et al., 2012; Strong et al., 2013; Choi et al., 2014; Pittman et al., 2014) and scarcely reported in bacterial models (Westermann et al., 2012). However, the improvement of high-throughput sequencing and the development of novel RNA capture/depletion methods offer a promising opportunity to also expand this approach to bacterial infections (Westermann et al., 2016).

Although most dual RNA-seq approaches applied in bacterial infections have been exploratory, some of them have unraveled novel mechanisms of host–pathogen interaction. For instance, a dual RNA-seq was used to discover a possible strategy employed by *Chlamydia trachomatis* for the *in vitro* infection of human epithelial cells based on an early iron acquisition and a host immune depletion strategy (Humphrys et al., 2013). Furthermore, the simultaneous transcriptome analysis of the Gram-negative bacterium *Haemophilus influenza* during the infection of mucosal epithelial cells revealed the importance of the host oxidative response and the mechanisms employed by the bacteria to overcome this adverse environment (Baddal et al., 2016). Likewise, the co-transcriptomic analysis of the uropathogenic *Escherichia coli* (UPEC) and its host evidenced that while host transcriptomic response was similar to different bacterial strains, different expression patterns were identified in UPEC strains with contrasting pathogenic effects (Mavromatis et al., 2015). These results were used to reveal novel insights into the mechanisms employed by the bacteria for the intra-macrophage survival. Moreover, a dual RNA-Seq was used to characterize the regulatory role of small RNAs (sRNAs) in *Salmonella enterica* infection. Here, researchers identified bacterial sRNA involved in the regulation of both host and pathogenic genes, revealing the hidden roles of *S. enterica* transcripts during the pathogenesis (Westermann et al., 2016). Simultaneous profiling of host-pathogen transcriptomes has become a powerful approach tool to unravel key aspects during the infection process. In the present study, we apply a dual RNA-Seq approach to reveal novel aspects of the infective process of the intracellular bacterium *Piscirickettsia salmonis* during the infection on the Atlantic salmon (*Salmo salar*).

Piscirickettsia salmonis is a facultative intracellular gram-negative bacterium that causes the salmonid rickettsial septicemia (SRS), a severe systemic disease responsible for up to 85% of the infectious moralities in farmed salmons in Chile. Just in this country, the economic losses associated with this pathogen has been estimated in around US$100 million per year (Smith et al., 1997; Bravo and Midtlyng, 2007; Pulgar et al., 2015), becoming one of the main concerns for the industry (Mauel and Miller, 2002). Beyond this negative impact, perhaps one of the most remarkable features of *P. salmonis* is its capability to infect and replicate within host immune cells, such as in macrophages (McCarthy et al., 2008; Rajas et al., 2009; Ramirez et al., 2015). In this context, the mechanism whereby this pathogen can evade host immune response are still unclear. Due to the high prevalence, negative impact and scientific interest on this pathogen, different efforts have tried to understand salmonids defensive mechanism against *P. salmonis* and how the bacteria overcome this response. Host transcriptomic response has been mainly associated with a regulation of genes involved in the innate immunity, apoptosis, different signaling pathways, endocytosis, non-coding RNAs and iron metabolism among others (Rise et al., 2004; Tacchi et al., 2011; Pulgar et al., 2015; Valenzuela-Miranda and Gallardo-Escarate, 2016; Valenzuela-Miranda et al., 2017). On the other hand, *P. salmonis* transcriptomic response has been assessed after the *in vitro* infection in Sf21 cell lines (Machuca and Martinez, 2016). Although different genes associated with the type IV secretion and iron acquisition system were identified, it remains unexplored how *P. salmonis* transcriptome is modulated during the infection. Due to this, we explore a dual RNA-Seq approach to unravel novel mechanisms of interactions during the infection of *P. salmonis* on the Atlantic salmon. A special emphasis was placed in bacterial gene expression, since transcriptional response of the Atlantic salmon against *P. salmonis* have been widely described previously (Tacchi et al., 2011; Pulgar et al., 2015; Valenzuela-Miranda and Gallardo-Escarate, 2016; Tarifeno-Saldivia et al., 2017). Beyond canonical pathogenic and immune related-genes, our results evidenced a common transcriptomic response between host and pathogen associated with the amino acid metabolism. Further analyses revealed a lack of *P. salmonis* genes associated with different amino acids biosynthetic pathways and the importance of the availability of some amino acids for the bacterial growth medium. We hypothesize metabolic amino acids dependency of *P. salmonis* on *S. salar*, which could imply novel mechanisms of pathogenesis based on the capacity to uptake nutrients from the host and capacity of the host to regulate the availability of free amino acids.

MATERIALS AND METHODS

Experimental Design

Atlantic salmons (154.7 ± 13.5g) were obtained from a commercial farm located at Puerto Montt, Chile and transferred to the Marine Biology Station of the University of Concepción (Dichato, Chile). Here, individuals were randomly screened to discard the presence of different pathogens commonly present salmonid aquaculture. After quarantine, individuals were randomly divided into two independent marine water-based recirculating lines, each containing five 370 L tanks. For each line, three tanks were used for sampling (six in total), one tank was used to record mortality (two in total) and the remaining tank was not considered in this experiment. A total of 50 individuals per tank were maintained during an acclimation period of 14 days before the challenge. After this period, each individual was anesthetized and intraperitoneally injected with 0.2 ml of *P. salmonis* (EM-90 strain) containing 1×10^6 bacteria per dose, as previously standardized. Later, samples were collected from infected individuals at 3, 7, and 14 days post

infection (dpi). Head kidney and spleen tissues were collected from two individuals of each sampling tank (12 individuals per point) and stored in RNA later solution (Ambion, United States) at −80°C. In the remaining tanks, mortalities were daily recorded (**Supplementary Figure S1**), clinically and molecularly confirmed as a result of SRS. All animal procedures were carried out under the guidelines approved by the Ethics Committee of University of Concepción.

RNA Isolation and Sequencing Strategy

Infected tissues stored at −80°C in RNA later solution were thawed at room temperature and total RNA (host and pathogen) was isolated from 10 different individuals using the TRIzol reagent kit (Thermo Fisher Scientific) according to manufactures instructions. RNA integrity was verified using the R6K screen tape 2200 on the TapeStation (Agilent Technologies, United States) platform. Thus, isolated RNAs with RNA Integrity Numbers (RIN) above 8 were considered for further analysis. Based on RNA quality, 3 different pools of RNA were prepared from 3 distinct individuals for each tissue and time (biological replicates). RNA pools were precipitated in absolute ethanol and shipped in dry ice to Macrogen Inc. (Korea). Here, two Ribo-Zero rRNA Removal Kit (Illumina, San Diego, CA, United States) were used to remove both bacterial and host rRNAs. Remaining RNA containing both *P. salmonis* and *S. salar* transcripts were used to prepare high-throughput sequencing libraries using the TrueSeq RNA sample preparation kit (Illumina, San Diego, CA, United States). Each library was sequenced on a HiSeq platform at 100 bp paired-end reads (Macrogen, Korea). All sequencing data will be available under the SRA accession number SUB4576220. Sequencing statistics for each RNA-seq data are presented in **Supplementary Table S1**.

Dual RNA-Seq and Differential Expression Analysis

Raw sequencing reads were filtered by quality and adapter/index were identified and removed from remaining reads using CLC Genomics Workbench (V10, CLC Bio, Denmark). In order to discriminate pathogen and host transcriptomes, cleaned reads were mapped against the last available version of the Atlantic salmon (*S. salar)*[1] and against *P. salmonis* available genomes[2]. Mapping parameters included a mismatch cost of 2, insertion/deletion cost of 3 and a similarity/length fraction of 0.8. Effectively mapped reads against both genomes were separated in different files and used for further RNA-Seq analysis. RNA-Seq analysis was conducted using CLC Genomics Workbench (V10, CLC Bio, Denmark). Previously discriminated reads from host and pathogen were used to perform RNA-Seq analysis using all coding sequences annotated in the Atlantic salmon and *P. salmonis* genomes. For RNA-Seq analyses, similarity/length fraction was set as 0.9 in order to minimize the probability to include misassigned reads for each species. Expression values were estimated as transcripts per million (TPM) and normalized by totals per million read. Expression values obtained at 3 dpi were used as baseline for gene expression comparison. We decided to use this dataset as reference because we needed transcriptomic data that contained reads from the pathogen to compare, since pathogenic reads in any type of control would not be present. Further, statistical differences were identified through a Baggerly's test adjusting p-values through a false discovery rate (FDR) correction. Genes with a fold change > 4 and FDR p-values < 0.01 were considered as differentially expressed.

Functional Annotation and qPCR Validations

Molecular annotation of the differentially expressed transcripts for both *P. salmonis* and *S. salar* was carried out to identify the most representative biological processes. For this purpose, the Gene Ontology (GO) annotation was conducted through the BLAST2GO software V 4.1.9 (Conesa et al., 2005) and the enrichment analysis was performed using as reference the genomes of *P. salmonis* and *S. salar*. Further, resulting GO enrichment analysis was visualized in REVIGO platform (Supek et al., 2011). Finally, KEGG pathway annotation analysis was also conducted using the KEGG automatic annotation server (Moriya et al., 2007) through the bidirectional best-hit assignment method. Furthermore, RT-qPCR were used to validate sequencing results. To do this, 10 genes from the bacteria and 10 genes from the fish were randomly selected and used to RT-qPCR primer design (**Supplementary Table S2**). After primer validation, each RT-qPCR was conducted in a thermocycler StepOne plus (Applied Biosystems, United States) using the Maxima SYBR Green/ROX kit according to manufactures instructions. Amplification cycles were used as following 95°C for 10 min, 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. All qPCRs were carried on five biological and three technical replicates and expression values were estimated through the 2Δ Ct method using 16s and elongation factor 1a as normalizer genes for *P. salmonis* and *S. salar,* respectively. Significant differences between 7 and 14 dpi regarding 3 dpi were estimated with the Student's t-test ($p < 0.05$). A comparison between fold-changes obtained through RT-qPCR and RNA-seq evidence a r^2 value above 0.8, evidencing a high correlation between the fold changes obtained by RT-qPCR and RNA-seq (**Supplementary Figure S2**). Individual fold changes were included as **Supplementary Figure S3**.

Exploring the Amino Acid Metabolism of *P. salmonis*

Dual RNA-Seq analysis revealed a large number of genes differentially expressed associated with amino acid metabolism during the infection process. Due to this, we further explored the importance of amino acids in *P. salmonis* metabolism. First, a genome-scale comparison was conducted between *P. salmonis*, a second salmonid pathogen such as *Aeromonas salmonicida* and a closely related bacterium as *Francisella tularensis*. For this purpose, coding genes were obtained from NCBI for *P. salmonis* (see text footnote 2), *F. tularensis*[3] and *A. salmonicida*[4]

[1]https://www.ncbi.nlm.nih.gov/assembly/GCF_000233375.1/

[2]https://www.ncbi.nlm.nih.gov/genome/genomes/11769

[3]https://www.ncbi.nlm.nih.gov/genome/genomes/511

[4]https://www.ncbi.nlm.nih.gov/genome/genomes/540

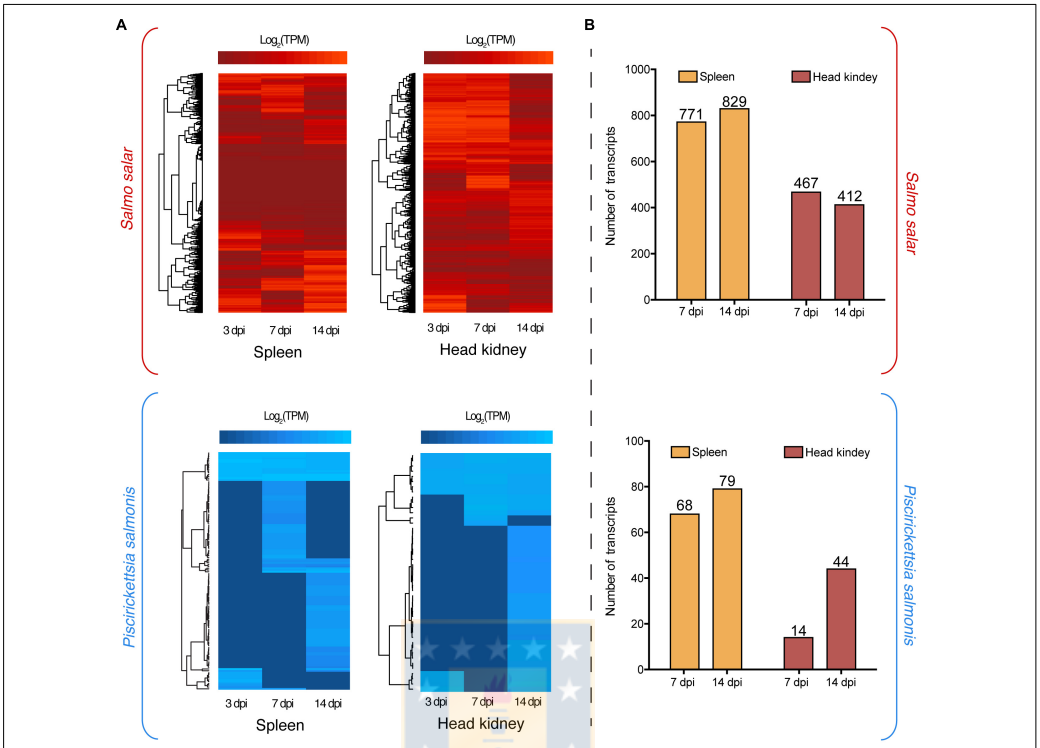

FIGURE 1 | (A) Global transcriptome analysis of *Piscirickettsia salmonis* (blue) and *S. salar* (red) at 3, 7, and 14 days post-infection (dpi) in spleen and head kidney tissues. Expression values were estimated as transcripts per million reads (TPM) and Log_2 transformed for heat map clustering. **(B)** Differentially expressed genes in spleen and head kidney at 7 and 14 dpi using as control the expression values at 3 dpi. Three days post-infection were used as baseline gene expression since no bacterial reads would be present in any type of control group.

genomes. All coding sequences were annotated through KAAS annotation server as described above, focusing our attention in Histidine metabolism (00340), valine, leucine and isoleucine degradation/biosynthesis (00280 and 00290), Arginine and proline metabolism (00330), Lysine biosynthesis/degradation (00300 and 00310), Cysteine and methionine metabolism (00270), Glycine, serine and threonine metabolism (00260), Phenylalanine, tyrosine and tryptophan biosynthesis (00400) and Alanine, aspartate and glutamate metabolism (00250). Furthermore, liquid cultures of *P. salmonis* were conducted considering different experimental culture media with distinct amino acid composition. A basal medium (BM) was prepared with Eugon (30.4 g/l) supplemented with $FeCl_3$ (2 mM), a complete medium (CM) prepared with Eugon (30.4 g/l) supplemented with $FeCl_3$ (2 mM) and Casamino acid (1%) and experimental cultures medias were prepared with BM + 1% of the desired amino acid (Valine, leucine, and Isoleucine). A bacterial inoculum previously obtained from CHSE-214 cells infected with *P. salmonis* at 90% of lysis was used as starting material for the growth of *P. salmonis* in CM medium. When the exponential phase was reached, 300 μL from the liquid culture was used to inoculate 2.7 ml of liquid culture containing the different experimental mediums. All cultures were carried on triplicates and maintained at 20°C with a constant agitation of 100 rpm. Bacterial growth was daily based monitored through the change in the optical density at an absorbance of 600 nm. A multiple *t*-test was carried out to identify statistically significant differences ($p < 0.01$) between treatments.

