Universitat Autònoma de Barcelona

EL USO DE ADITIVOS FITOGÉNICOS PARA PROMOVER EL ESTADO DE LA SALUD Y LA RESISTENCIA A ENFERMEDADES EN PECES DE CULTIVO

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Tesis Doctoral 2022

EL USO DE ADITIVOS FITOGÉNICOS PARA PROMOVER EL ESTADO DE LA SALUD Y LA RESISTENCIA A ENFERMEDADES EN PECES DE CULTIVO

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THE USE OF PHYTOGENIC ADDITIVES TO PROMOTE HEALTH STATUS AND RESISTANCE TO DISEASES IN FARMED FISH

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L'ÚS D'ADITIUS FITOGÈNICS PER PROMOURE L'ESTAT DE LA SALUT I LA RESISTÈNCIA A MALALTIES EN PEIXOS DE CULTIU

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Bellaterra, mayo 2022

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Tesis presentada para la obtención del título de
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FINANCIACIÓN

La investigación realizada para el desarrollo de esta tesis fue apoyada económicamente tanto por el proyecto "*AQUOLIVE by NATAC*", financiado por el Programa de Investigación e Innovación Horizonte 2020 de la Unión Europea (Acuerdo de subvención nº 830202), como también por el proyecto *Estrategias nutricionales para la mejora en el rendimiento productivo: uso de piensos funcionales y dietas saludables en acuicultura* (DIETAplus), financiado por JACUMAR (Ministerio de Agricultura, Pesca y Medio Ambiente de España, MAPAMA) y FEMP (UE), y parcialmente por la Agencia Española de Investigación (subvención AGL2016-76069-C2-2-R a la *Universitat Autònoma de Barcelona*).

El autor, Ricardo Salomón Figueredo, com NIE: Y4968259C, fue beneficiario de una beca financiada por el Gobierno de Paraguay, Programa Nacional de Becas de Postgrado en el Exterior "Don Carlos Antonio López" (BECAL), mediante la resolución del 28 de diciembre del 2017, correspondiente a la "Quinta Convocatoria".

AGRADECIMIENTOS

A mi director de tesis, el Dr. Enric Gisbert, quiero expresarle mi mayor y profundo agradecimiento por la orientación y el apoyo absoluto a lo largo de estos cuatro años, que contribuyeron en gran medida a enriquecer mi crecimiento como joven científico y como persona. Gracias por tu profesionalidad, la dedicación, la amistad sincera y la plena disponibilidad que siempre me brindaste. Tu apoyo y tus enseñanzas fueron vitales para que este trabajo llegara a su fin con una enorme sensación de satisfacción.

A mi co-directora de tesis, la Dra. Eva Vallejos-Vidal y al Dr. Felipe E. Reyes López, a ambos, mis más sinceros agradecimientos por enseñarme e introducirme al mundo de los "*microarrays*" y sus respectivos análisis. Por el apoyo y la disponibilidad brindada en el transcurso de este doctorado, y por los momentos compartidos en el laboratorio (que no han sido pocas), muchas gracias.

A todos los co-autores que en gran medida siempre han estado ahí colaborando y transmitiendo conocimiento. En especial a José Carlos Quintela y José María Pinilla, quienes siempre estuvieron presentes apoyando en todo y participando activamente en el proyecto.

A toda la comunidad del centro de investigación IRTA de La Ràpita, investigadores y técnicos, quienes durante estos largos cuatros años estuvieron siempre presente apoyándome y ayudándome en todo lo necesario. Gracias gente.

A mis colegas del IRTA, los "*doctorands*", con quienes he forjado una gran amistad durante todos estos años, que estoy seguro de que perdurará para toda la vida. En especial a Joana, quien fue más que una colega, sino una compañera de vida que me

ayudó en absolutamente todo lo que fuese respecto a la tesis y la vida durante este "*PhD journey*". A vos Joana, muchísimas gracias, de corazón.

Por supuesto, a mi familia, a mis padres, y en especial a mis hermanos, mis queridos hermanos que son mis pilares en la vida, sin su aprobación y apoyo no hubiera llegado a estas instancias. Esta tesis va dedicada a todos ustedes (mami, papi, Rodri y Rauli). ¡Gracias!

"El secreto de la felicidad no es hacer siempre lo que se quiere,
sino querer siempre lo que se hace."

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ABSTRACT

Aquaculture growth will unavoidably involve the implementation of innovative and sustainable production strategies, whereas functional feeds are among the most promising strategies in terms of animal health and welfare management. A broad spectrum of phytogenics has been gaining interest in aquafeeds due to their growth-promoting, antimicrobial, immunostimulant, antioxidant and anti-inflammatory properties. Although the impact of phytogenics on fish immunity has been extensively evaluated, most studies fail in addressing the mechanisms underlying their beneficial effects on the animal. In this context, the set of studies presented in this thesis aims to provide insights on the impact of a combination of phytogenic additives obtained from two medicinal plants, the sage and the lemon verbena (10% ursolic acid; 3% other triterpene compounds; 2% verbascoside; <1% polyphenols), and a third one obtained from olive fruit (10% olive bioactive compounds; 8% triterpenic compounds; 2% polyphenols), on growth performance, immune response, both systemic and local, and resistance to infectious diseases in fish. For this purpose, two model aquaculture species were used, the gilthead seabream (*Sparus aurata*), the main Mediterranean farmed species, and the Atlantic salmon (*Salmo salar*), the most important aquaculture species in the world in terms of economic value. The overall analysis of the results indicated that dietary supplementation of extracts of a mixture of sage and lemon verbena (0.1%) and olive fruit (0.15%) promoted both innate and adaptive immunity in gilthead seabream and Atlantic salmon. Significant improvements in productive performances such as weight gain were observed in gilthead seabream, whereas concerning feed conversion ratio, they were observed in both species. In addition, both phytogenics protected Atlantic salmon smolts by improving the cumulative survival when fish were challenged with *Aeromonas salmonicida*, the causative agent of furunculosis in salmonids. On the other hand, it has also been shown that, in the gilthead seabream, the combined administration of sage and lemon verbena promoted intestinal

integrity, while at the level of local immunity, both phytogenics proved to be beneficial. The overall results presented in this doctoral thesis indicate that both phytogenics, the combination of sage and lemon verbena extracts and the olive fruit extract, can be used by the aquaculture industry as zootechnical additives with immunomodulatory properties, besides being an effective, safe, and environmentally eco-friendly tool to be used as a prophylactic strategy against infectious diseases and as an alternative to antimicrobial compounds.

Keywords: Feed additives, phytogenics, sage, lemon verbena, olive fruit, aquaculture, *Sparus aurata*, *Salmo salar*, *Salvia officinalis*, *Lippia citriodora, Olea europaea*, functional networks, interactome, *Aeromonas salmonicida*, challenge

INTRODUCCIÓN

Introducción

1. Crecimiento y retos del sector acuícola

1.1 Tendencias mundiales de la acuicultura, retos y sostenibilidad

La creciente demanda mundial de alimentos sanos y nutritivos, como el pescado y los mariscos, derivado del crecimiento demográfico mundial, el aumento de renta per cápita del consumidor y los cambios en los patrones de consumo, es un reto actual y a futuro que depende en gran medida del crecimiento del sector de la producción acuícola. Según en el último informe publicado por la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), la producción acuícola mundial en 2019 fue de 120,1 millones de toneladas, un 3,6% más que en el año anterior (FAO, 2021). La producción acuática ha crecido continuamente durante las últimas tres décadas a una tasa media anual del 2,5%, superando incluso la tasa de crecimiento de la población mundial del 1,6% (FAO, 2020). Según esta organización, el consumo mundial de productos acuáticos per cápita ha pasado de 9,9 kg en 1961 a 20,5 kg en 2018, creciendo aproximadamente un 1,5%. Por sexto año consecutivo, la producción acuícola en 2018 superó a los productos pesqueros en el mercado en 17,1 millones de toneladas. En el contexto del aumento previsto del consumo de productos acuáticos, la producción acuícola tendrá que crecer en paralelo durante las próximas décadas, al tiempo que se cumplan los Objetivos de Desarrollo Sostenible (ODS) de la Agenda 2030. Para proporcionar beneficios sociales y medioambientales, es aconsejable y necesaria la adopción de nuevos sistemas de producción acuícola, así como promover su sostenibilidad. La rápida expansión de la acuicultura aumenta su propia vulnerabilidad con muchos desafíos que obstaculizan el desarrollo del sector, en algunos casos socavando su capacidad para lograr los resultados sostenibles necesarios, y afectando así negativamente a la opinión de los consumidores y redundando a la vez sobre la credibilidad del sector

(Naylor et al., 2021). Esto viene dado por una serie de factores que terminan contribuyendo a esta situación, entre ellos la mayor adopción y aplicación de la normativa medioambiental, la disminución de las ganancias de productividad, el aumento de los brotes de enfermedades de los animales acuáticos relacionados con las prácticas de producción intensiva, y todas las limitaciones asociadas al cambio climático (FAO, 2020).

En este contexto, los expertos consideran que factores como los sistemas de producción intensivos y el cambio climático favorecen la aparición de brotes de enfermedades donde los animales son más propensos al estrés y a la inmunosupresión, lo cual conlleva a la propagación de patógenos más virulentos. Esto hace que las enfermedades de los animales acuáticos sean uno de los principales riesgos para la industria y uno de los principales factores limitantes para el crecimiento y desarrollo sostenible de la acuicultura (Reverter et al., 2020; Naylor et al., 2021). Además, los modelos asociados al cambio climático estiman que está previsto que patógenos que causan enfermedades nuevas y/o desconocidas, surjan y se propaguen rápidamente, causando brotes y grandes pérdidas de producción cada tres o cinco años (FAO, 2020). Además, los cambios de temperatura y los fenómenos meteorológicos pueden afectar negativamente a la calidad del agua y aumentar la transmisión de enfermedades infecciosas, además de contribuir a la expansión de su distribución geográfica (Reverter et al., 2020; Naylor et al., 2021).