RESULTS

Exploring Host and Pathogen Transcriptome During Pathogenesis

Dual RNA-Seq analysis evidenced the modulation of *P. salmonis* and *S. salar* transcriptomes during the infection. This modulation is represented in two heat maps, one for the fish host (red) and another one for the pathogen (blue), where different clusters

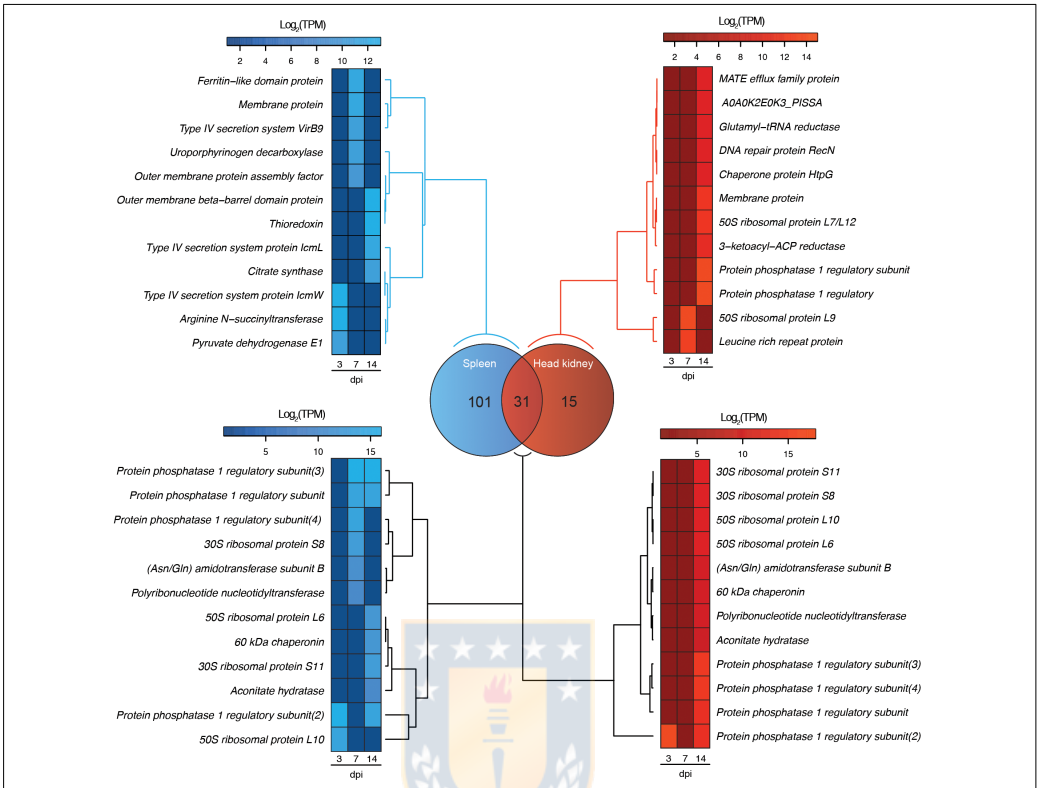

FIGURE 2 | Venn diagram showing the number of exclusive and shared genes in the transcriptome of *P. salmonis* during the infection in spleen (blue) and head kidney (red) tissues of Atlantic salar. The heat maps show a subset of exclusive and shared genes expressed by *P. salmonis* in both tissues.

of expression profiles were identified (**Figure 1A**). Regarding *S. salar* transcriptome, 771 and 829 genes were differentially expressed in spleen at 7 and 14 dpi, respectively (**Figure 1B**). Meanwhile, 412 and 467 genes were differentially modulated in the head kidney at the same time-points (**Figure 1B**). On the other hand, 68 and 79 *P. salmonis* genes were differentially expressed in the spleen at 7 and 14 days, respectively, while 14 and 44 were identified in the head kidney (**Figure 1B**). Thus, the number of bacterial genes differentially expressed were increased together with the course of the infection. Since transcriptional responses for the host have been previously reported, a special focus was placed on bacterial gene expression. Herein, a Venn diagram analysis revealed that 31 *P. salmonis* genes were differentially regulated in both spleen and head kidney, while 101 and 15 transcripts were exclusively regulated in spleen and head kidney, respectively. Genes exclusively regulated in spleen included a *ferritin-like domain protein*, genes associated with the type IV secretion system (*VirB9*, *IcmL*, and *IcmW*) and several outer membrane proteins (**Figure 2**). On the other hand, the genes exclusively regulated in head kidney included a *MATE efflux family protein*, *DNA repair protein RecN*, *chaperone protein HtpG* and membrane proteins among others (**Figure 2**). Among shared genes, different Protein phosphatase 1, ribosomal protein, chaperones and Asn/Gln aminotransferase subunits were also found (**Figure 2**). The complete list of differentially expressed genes for the Atlantic salmon and *P. salmonis* is included as **Supplementary Table S3**.

Amino Acid Metabolism: A Common Response Between *P. salmonis* and *S. salar*

A functional annotation of the different differentially expressed genes for both *S. salar* and *P. salmonis* was conducted in order to identify key molecular pathways regulated during the infective process. GO enrichment analysis evidenced that a large percent of differentially expressed genes in *P. salmonis* belonged to biological processes related with the metabolism of

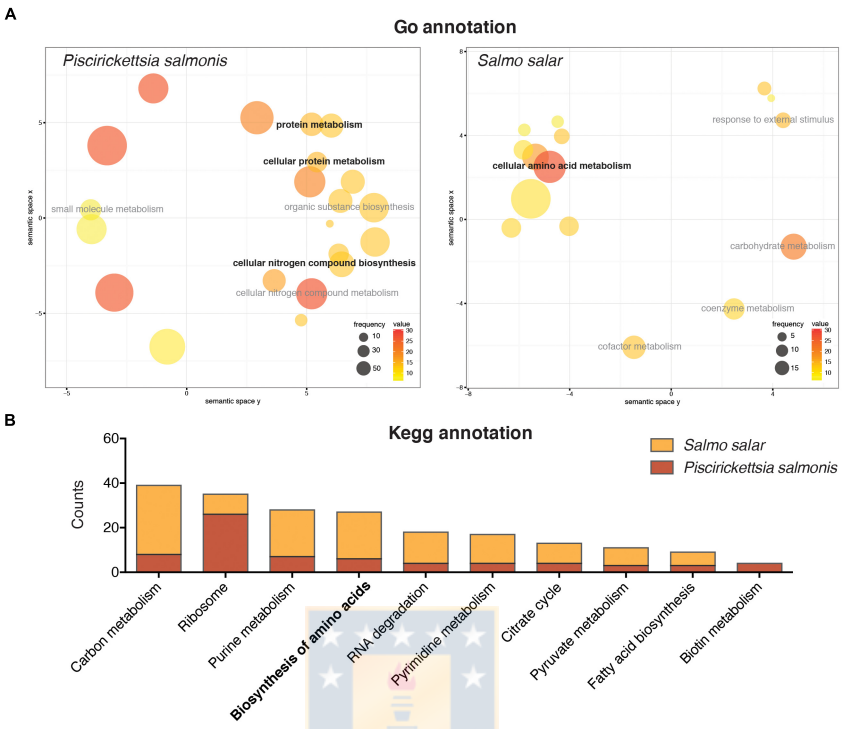

FIGURE 3 | Gene ontology annotation **(A)** and KEGG pathway annotation **(B)** of the differentially expressed genes in *Salmo salar* and *P. salmonis* during the infection. The size of each circle represents the frequency of the GO term in the underlying GO database (larger circles represent more general terms) and the color represents the number of genes in each term (value). Both host and pathogen displayed a large number of genes associated to amino acid biosynthesis and degradation.

proteins and nitrogen compounds, such as the terms "protein metabolism," "cellular protein metabolism" and "cellular nitrogen compound biosynthesis" among others (**Figure 3A**). On the other hand, a more complex transcriptomic response was found in Atlantic salmon. Here, multiple biological processes were represented, such as organic acid metabolic process, oxidation-reduction process, response to external stimulus, chemotaxis among others (**Supplementary Table S2**). However, terms related to the metabolism of amino acids was also represented within differentially expressed transcripts, such as the "cellular amino acid metabolism" (**Figure 3A**). Enrichment of genes associated with protein metabolism was also found through KEGG annotation. Furthermore, one of the most represented pathways among differentially regulated genes in *P. salmonis* transcriptome included different metabolic pathways, among them, the "biosynthesis and degradation of amino acids" (**Figure 3B**). Regarding host transcriptome, the response of the Atlantic salmon was associated not just associated with endocytosis, cytokine-cytokine receptor interaction, apoptosis, phagosome and Nod-like receptor signaling pathways (**Supplementary Table S4**), but also with key metabolic pathways, including the "biosynthesis of amino acids" (**Figure 3B**). Overall, the results evidenced that although the metabolism of amino acids was not the predominant transcriptomic response in the host, both *P. salmonis* and *S. salar* displayed a large number of genes involved with biosynthesis and degradation of amino acids. Due to this common response, we further investigate the role of amino acids in *P. salmonis* metabolism.

Role of Amino Acids in *P. salmonis* Metabolism

To explore the importance of amino acids in *P. salmonis* metabolism, a genome-scale comparison was used to identify the presence/absence of genes directly involved in the degradation/biosynthesis of different bacterial pathogens. Thus, *P. salmonis* genome was compared with *F. tularensis* and *A. salmonicida* available genomes. Results showed that all three pathogens dispose of a similar genetic background of genes

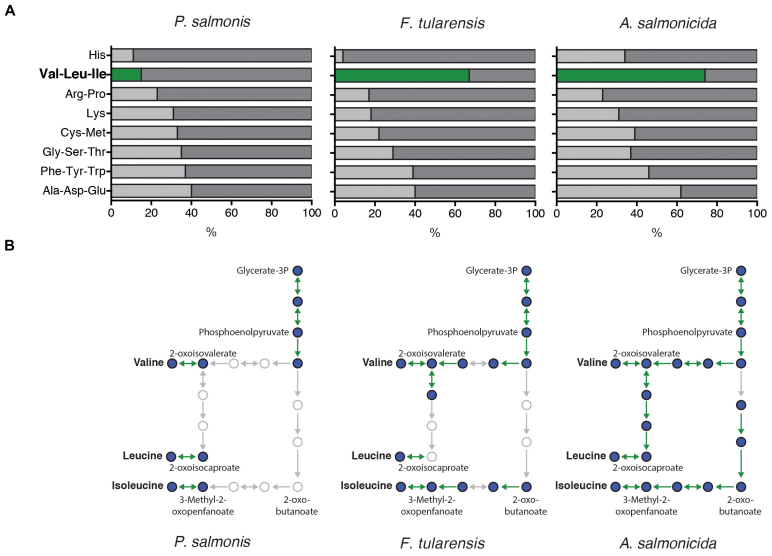

FIGURE 4 | (A) Genomic-wide comparison of genes involved in the biosynthesis and degradation of amino acid in *P. salmonis*, a close related bacterium (*F. tularensis*) and another fish Gram negative bacterium (*A. salmonicida*). Bars represent the percentage of genes identified for each pathogen underlying all the possible genes for the pathway in KEGG pathway database. **(B)** Subset of the biosynthesis and degradation pathway for valine, leucine, and isoleucine in *P. salmonis*, *F. tularensis*, and *A. salmonicida*. Circles represent intermediate molecules and arrows the genes catalyzing each reaction. Thus, a green arrow represents a gene that was found in the genome of each pathogen and gray ones for those absent. Thus, *P. salmonis* evidenced a smaller number of genes involved in valine, leucine, and isoleucine biosynthesis/degradation.

involved in biosynthesis/degradation of amino acids (**Figure 4**). However, a significant lack of genes related with valine, leucine, and isoleucine metabolism was found in *P. salmonis* compared with *F. tularensis* and *A. salmonicida*, where over 60% of all possible genes were found (**Figure 4A**). However, a deeper look into this metabolic pathway evidenced that although *P. salmonis* lacks the majority of the genetic background for the biosynthesis of this amino acids, it was possible to found the gene that encodes for the primary degradation of valine, leucine, and isoleucine (**Figure 4B**). These results suggest that the bacterium is not able to biosynthesize these amino acids and therefore a metabolic dependency of *P. salmonis* on environmental host amino acid availability can be expected. To further explore the importance of amino acid availability to the bacteria, *P. salmonis* was growth in different liquid culture mediums with different amino acidic availability. A control medium (CM) fully supplemented with amino acids, a basal medium (BM) with no supplementation of amino acids and basal mediums supplemented with 1% of either valine (BM + V), leucine (BM + I) or isoleucine (BM + L) were used as experimental mediums. The results evidence that the bacteria are not able to grow when no amino acid is supplemented. However, when BM is supplemented with either valine, leucine or isoleucine, the growth kinetics of *P. salmonis* resembles the one observed in a fully amino acidic supplemented condition (**Figure 5**).

DISCUSSION

Dual RNA-Seq has emerged as a promising approach to elucidate host–pathogen interaction. Although this approach was previously limited to pathogens that resembles host transcripts (Westermann et al., 2017), the development of high-throughput sequencing technologies has allowed to expand this approach to bacterial infections. In this context, we applied a dual RNA-Seq approach to explore novel means of interaction between the intracellular bacterium *P. salmonis* and its main host the Atlantic salmon (*S. salar*). In addition to being the main threat to Chilean salmonid aquaculture (Rozas and Enriquez, 2014), the intracellular nature of this pathogen makes it an interesting model to study host immune evasion strategies and intracellular survival mechanisms employed by intracellular pathogens.

The dual RNA-Seq analysis revealed the presence of different bacterial genes among the spleen and head kidney transcriptome. Thus, the spleen showed the largest number of bacterial genes regarding the ones found in head kidney data. This can be attributable due to when IP injection is used as infection method in previous IP challenges with *P. salmonis*, the spleen is one of the first tissues to be infected, followed by head kidney (Valenzuela-Miranda and Gallardo-Escarate, 2016). Therefore, the differences between

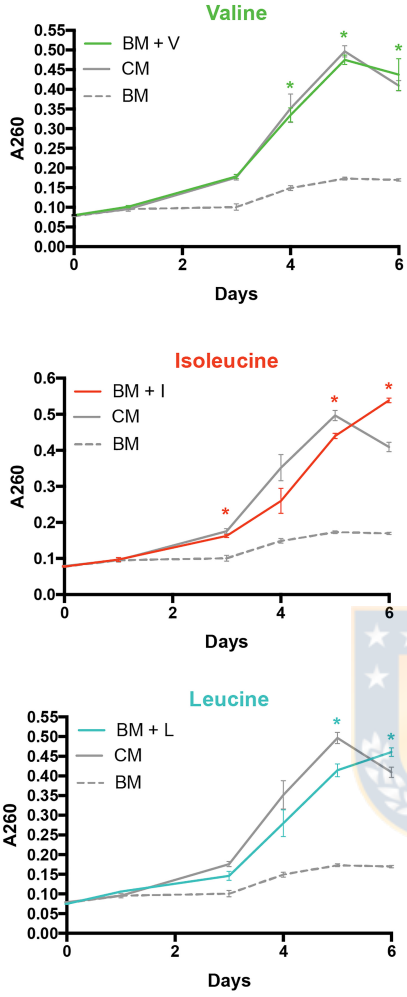

FIGURE 5 | Growth kinetics of *P. salmonis* in liquid culture media supplemented with different amino acids. Bacteria was growth in a control medium (CM), a basal medium without amino acids (BM) and in basal medium supplemented with valine (BM + V), leucine (BM + L), and isoleucine (BM + I) amino acids at 1%. *Represents statistically significant differences ($p < 0.01$) between the supplemented medium (Val, Leu, or Ile) regarding control group (no amino acids).

the transcriptional modulation of pathogen genes among this tissue could be as a result of different infection stages rather than a tissue-specific transcriptomic response of the pathogen. Nevertheless, different classical pathogenic related genes were identified in transcriptomic data. Such as the case of several members of the type IV secretion system, including *VirB9*, *IcmL*, and *IcmW*. The type IV secretion system it has been described as a conserved mechanism for the delivery of virulent factors from host to pathogen (Thanassi and Hultgren, 2000). This system has been previously described in *P. salmonis* (Gomez et al., 2013) and even the directed mutagenesis of this locus has resulted in the attenuation of the pathogenesis of *P. salmonis* (Mancilla et al., 2018). On the other hand, the repertory of genes displayed by the Atlantic salmon in response to infection was classified into different molecular pathways. These processes included endocytosis, cytokine-cytokine receptor interaction, apoptosis, phagosome and Nod-like receptor signaling pathways, which has been previously described as key responses triggered during the infection of *P. salmonis* in *S. salar* tissues (Valenzuela-Miranda and Gallardo-Escarate, 2016).

However, beyond the canonical pathogenic genes commonly associated with bacterial pathogenesis and the Atlantic salmon immunity (Valenzuela-Miranda and Gallardo-Escarate, 2016; Tarifeno-Saldivia et al., 2017), a common response associated with protein metabolism and particularly the biosynthesis/degradation of amino acids was present in both *S. salar* and *P. salmonis* transcriptomic response during the infection process. Due to this, a genome-scale comparison was performed in order to evidence the genetic background of genes involved in biosynthesis degradation of amino acids in *P. salmonis*, a close related bacterium *F. tularensis* and another salmonid bacterial pathogen *A. salmonicida*. The genomic background has been previously used to predict essential and non-essential amino acids in different pathogens (Meibom and Charbit, 2010). Based on our results, we found a lack of biosynthetic genes associated with the metabolism of valine, leucine, and isoleucine for *P. salmonis* when compared with *F. tularensis* and *A. salmonicida* genomes. Therefore, the availability of these amino acids for *P. salmonis* might rely upon the presence of these resources in the host intracellular environment.

During infection, intracellular pathogens must overcome different adverse condition, such as the entrance to host cells, host immune response, free radicals and also nutrient deprivation. Although host cytosol was previously considered as an abundant source of nutrients for invading pathogens (Ray et al., 2009), recent evidence suggests that hosts can reduce the intracellular availability of certain nutrients as a protective response against the invading pathogens (Abu Kwaik and Bumann, 2013; Barel and Charbit, 2013). This deprivation results in a struggle between host and pathogen for the limited nutrient availability, which commonly known as nutritional immunity (Hood and Skaar, 2012). Thus, it has been suggested that part of the immune response of the Atlantic salmon to *P. salmonis* infection relies on the nutritional immunity. This has been exclusively explored due to the struggle for iron availability. From *P. salmonis* perspective, it has been suggested the importance of a siderophore-based mechanism to capture iron from different host sources (Calquin et al., 2018). On the other hand, the infection of *P. salmonis* induces a

strong transcriptomic modulation of genes involved in iron availability in *S. salar* (Pulgar et al., 2015; Valenzuela-Miranda and Gallardo-Escarate, 2016). In this context, our results evidence that both host and pathogen display a large number of genes involved in the biosynthesis/degradation of amino acids. Considering the lack of biosynthetic pathways in leucine, valine and isoleucine in *P. salmonis* and that these same amino acids are defined as essential amino acids for salmonids (Helland et al., 2010), we suggest that this transcriptional modulation can be reflecting an amino acid-based nutritional immunity triggered by *S. salar* to overcome *P. salmonis* infection. In turn, *P. salmonis* displayed a transcriptional modulation of different genes associated with amino acid metabolism to deal with host response.

Intracellular pathogens have developed different strategies to overcome amino acids starvation triggered by the host during infection. Some of these adaptations include the growth arrest and differentiation, an amino acid self-sufficiency and to exploit host machinery to obtain amino acids form host cell (Zhang and Rubin, 2013). Based on our results and considering the recent reconstruction of metabolic models for the bacteria (Cortes et al., 2017), the self-sufficiency strategy for amino acids in the *P. salmonis* can be rejected. Regarding growth arrest and differentiation, it has been reported that during an amino acid restricted scenario *C. trachomatis* morphologically changes into an aberrant form that is unable to grow but protects them from a nutrient restricted environment (Leonhardt et al., 2007). However, the addition of tryptophan or isoleucine can restore these aberrant forms and reactivate bacterial growth (Hatch, 1975; Ibana et al., 2011). Previously, it has been reported the existence of morphological small variants of *P. salmonis*, which were suggested as a survival mechanism employed by the bacteria to overcome adverse scenarios (Veronica Rojas et al., 2008). However, the role of these variants during an amino acid restricted scenario and its relation with virulence remains unexplored. On the other hand, pathogens like *Legionella pneumophila* have devolved mechanism that grants the access to host nutrients by promoting the expression of host amino acid transporters and taking advantage of proteasomes of infected cells to generate free amino acids for bacterial growth (Wieland et al., 2005; Price et al., 2011). In this context, it has been suggested that one of the mechanisms employed by *P. salmonis* to evade immune response can be related with the regulation of host transcriptional response through non-coding RNAs (Valenzuela-Miranda and Gallardo-Escarate, 2016; Valenzuela-Miranda et al., 2017), therefore, the idea that *P. salmonis* is able to hijack host machinery to obtain amino acids should also be explored.

The importance of amino acids in pathogenic cell cycle has not only be associated as a primary resource for the biogenesis of proteins, but also as alternative sources of carbon and nitrogen (Steeb et al., 2013). Therefore, a metabolic plasticity to obtain carbon and energy from multiple amino acid sources could become an advantage in a resource-limited scenario. This metabolic plasticity has been reported in other intracellular pathogens like *F. tularensis*, where the intracellular survival of this bacteria relies on their availability to exploit multiple host amino acids (Meibom and Charbit, 2010; Barel et al., 2015). Based in our results, and considering the recently proposed metabolic models for *P. salmonis* (Cortes et al., 2017; Fuentealba et al., 2017), we can hypothesize that this bacteria is capable to use different amino acids as a carbon an energy resource. This strategy has been adopted by others intracellular bacterium, where this capability has been described as a crucial factor for virulence development (Eisenreich et al., 2010; Meibom and Charbit, 2010; Barel et al., 2015). In this scenario, the implications of a metabolic plasticity in *P. salmonis* and its link to virulence of different bacterial strains must be further explored.

Overall, our results showed how a dual RNA-Seq approach can lead us to the understanding of novel means of interaction between host and pathogens. However, the importance of an amino acid-based nutritional immunity of *S. salar* in response to *P. salmonis* infection must be further investigated. This information will not just lead us to the development of novel treatments for the pathogen, but also to the understanding of the pathogenesis process from a different perspective, beyond canonical immunological mechanisms.

AUTHOR CONTRIBUTIONS

DV-M and CG-E conceived the study and drafted the manuscript. DV-M performed the experiments and analyzed the data under CG-E extensive supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02877/full#supplementary-material

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CAPÍTULO IV (H2, OE3). Long-term serial culture of *Piscirickettsia salmonis* leads to a genomic and transcriptomic reorganization affecting bacterial in vitro virulence

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Abstract

The propagation of bacteria in artificial mediums implies and adaptation to this non-natural environment. If these conditions are persistent enough time, a permanent adaptation to this new environment can be expected. In this study we explore the genomic and transcriptomic rearrangement that the salmonid pathogen *Piscirickettsia salmoni*s undergo after a serial propagation for 200 passages (~2 years) in a cell-free culture medium. One of the most remarkable genomic changes that *P. salmonis* undergo during the culture period was the migration of a 35 Kb segment form the original (P0) genome to a P200 plasmid. Interestingly, the genomic content of this block revealed the presence of the Dot/Icm secretion system, which has been previously associated to *P. salmonis* pathogenesis. On the other hand, a reduced transcriptomic response was evidence in *P. salmonis* after 200 passages affecting the expression of different pathways including the Iron acquisition and metabolism. Further *in vitro* infections revealed that after 200 passages *P. salmonis* is less capable of generate cytopathic effects than the original P0 form. Overall, our results evidence that the continuous propagation of *P. salmonis* lead to genomic and transcriptomic rearrangement that impact on bacterial *in vitro* pathogenesis. These results open new perspective about the adaptation of *P. salmonis* to artificial cultures providing useful information for the development of novel mitigations strategies, such as live attenuated vaccines against *P. salmonis*.

Long-term serial culture of *Piscirickettsia salmonis* leads to a genomic and transcriptomic reorganization affecting bacterial *in vitro* virulence

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Abstract

The propagation of bacteria in artificial mediums implies and adaptation to this non-natural environment. If these conditions are persistent enough time, a permanent adaptation to this new environment can be expected. In this study we explore the genomic and transcriptomic rearrangement that the salmonid pathogen *Piscirickettsia salmoni*s undergo after a serial propagation for 200 passages (~2 years) in a cell-free culture medium. One of the most remarkable genomic changes that *P. salmonis* undergo during the culture period was the migration of a 35 Kb segment form the original (P0) genome to a P200 plasmid. Interestingly, the genomic content of this block revealed the presence of the Dot/Icm secretion system, which has been previously associated to *P. salmonis* pathogenesis. On the other hand, a reduced transcriptomic response was evidence in *P. salmonis* after 200 passages affecting the expression of different pathways including the Iron acquisition and metabolism. Further *in vitro* infections revealed that after 200 passages *P. salmonis* is less capable of generate cytopathic effects than the original P0 form. Overall, our results evidence that the continuous propagation of *P. salmonis* lead to genomic and transcriptomic rearrangement that impact on bacterial *in vitro* pathogenesis. These results open new perspective about the adaptation of *P. salmonis* to artificial cultures providing useful information for the development of novel mitigations strategies, such as live attenuated vaccines against *P. salmonis*.

Keywords: Piscirickettsia salmonis; RNA-seq; Whole genome sequencing; attenuation; long-term culture; serial passages.

1. Introduction

Bacteria are highly dynamic organisms that can survive and adapt to a wide variety of environments. In laboratory conditions, these microorganisms are routinely propagated in different culture settings. If these conditions are persistent enough time, bacteria will eventually become adapted (domesticated) to this non-natural environment (Eydallin et al., 2014;Kram et al., 2017). Together with their easy handling and short generation times, bacteria have been used as study models to address key aspects about adaptation and evolution in response to challenging environments. Medium adaptation has been mainly explored in *Escherichia coli*, where this bacterium has been grown through serial passages in different stressful condition, including nutrient, acid or antibiotic stress (Herring et al., 2006;Conrad et al., 2009;Toprak et al., 2012;Barrick and Lenski, 2013;Wiser et al., 2013;Spagnolo et al., 2016). These studies evidenced that *E. coli* undergo different genotypic and phenotypic modifications that provides them a comparative advantage to survive or fully explode the conditions where they are maintained. For instance, through whole genome sequencing, 13 *de novo* mutations were identified in *E. coli* grown in a glycerol-based growth medium for a 44 days period (Herring et al., 2006). Here, authors demonstrate that these spontaneous modifications conferred the mutants and improved fitness when compared with wild type population. Even during a short period of adaptation (2-3 days) *E. coli* can display phenotypic changes as a domestication response (Eydallin et al., 2014). However, every adaptation to a new environment comes with a cost.