Con el fin de prevenir los brotes de enfermedades, el uso profiláctico indiscriminado de antibióticos y productos químicos asociados a las prácticas de la acuicultura intensiva aún persiste en algunos de los principales países productores de acuicultura, si bien es notable mencionar que se ha observado un cierto descenso en su uso en los últimos años (Lulijwa et al., 2020; Schar et al., 2020). El problema radica que en el uso recurrente de estas terapias trae consigo graves efectos secundarios en el sistema acuícola, no sólo por comprometer la inmunidad de los animales, sino también por la potenciación a la aparición de bacterias resistentes a los antibióticos, así como también efectos negativos relacionados con la seguridad alimentaria y salud del consumidor (Reverter et al., 2020). En la Unión Europea (Reglamento

1831/2003/CE), la prohibición de los antibióticos como promotores del crecimiento en los alimentos para animales debido a las amenazas de resistencia a los antibióticos ha obligado a la industria de producción animal a adoptar producciones libres de uso de antibióticos (Vincent et al., 2019; Reverter et al., 2020). Esto unido al desarrollo de estrategias alternativas y/o complementarias más sostenibles para reducir el uso de fármacos quimioterapéuticos en la acuicultura (Dawood et al., 2018).

Este escenario actual en el cual se encuentra el sector acuícola trae consigo una responsabilidad hacia la mejora de su sostenibilidad ambiental y económica de esta industria. Por lo tanto, para resolver con éxito los grandes retos a los que se enfrenta el sector; la investigación y las iniciativas innovadoras deben dirigirse a optimizar su eficiencia y productividad, tanto en sistemas pequeños, de medio y de gran escala, como así también fuerzas impulsoras que apoyen el crecimiento sostenible de esta industria.

2. Aditivos funcionales para piensos como profilácticos sostenibles en la acuicultura

El uso tradicional de quimioterapéuticos en la acuicultura está disminuyendo y evolucionando hacia alternativas más sostenibles, pues el uso sostenido en el tiempo de agentes antimicrobianos genera la aparición de cepas patógenas resistentes, tienen un notable impacto medioambiental y genera residuos de estos compuestos en los peces lo que conlleva problemas de seguridad alimentaria (Romero et al., 2012; Salin & Ataguba, 2018; Asif et al., 2018). Estas preocupaciones han fomentado el desarrollo de métodos fiables, seguros y respetuosos con el medio ambiente para prevenir las enfermedades, como por ejemplo son los piensos funcionales y las vacunas. Sin embargo, y aunque la vacunación ha demostrado ser una excelente herramienta de prevención en algunos sectores de la acuicultura, aún es una técnica utilizada en casos específicos para un patógeno en concreto y que a su vez requiere de un claro diagnóstico de la enfermedad, presentando una eficacia

limitada contra las infecciones por múltiples agentes. Además, el tiempo y los costes asociados al desarrollo de vacunas pueden limitar su disponibilidad y aplicación a un amplio repertorio de organismos patógenos, lo que hace que en la actualidad no existan vacunas eficaces contra varias enfermedades de importancia económica, como las infecciones víricas y parasitarias (Miccoli et al., 2021).

Dado que los brotes de enfermedades están íntimamente relacionados con el estado fisiológico e inmunológico del animal, la aplicación de estrategias nutricionales funcionales, como el uso de aditivos zootécnicos con propiedades inmunoestimulantes como herramientas profilácticas sostenibles para mejorar la gestión de la salud de los peces en las piscifactorías, ha ganado una considerable atención en la última década (Vallejos-Vidal et al., 2016; Pérez-Sánchez et al., 2018). Además de beneficiar el crecimiento de los peces y la eficiencia de la alimentación, los alimentos funcionales pueden promover la inmunidad y el bienestar de los peces. Estos beneficios se obtienen a partir de la inclusión de ingredientes específicos y/o compuestos bioactivos que tienen como objetivo promover funciones específicas en el organismo que mejoran la capacidad inmunitaria de los peces frente a brotes infecciosos (Dawood et al., 2018). En la actualidad hay un gran número de aditivos o suplementos para piensos, como probióticos, prebióticos, simbióticos, inmunoestimulantes postbióticos, nucleótidos, fitogénicos y vitaminas y minerales, que se pueden incluir en los piensos funcionales (*Figura 1*). A continuación, se describe un breve resumen de sus propiedades y funciones en el organismo de las especies acuáticas.

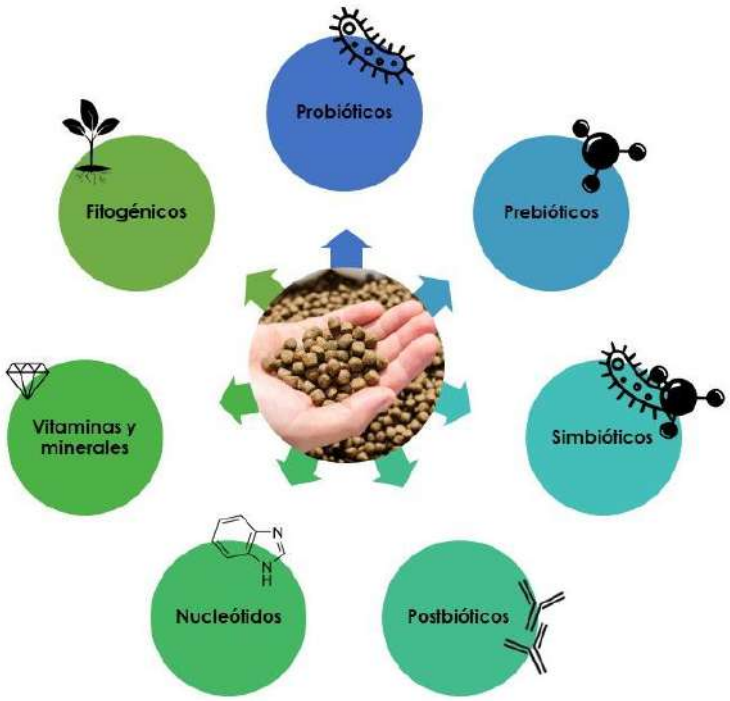


Figura 1. En el diseño de los piensos funcionales, se puede utilizar una amplia gama de aditivos zootécnicos para mejorar el rendimiento de los peces más allá de los requisitos nutricionales de la especie para mejorar el crecimiento y la utilización del alimento. Estos piensos funcionales están especialmente diseñados para favorecer la salud y la resistencia al estrés de las especies acuáticas de cultivo. Los probióticos, los prebióticos, los simbióticos, los inmunoestimulantes postbióticos, los nucleótidos, los fitogénicos y las vitaminas y los minerales son algunos de los aditivos funcionales para piensos actualmente disponibles para su inclusión en los alimentos acuícolas.

En la acuicultura, los **probióticos** son microorganismos vivos, muertos o un componente de los mismos que se administran por vía oral a través de la dieta o directamente en el agua de cultivo, lo que confiere beneficios para la salud del huésped al mejorar la resistencia a las enfermedades, el estado de la salud, el rendimiento del crecimiento, la utilización de los alimentos, la respuesta al estrés o el vigor general del animal. Estos beneficios para la salud se consiguen, al menos en parte, mediante la modificación de las comunidades de la microbiota del huésped y del entorno (Simón et al., 2021). La categoría de probióticos puede incluir diferentes

bacterias, bacteriófagos, microalgas y levaduras, que se han utilizado ampliamente en la acuicultura como soluciones mono o multicepas a través de suplementos en la dieta o aplicaciones en el agua. Los probióticos comúnmente utilizados en la acuicultura incluyen miembros de los géneros *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium*, *Shewanella*, *Bacillus*, *Aeromonas*, *Vibrio*, *Enterobacter*, *Pseudomonas*, *Clostridium* y *Saccharomyces*. Los probióticos multiespecies pueden ser más eficaces que los probióticos de una sola cepa, ya que las diferentes cepas presentes en los probióticos multiespecies aumentan la posibilidad de que sobrevivan en el intestino y por ende ejercer un efecto sinérgico más fuerte en el huésped (Butt & Volkoff, 2019).

Los beneficios para la salud promovidos por los probióticos en la acuicultura han sido ampliamente revisados (Hoseinifar et al., 2018; Dawood et al., 2019; 2020). La capacidad de los probióticos para promover y/o mejorar la salud está relacionada con su capacidad para estimular la respuesta inmunitaria del huésped e inhibir el crecimiento de bacterias patógenas. Los probióticos pueden interactuar con otras bacterias entéricas o antagonizarlas resistiendo la colonización o inhibiendo y reduciendo directamente la incidencia de patógenos oportunistas. Se ha sugerido que la exclusión competitiva es el principal modo de acción de los probióticos. Mediante la competencia por los sitios de fijación y los nutrientes, como el hierro, la ocupación por parte de las cepas probióticas del epitelio de la mucosa gastrointestinal y de la mucosidad impide una mayor colonización del patógeno (Hoseinifar et al., 2018). Los probióticos pueden ejercer también efectos antibacterianos mediante la producción de moléculas con actividad bactericida, como bacteriocinas, sideróforos, lisozimas, proteasas y/o peróxido de hidrógeno. Además, la alteración del pH intestinal debido a la generación de ácidos orgánicos también puede inhibir el crecimiento de bacterias patógenas (Dawood et al., 2018).

Los probióticos también pueden mejorar la salud a través de la modulación fisiológica o inmunológica del huésped (Chauhan & Singh, 2019; Simón et al., 2021). Se ha estudiado que los probióticos pueden afectar a elementos del sistema

inmunitario no específico, como el aumento de los leucocitos tras el tratamiento. Además, los probióticos mejoran el rendimiento del crecimiento y la utilización del alimento en los animales acuáticos a través de la promoción de la actividad de las enzimas digestivas, como las alginato liasas, las amilasas y las proteasas (Hoseinifar et al., 2018). Los probióticos también proporcionan un entorno más favorable para los peces mediante la reducción de la proliferación de bacterias patógenas en el agua de cultivo, así como mediante la biorremediación de los residuos orgánicos (Dawood et al., 2018).