For pathogenic agents it has been reported that medium adaptation through serial passages improves the performance in new culture conditions but comes with the development of a decreased virulence. This has been extensively studied in virus, where serial passages has been used as a classical approach for the development of live attenuated vaccines against

viral infections (Hanley, 2011). For instance, vaccines against measles virus, yellow fever and varicella virus among others, have been developed through the serial passage of the virus in a non-natural host (Hanley, 2011). A reduced virulence after media adaptation has also been reported in bacteria. A reduction in the pathogenesis of *Staphylococcus aureus* after a 6 week *in vitro* serial passage has been reported, probably associated with point mutations occurred in the coding region of the *agrC* gene (Somerville et al., 2002). Other classical examples of pathogenic attenuation through serial passages includes the development of live attenuated vaccines against *Salmonella typhi* and *Mycobacterium bovis* infections (Germanier et al., 1975;Toida, 2000). However, most of the time these adaptations are focused into the occurrence of random point mutations that will modify a restricted number of proteins, conferring mutants a comparative advantage when compared with the wild type populations. Due to this, we explore the landscape of genomic and transcriptomic adaptation that *Piscirickettsia salmoni*s undergo after a long-term medium adaptation experiment.

Piscirickettsia salmonis is defined as a Gram-negative, non-motile and coccoid bacteria responsible for the salmonid rickettsial septicemia (SRS), a systemic disease associated with high mortalities in salmon aquaculture (Rozas and Enriquez, 2014). Besides being one of the main treats for the sustainable development of aquaculture in Chile, *P. salmonis* has gain scientific attention due to its ability to infest and replicate within host immune cells (Rojas et al., 2010;Ramirez et al., 2015), becoming an interesting model for the understanding of immune evasion strategies during host-pathogen interactions (Valenzuela-Miranda and Gallardo-Escarate, 2016;Valenzuela-Miranda et al., 2017;Valenzuela-Miranda and Gallardo-Escarate, 2018). For many years, *P. salmonis* was considered as an obligate intracellular bacterium, however, the development of cell-free culture media changed the paradigm about the nutritional requirements for this pathogen (Yanez et al., 2012;Henriquez et al., 2013). In

this scenario, the aim of this study was to evaluate the genomic and transcriptomic changes that *P. salmonis* undergo after a serial propagation for 200 passages (~2 years) in a cell-free culture medium. Our results evidenced a genomic rearrangement and a bounded transcriptomic response in media adapted bacteria (P200), affecting some relevant pathogenic pathways, such as the Dot/Icm secretion system and mechanisms involved in iron acquisition. We suggest that these modifications can be responsible for the *in vitro* pathogenic attenuation exhibit by the P200 bacteria.

2. Materials and methods

2.1 Serial propagation of P. salmonis in cell free medium

Eugon broth culture was selected as a cell free medium for the serial propagation of *P. salmonis*. This medium was prepared by dissolving 30,4 g of Eugon broth (Bacto™) in 1 liter of autoclaved (121ºC for 20 minutes) distillated water. The dissolved Eugon was autoclaved again and let cool to room temperature. At this point, the media was supplemented with 1% (m/v) of Casamino acids (Bacto™) and with $FeCl_3$ (MERCK) at final concentration of 2 mM. Both Casamino acids and $FeCl_3$ were filtered through a 0,22um membrane and aseptically added to supplement the media.

A previously field isolated *P. salmonis* (EM-90 strain) was used in this experiment (Valenzuela-Miranda and Gallardo-Escarate, 2018). The isolated strain was first propagated in salmon cell lines. For this, CHSE-214 cells were culture in T75 flasks at 20 ºC with 20 ml of DMEM (HyClone) supplemented with 10% of heat inactivated fetal bovine serum (Biological Industries) and 1% of non-essential amino acids (HyClone). When cell cultures reached 70% confluency, the flasks were inoculated with 10^6 *P. salmonis*. The cultures were incubated at 20ºC until the appearance of cytopathic effects (CE) produced by *P. salmonis*

120 infection in around 80% of the CHSE-214 cells. At this point the culture was disrupted with
121 a cell scraper and the supernatant was collected and centrifugated to pellet CHSE-214 cell
122 debris. The collected supernatant containing *P. salmonis* was considered as P0 passage (Fig.
123 1). 1 ml of P0 bacteria was used to inoculate a 15 mL falcon containing 2 ml of Eugon broth
124 culture. The cultures were maintained at 20º C with a constant shaking of 100 rpm. When
125 bacteria culture reached the exponential phase (A_{600} 0,3-0,4) 300 µl were transferred to a new
126 15 ml falcon containing fresh 2 ml of Eugon broth culture (P1). This was considered as one
127 passage and the process was repeated 200 times (until P200) every time that the culture
128 reached the exponential phase (Fig.1). Given the slow growth rates of *P. salmonis* the
129 exponential phase was achived every 3 to 4 days, thus it was necessary to maintain a
130 continuous culture of the bacteria for around 2 years. Samples for DNA and RNA isolation
131 were collected at P0, P1 and P200.

132 *2.2 Whole genome sequencing*

133 Whole genome sequencing was performed in order to compare the genome dynamics
134 between the original bacteria (P0) and after 200 passage (P200) in Eugon broth culture. For
135 this, whole DNA were isolated form P0 and P200 cultures using the DNeasy Blood & Tissue
136 Kits (Qiagen) according to manufactures instructions. Integrity of the isolated DNA was
137 confirmed through a 1% agarose gel and purity was evaluated through the 260/280 and
138 260/230 absorbance ratio estimated through NanoDrop 1000 Spectrophotometer (Thermo
139 Scientific). DNA samples with no smear and absorbance ratios above 1.8 were send
140 to Macrogen Inc. (Korea) for whole genome PacBio Single Molecule Real Time (SMRT)
141 sequencing. After quality control and trimming, long reads were *de novo* assembled using
142 the Hierarchical Genome Assembly Process (HGAP3) workflow with default options. The
143 genomes and plasmids obtained for both P0 and P200 were then annotated with RAST server

144 (http://rast.nmpdr.org/rast.cgi) (Overbeek et al., 2014) using as reference the *P. salmonis*
145 genomes (NCBI taxonomy ID: 1238) and default parameters. In order to identify genomic
146 rearrangement, synteny blocks were detected between P0 and P200 genomes and plasmids.
147 For this purpose, the Synteny Block ExpLoration tool (Sibelia)
148 (http://bioinf.spbau.ru/sibelia) was used to identify shared regions between both genomes.
149 The results were then visualized using the Circos software version 0.69-6 (http://circos.ca/)
150 (Krzywinski et al., 2009).

151 *2.3 Transcriptome sequencing*

152 RNA-seq analysis were conducted in order to identify differentially expressed genes among
153 P0, P1 and P200 bacteria. For this purpose, total RNA was isolated from the three passages
154 obtained as described above using RiboPureTM Kit (Ambion) and treated with DNAse I,
155 RNAse-free (Thermofisher) according to manufacture instruction. The quality and purity of
156 the isolated RNA was confirmed using the 2200 TapeStation (Agilent Technologies, USA)
157 using R6K screen tape and through NanoDrop 1000 Spectrophotometer (Thermo Scientific).
158 Samples with RNA Integrity Values (RIN) above 8, with 260/280 and 260/230 absorbance
159 ratios above 1,8 were shipped to Macrogen Inc. (Korea) for library preparation and Hi-seq
160 illumina sequencing. Three biological replicates were considered for sequencing in each
161 group. Bioinformatic analyses were conducted using CLC genomics Workbench software
162 (V10, CLC Bio, Denmark). Raw reads were trimmed and filter by quality and adapters were
163 removed. Filtered reads were used for RNA-seq analysis using as reference the previously
164 annotated genomes for P0 and P200 respectively. Considerer parameters for RNA-seq
165 including minimum read length fraction and a minimum read similarity fraction of 0.8,
166 unspecific read match limit of 10 and expression values were calculated as transcripts per
167 million reads (TPM). Differential expression analysis was conducted by comparing TPM

means between all replicates for each pair of groups and an ANOVA test was used to identify statistically significant differences between all groups. P-values were FDR corrected and genes with an absolute fold change above 4 and with FDR corrected p-value above 0,05 were considered as differentially expressed transcripts.

2.4 in vitro attenuation

An *in vitro* experiment was performed in order to test the potential impact of a serial passage culture of *P. salmonis* in a cell-free medium. For this the salmon head kidney cells (SHK-1) were grown in T75 flasks at 20 ºC with 20 ml of Leibovitz L-15 medium (HyClone) supplemented with 10% of heat inactivated fetal bovine serum (Biological Industries) and 1% of non-essential amino acids (HyClone). When cell cultures reached 70% confluency, the flasks were inoculated with 10^6 *P. salmonis* of either P0 or P200 bacteria. The flasks were incubated during 14 days at 20 ºC and cytopathic effects were registered after this period.

3. Results

Medium adaptation is defined as the process were bacteria might undergo genotypic and phenotypic changes that provides them a comparative advantage to fully explode culture conditions. The aim of this study was first to describe genomic changes that the salmonid pathogen *Piscirickettsia salmonis* undergo during 200 serial passage experiment. After whole genome sequencing of P0 and P200, a full-length chromosome and each one of the 4 natural plasmids commonly associated with *P. salmonis* were identified (Table 1). In general, a slight diminution on both the genome size and coding sequences were evidenced in P200 genetic material regarding the original P0 bacteria. The P200 genome size was estimated as 24965 bp smaller than the P0 genome and with 93 less coding sequences than the original bacteria (Table 1).

192 Through synteny analysis it was possible to identify all the different conservation blocks
193 composing *P. salmonis* genome. Interestingly, when the original (P0) and the medium
194 adapted (P200) bacterial genomes are compared, an exchange in the position between the
195 third and fifth synteny block was evidenced (Fig 2A), while the remaining blocks maintained
196 their position on both genomes. The genetic content of both blocks revealed that most of the
197 annotated genes within these regions corresponded to mobile genetic elements or
198 transposases, following by flagellar related proteins, metabolic genes and secretion system
199 proteins (Fig. 2B). RNA-seq analysis was perform in order to identify differentiall expression
200 patterns among these genes. Results evidence that just 57 of these genes were differentially
201 regulated and that the major differences were found against P200 bacteria (Fig. 2C). Mostly
202 respiration and metabolic genes were found in these genes, including *Cytochrome d ubiquinol*
203 *oxidase*, *Fatty acid desaturase*, *Lactoylglutathione lyase* and *ADP-ribose pyrophosphatase*
204 among others (Table S1).
205 A second comparative genome analysis was performed including the 4 natural plasmids of
206 *P. salmonis*. The results evidenced that translocation of a DNA segment from the genome to
207 one of the plasmids of *P. salmonis* after the two years of continuous culture of the bacteria
208 in the cell free medium (Fig. 3A). The genomic content of this region evidenced that most of
209 the genes associated with this region where related with the Dot/Icm secretory system,
210 phosphate metabolism, respiration and transposases (Fig. 3B). Although multiple genes
211 associated with the secretion system of *P. salmonis* were found (Fig. 3C) just two genes were
212 differentially expressed in the translocated segment from the genome to the plasmid when
213 comparing the different passages of the bacteria.
214 Global transcription patterns were also studied in order to understand the transcriptomic
215 reorganization behind media adaptation in *P. salmonis*. In general, reduced transcriptomic

activity was found in media adapted bacteria regarding P0 and P1 (Fig. 4A). Among them, a total of 499 genes were differentially expressed in at least one of the tested conditions (P0, P1 and P200). In general, most of these changes occurred in P200 bacteria. Thus, a total 378 genes were differentially regulated in P200 compared with P0 and 231 when the comparison is made against P1 bacteria (Fig. 4B). The distribution between up and down regulated genes evidenced that most of this differential expression is explained due to a down-regulation in gene expression in media adapted bacteria (P200), where 274 and 182 genes were down regulated in P200 regarding P0 and P1 respectively (Fig. 5). The differentially expressed genes for each comparison were further classified by functions (subsystem) using RAST annotations. Here, different pathways were regulated in response to media adaptation, including respiration and metabolism of different molecules including DNA, Lipids and carbohydrates. When considering the percentage of differentially expressed genes regarding to total amount of genes for the pathway (enrichment), iron acquisition and metabolism was the most regulated pathways (Fig. 5). Due to the importance of iron in bacterial pathogenesis, we further investigate this process. Thus, different genes associated with the iron siderophore vibrioferrin were identified and differentially regulated during the experiment (fig. 6A) including *PvuA*, *PvsA*, *PvsB*, *PvsC*, *PvsD*, *PvsE* and *TonB*. When analyzing the individual expression patterns for each one of these genes, the results shown a marked and gradual decrease in the transcription of these genes. Due to the reorganization and regulation of pathogenic related genes in media adapted regarding the original bacteria, we further explored the capacity of both passages to produce cytopathic effects and lysis in salmon cell lines (Fig. 7). The results evidenced a clear difference in the lysis of SHK-1 cells as a result of *P. salmonis* infection. While a 70% of lytic effects was estimated in cells infected with P0 bacteria, no major lysis was evidenced in groups infected with the media adapted bacteria.