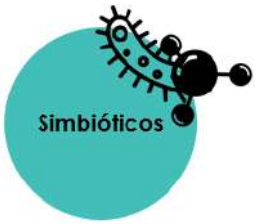
Desde principios de la década de 1980 se han utilizado probióticos comerciales en la acuicultura, que proceden en su mayoría de fuentes terrestres y no del entorno en el que viven los animales acuáticos o del propio huésped. Estudios recientes se han centrado en el uso de la microbiota relacionada con el hospedador como fuente de probióticos, ya que se establecen de forma natural en el sistema de defensa del hospedador y han revelado otros efectos beneficiosos (Van Doan et al., 2020).

Los **prebióticos** son carbohidratos no digeribles que tienen efectos beneficiosos en el crecimiento y/o la actividad de la microbiota intestinal de los peces, así como en la inmunidad de éstos, mejorando así la salud del huésped. La actividad inmunomoduladora de los prebióticos está mediada por interacciones directas con el sistema inmunitario innato del huésped o por la modulación del crecimiento de la microbiota intestinal comensal, ya que se utilizan como fuentes de energía para las bacterias entéricas. Para ser considerados como prebióticos, estos ingredientes funcionales deben ser resistentes a la digestión, fermentables por las bacterias intestinales beneficiosas y capaces de promover el crecimiento y/o la actividad de grupos de microbiota relacionados con la salud y el bienestar del huésped. Varios productos prebióticos se aplican ampliamente en la acuicultura, como la inulina, el fructo-oligosacárido (FOS), el manano-oligosacárido (MOS), el galactooligosacárido

(GOS), el arabinoxilano-oligosacárido (AXOS) y el isomaltooligosacárido (IMO) (Dawood et al., 2020).

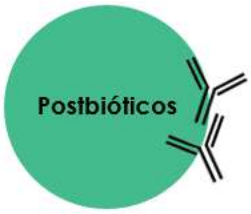
Se encuentran de forma natural en numerosas plantas, en particular las ricas en fructanos, como por ejemplo la cebolla, el ajo, la alcachofa, el kiwi y la soja, además de la avena y el trigo, y las plantas ricas en inulina, como la jícama y la raíz de achicoria (Amenyogbe et al., 2020). Los prebióticos favorecen el aumento de los probióticos en el intestino debido a su capacidad para digerirlos, mientras que los patógenos carecen de las enzimas sacrolíticas necesarias (Nawaz et al., 2018).

Además de beneficiar a la población microbiana del intestino, estas moléculas funcionales también afectan a las bacterias responsables de la producción de metabolitos intermedios. Estos compuestos, los ácidos grasos de cadena corta (AGCC), son bien conocidos por sus propiedades antiinflamatorias, ayudando a la regulación del sistema inmunitario. También mejoran directamente la función de barrera epitelial del intestino y su capacidad fagocítica. Por ello, también se les denomina inmunosacáridos. Los prebióticos también modulan la respuesta inmunitaria al interferir en la unión bacteria-epitelio y, por tanto, impidiendo la adhesión de patógenos (Nawaz et al., 2018).



Los **simbióticos** consisten en una aplicación combinada de prebióticos y probióticos. El concepto de simbiótico implica conferir el beneficio de los probióticos y los prebióticos a los animales acuáticos, principalmente debido a su efecto sinérgico. Su uso combinado tiene como objetivo mejorar la supervivencia del organismo probiótico y su eficiencia de fermentación, ya que el sustrato específico requerido está fácilmente disponible cuando se administra conjuntamente. Por lo tanto, la presencia simultánea de probióticos y prebióticos engrandece sus resultados beneficiosos sobre el huésped, proporcionando más beneficios para los animales acuáticos en comparación con la adición individual de probióticos o prebióticos (Pérez-Sánchez et al., 2018; Dawood et al., 2020).

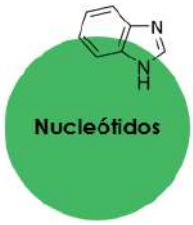
Los simbióticos se utilizan desde 2005 en la acuicultura para promover el crecimiento y la inmunidad de los animales acuáticos. Como parte de un simbiótico, el prebiótico (es decir, FOS, MOS, GOS, AXOS, IMO o inulina) se hidroliza a mono o disacáridos, aumentando selectivamente la biomasa probiótica y la colonización que se establece por la interacción específica entre las bacterias y las células epiteliales intestinales del huésped, mejorando en última instancia el bienestar y el rendimiento de éste (Huynh et al., 2017).



Los **postbióticos** incluyen cualquier sustancia liberada o producida a través de la actividad metabólica de los microorganismos probióticos que ejercen efectos beneficiosos directos o indirectos sobre el huésped. Los probióticos inactivados por calor también funcionan como postbióticos, ya que conservan importantes estructuras bacterianas con potencial para ejercer una actividad biológica en el huésped (Cuevas-González et al., 2020). Como los postbióticos no contienen microorganismos vivos, los riesgos medioambientales asociados a su administración se reducen al mínimo (Dawood et al., 2019).

Durante su crecimiento, los microorganismos producen biopolímeros con diferentes propiedades químicas que pueden ser liberados fuera de la pared celular bacteriana, formando un grupo heterogéneo de sustancias denominadas exopolisacáridos (EPS) (Peluzio et al., 2021). Entre la amplia lista de compuestos producidos por los microorganismos y reputados como postbióticos, un ejemplo popular de EPS son los β-glucanos. Los β-glucanos son polisacáridos complejos que se encuentran en las levaduras, los hongos, las algas o los cereales, como la avena y la cebada, y que a menudo se mencionan por sus propiedades inmunomoduladoras, utilizándose como parte de estrategias tanto profilácticas como terapéuticas. Existe una variabilidad estructural entre las moléculas de β-glucano que influye en sus propiedades químicas y en su actividad funcional. Los efectos inmunomoduladores suelen asociarse a los β-glucanos de mayor peso molecular, aunque la relación

directa entre estructura y actividad sigue sin estar clara (Meena et al., 2013). Los principales beneficios reportados de los β-glucanos en los peces son la estimulación y modulación inmunológica, la mejora de la resistencia al estrés y el aumento del crecimiento y la supervivencia. De todos modos, estos resultados pueden variar entre los estudios dependiendo de la fuente de glucano, la dosis, la especie de pez y la edad (Żółkiewicz et al., 2020; Pogue et al., 2021).



Los **nucleótidos** (NT) son compuestos intracelulares de bajo peso molecular que representan los componentes químicos básicos de los ácidos nucleicos y desempeñan un papel clave en casi todos los procesos bioquímicos. Los NT se producen a través del reciclaje de las células muertas y la degradación del ARN y el ADN; o a través de la síntesis directa *de novo* a partir de aminoácidos precursores, como la glutamina, el formiato, la glicina y el ácido aspártico. Sin embargo, debido a su alto requerimiento energético, la síntesis de NT puede llegar a ser limitante en condiciones de estrés, como una infección o durante periodos de crecimiento y desarrollo rápidos (Hossain et al., 2020).

Además de su posible implicación en la palatabilidad de la dieta, el comportamiento alimentario de los peces y las vías biosintéticas, la suplementación dietética de NT ha mostrado resultados prometedores en la mejora de la inmunidad y la resistencia a las enfermedades en especies relevantes para la acuicultura (Ringø et al., 2012). La investigación sobre los NT en la dieta ha demostrado que pueden mejorar el crecimiento de las especies acuáticas en las primeras etapas de desarrollo, mejorar la calidad de las larvas a través del enriquecimiento de los reproductores, mejorar la morfología intestinal, aumentar la tolerancia al estrés, así como modular las respuestas inmunes innatas y adaptativas, mostrando una mayor resistencia a las infecciones virales, bacterianas y parasitarias. Además, se ha visto que los NT reducen los efectos negativos de las proteínas alternativas de origen vegetal, lo que

conduce a un aumento de la eficiencia de la alimentación, incrementando el crecimiento y la salud de las especies acuícolas (Hossain et al., 2020).

Los animales no pueden sintetizar las vitaminas en cantidades suficientes para satisfacer sus necesidades nutricionales y fisiológicas, por lo que deben obtenerlas de la dieta. Las **vitaminas** desempeñan un papel esencial en el mantenimiento de las funciones metabólicas normales, actuando principalmente como cofactores de las enzimas, cuyo suministro inadecuado conduce a una reducción de las actividades enzimáticas que da lugar a un crecimiento deficiente, una baja supervivencia y una mayor susceptibilidad a las infecciones, entre otros signos y síntomas de deficiencia (Oliva-Teles, 2012).

Se sabe que las vitaminas C y E poseen un efecto antioxidante e inmunomodulador importante, que se correlaciona con un mayor rendimiento de los animales acuáticos (Rayman, 2000; Hamilton, 2004). La vitamina C, por ejemplo, es un antioxidante hidrosoluble capaz de eliminar los radicales libres, incluidos las especies de oxígeno reactivo (EOR o ROS del inglés "*reactive oxygen species*") y las especies reactivas de nitrógeno, evitando los daños celulares provocados por los componentes radicales. También se ha propuesto que aumenta la respuesta inmunitaria de los peces, promoviendo la actividad bactericida del suero, la actividad fagocítica, los niveles de inmunoglobulinas y la actividad de la lisozima (Dawood, 2021).

Los oligoelementos, como el Zn, el Mn, el Cu y Se, se requieren en pequeñas cantidades, pero participan en una gran variedad de procesos fisiológicos bioquímicos. La función general de los minerales incluye ser componentes de enzimas y vitaminas clave, constituyentes estructurales de los tejidos, equilibrio de la presión osmótica y transmisión del impulso nervioso y las contracciones musculares. Los referidos, han sido particularmente asociados con una mejora de la

inmunidad o función que apoya la inmunidad, y la protección antioxidante en animales acuáticos cultivados (Dawood, 2021).