4. Discussion

The continuous culture of bacteria in artificial medium can lead to genotypic and phenotypic changes that will allow the microorganism to fully explode the available nutrients in the media. If these conditions are persistent enough time, some of these changes can become permanent (Eydallin et al., 2014;Kram et al., 2017). For intracellular pathogens, it has been well stablished that serial propagation in a cell free media can lead to a decreased pathogenesis. However, most of the time this studies relay on the identification of point mutations as a result of media adaptation. Here, the aim of this study was to evaluate the genomic and transcriptomics rearrangements that salmonid pathogen *Piscirickettsia salmonis* undergo after a serial propagation experiment after 200 passages in a cell free culture media. Our whole genome comparative analysis revealed a genome size and genetic content reduction after serial passage. Its known that bacterial genomes are highly dynamic both in size and composition (Puigbo et al., 2014). Here, genetic loss and genome reduction has been previously reported as a result of experimental evolution both in extracellular and intracellular pathogens (Dufresne et al., 2005;Nilsson et al., 2005;Koskiniemi et al., 2012). The serial culture of *E. coli* in a nutrient rich environment have demonstrated to drive a genetic loss and the development of metabolic dependency on the media supplemented metabolites (D'Souza and Kost, 2016). This phenomenon has been explained from the cellular energetic efficiency perspective, where loosing no longer required genes might imply an increased cellular fitness and saving in replication and production costs (Zamenhof and Eichhorn, 1967;Dufresne et al., 2005;D'Souza et al., 2014;D'Souza and Kost, 2016).

Together with the genome size reduction, a genomic reassortment was also evidenced after the serial culture of *P. salmonis*. These modifications included the translocation of two

genomic blocks and the migration of a segment of genomic DNA to the plasmid in medium adapted bacteria. The genetic content of these segments revealed the presence of genetic mobile elements, transposases, flagellar related proteins and secretion system related genes among others. Mobile genetic elements (MGEs) promote gene exchange and reassortment (Craig et al., 2002) and *Rickettsia* genomes are known to be composed of a large amount of these elements (Gillespie et al., 2012). More than 40 MGEs have been described to be expressed in *P. salmonis* transcriptome (Machuca and Martinez, 2016) and some MGEs like ISPsa2 genes have been well characterized in *P. salmonis* genome (Marshall et al., 2011). Although these evidences suggested that *P. salmonis* genome was a highly dynamic and fluid entity, this is the first report confirming that these rearrangements are actually occurring in *P. salmonis* genome and what is the extent of these changes. Further investigation must be destined to evaluate how frequent these changes occur and what is the actual role MGEs in this reassortment.

It has been evidenced that genome reorganizations can directly impact in the function, evolution and regulation of gene expression in bacterial models (Ptacin and Shapiro, 2013;Hendrickson et al., 2018;Krogh et al., 2018). Although DNA translocation between genome and plasmids is a well-known phenomenon (Heffron et al., 1975;Russell and Dahlquist, 1989;Frost et al., 2005;Wozniak and Waldor, 2010), most of the time are studied under the scope of antibiotic resistance, however the role of this recombination and the impact in pathogenesis process remains largely unknown. Flagellar related proteins and secretion system related genes were also found in the translocated DNA. *P. salmonis* is defined as a non-motile bacterium and no structural flagellum has been reported for the bacteria (Fryer and Hedrick, 2003). However, the presence of flagellar related proteins has been suggested as a possible immunomodulator of host response to promote *P. salmonis*

288 macrophage infection (Carril et al., 2017). On the other hand, the secretion system is a well-
289 known and conserved mechanism of secretion of virulence factors among pathogenic
290 bacteria (Fronzes et al., 2009). Particularly in *P. salmonis*, a functional Dot/Icm type IV-B
291 secretion system has been reported (Gomez et al., 2013) and recent evidence suggest that the
292 mutagenesis of this system in *P. salmonis* leads to a pathogenic attenuation of the bacteria
293 (Mancilla et al., 2018). Our results evidenced a change in the loci of both pathogenic related
294 genes, probably affecting the genomic architecture in media adapted *P. salmonis*. Although
295 no major differences in gene expression were evidenced in these genes during broth culture
296 for P0 and P200 bacterium, it is unknown how different becomes this scenario during the
297 infective process of *P. salmonis* in host cells.
298 Global gene expression patterns between different passages of *P. salmonis* (P0, P1 and P200)
299 evidence a smaller transcriptomic repertory in media adapted bacteria when compared with
300 the first passages in the cell free media. We hypothesize that in the absence of a host to infect
301 and struggle for nutrient availability for more than 200 generation, *P. salmonis* displays a
302 reduced and cost-effective transcriptomic response that allows the bacteria to explode the
303 nutrient availability in the media. We have previously shown that nutritional immunity plays
304 a pivotal role in *P. salmonis*-Salmon interaction, where host and pathogen can compete for
305 the amino acid and iron availability (Valenzuela-Miranda and Gallardo-Escarate,
306 2016;Valenzuela-Miranda et al., 2017;Valenzuela-Miranda and Gallardo-Escarate, 2018).
307 This reduced transcriptomic response involved the negative regulation of iron homeostatic
308 related genes, including the group of vibrioferrin. Vibrioferrin (VF) is a class of carboxylate
309 marine siderophore involved in iron transport (Amin et al., 2009). Siderophores have been
310 characterized as key elements for pathogenic bacterium in order to compete for iron
311 acquisition from their hosts (Miethke and Marahiel, 2007;Wilson et al., 2016). Previously, it

312 has been reported that *P. salmonis* produces siderophore-like molecules and encodes
313 vibrioferrin related genes (Machuca and Martinez, 2016;Calquin et al., 2018). It is likely to
314 argue that with no host to infest and no competition for the availability of iron, vibrioferrin
315 is no longer required to compete for iron uptake, therefore explaining the transcriptomic
316 downregulation of these genes.
317 Due to all the important changes observed in media adapted bacteria regarding the
318 reorganization and transcriptomic regulation of pathogenic genes, we further compare the
319 virulence of P0 and P200 *P. salmonis* in the salmon head kidney cell lines SHK-1. Although
320 exploratory, our experiment showed that cells infected with the media adapted bacteria
321 evidenced less cytopathic effects and lysis than cells infected with the original P0 bacteria.
322 These results agree with the extinguish evidence suggesting that multiple passages
323 pathogenic bacteria leads to an attenuated virulence (Cabral et al., 2017). In fact, until the
324 development of effective bacterial genome editing, the serial passage of pathogenic bacteria
325 was the gold standard method for the development of live attenuated vaccines (Hanley,
326 2011). Considering that almost all developed vaccines to control *P. salmonis* infection rely
327 on inactivated bacterium (bacterin) (Maisey et al., 2017) and that no mayor advances have
328 been made to control the pathogen, it is necessary to further investigate how media adapted
329 *P. salmonis* can be useful for the generation of an effective live attenuated vaccine against
330 this pathogen.
331
332 **5. Conclusions**
333 Our results evidenced that the serial propagation of *P. salmonis* in a cell free broth culture
334 leads to a genomic and transcriptomic reorganization affecting the organization and
335 regulation of pathogenic related genes, including the Dot/Icm secretion system and iron

homeostasis pathways among others. Our results also suggest that these modifications are responsible for the reduced virulence evidenced by media adapted bacteria. We expect that these results will help to the development of novel live attenuated vaccines against for the control of pathogens affecting salmon aquaculture and to improve our understanding of how the pathogenic attenuation is developed in *P. salmonis*.

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7. Authors contribution

The study was conceived by DV and CG. DV performed the experiments and analyzed the data under CG extensive supervision. Cell infection experiment and qPCR were conducted by CC. DV and CG drafted the manuscript.

8. Conflict of interest

The authors declare that they have no competing financial interests

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Figure List

Figure 1. Experimental design for the serial passages of *Piscirickettsia salmonis*. CHSE-214 cells were infected with 10^6 P. salmonis (P0). When 90% of lysis was achieved, bacterial cells were collected and growth in the cell free Eugon medium. When the culture reached the exponential phase at $A_{600} > 0.3$ (P1), 300 µl of that culture where transferred to 2.7 ml of fresh medium. The process was repeated 200 times (P200) for a total period of broth culture of around 2 years

Figure 2. A) Circos image comparing the organization of P. salmonis P0 and P200 genome blocks. Blocks with different position between the two passages are highlighted. **B)** Genomic content of the translocated blocks. Genes were classified according to the predicted function. **C)** Venn diagram showing the differentially expressed genes (Absolute fold change above 4 and p value < 0.05) in the translocated blocks among sequenced passages (P0, P1, P200)

Figure 3. A) Circos image highlighting a block of DNA that was originally located in the genome of P0 but that after 200 passages was found in one of the 4 natural plasmids of P. salmonis. **B)** Genomic content of the translocated region. Genes were classified according to the predicted function. **C)** List of the different genes belonging to the Dot/Icm secretion system identified within this region.

Figure 4. A) Global transcriptomic patterns during different passages of *P. salmonis* in broth culture. **B)** Venn diagram showing the differentially expressed genes (Absolute fold change above 4 and p value < 0.05) among sequenced passages (P0, P1, P200). **C)** Distribution between differentially up and down regulated genes for each one of group comparisons. Red bars represent transcripts with and absolute fold change value between 4 to 10 and yellow bars represents absolute fold change values above 10.

Figure 5. Functional classification of the differentially regulated transcripts (Absolute fold change above 4 and p value < 0.05) between P1vsP0 **(A)** P200vsP1 **(B)** and P200vsP0 **(C)**. Red bars represent up regulated genes while the blue bars represent down regulated genes. Each functional classification is sorted by the percentage of differentially regulated genes regarding the total number of genes annotated for the pathway (Enrichment).

Figure 6. A) Vibrioferrin related genes identified in *P. salmonis* genomes. **B)** Individual gene expression of each vibrioferrin related gene differentially expressed among the sequenced passages (P0, P1 and P200)

Figure 7. In vitro infection of SHK-1 cells line with the media adapted (P200) bacteria (A) and the original (P0) P. salmonis (B). Both images were capture at 40x magnification.

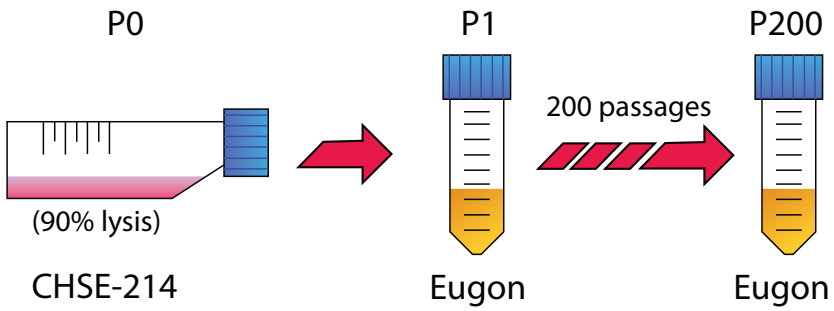


Figure 1

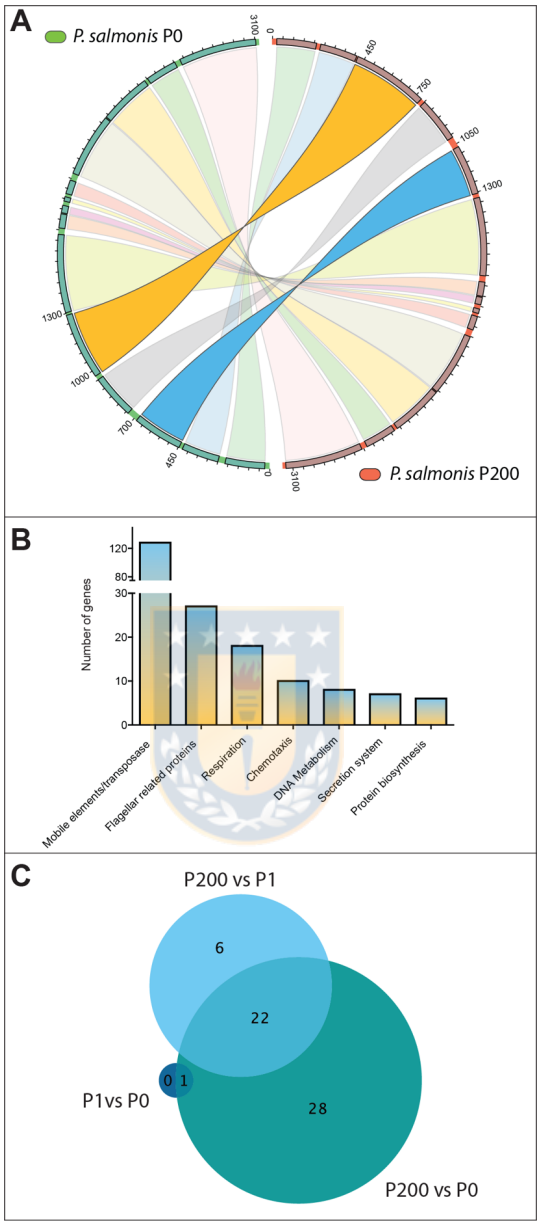

A
P. salmonis P0
P. salmonis P200
3100
0
450
750
1050
1300
1300
1000
700
450
0
3100
B
Number of genes
120
80
30
20
10
0
Mobile elements/transposase
Flagellar related proteins
Respiration
Chemotaxis
DNA Metabolism
Secretion system
Protein biosynthesis
C
P200 vs P1
6
22
0 1
P1vs P0
28
P200 vs P0

Figure 2.

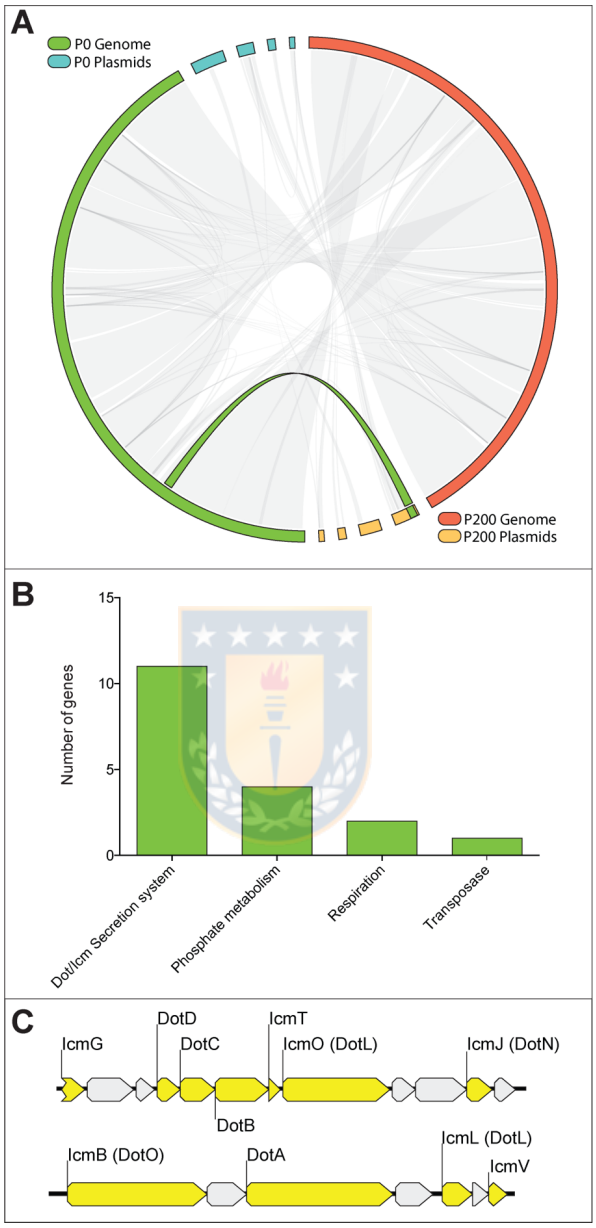

A
P0 Genome
P0 Plasmids
P200 Genome
P200 Plasmids
B
Number of genes
15
10
5
0
Dot/Icm Secretion system
Phosphate metabolism
Respiration
Transposase
C
IcmG
DotD
DotC
IcmT
IcmO (DotL)
IcmJ (DotN)
DotB
IcmB (DotO)
DotA
IcmL (DotL)
IcmV

Figure 3.

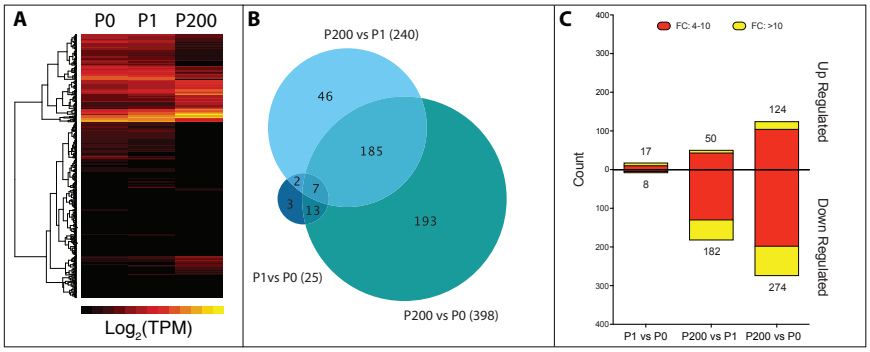
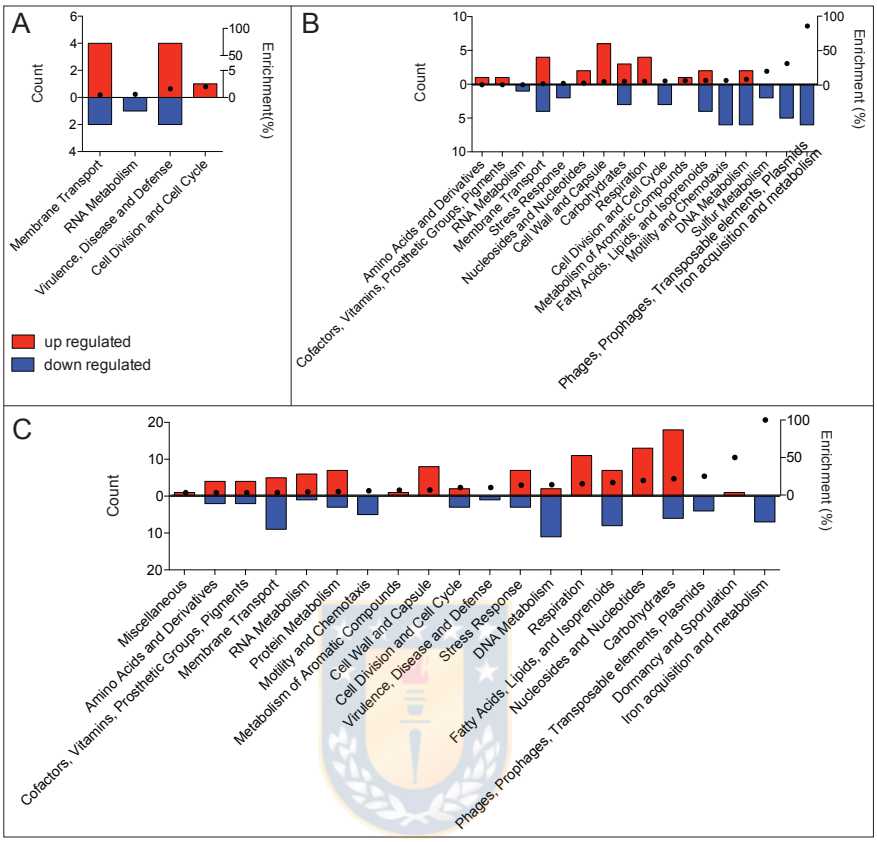

A
P0
P1
P200
Log2(TPM)
B
P200 vs P1 (240)
46
185
2
7
3
13
193
P1vs P0 (25)
P200 vs P0 (398)
C
FC: 4-10
FC: >10
Count
Up Regulated
Down Regulated
17
8
50
182
124
274
P1 vs P0
P200 vs P1
P200 vs P0

Figure 4.



563
564 **Figure 5.**

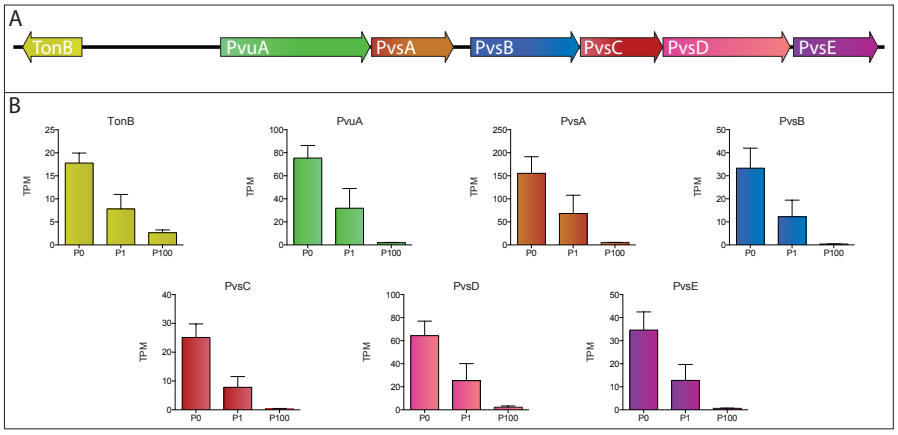

A
TonB
PvuA
PvsA
PvsB
PvsC
PvsD
PvsE
B
TonB
PvuA
PvsA
PvsB
PvsC
PvsD
PvsE
TPM
P0
P1
P100

Figure 6.

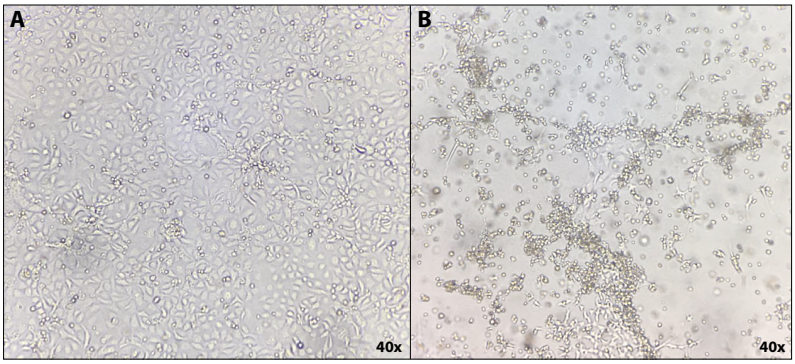


Figure 7.

6. DISCUSIÓN

7.1 Respuesta transcriptómica codificante del salmón durante la infección con *P. salmonis*

La anotación de los genes diferencialmente expresados por parte del salmón Atlántico durante la infección por *P. salmonis* reveló los diferentes procesos biológicos regulados durante la infección. En el cerebro, los procesos regulados se asociaron principalmente con las transmisiones de impulsos nerviosos, la sinapsis glutamatérgica y el ciclo de vesículas sinápticas. Evidencia previa en mamíferos sugiere que infecciones producidas por *Streptococcus pneumoniae* alterar la regulación del glutamato, lo que lleva a un daño sináptico en el cerebro (Wippel et al., 2013). Por otra parte, *S. salar* infectados con otro organismo de tipo rickettsial (RLOs) evidenciaron signos de meningitis granulomatosa e hipercelularidad mononuclear (Olsen et al., 1997). Uno de los síntomas clásicos asociados a un brote de SRS se asocia al nado errático de los salmones infectados (Rozas and Enriquez, 2014). En este sentido, la sobreregulación de genes vinculados a la sinapsis neuronal podría alterar el correcto funcionamiento cerebral, lo que podría explicar la sintomatología observada.

Entre los tejidos analizados, una respuesta común asociada con los procesos de oxidación-reducción, endocitosis y homeostasis de iones fue evidenciada en cerebro, el bazo y riñón cefálico. Entre estos, los procesos de oxidación-reducción son componentes importantes conocidos de la respuesta inmune innata del salmón del Atlántico contra *P. salmonis* (Tacchi et al., 2011). Por otra parte, la endocitosis ha sido descrito como el mecanismo de entrada para diversas bacterias intracelulares (Gruenberg and van der Goot, 2006). Uno de los signos celulares más comunes de la infección por *P. salmonis* es la presencia de vacuolas citoplasmáticas en las que las bacterias se desarrolla y se replican (Fryer et al., 1990;McCarthy et al., 2008b;Rojas et al., 2009), lo que ha sugerido que la bacteria emplea este mecanismo para lograr invadir el entorno intracelular. Nuestros resultados evidencian que durante la infección con *P. salmonis* induce la expresión de distintos factores promotores de endocitosis, tales como el ARF6. Este tipo de respuesta ha sido reportado previamente para otros patógenos intracelulares como *Chlamydia* (Balana et al., 2005). Aquí, los autores plantean que la infección con esta bacteria promueve la activación de ARF6 endógena, facilitando así la endocitosis bacteriana a través de la remodelación de actina (Balana et al., 2005). Esta regulación del

citoesqueleto también se puede observar durante infecciones con *P. salmonis*, donde la interacción con actina endógena y clatrina se ha sugerido como un proceso clave para la internalización bacteriana (Ramirez et al., 2015). Curiosamente, nuestros resultados mostraron que clatrina aumentaba su expresión génica significativamente en individuos infectados, reafirmando la importancia de la endocitosis durante el proceso infectivo. Sin embargo, nuestros resultados también sugieren una regulación negativa de la expresión de distintos receptores y proteínas adaptadoras que darán paso a la maduración de endosomas y su posterior lisis. La represión de la expresión génica de distintos de receptores de salmón durante la infección con la bacteria ha sido reportada previamente (Rise et al., 2004;Tacchi et al., 2011). Debido a esto se sugiere que *P. salmonis* puede poseer mecanismos moleculares que incidan en la expresión de genes de su hospedador, los que podrían evitar la maduración de endosomas a fin de evadir la respuesta inmune del hospedador. Sin embargo, futuras investigaciones deben ser destinadas para esclarecer la existencia de estos mecanismos y su funcionamiento.

Otra respuesta transcriptómica común entre los tejidos analizados estuvo relacionada a la homeostasis de iones, particularmente del hierro, elemento vital para casi todos los organismos vivos, especialmente las bacterias (Wooldridge and Williams, 1993;Krewulak and Vogel, 2008;Skaar, 2010). En consecuencia, una de las primeras líneas de defensa del hospedador contra patógenos corresponde al secuestro de hierro, o la limitación de su biodisponibilidad (Skaar, 2010;Cherayil, 2011). Esta estrategia es conocida como inmunidad nutricional y puede ser la clave entre familias de salmones resistentes y susceptibles a *P. salmonis* (Pulgar et al., 2015). Nuestros resultados mostraron un aumento significativo en la expresión de genes asociados con la acumulación intracelular de hierro, tales como la haptoglobina, la hepcidina-1, el receptor de transferrina y ferritina. Aunque la haptoglobina no participa directamente en la captación de hierro, se ha demostrado que en peces óseos es capaz de unirse a hemoglobina libre (Wicher and Fries, 2006), lo que evita que las bacterias utilicen esta molécula para la obtención de hierro (Hood and Skaar, 2012b). A su vez, aunque la hepcidina ha sido ampliamente estudiada como un péptido antimicrobiano en peces óseos (Douglas et al., 2003;Shike et al., 2004;Chen et al., 2007;Wang et al., 2009a;Alvarez et al., 2013a;Alvarez et al., 2014), se ha reportado que sobrecargas experimentales de hierro experimentales pueden inducir la expresión de hepcidina (Rodrigues et al., 2006) (Wang and Wang, 2016) (Alvarez et al., 2013b). Estos

antecedentes sugieren una participación en la homeostasis de hierro similar al observado en mamíferos. En contraparte, genes asociados a la movilización de hierro hacia el espacio extracelular tales como el feline leukemia virus receptor y ABCg2 redujeron significativamente su expresión. Estos resultados sugieren una tendencia a la acumulación de hierro a nivel intracelular durante la infección, lo que podría favorecer el desarrollo de patógenos intracelulares como *P. salmonis*.