Los aditivos **fitogénicos**, a veces también denominados fitobióticos, son un grupo de sustancias naturales de origen vegetal, derivadas de hierbas, especias y partes enteras o extractos de plantas. Estas sustancias se utilizan como aditivos para piensos en la nutrición animal y en la última década han cobrado un interés creciente en el sector de la acuicultura (Reverter et al., 2021). Entre otros aditivos como los mencionados anteriormente, los fitogénicos son importantes herramientas profilácticas y terapéuticas que ejercen un impacto positivo en la salud y el bienestar de los animales de granja, sin que se conozcan problemas ambientales y de peligrosidad asociados a su administración. En este contexto, los fitogénicos se consideran soluciones sostenibles y prometedoras para la nutrición animal convencional, sobre todo dentro del contexto de reducción del uso de agentes antimicrobianos en los sistemas de producción animal (Reverter et al., 2020; 2021).

Las propiedades promotoras de la salud de los compuestos fitogénicos han sido ampliamente revisadas en diferentes especies acuícolas (Van Hai, 2015; Reverter et al., 2021; Elumalai et al., 2021; Firmino et al., 2021a; 2021b). Varios estudios han mostrado de que los compuestos fitogénicos mejoran la respuesta inmunitaria no específica en peces (Dawood et al., 2018; Reverter et al., 2021). También se ha visto que los fitogénicos mejoraban eficazmente el crecimiento de los peces y la supervivencia a las enfermedades, independientemente del nivel trófico de las especies de peces estudiadas, la duración del tratamiento o el tipo de material utilizado (Reverter et al., 2021).

La funcionalidad de los fitogénicos se debe en gran parte por su contenido en metabolitos secundarios de los vegetales de los que se derivan. En particular, los metabolitos secundarios de las plantas tienen papeles funcionales independientes del crecimiento y el desarrollo de la planta; así, éstos protegen a las plantas de los

organismos herbívoros y las plagas, o actúan como quimioatrayentes para los polinizadores (Wink, 2018). Estos compuestos bioactivos se encuentran ampliamente en los extractos de plantas aromáticas, como los aceites esenciales (AE), y suelen estar presentes en forma de mezclas, representadas principalmente por compuestos fenólicos y terpenos que se caracterizan químicamente por sus anillos aromáticos (Christaki et al., 2020). Por lo tanto, sus beneficios como suplementos dietéticos están sujetos a la variabilidad y complejidad de la mezcla de compuestos aromáticos, además de su efecto sinérgico, su origen, el nivel de inclusión en la dieta y su farmacocinética (Figueiredo et al., 2008). Estos compuestos se utilizan por sus reconocidas propiedades promotoras del crecimiento, antimicrobianas, inmunoestimulantes, antioxidantes, antiinflamatorias y sedantes. En particular, los fitogénicos derivados de la familia Lamiaceae, Verbenaceae y Oleaceae se encuentran entre los aditivos de origen vegetal más administrados en acuicultura (Elumalai et al., 2021) y ganadería (Franz et al., 2020; Napoli et al., 2020). Aunque pueden encontrarse en todo el mundo, algunos representantes de este grupo de plantas aromáticas -es decir, la salvia, la hierbaluisa, el olivo, el orégano, el tomillo, la albahaca, la menta, el romero, el ajo y la cebolla, entre otros- están especialmente presentes y se consumen tradicionalmente en el área mediterránea y de Sudamérica, donde son apreciados en términos de nutrición humana (Christaki et al., 2012; Grigoriadou et al., 2020). Las combinaciones de diferentes fitogénicos son también estrategias prometedoras para la formulación de alimentos funcionales debido a sus potenciales efectos sinérgicos. Dentro de la amplia gama de fitogénicos que se han probado en los piensos para acuicultura, esta tesis se centra en la evaluación de un extracto de dos plantas medicinales como la hierbaluisa y la salvia, así también como también en un extracto obtenido de la fruta del olivo. Las propiedades y aplicaciones nutricionales de los distintos compuestos fitogénicos se describen a continuación.

3. Origen botánico de los fitogénicos utilizados en la presente tesis

3.1. Consideraciones previas sobre los fitogénicos: normas de calidad en su preparación

Si bien todos los aditivos derivados de extractos de plantas reciben el nombre de fitogénicos, es difícil establecer un marco comparativo sobre la funcionalidad de estos, ya que todos ellos deben sus propiedades funcionales en base a su origen, proceso de producción y su especificación. Según Länger et al. (2018) y la *European Medicines Agency* (EMA), con relación a su proceso de producción, los extractos de plantas y por consiguiente los fitogénicos se pueden clasificar en:

- **Extractos estandarizados**, los cuales se ajustan a un contenido definido de uno o más constituyentes con actividad terapéutica conocida. Esto se consigue ajustando el extracto con excipientes inertes o mezclando lotes del extracto.

- **Extractos cuantificados**, los cuales se ajustan a uno o más compuestos activos, cuyo contenido se controla dentro de un rango limitado y especificado. Los ajustes se realizan mezclando distintos lotes del extracto.

- **Otros extractos** no se ajustan a un contenido concreto de constituyentes. A efectos de control, se utilizan uno o varios constituyentes como marcadores analíticos.

En la presente tesis, ambos extractos, por un lado, el fitogénico obtenido de la combinación de dos plantas medicinales, tales como la salvia y la hierbaluisa, y por el otro lado, el obtenido del fruto del olivo (la aceituna), integran la categoría de extractos cuantificados, donde ciertos compuestos activos son cuantificados, pero a su vez no son los únicos compuestos presentes en las plantas, lo cual abre un abanico de posibles diferentes efectos en el organismo independientemente de la posible similitud en cuanto a compuestos bioactivos entre aditivos, como es en nuestro caso, donde ambos aditivos comparten ciertos compuestos bioactivos, como

son los triterpenos y polifenoles, sin embargo, no son los únicos presentes en las plantas, como hemos mencionado anteriormente (Länger et al., 2018).

3.2. La salvia (*Salvia officinalis*)

En la medicina tradicional, la *Salvia officinalis* conocida coloquialmente como salvia, goza desde hace tiempo de una gran reputación por sus beneficios para la salud y el tratamiento de todo tipo de dolencias. La salvia es una planta herbácea común perteneciente a la familia Lamiaceae, ampliamente cultivada en diversas partes del mundo, y nativa de la región mediterránea. Es sabido que es muy rica en compuestos fenólicos como flavonoides, taninos, cumarinas (Ghorbani et al., 2017) y triterpenos, que son un grupo muy diverso de componentes naturales que se encuentran ampliamente en una variedad de plantas y frutos (Vincken et al., 2007; Babalola & Shode et al., 2013). Debido al contenido en compuestos funcionales de la salvia, esta planta ha recibido la atención de la industria ganadera y acuícola. Por ejemplo, Simonová et al. (2010) han descrito que la suplementación en la dieta con extracto de salvia (10 µl/animal/día) era eficaz para mejorar el valor energético y la composición de aminoácidos de la carne de conejo, además de promover un buen estado de salud de los animales. Del mismo modo, Placha et al. (2015) han demostrado que el aceite esencial de la salvia incluidos al 0,1 y 0,25 g/kg en la dieta promueve la integridad de la pared duodenal en gallinas ponedoras. En cuanto a las especies acuáticas, Sönmez et al. (2015) han indicado que el aceite esencial de la salvia con tres inclusiones diferentes en la dieta (500, 1,000 y 1,500 mg kg^{-1}) tiene un efecto positivo sobre el crecimiento y las actividades de las enzimas antioxidantes en los juveniles de trucha arco iris (*Oncorhynchus mykiss*). En este sentido, se han aislado compuestos bioactivos de plantas pertenecientes al género *Salvia* como flavonoides (Lu & Foo et al., 2000), y triterpenos pentacíclicos (Masterová et al., 1989), entre otros. Los flavonoides, por ejemplo, son uno de los ingredientes más eficaces de las plantas

medicinales con conocidas propiedades antiinflamatorias (Hämäläinen et al., 2007). Estudios *in vitro*, han demostrado que los flavonoides de varias fuentes vegetales poseen una eficacia respecto a la eliminación de radicales libres y protección contra el estrés oxidativo (Vosoughi et al., 2018). También se ha visto que los flavonoides tienen actividad antiviral contra virus patógenos de peces *in vitro* (Kang et al., 2012), además de mejorar el crecimiento somático, la respuesta antioxidante e inmunitaria, regulando la expresión de los genes relacionados con la inmunidad, aumentando así la resistencia a la enfermedad contra *Aeromonas hydrophila* en los peces cabeza de serpiente (*Channa argus*) (Li et al., 2019). Por otro lado, en cuanto a los ácidos triterpénicos nos referimos, el ácido ursólico (*Figura 2*), el cual es un terpenoide pentacíclico, ha mostrado tener propiedades beneficiosas en la salud humana (Woźniak et al., 2015) e incluso en teleósteos (Ding et al., 2015; Li et al., 2019). En el pez cebra (*Danio rerio*), se ha visto que el ácido ursólico tiene actividad antiinflamatoria (Ding et al., 2015), mientras que en la trucha arco iris se visto que tiene una fuerte actividad antiviral tanto *in vitro* como *in vivo* (Li et al., 2019).

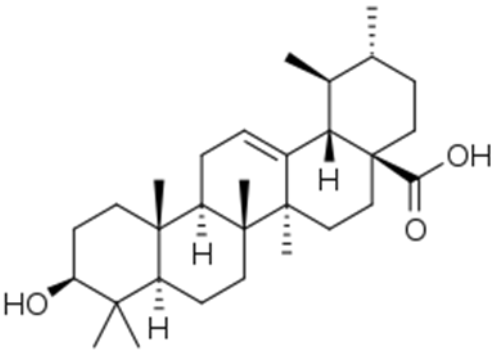


Figura 2. Estructura química del ácido ursólico.