7.2 lncRNAs y su rol emergente en la regulación de procesos biológicos

Debido al rol emergente de los lncRNAs en la regulación de diversos procesos biológicos (Rinn and Chang, 2012), el presente trabajo buscó la caracterización de lncRNAs en salmón y su regulación durante la infección con *P. salmonis*. Aunque estos transcritos han sido estudiados mayormente en mamíferos (Ponting et al., 2009;Chang, 2013), existen algunos trabajos reportando su caracterización en peces (Kaushik et al., 2013;Al-Tobasei et al., 2016;Boltaña et al., 2016;Wang et al., 2016). Dentro de estos reportes, se ha planteado que los lncRNAs podrían tener un rol durante la respuesta inmune del salmón Atlántico frente a una infección con el virus de la anemia infecciosa del salmón (ISAv) (Boltaña et al., 2016). Al comparar los lncRNAs caracterizados durante la respuesta a ISAv y los identificados en este trabajo, se evidenció una divergencia aproximada del 50%. Esto podría ser indicativo de que una parte importante de la respuesta transcriptómica basada en lncRNAs puede ser patógeno especifica, tal como lo observado previamente para miRNAs (Zhang and Li, 2013).

Al igual que los microRNAs, la modulación de lncRNAs puede impactar la expresión de genes codificantes de proteínas. Mientras que los miRNAs se asocian principalmente con el silenciamiento génico post-transcripcional, los lncRNAs poseen un espectro más amplio de regulación, incluso llegando a tener incidencia a nivel epigenético (Mercer et al., 2009). Dado que los lncRNAs pueden actuar como reguladores *cis* de expresión génica (Cabili et al., 2011). Enfoques de co-localización y co-expresión han sido utilizados para la predicción de la interacción lncRNA-gen. Dada la importancia previamente señalada de genes clatrina, hepcidina y haptoglobina durante la respuesta del salmón, se buscaron lncRNAs que podrían estar vinculados con la regulación de estos genes. A nivel del genoma, se encontraron distintos lncRNAs adyacentes a estos transcritos, dentro de los cuales lncRNAs particulares para cada

gen mantuvieron una correlación significativa en sus niveles de expresión. Así, una correlación negativa fue encontrada entre clatrina y Ss_lncRNA_2892, mientras que para hepcidina existió una correlación positiva con el lncRNA adyacente SS_lncRNA_3122 en todos los tejidos analizados. A diferencia de los miRNAs que son asociados principalmente al silenciamiento génico (Fabian and Sonenberg, 2012), los mecanismos de regulación de expresión mediados por lncRNAs son más diversos (Nie et al., 2012) y por ende, constituyen un mecanismo de regulación que puede promover o reprimir la expresión génica (Nie et al., 2012). En efecto, se ha determinado incluso que un solo lncRNA es capaz de mediar tanto la activación como represión de un proceso biológico (Carpenter et al., 2013). Si bien estos resultados dan cuenta de posibles lncRNAs asociados a la regulación de genes claves durante el desarrollo de la enfermedad, es necesario tener presente que una correlación no implica necesariamente causalidad. Sin embargo, nuestros resultados presentan evidencia promisoria sobre la posible participación de lncRNAs en la regulación de procesos biológicos, particularmente durante la respuesta de salmones frente a infecciones bacterianas.

7.3 Rol de los miRNAs durante la infección con *P. salmonis*

Otro tipo de RNA no-codificante con un conocido rol en la regulación post-transcripcional corresponde a los miRNAs. Sin embargo, a pesar de su importancia, poco se conoce sobre su rol en la regulación de la respuesta inmune de salmónidos. Utilizando la ultima versión de miRBase, se logró la caracterización de 595 miRNAs desde los transcriptomas provenientes de hígado y riñón de salmón. Basado en criterios estructurales y de secuencia (Ding et al., 2011;Kozomara and Griffiths-Jones, 2014b), estos miRNAs fueron clasificaron en diferentes familias. Acorde a lo descrito previamente en teleósteos (Gong et al., 2015; Sha et al., 2014; Wang et al., 2016; Xu et al., 2016; Yuhong et al., 2016; Zhao et al., 2016, la infección bacteriana en *S. salar* promovió un cambio en la diversidad de familias de miRNAs en el transcriptoma de los peces infectados. Las familias más diversas en el grupo control incluyo la mir-181, mir-143 y mir-21, mientras que, en el grupo desafiado las familias mir-21, mir-181 y mir-30 fueron las más abundantes. Estos últimos grupos de familias han sido asociados previamente a la regulación de la respuesta inmune en diferentes especies de teleósteos (Andreassen and Hoyheim, 2017). Entre ellos, miRNA-181 se ha propuesto como un miRNA conservado

evolutivamente entre especies con roles en la respuesta inmune de los teleósteos y vertebrados superiores (Zhou et al., 2011;Forster et al., 2015).

Los análisis de expresión diferencial para estos miRNAs revelaron 84 y 25 miRNAs diferencialmente expresados en riñón y bazo respectivamente. Si bien algunos miRNAs cumplen funciones comunes en el mantenimiento de las funciones en diferentes tejidos, también se ha reportado que la especificación de algunos tejidos en teleósteos implica una regulación basada en miRNA (Wienholds et al., 2005;Bizuayehu and Babiak, 2014;Juanchich et al., 2016). Una caracterización reciente de la respuesta transcriptómica de miRNAs de trucha arco iris en múltiples tejidos reveló que una gran parte de estas transcritos se expresan de manera tejido-especifica (Juanchich et al., 2016), de una manera similar a lo observado en vertebrados superiores (Clop et al., 2006). El mir-21 se ha caracterizado como un interruptor clave en la respuesta inflamatoria (Sheedy, 2015). Se ha sugerido que esta función estaría presente también en teleósteos, pero caracterizada durante respuestas (Andreassen and Hoyheim, 2017). Nuestros resultados muestran que una infección bacteriana también induce la expresión de diferentes isoformas mir-21, pudiendo estar cumpliendo roles similares en la respuesta inmune a los demostrados en vertebrados superiores.

De forma relevante, el mir-21 también se ha asociado con estrategias de evasión inmunológica empleadas por algunos patógenos. Al igual que *P. salmonis*, las micobacterias son bacterias intracelulares que persisten y se replican en los macrófagos del hospedador (Ernst, 1998;McCarthy et al., 2008a). Se ha demostrado que las especies de micobacterias inducen mir-21 para regular las múltiples vías requeridas para la infección por patógenos, incluyendo la supresión de la producción de IL-12 (Liu et al., 2012;Wu et al., 2012). Debido a esto, el bloqueo temporal y específico de mir-21 se ha propuesto como una estrategia ideal para el desarrollo de vacunas en respuesta a este tipo de patógeno (Sheedy, 2015). Por lo tanto, una mayor investigación sobre el papel de la familia mir-21 en los salmónidos podría ayudarnos a comprender mecanismos de evasión de respuesta inmune empleados por *P. salmonis*, así también como la identificación de potenciales candidatos para el desarrollo de vacunas contra *P. salmonis*.

Utilizando la lista completa de miRNAs se realizó la predicción *in silico* de los targets para determinar que genes estarían regulando. Esta se llevó a cabo sobre las regiones 3' UTR de los mRNAs diferencialmente expresados que fueron descritos en este trabajo. Así, los principales targets identificados se asociaron con procesos del sistema inmunitario, como la vía de señalización mediada por quimiocinas, el metabolismo del cortisol, la quimiotaxis de los neutrófilos y el desarrollo del sistema inmunitario, que desempeñan un papel fundamental en la respuesta inmunitaria del teleósteo a la infección bacteriana (Uribe et al., 2011;Bird and Tafalla, 2015;Philip and Vijayan, 2015). Estos genes incluyeron C-C motif chemokine 19-like, stromal cell-derived factor 1-like, myxovirus resistance protein 2 y nuevamente hepcidina-1. Los altos valores de ΔG estimados durante la interacción miRNA-mRNA, así también como sus valores de expresión contrastante otorgan evidencias de que estos genes estarían siendo regulados por miRNAs. En el caso de hepcidina, esta regulación podría estar vinculada a dos tipos de RNAs no codificantes (miRNAs y lncRNAs). Sin embargo, se desconoce si estas regulaciones pueden ser contrastantes o aditivas.

7.4 Entendiendo la patogénesis desde el punto de vista de la bacteria

El entendimiento de la patogénesis de *P. salmonis* ha sido abarcado desde la perspectiva del salmón o de la bacteria, dejando de lado que en un proceso infectivo existe una interacción constante entre ambos agentes involucrados. Debido a esto, se utilizó una aproximación Dual RNA-seq para estudiar el transcriptoma de la bacteria durante la infección, teniendo en consideración de manera paralela la expresión de genes del hospedador.

De esta manera, se identificó que la bacteria expresa distintos genes vinculados clásicamente a patogénesis, tales como miembros del sistema de secreción tipo IV, incluidos VirB9, IcmL e IcmW. El sistema de secreción de tipo IV se ha descrito como un mecanismo conservado para la administración de factores virulentos desde el patógeno al hospedador (Thanassi and Hultgren, 2000). Este sistema había sido descrito previamente en *P. salmonis* (Gomez et al., 2013a), e incluso se ha reportado que la mutagénesis de esta región provoca una atenuación de la patogenicidad de *P. salmonis* (Mancilla et al., 2018). Sin embargo, más allá de los genes clásicos asociados con patogenicidad, una respuesta común relacionada con el metabolismo de proteínas y en particular la biosíntesis/ degradación de los aminoácidos estuvo presente tanto en

S. salar como en *P. salmonis*. Debido a esto, se realizó una comparación a escala genómica para evidenciar la batería de genes implicados en la degradación y biosíntesis de aminoácidos en *P. salmonis*, y se comparó con los descritos para *A. salmonicida* y *F. tularensis*. De acuerdo con nuestros resultados, *P. salmonis* presentó una carencia de genes relacionados con la biosíntesis y degradación de valina, leucina e isoleucina en comparación con los genomas utilizados. Por lo tanto, la disponibilidad de estos aminoácidos para *P. salmonis* podría depender de la presencia de estos recursos en el entorno intracelular del huésped.

Durante la infección, los patógenos intracelulares deben superar diferentes condiciones adversas, incluyendo la invasión intracelular, la respuesta inmune del hospedador, la producción de radicales libres y también la privación de nutrientes. Aunque el citosol del hospedador se consideraba anteriormente como una fuente abundante de nutrientes para los patógenos invasores (Ray et al., 2009), la evidencia reciente sugiere que los hospedadores pueden reducir la disponibilidad intracelular de ciertos nutrientes como respuesta protectora contra los patógenos invasores (Abu Kwaik and Bumann, 2013;Barel and Charbit, 2013). Esta privación da como resultado una lucha entre el hospedador y el patógeno por la disponibilidad limitada de nutrientes, lo que comúnmente se conoce como inmunidad nutricional (Hood and Skaar, 2012a). Teniendo en cuenta la falta de rutas biosintéticas para leucina, valina e isoleucina en *P. salmonis* y que estos mismos aminoácidos se definen como aminoácidos esenciales para los salmónidos (Helland et al., 2010), es posible sugerir que la inmunidad nutricional desplegada por el hospedador frente a la bacteria no estaría solo limitada a la captación de hierro, sino que además podría incorporar a los aminoácidos.

Los patógenos intracelulares han desarrollado diferentes estrategias para enfrentarse a condiciones limitantes de aminoácidos. Algunas de estas adaptaciones incluyen la detención del crecimiento y la diferenciación, una autosuficiencia de aminoácidos y la explotación de la maquinaria del hospedador para obtener aminoácidos (Zhang and Rubin, 2013). Basándonos en nuestros resultados y considerando la reciente reconstrucción de modelos metabólicos para *P. salmonis* (Cortes et al., 2017), la estrategia de autosuficiencia para los aminoácidos en el *P. salmonis* puede ser rechazada. Con respecto a la detención del crecimiento y la diferenciación, se ha reportado que, durante un escenario de restricción de aminoácidos, *Chlamydia trachomatis*

se diferencia morfológicamente en una forma aberrante que no puede crecer pero que los protege de un ambiente restringido de nutrientes (Leonhardt et al., 2007). (41). Sin embargo, basta con la adición de triptófano o isoleucina revertir esta transformación y reactivar el crecimiento bacteriano (Hatch, 1975;Ibana et al., 2011). Anteriormente, se ha reportado la existencia de pequeñas variantes morfológicas de *P. salmonis*, las que fueron sugeridas como un mecanismo de supervivencia empleado por las bacterias para superar escenarios adversos (Veronica Rojas et al., 2008). Sin embargo, el papel de estas variantes durante un escenario de restricción de aminoácidos y su relación con la virulencia permanece sin explorar. Por otro lado, patógenos como *Legionella pneumophila* han desarrollado mecanismos que garantizan el acceso a los nutrientes del hospedador al promover la expresión de los transportadores de aminoácidos del huésped y al aprovechar los proteasomas de las células infectadas para generar aminoácidos (Wieland et al., 2005;Price et al., 2011). En este contexto, se ha sugerido que uno de los mecanismos empleados por *P. salmonis* para evadir la respuesta inmune puede estar relacionado con la regulación de la respuesta transcripcional del hospedador a través de RNAs no-codificantes (Valenzuela-Miranda and Gallardo-Escarate, 2016;Valenzuela-Miranda et al., 2017). Por lo tanto, la idea de que *P. salmonis* sea capaz de secuestrar la maquinaria del huésped para obtener aminoácidos también deben ser explorados.

La importancia de los aminoácidos en el ciclo celular patógeno no solo radica en su utilización para biogénesis de las proteínas, sino también como fuentes alternativas de carbono y nitrógeno (Steeb et al., 2013). Por lo tanto, una plasticidad metabólica para obtener carbono y energía de múltiples fuentes de aminoácidos podría convertirse en una ventaja en un escenario de recursos limitados. Esta plasticidad metabólica se ha reportado en otros patógenos intracelulares como *F. tularensis,* donde la supervivencia intracelular de esta bacteria se basa en su disponibilidad para explotar múltiples fuentes de aminoácidos del huésped (Meibom and Charbit, 2010;Barel et al., 2015). El crecimiento de *P. salmonis* en medios suplementado con distintos aminoácidos reveló que la bacteria es capaz de crecer independiente del tipo de aminoácido suplementado en el medio. Basándonos en nuestros resultados, y considerando los modelos metabólicos propuestos recientemente para *P. salmonis* (Cortes et al., 2017;Fuentealba et al., 2017), podemos argumentar que esta bacteria es capaz de usar diferentes fuentes de aminoácidos como carbono y como recurso energético. Esta estrategia ha sido adoptada por otras bacterias

intracelulares, donde esta capacidad es un factor crucial para el desarrollo de la virulencia (Eisenreich et al., 2010;Meibom and Charbit, 2010;Barel et al., 2015). El comprender la patogénesis del punto de vista de la bacteria nos permitió identificar que la inmunidad nutricional gatillada por el salmón durante la infección con *P. salmonis* no solo está limitada a la movilización de hierro, sino que además podría estar vinculada a la regulación de los aminoácidos disponibles. Futuras investigaciones deben ser destinadas para entender la verdadera plasticidad de *P. salmonis* para explotar los aminoácidos como fuente de carbono y como esta herramienta puede incidir en la patogénesis bacteriana

7.5 En busca de la atenuación de los mecanismos de patogénesis de *P. salmonis*

El cultivo prolongado de bacterias en medios artificiales puede provocar cambios genotípicos y fenotípicos que permitirán que el microorganismo utilice completamente los nutrientes disponibles en los medios. Si estas condiciones son lo suficientemente persistentes, algunos de estos cambios pueden volverse permanentes (Eydallin et al., 2014;Kram et al., 2017). Para los patógenos intracelulares, se ha establecido que la propagación en serie en un medio libre de células puede conducir a una disminución de la patogénesis. Sin embargo, la mayoría de las veces, estos estudios se limitan a la identificación de mutaciones puntuales como resultado de la adaptación de los medios. Aquí, el objetivo de este estudio fue evaluar si es posible lograr una atenuación de *P. salmonis* mediante el cultivo prolongado de la bacteria en un medio liquido libre de células y evaluar las bases moleculares que explicarían este cambio.

Al comparar los genomas se evidenció una reducción del tamaño y del contenido genético después del pasaje en serie. Se sabe que los genomas bacterianos son altamente dinámicos tanto en tamaño como en composición (Puigbo et al., 2014). La pérdida genética y la reducción del genoma se han reportado previamente como resultado de la evolución experimental tanto en patógenos extracelulares como intracelulares (Dufresne et al., 2005;Nilsson et al., 2005;Koskiniemi et al., 2012). El cultivo en serie de *E. coli* en un ambiente rico en nutrientes ha demostrado conducir una pérdida genética y el desarrollo de la dependencia metabólica en los metabolitos suplementados con los medios (D'Souza and Kost, 2016). Este fenómeno se ha explicado desde la perspectiva de la eficiencia energética celular, donde perder los genes que ya

no son requeridos podría implica un menor costo energético (Zamenhof and Eichhorn, 1967;Dufresne et al., 2005;D'Souza et al., 2014;D'Souza and Kost, 2016).

Junto con la reducción del tamaño del genoma, también se evidenció una reorganización del genoma bacteriano. Estas modificaciones incluyeron la translocación de dos bloques genómicos y la migración de un segmento de ADN genómico al plásmido en bacterias adaptadas al medio. El contenido genético de estos segmentos reveló la presencia de elementos genéticos móviles, transposasas, proteínas relacionadas con flagelos y genes relacionados con el sistema de secreción, entre otros. Los elementos genéticos móviles (MGE) promueven el intercambio y la redistribución de genes (Craig et al., 2002) y se sabe que los genomas de Rickettsias están compuestos por una gran cantidad de estos elementos (Gillespie et al., 2012). Se ha descrito que más de 40 MGE se expresan en el transcriptoma de *P. salmonis* (Machuca and Martinez, 2016) y algunos MGE como ISPsa2 se han caracterizado en el genoma de *P. salmonis* (Marshall et al., 2011b). Aunque estas evidencias sugirieron que el genoma de *P. salmonis* correspondía a una entidad altamente dinámica y fluida, este es el primer reporte que confirma que estos reordenamientos están ocurriendo en realidad en el genoma de *P. salmonis* y cuál es el alcance de estos cambios. Investigaciones futuras deben ser dirigidas a evaluar la frecuencia con la que se producen estos cambios y cuál es el rol real de los MGE en esta reorganización.

Se ha demostrado que las reorganizaciones del genoma pueden tener un impacto directo en la función, evolución y regulación de la expresión génica en modelos bacterianos (Ptacin and Shapiro, 2013;Hendrickson et al., 2018;Krogh et al., 2018). Si bien la translocación de DNA genómico y plásmidos es un fenómeno conocido (Heffron et al., 1975;Russell and Dahlquist, 1989;Frost et al., 2005;Wozniak and Waldor, 2010), la mayoría de las veces es estudiado desde la perspectiva de la resistencia a los antibióticos. Sin embargo, el rol de esta recombinación y su impacto en la patogénesis sigue siendo en gran parte desconocido. Proteínas relacionadas con flagelos y genes del sistema de secreción también se encontraron en las regiones translocadas. *P. salmonis* se define como una bacteria no móvil, además no se ha reportado la existencia de un flagelo estructural en esta bacteria (Fryer and Hedrick, 2003). Sin embargo, se ha sugerido que la presencia de proteínas relacionadas flagelos cumplen un rol inmunomodulador de la respuesta del huésped para promover la infección en macrófagos de *P. salmonis* (Carril et al.,

2017). Por su parte, el sistema de secreción es un mecanismo bien conocido y conservado de la secreción de factores de virulencia entre bacterias patógenas. Nuestros resultados evidenciaron un cambio en los loci de ambos grupos de genes relacionados con patogenicidad, que probablemente afectan la arquitectura genómica de las bacterias cultivadas continuamente en medio libre de células. Aunque no se evidenciaron diferencias importantes en la expresión génica en estos genes durante el cultivo en caldo de los pasajes 0 y 200, se desconoce qué tan diferente puede ser este escenario durante el proceso infeccioso de *P. salmonis* en las células de salmón.

Los patrones globales de expresión génica entre diferentes pasajes de *P. salmonis* (P0, P1 y P200) evidenciaron una disminución en el transcriptoma conforme se progresaba en los pasajes. Nuestra hipótesis es que, en ausencia de un huésped para infectar y luchar por la disponibilidad de nutrientes durante más de 200 generaciones, *P. salmonis* reduce su repertorio transcriptómico a un set de genes que le permita explotar los recursos disponibles en el medio. Anteriormente demostramos que la inmunidad nutricional desempeña un papel fundamental en la interacción *P. salmonis*-Salmón. Nuestros resultados demuestran que dentro de los genes que reducen su expresión, se encontraron aquellos vinculados a la captación de hierro mediante vibrioferrina. La vibrioferrina (VF) es una clase de sideróforos marinos de carboxilato involucrados en el transporte de hierro (Amin et al., 2009). Los sideróforos se han caracterizado como elementos claves empleados por las bacterias para competir por la adquisición de hierro de sus hospedadores (Miethke and Marahiel, 2007;Wilson et al., 2016). Por otra parte, se ha demostrado que *P. salmonis* produce moléculas similares a sideróforos y codifica genes relacionados con vibrioferrina (Machuca and Martinez, 2016;Calquin et al., 2018). Debido a esto, es posible argumentar que en ausencia de un huésped con el cual no competir por el hierro, la bacteria ya no requiere la producción de vibrioferrina y disminuye la expresión de estos genes.

Adicionalmente, nuestro experimento sugieren que las bacterias mantenidas en un crecimiento continuo por 200 pasajes perdieron en gran medida su capacidad de generación de efectos citopáticos y lisis en células de salmón en comparación a la bacteria original. Estos resultados concuerdan con la evidencia previa demostrando la atenuación de bacterias patógenas mediante cultivo serial (Cabral et al., 2017). Antes del desarrollo de herramientas de edición genética

dirigida, el pasaje en serie de bacterias patógenas fue el método de referencia para el desarrollo de vacunas atenuadas vivas (Hanley, 2011). En la actualidad las vacunas desarrolladas para controlar la infección por *P. salmonis* se basan en el uso de bacterias inactivadas (bacterina) (Maisey et al., 2017). Sin embargo, estas no han logrado generar el nivel de protección deseado. En este escenario, nuestro trabajo demuestra que el cultivo continuo de *P. salmonis* conlleva cambios genómicos y transcriptómicos profundos que repercuten en la virulencia de la bacteria. Estos resultados resultan promisorios con miras al desarrollo de una vacuna viva atenuada contra *P. salmonis*.

7. CONCLUSIONES

El desarrollo de herramientas efectivas para el control de SRS se ha visto dificultado por distintas brechas de conocimiento asociadas a diversos aspectos de la enfermedad. El presente trabajo tuvo como objetivo la generación de nuevos conocimientos sobre la interacción patógeno-hospedero en salmones Atlánticos infectados con la bacteria *P. salmonis*. Mediante una aproximación genómica funcional se lograron identificar distintos procesos moleculares que se regulan durante la infección. Uno de estos procesos incluyo la endocitosis mediada por clatrina. Mientras los factores promotores de esta ruta se encontraron sobreregulados, la cadena de genes vinculada al desarrollo de endosomas reprimieron su expresión. Esto puede deberse a la existencia de un mecanismo empleado por la bacteria que le permita ingresar a la célula por endocitosis, pero evitar la maduración de endosomas y reprimir su posterior lisis. Otra ruta regulada durante la infección incluyó la de homeostasis de hierro. Nuestros resultados nos permiten inferir que el salmón gatilla de manera innata una respuesta inmune nutricional ante la presencia de un agente patógeno. Esta respuesta promovería la acumulación de hiero a nivel intracelular, contradictoriamente esto podría generar un ambiente intracelular rico en hierro, lo que es aprovechado por patógenos intracelulares como *P. salmonis*.

Nuestros resultados nos permiten inferir que los RNAs no-codificantes tendrían un rol fundamental en la regulación de la respuesta del salmón durante la infección. Del total de lncRNAs identificados en este trabajo, alrededor del 50% correspondieron a nuevas secuencias caracterizadas para la especie en comparación con lo reportado previamente en *S. salar* durante una infección viral. Esto sería indicio de que un porcentaje importante de la respuesta basada en lncRNAs dependería del tipo de patógeno enfrentado. De esta forma, se lograron identificar lncRNAs candidatos que podrían participar en la regulación de genes como clatrina y hepcidina. Por otra parte, distintos miRNAs fueron diferencialmente expresados en tejidos de salmón infectados con *P. salmonis*. Estos pertenecieron a familias de miRNAs comúnmente asociadas a regulación de respuesta inmune en teleósteos. La predicción *in silico* de los targets de estos miRNAs incluyo la regulación de hepcidina, gen clave durante la respuesta del salmón. El presente estudio sugiere que los RNAs no-codificantes son parte importante de la respuesta de *S. salar* frente a la bacteria, posiblemente como entes reguladores de la respuesta.

El estudiar la patogenicidad desde el punto de vista de la bacteria nos permitió identificar procesos gatillados por el patógeno durante la infección, esta respuesta se basó en genes clásicos de patogénesis incluyendo la regulación de genes del sistema de secreción tipo IV. Sin embargo, el integrar en el análisis el transcriptoma del hospedador nos permitió identificar una respuesta común entre *P. salmonis* y *S. salar* asociada al metabolismo de aminoácidos. Posteriores análisis confirmaron la plasticidad de *P. salmonis* para usar aminoácidos posiblemente como fuente de carbono. De este modo, se sugiere que la respuesta inmune nutricional empleada por el salmón frente a la bacteria no estaría limitada a una competencia por el hierro, sino que además los aminoácidos podrían jugar un rol fundamental en la determinación del éxito o fracaso de la infestación. Estos resultados resaltan la importancia de considerar que el desarrollo de una enfermedad implica la constante interacción entre patógeno y hospedero, y que abordar estos procesos sólo desde una de las perspectivas puede dejar fuera procesos que resulten de igual o mayor importancia. La sumatoria de resultados nos permite afirmar que se valida la hipótesis de trabajo 1, ya que mediante la utilización de genómica funcional fue posible identificar mecanismos moleculares claves de la interacción entre *Piscirickettsia salmonis* y el salmón Atlántico (*Salmo salar*), así como los elementos no codificantes que participarían en la regulación de estos procesos

Finalmente, se exploró como el cultivo continuo de *P. salmonis* en un medio liquido libre de células puede impactar en la patogénesis bacteriana. Luego de aproximadamente 2 años de cultivo, *P. salmonis* evidenció cambios genómicos y transcriptómicos asociados a genes como el sistema de secreción tipo IV, genes relacionados a flagelos y captura de hierro entre otros. Dada la profundidad de los cambios observados, estos resultados nos permiten concluir que el genoma de *P. salmonis* es una entidad altamente dinámica, la cual sufrió considerables cambios estructurales luego del cultivo continuo de la bacteria. Por otra parte, nos permite inferir que los cambios observados serían responsables de la atenuación de *P. salmonis* propagada en forma continua durante la infección en células de salmón. Estos resultados nos permiten validar la segunda hipótesis planteada puesto que la propagación de *P. salmonis* a largo plazo en un medio de cultivo libre de células generó modificaciones genómicas/transcriptómicas en respuesta a la adaptación al medio, lo que resultó en la atenuación de la patogenicidad de la bacteria en células de salmones.

La información generada durante la tesis pueda ser aplicada en la generación de nuevas estrategias de control de *P. salmonis*. Estas podrían incluir el uso de dietas funcionales con distintos niveles de suplementación de hiero o distintos tipos de aminoácidos y determinar la existencia de alguna condición que minimice la disponibilidad de estos nutrientes para la bacteria. Con relación al hierro, esto resulta de vital importancia puesto que una de las alternativas para el remplazo de harina de pescado en la industria de alimentos para salmón incluye el uso de insectos o la incorporación de grasas animales (tallow). Sin embargo, el alto contenido de hierro en estos reemplazos podría resultar contraproducente y beneficiar a la bacteria por sobre el hospedador. Por otra parte, la obtención de *P. salmonis* atenuada mediante pasajes seriales puede servir como base para la generación de una nueva vacuna viva atenuada, la cual podría contribuir al control de la enfermedad, disminuir el uso de antibióticos y promover un desarrollo mas sustentable de la acuicultura nacional.

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