3.3. La hierbaluisa (*Lippia citriodora*)

La *Lippia citriodora*, conocida coloquialmente como hierbaluisa, es una especie vegetal de la familia de las Verbenáceas que crece principalmente en Sudamérica y se cultiva en el

norte de África y el sur de Europa. El extracto de hoja de hierbaluisa contiene polifenoles, incluyendo fenilpropanoides como el verbascósido, iridoides como el gardósido y flavonoides como la luteolina-7-diglucorónido. Entre ellos, el ácido verbascósido (*Figura 3*) es el compuesto más abundante en las hojas de hierbaluisa, por lo que la mayoría de sus efectos beneficiosos se atribuyen a este fitoquímico (Sánchez-Marzo et al., 2019). Hace casi dos décadas, Avila et al. (1999) ya reportaron en la literatura aspectos sobre la bioactividad y fitoquímica de la hierbaluisa, indicando que el ácido verbascósido aislado de la misma y de otras especies del género *Lippia* parecía ejercer una actividad antimicrobiana más potente contra bacterias Gram-positivas que contra bacterias Gram-negativas. En este sentido, varios estudios han indicado que el ácido verbascósido es responsable de múltiples propiedades beneficiosas de la hierbaluisa como, por ejemplo, sus propiedades antioxidantes (Mosca et al., 2014; Martino et al., 2016), antiinflamatorias y antineoplásicas, además de numerosas propiedades cicatrizantes y neuroprotectoras (Funes et al., 2009; Caturla et al., 2011; Alipieva et al., 2014).

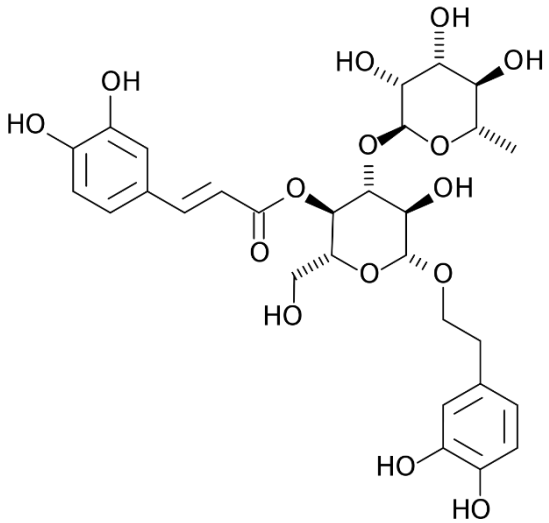


Figura 3. Estructura química del ácido verbascósido.

El verbascósido ha sido descrito ampliamente como antioxidante tanto en estudios *in vitro* como *in vivo*. La actividad antioxidante de los compuestos fenólicos deriva

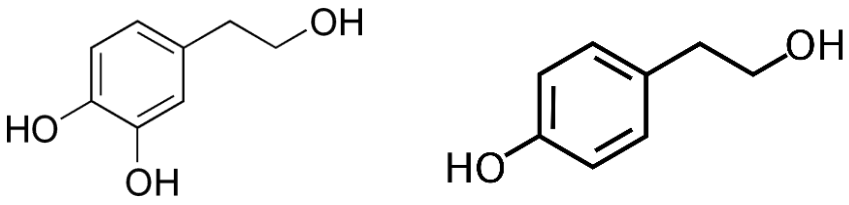
de su capacidad para transferir electrones (Li et al., 2016). La capacidad captadora de radicales libres del verbascósido protege a las biomoléculas, como lípidos y ADN, del daño oxidativo. Se ha comprobado que el verbascósido presente en un extracto de hierbaluisa es capaz de inhibir la peroxidación lipídica mitocondrial. Se ha demostrado que cuando el extracto de la hierbaluisa que contenía un 25% de verbascósido en una dosis de 2000 mg/kg administrado por vía oral en ratas se obtenía una alta correlación entre la concentración máxima de este compuesto, en este caso el verbascósido circulante y la máxima capacidad antioxidante en tejidos, sin mostrar toxicidad en los animales (Funes et al., 2009). También se ha visto en un estudio *in vitro* que la adición de extracto de verbascósido de 1 nM en blastocistos, utilizado en un modelo de ovejas jóvenes favorece el desarrollo del embrión al proteger el ovocito contra el estrés oxidativo (Martino et al., 2016). Además, otro estudio demostró que cerdos alimentados con una dieta enriquecida con aceites esenciales de verbascósido con 5 mg/kg en la dieta, influyeron positivamente en el estado antioxidante como también en una mejora en sus rendimientos de crecimiento y eficiencia alimentaria (Pastorelli et al., 2012). A pesar de la literatura existente, la información sobre la función de estos compuestos bioactivos derivados de la salvia y la hierbaluisa, es todavía escasa en lo que respecta a sus aplicaciones en la producción de organismos acuáticos, y especialmente sobre sus efectos inmunomoduladores y su potencial uso como aditivo zootécnico para piensos con el fin de promover la salud y la resistencia a las enfermedades en los peces.

3.4. El fruto del olivo, la aceituna

El olivo tal como lo conocemos (*Olea europaea* L.) pertenece al género *Olea*, de la familia Oleaceae. Se asume que es un árbol originario de la región sirio-palestina, aunque también se ha sugerido que procede del cruce de especies próximas: *Olea africana*, originaria de Arabia y Egipto; *Olea ferruginosa*, procedente del área asiática, y *Olea laperrini*, de

las montañas del sur de Marruecos. Otros autores consideran que procede del acebuche (*Olea europaea* ssp. *europaea* var. *sylvestris*), conocido desde el Neolítico (Fogher et al., 2010).

Durante muchos siglos, los extractos de hoja y el fruto del olivo conocido como aceituna han sido asociados con beneficios para la salud. En la aceituna, los principales compuestos fenólicos presentes son ácidos fenólicos, alcoholes fenólicos, flavonoides, lignanos y secoiridoides. El 3,4-dihidroxifeniletanol (hidroxitirosol) y el p-hidroxifeniletanol (tirosol) son los alcoholes fenólicos más abundantes en las aceitunas (Ghanbari et al., 2012) (*Figura 4*). El hidroxitirosol es uno de los componentes hidroxiaromáticos de los secoiridoides. Es un ortodifenol alcohólico muy bioactivo. En varios estudios específicos, se ha demostrado que el hidroxitirosol es antioxidante y antimicrobiano (De Leonardis et al., 2007), y que tiene efectos beneficiosos en el sistema cardiovascular y en varias enfermedades humanas (De Leonardis et al., 2008). Por otro lado, el tirosol, también es conocido como uno de los principales representantes de los compuestos fenólicos simples en las hojas de olivo y en las aceitunas (Özcan et al., 2017). Se ha demostrado que el tirosol es capaz de impedir la generación de especies reactivas de oxígeno por parte de los leucocitos, y más importante aún, sin demostrar evidencia de toxicidad (Özcan et al., 2017).



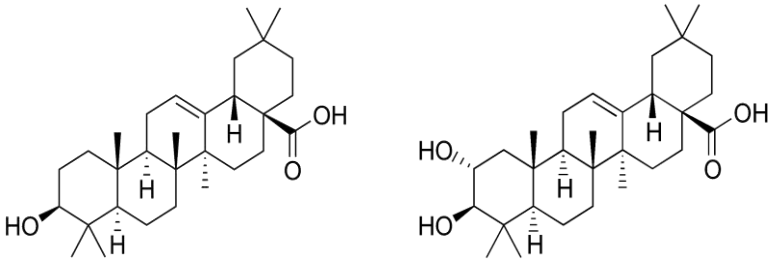
Hidroxitirosol Tirosol

Figura 4. Hidroxitirosol y tirosol, los dos alcoholes más abundantes en la aceituna.

Por otro lado, se encuentran los triterpenos pentacíclicos, los cuales son metabolitos secundarios ampliamente distribuidos en el reino vegetal, que destacan por su gran variedad estructural. Los ácidos, ursólico, oleanólico y betulínico, y los alcoholes α-

amirina, β-amirina y lupeol, son los triterpenos pentacíclicos con mayor presencia en las plantas (Peng et al., 2014). La utilización de plantas conteniendo triterpenos ha sido práctica habitual desde tiempos inmemoriales en la medicina popular, especialmente la asiática. Se han descrito más de 200 especies vegetales portadoras de cantidades relevantes de compuestos triterpénicos (Bianchi et al., 1994), pero pocas de ellas representan un cultivo socio-económicamente tan significativo como el del olivo.

Actualmente, los ácidos oleanólico y maslínico, (*Figura 5*) y sus análogos estructurales, se encuentran en el punto de mira de múltiples estudios por su actividad biológica multifactorial con efectos favorables en la salud humana, y están siendo considerados como productos naturales que pueden sustituir o complementar la acción de fármacos convencionales en el tratamiento de muy diversas patologías (Blanco-Cabra et al., 2019).



Ácido oleanólico Ácido maslínico

Figura 5. Triterpenos pentacíclicos en la aceituna.

Además, la aceituna y la hoja del olivo han sido largamente conocidos por contener una amplia variedad de esteroles y triterpenoides pentacílicos en su epidermis (Vázquez-Roncero & Janer, 1969). En partes de la planta, los ácidos triterpénicos se encuentran en forma de ácidos libres, mientras que los triterpenoles pentacíclicos pueden encontrarse libres o esterificados con ácidos grasos (Pérez-Camino & Cert, 1999). El análisis de las ceras de la cutícula de la aceituna ha demostrado que los triterpenoides de tipo oleanano son, con mucho, los predominantes (Bianchi et al.,

1994). El ácido maslínico puede llegar a representar hasta el 80% de las ceras de la piel de este fruto (Reyes et al., 2006).

Resultados recientes han demostrado que extractos del fruto del olivo gozan de propiedades antiinflamatorias, antioxidantes y antimicrobianas, indicando así que los fitogénicos derivados del olivo tienen efectos beneficiosos para la salud humana (Caramia et al., 2012; Gorzynik-Debicka et al., 2018; George et al., 2019) y del ganado (Morrison et al., 2017; Liehr et al., 2017; Cangiano et al., 2019). Sin embargo, se dispone de poca información sobre sus efectos en los peces de crianza (Gisbert et al., 2017). En cerdos (Liehr et al., 2017) y peces (Gisbert et al., 2017), un extracto bioactivo de aceite de oliva, que contiene una mezcla de ácido triterpénico y polifenoles, produciendo así una respuesta antiinflamatoria e inmunomoduladora en el intestino, mejorando al mismo tiempo la integridad del epitelio. Además, otro estudio reciente demostró que estos compuestos fueron capaces de reducir la inflamación sistémica en vacunos recién destetados (Cangiano et al., 2019). A pesar de estos resultados, se sabe poco sobre los efectos inmunomoduladores del extracto bioactivo de la aceituna en la respuesta inmunitaria y sobre su posible uso como aditivo funcional en piensos para promover la salud y resistencia a las enfermedades en los peces. Lo cual lo sitúa como un perfecto candidato a ser estudiado en son de proveer más información sobre sus posibles propiedades inmunomoduladoras para ser utilizado en el sector acuícola.

4. El sistema inmunitario de los teleósteos como objetivo de los aditivos funcionales

Las enfermedades de los peces se consideran una de las principales amenazas persistentes para la acuicultura, que representa una pérdida anual estimada de US$6 mil millones a nivel mundial. Si bien en los últimos años se ha producido una drástica reducción en el uso de antimicrobianos debido a la vacunación y la mejora de prácticas de cultivo, el uso de antibióticos sigue siendo una práctica común en algunos países para evitar y mitigar las posibles pérdidas productivas y económicas

derivadas de enfermedades (Stentiford et al., 2017). Es ahí, donde entran a jugar un papel de suma importancia los aditivos funcionales con propiedades inmunomodultorias, y dentro de ese abanico de posibilidades, los fitogénicos se presentan como herramientas profilácticas sostenibles para mejorar la gestión de la salud de los peces en las piscifactorías (Reverter et al., 2020; 2021).

Los mecanismos de inmunidad existen en múltiples y variadas formas a lo largo de la evolución, desde los organismos pluricelulares más primitivos hasta los organismos superiores. Las células fagocíticas y algunas moléculas en circulación son parte importante de la inmunidad innata que aparece temprano y se conserva en especies superiores Los peces no mandibulados presentan sólo inmunidad innata. A partir de los teleósteos aparece un nuevo sistema de defensa mucho más complicado, la inmunidad adaptativa, que incluye mayor número de células participantes y una gran diversidad de ligandos y receptores celulares. La inmunidad innata provee la primera línea de defensa y es responsable por la eliminación de la mayor parte de microorganismos infectantes. Esta respuesta de contención da tiempo a que se desarrolle la inmunidad adaptativa, que a su vez aumenta y potencia los mecanismos de inmunidad innata, haciendo más eficiente la respuesta inmunológica (Secombes & Ellis, 2012). La hipótesis actual sobre la evolución del sistema inmunitario es que los organismos unicelulares primitivos ya eran capaces de identificar sustancias tóxicas y otros microorganismos que querían invadirlos, además estos organismos primitivos también eran capaces de defenderse contra ellos. Actualmente las bacterias y protozoos siguen mostrando esta primera línea de defensa que forma la base de la inmunidad innata o inespecífica. Este sistema está presente en plantas y animales, y es capaz de reconocer partes comunes de los patógenos más frecuentes sin haber estado expuesto nunca al patógeno (Castro & Tafalla, 2015).

El gran cambio en este sistema de defensa tuvo lugar en los peces óseos y cartilaginosos, con la aparición de un segundo tipo de inmunidad basado en las células blancas o leucocitos (del griego *leucos* = blanco y *citos* = células). Estas células son capaces de reconocer partes concretas de los patógenos llamados antígenos,

activarse en su presencia y formar una memoria inmunológica contra este antígeno concreto durante un tiempo más o menos largo. Este sistema es el sistema adaptativo o específico (Secombes & Ellis, 2012; Rauta et al., 2012).

Por lo tanto, en los peces óseos el sistema inmunitario se divide en inmunidad innata o inespecífica e inmunidad adaptativa o específica. Cada una de ellas tiene dos componentes, la parte humoral y la parte mediada por células. En este apartado de la tesis vamos a explicar a continuación las características principales de cada una, de manera resumida (*Figura 6*), así como su vinculación con los trabajos desarrollados.

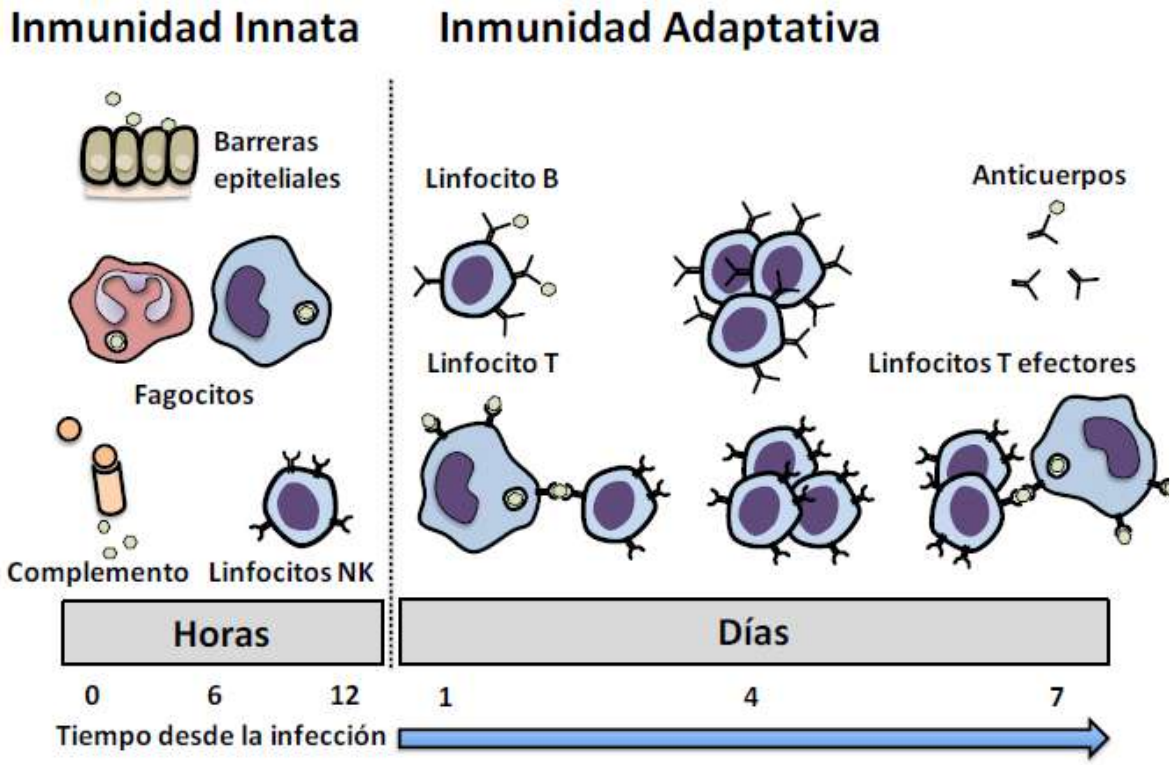


Figura 6. Representación gráfica del desencadenamiento de una respuesta clásica inmunitaria en peces en ambos tipos de inmunidad, innata y adaptativa. **Fuente:** (Toche, 2012).

4.1. La respuesta inmune innata

El sistema inmunitario innato es arma fundamental de defensa de los invertebrados y un mecanismo de defensa primordial de peces. La inmunidad innata es la primera

línea de defensa contra las infecciones en vertebrados. Las células responsables de esta respuesta poseen receptores codificados en línea germinal, que no están sujetos a recombinación genética, y que reconocen estructuras muy conservadas, denominadas patrones moleculares asociados a patógenos (del inglés *pathogen associated molecular patterns*, PAMP). Este reconocimiento se realiza mediante receptores de reconocimiento de patrones (PRR, por sus siglas en inglés: *pattern recognition receptors*) que no requieren de exposición previa a los PAMPs para interaccionar con ellos y generar una respuesta ante las glicoproteínas bacterianas y fúngicas y los lipopolisacáridos, así como los componentes intracelulares liberados en caso de lesión o infección. El sistema inmunitario innato se divide en barreras físicas, componentes celulares y humorales. Los parámetros humorales incluyen inhibidores del crecimiento, varias enzimas líticas y componentes de las vías del complemento, aglutininas y precipitinas (opsoninas, principalmente lectinas), anticuerpos naturales, citocinas, quimiocinas y péptidos antibacterianos. Varios factores externos e internos pueden influir en la actividad de los parámetros inmunitarios innatos. Los cambios de temperatura, la manipulación y el estrés por hacinamiento pueden tener efectos negativos sobre los parámetros innatos, mientras que varios aditivos funcionales pueden potenciar diferentes factores innatos (Secombes & Wang, 2012).

4.2. La respuesta inmune adaptativa

En los peces teleósteos, las respuestas inmunitarias adaptativas están mediadas por los linfocitos (células T y células B) y los leucocitos, células que tienen receptores especiales en su superficie para detectar moléculas extrañas. Cuando estos receptores se unen a su ligando (o antígeno), se desencadena una proliferación de las células que sufren una expansión clonal dando lugar a una población más grande capaz de luchar contra cualquier posible agente infeccioso. Además, algunas de las células permanecen a largo plazo como células de memoria, de modo que un contacto posterior con el mismo antígeno puede provocar una respuesta

inmunitaria más rápida, específica y eficaz, lo que conduce a la protección. Los receptores de antígenos de las células T y B se forman a partir de genes diferentes y tienen una estructura distinta, aunque el mecanismo genético que genera la diversidad de receptores es similar. Curiosamente, el receptor de antígeno de las células T (TCR) en la mayoría de las células T requiere la presentación del antígeno, en forma de péptidos procesados de la proteína original, y entregados por las moléculas del complejo mayor de histocompatibilidad (MHC). Los linfocitos B, en cambio, pueden reconocer antígenos solubles y unirse a ellos directamente a través de su receptor de antígenos de células B (BCR). Además, los linfocitos B pueden producir una forma soluble de este receptor que se segrega como anticuerpo o inmunoglobulina (Ig) que puede actuar directamente contra el patógeno (anticuerpo neutralizante) o ayudar a la internalización de un patógeno por un fagocito (anticuerpo opsonizante) (Secombes & Belmonte, 2016).

4.3. Diferentes tejidos inmunes como objetivo de los aditivos funcionales

La carencia de médula ósea y de ganglios linfáticos es una de las principales características del sistema inmune en peces teleósteos. Es por ello que resulta difícil distinguir una clara diferencia entre órganos hematopoyéticos y órganos linfoides primarios y secundarios. En consecuencia, se ha dado la denominación de órganos linfo-hemato-poyéticos, siendo cuatro los principales. El timo es un órgano de diferenciación y selección de linfocitos T. El riñón anterior o riñón cefálico contiene un gran número de macrófagos y de linfocitos B, y debido a su gran capacidad hematopoyética se le considera como un análogo de la médula ósea de los mamíferos. El bazo tiene funciones semejantes a las del riñón anterior con énfasis en la presentación de antígenos y la inducción de la respuesta inmune adaptativa. En el riñón cefálico y en el bazo se pueden encontrar también agregados de macrófagos, conocidos como centros melanomacrófagos (MMC), que se forman debido a lesiones inflamatorias crónicas. El tejido linfoide asociado a mucosas (MALT, por sus siglas en inglés: *mucosa-associated lymphoid tissue*) está conformado

por linfocitos de diferente tamaño, células plasmáticas, macrófagos, algunos tipos de granulocitos, células positivas al ácido periódico de Schiff (PAS, por sus siglas en inglés: *periodic acid-Schiff*) y eosinófilos. En los teleósteos, las células intraepiteliales del intestino son el equivalente a los linfocitos T de los mamíferos, mientras que las áreas linfoides en la lámina propia contienen principalmente linfocitos B (*Figura 7*).

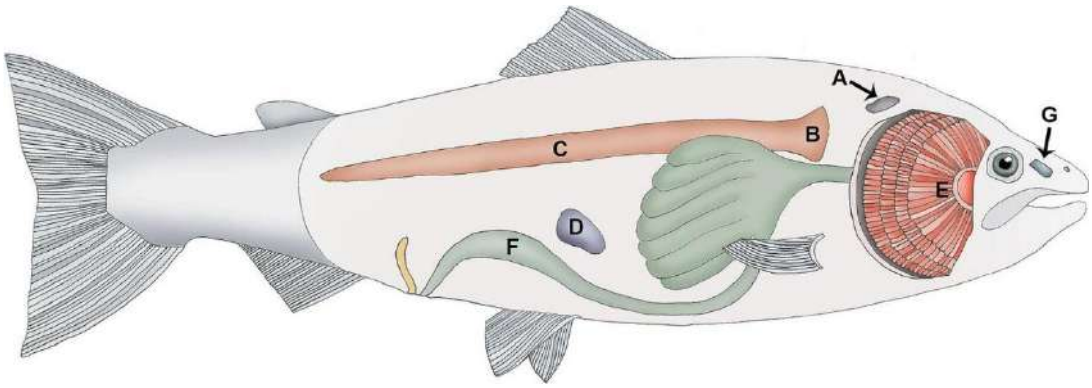


Figura 7. Localización de los principales órganos linfoides en los peces teleósteos. **(A)** Timo; **(B)** riñón cefálico; **(C)** riñón del tronco; **(D)** bazo; **(E)** branquias; **(F)** intestino; **(G)** órgano olfativo. **Fuente:** (Bjørgen & Koppang, 2021).

A continuación, se presenta una pequeña revisión más detallada de cada uno de los tejidos diana utilizados para la evaluación de los aditivos basados en fitogénicos en base a los trabajos realizados dentro de la presente tesis doctoral. Todo esto, teniendo en cuenta la funcionalidad del tejido, las características morfológicas básicas, el papel desempeñado en la defensa del organismo, y el potencial como objetivos de las estrategias nutricionales.

4.3.1. El bazo

El bazo de los mamíferos es el mayor órgano inmunitario secundario del cuerpo. Además de ser el responsable de iniciar las reacciones inmunitarias a los antígenos transmitidos por la sangre, de filtrar la sangre de material extraño y de glóbulos rojos viejos o dañados. Estas funciones las llevan a cabo los dos compartimentos

principales del bazo, la pulpa blanca (incluida la zona marginal) y la pulpa roja, que son muy diferentes en su arquitectura, organización vascular y composición celular (Cesta, 2006). En los peces, el bazo contiene los mismos elementos que los demás vertebrados: vasos sanguíneos, elipsoides, pulpa roja y pulpa blanca. Sin embargo, la pulpa roja y blanca en los peces está menos claramente definida que en los vertebrados homeotermos. La pulpa ocupa la mayor parte del órgano y consiste en una red celular reticular que soporta sinusoides llenos de sangre que albergan diversas poblaciones celulares, incluidos macrófagos y linfocitos. La pulpa blanca suele estar poco desarrollada y suele tener dos componentes principales: los acúmulos de melano-macrófagos y los elipsoides. El bazo de los peces cumple con un papel clave en los procesos de presentación de antígenos y activación de linfocitos, promoviendo la inmunidad humoral, mediante la coordinación celular de las células dendríticas (DC) y con la inducción específica de la proliferación de células T (Lugo-Villarino et al., 2010; Neely & Flajnik, 2016). Además, en salmónidos como la trucha arco iris, ya se ha descrito que los peces alimentados con dietas funcionales fueron capaces de regular el perfil inflamatorio en el bazo, reduciendo las posibles respuestas perjudiciales tras una incubación con lipopolisacárido (LPS) (Djordjevic et al., 2009). Esto podría deberse a que las células presentadoras de antígenos (APC) en el bazo polarizarían las células T hacia fenotipos reguladores, que son importantes para controlar las respuestas inmunitarias y en el mantenimiento de la homeostasis de los peces (Morales-Lange et al., 2021). A modo particular, el efecto de los compuestos fitogénicos sobre la respuesta inmunitaria a nivel del bazo ha sido explorada en el Capítulo I de la presente tesis doctoral.

4.3.2. El intestino

El tracto gastrointestinal de los vertebrados es un órgano multifuncional que desempeña muchas funciones fisiológicas importantes y diversas, como la digestión, la lubricación del quimo, la absorción de nutrientes, la osmorregulación, el metabolismo del nitrógeno, la regulación del apetito y la defensa (Ray & Ringø,

2014). El epitelio intestinal de los peces está formado por una sola capa de células epiteliales columnares, llamadas enterocitos. La membrana apical de los enterocitos se caracteriza por la presencia de microvellosidades, que forman un borde en cepillo que contribuye a casi el 90% de la superficie total del intestino, dependiendo de la zona del intestino y de la especie de pez. Las células epiteliales también son responsables de la actividad enzimática en la membrana del borde en cepillo.

El tracto gastrointestinal de los peces se enfrenta continuamente a antígenos alimentarios, así como también a bacterias, virus, parásitos y toxinas. Por ello, las células epiteliales del intestino están en continua renovación, además de estar protegidas por una capa de moco que crea una barrera física y química contra las amenazas externas. Las células caliciformes son el tipo de célula mucosa dominante en el epitelio del intestino de los peces, y son las responsables de la secreción de moco. Las principales moléculas que se encuentran en el moco son las mucinas, que desempeñan un papel importante en el mantenimiento de la barrera epitelial contra los patógenos. La mucosa intestinal también está implicada en la captación de nutrientes y la digestión, o incluso en la captación de oxígeno en el caso de los peces que respiran aire. Así, la mucosa intestinal es una barrera semipermeable que permite la captación de macromoléculas necesarias para la nutrición, pero que actúa al mismo tiempo como una barrera eficaz contra las partículas y los microorganismos. Además, esta capa de moco actúa como un importante mecanismo de defensa innata que mantiene la homeostasis del tejido de la mucosa, además de la microbiota comensal (Gomez et al., 2013).

Entre las diversas funciones del intestino, la defensa es una de las más importantes, ya que el intestino es una de las barreras físicas que los patógenos encuentran y tienen que superar para invadir el organismo. El tracto gastrointestinal de los peces contiene un tejido linfoide asociado al intestino (GALT, por sus siglas en inglés *gut-associated lymphoid tissue*) que tiene la notable capacidad de diferenciar entre el material beneficioso y el potencialmente peligroso, promoviendo respuestas inmunitarias protectoras contra los microorganismos y toxinas perjudiciales, al

mismo tiempo que acepta los antígenos alimentarios y la microbiota comensal (Salinas & Parra, 2015).

Al igual que los vertebrados superiores, el intestino de los peces también se caracteriza por tener sistemas neurales y endocrinos. La mayoría de las actividades que intervienen en el control fisiológico de la función gastrointestinal durante los periodos de alimentación o ayuno están mediadas por sistemas neuroendocrinos, que desempeñan un papel importante en la regulación general de la digestión. Las hormonas gastrointestinales son sintetizadas y secretadas por células enteroendocrinas distribuidas por todo el tracto intestinal. Este sistema neuroendocrino también puede interactuar con el sistema inmunitario local, en el que los cambios en la secreción de hormonas, como el cortisol, y a su vez podrían modular las respuestas inmunitarias del intestino, la motilidad intestinal y su microbioma (Webster et al., 2019; Serna-Duque & Esteban, 2020).

Este MALT es de especial relevancia para la industria de la acuicultura, ya que los peces de cultivo se alimentan generalmente con piensos comerciales, lo que permite manipular la salud de los peces a través de piensos funcionales (Rombout et al., 2011; Salinas & Parra, 2015). A pesar de su intrincado sistema inmunitario, el tejido intestinal se convirtió en un objetivo atractivo para los alimentos funcionales en la acuicultura. Sin embargo, las reglas que rigen la inmunidad local son muy diferentes de las que rigen la inmunidad sistémica. Por ello, la aplicación de inmunoterapias eficaces las especies acuícolas requiere un conocimiento profundo del sistema inmunitario de los peces. En el contexto de la presente tesis doctoral, el efecto de los compuestos fitogénicos sobre la respuesta inmunitaria a nivel de intestino ha sido explorada en el Capítulo II

4.3.3. El riñón cefálico

El riñón cefálico, es un órgano análogo a la glándula suprarrenal de los mamíferos, un importante órgano endocrino y hematopoyético-linfoide en los peces teleósteos.

El riñón cefálico carece de la estructura clara de su homólogo en los mamíferos, ya que no se puede distinguir una corteza y una médula zonales. En su lugar, las células interrenales productoras de cortisol y las células cromafines productoras de catecolamina están incrustadas y rodeadas de tejido hematopoyético productor de anticuerpos y citocinas (Geven, 2017). Además de desempeñar un papel central en la organización de la respuesta al estrés que implica una estrecha comunicación entre los sistemas reguladores. Esto ayuda a explicar por qué el estrés se caracteriza por una participación central y periférica de células inmunitarias y neuroendocrinas y sus respectivos mensajeros (Tort, 2011).

En el riñón cefálico se generan las células pluripotenciales a partir de células troncales y de ahí, dos líneas principales: la linfoide y la mieloide. De ellas, la gran diversidad celular que tiene implicación directa e indirecta con el sistema inmunitario. En específico, los leucocitos o células blancas de la sangre desempeñan un papel principal en la defensa del hospedero ante infecciones, y su actividad y composición cambia drásticamente en respuesta a la infección (Secombes, 2001), y por ello, este órgano es considerado un excelente modelo para estudiar los efectos a nivel de inmunidad sistémica (Tort, 2011). A modo particular, el efecto de los compuestos fitogénicos de la presente tesis sobre la respuesta inmunitaria a nivel de riñón cefálico ha sido explorada en los Capítulos III y IV de la presente tesis doctoral.

5. Herramientas para evaluar los aditivos funcionales

Si bien, numerosos estudios *in vivo* han demostrado una mejora de las respuestas inmunitarias de los peces tras la administración de piensos funcionales, la información actual sobre los efectos inherentes de los aditivos para piensos en la inmunidad es limitada en condiciones *in vitro*. De hecho, la mayoría de los estudios sobre aditivos funcionales para piensos, en particular los fitogénicos, se centran exclusivamente en las respuestas fisiológicas o bioquímicas y en los desafíos bajo

condiciones experimentales controladas. Siendo muy pocos los que realmente intentan dilucidar los mecanismos celulares y moleculares que subyacen a su capacidad inmunoestimuladora de un compuesto (Firmino et al., 2021c).

Sin embargo, en los últimos años se ha dispuesto de una serie de nuevas herramientas celulares y moleculares que permiten una comprensión más profunda y amplia de esos mecanismos. Estos datos, combinados con los enfoques clásicos, como los estudios histológicos, los parámetros inmunitarios humorales y los desafíos bióticos o abióticos, pueden proporcionar información útil para comprender adecuadamente el modo de acción de los aditivos funcionales para piensos, un enfoque que contextualiza las respuestas de los órganos y los tejidos a nivel celular y de expresión génica. En este sentido, las herramientas ómicas pueden proporcionar respuestas significativas para caracterizar las complejas respuestas inmunitarias de los teleósteos a la administración de fitogénicos (Salinas & Magadan, 2017; Natnan et al., 2021). Las tecnologías ómicas modernas incluyen la genómica, la proteómica, la transcriptómica, y la metabolómica, entre otras. Los estudios transcriptómicos en acuicultura han progresado gradualmente desde el enfoque tradicional del análisis de la expresión de un solo gen hasta las técnicas más recientes de secuenciación de alto rendimiento, incluyendo los microarrays y la secuenciación del transcriptoma (RNA-seq) (Martin & Król, 2017). En la actualidad, los análisis de redes funcionales basados en el transcriptoma de los peces teleósteos alimentados con piensos funcionales han ganado mucha atención, ya que proporcionan una mayor comprensión del modo de acción de los aditivos zootécnicos con propiedades inmunomoduladoras en el huésped, tal y como han puesto de manifiesto distintos trabajos de nuestro grupo (Firmino et al., 2020; 2021a; 2021b; Reyes-López et al., 2021). Para obtener un mayor conocimiento biológico de la regulación transcripcional que podría ser modulada por las dietas funcionales, se pueden realizar varios análisis, como: a) determinar el enriquecimiento de funciones biológicas conocidas, interacciones o vías; b) identificar la participación de los genes en vías o redes agrupando los genes en función de tendencias similares; y c) utilizar los cambios globales en la expresión de los genes, como por ejemplo

visualizando todos los genes significativamente regulados al alza o a la baja en el contexto del escenario experimental. Para ello, en los últimos años se han puesto a disposición varias herramientas bioinformáticas para el análisis funcional de los datos transcriptómicos de los genes (Shi et al., 2015).

La nutrigenómica, en particular, no sólo ha mejorado la comprensión de los marcadores biológicos de las enfermedades relacionadas con la nutrición, sino que también ha mejorado el desarrollo de aditivos alimentarios (Martin & Król, 2017). Este útil enfoque permite obtener un conocimiento exhaustivo de los diferentes componentes inmunitarios humorales y celulares presentes en los peces, así como su regulación tras diferentes estímulos, incluyendo infecciones naturales o experimentales, y/o diferentes estresores bióticos o abióticos. En general y en ámbito de la acuicultura, las herramientas ómicas funcionales proporcionan aplicaciones multifacéticas que van desde la monitorización de la fisiología del huésped, hasta la optimización de las formulaciones de los piensos y la evaluación a mayor profundidad de los aditivos para la acuicultura (Dawood et al., 2020). En esta tesis, se seleccionó el enfoque de microarrays como base central para proporcionar información sobre el perfil transcriptómico relacionado con la inmunidad local y sistémica de los tejidos de interés (Capítulos II, III y IV). Además de ello, se evaluó la respuesta inmunitaria sistémica mediante un ensayo *ex vivo*, donde en la expresión génica de un repertorio de marcadores de genes inmunitarios fueron analizados vía PCR cuantitativa (qRT-PCR, por sus siglas en inglés, *quantitative real time polymerase chain reaction*) (Capítulo I).

Además, la combinación de herramientas ómicas y otros análisis complementarios, como la histoquímica, la evaluación de parámetros inmunitarios humorales en plasma, la medición de la actividad enzimática, los desafíos con patógenos (retos bacterianos *in vivo* y/o modelos *ex vivo*), permite contextualizar las respuestas fisiológicas e inmunológicas que pueden obtenerse a través de los análisis sobre los mecanismos que subyacen a las propiedades inmunomoduladoras de los aditivos para piensos que se están desarrollando con fines comerciales. Estas

aproximaciones han sido empleadas en los Capítulos I, II, III y IV, con el fin de validar y caracterizar el efecto de los compuestos fitogénicos objeto de interés en esta tesis doctoral.

6. Especies modelo usadas en la presente tesis

Con el fin de evaluar el potencial efecto promotor de la salud de los compuestos fitogénicos objeto de estudio de la presente tesis, éstos han sido evaluados en dos especies de peces de crianza de gran importancia económica a escala europea y mundial, como son la dorada (*Sparus aurata*) y el salmón del Atlántico (*Salmo salar*), respectivamente. En este contexto, evaluar aditivos zootécnicos con propiedades inmunomodulatorias en especies acuícolas importantes, tanto desde un punto de vista de producción como de valor económico, supone una gran ventaja frente a otros modelos existentes como el pez cebra, pues posiciona el producto más cerca del mercado, facilitando y acelerando la transferencia de los resultados a la industria.

6.1. El cultivo de la dorada (*Sparus aurata*)

6.1.1. Relevancia de la especie

La dorada (*Sparus aurata*) (*Figura 8*) se encuentra comúnmente en todo el mar Mediterráneo, aunque es menos frecuente en las regiones del este y sureste del Mediterráneo y muy rara en el mar Negro. También está presente en el Océano Atlántico desde las islas británicas hasta Cabo Verde y alrededor de las Islas Canarias.

Figura 8. Dorada (*Sparus aurata*).

Es un pez bentopelágico que se encuentra de forma natural en entornos costeros, habitando en lechos de praderas de fanerógamas marinas, fondos rocosos y en la zona de oleaje hasta profundidades de unos 30 m. Los adultos pueden encontrarse hasta 150 m de profundidad. La dorada es una especie eurihalina, que suele adentrarse en aguas salobres donde se producen los cambios regulares de salinidad. Es un pez carnívoro, pero casualmente herbívoro, que puede ser sedentario, solitario o formar pequeños grupos. En cuanto a su biología reproductiva, esta especie es un hermafrodita protándrico, que madura como machos funcionales en los dos primeros años (20-30 cm) y posteriormente se convierten en hembras (33-40 cm). El desove se produce de forma natural de diciembre a abril, cuando la temperatura del agua es de 13-17 ^{o}C (Basurco et al., 2011).

La dorada es una especie relevante para la acuicultura, representando una de las principales especies cultivadas en el área del Mediterráneo, tanto a nivel de volumen de producción como en valor económico. Se cría principalmente de forma intensiva en jaulas marinas, y ocasionalmente en estanques en tierra en casi todos los países mediterráneos. Los principales países productores de dorada en Europa son Turquía, con 85.000 toneladas (que representan el 33,7% de la producción total), Grecia, con 65.300 toneladas (25,9%), Egipto, con 36.000 toneladas (14,3%), Túnez, con 16.000 toneladas (6,3%), y España, con 13.521 toneladas (5,4%). También se cultiva en Italia, Chipre y Croacia. (*Figura 9*). El periodo de cultivo varía en función de la ubicación y la temperatura del agua, pero normalmente se necesitan entre 18 y 24 meses para que un ejemplar alcance los 400 g a partir de las larvas nacidas. El tamaño comercial puede variar desde 250 g hasta más de 2,0 kg, dependiendo del mercado de destino y de las preferencias de los consumidores (APROMAR, 2020).

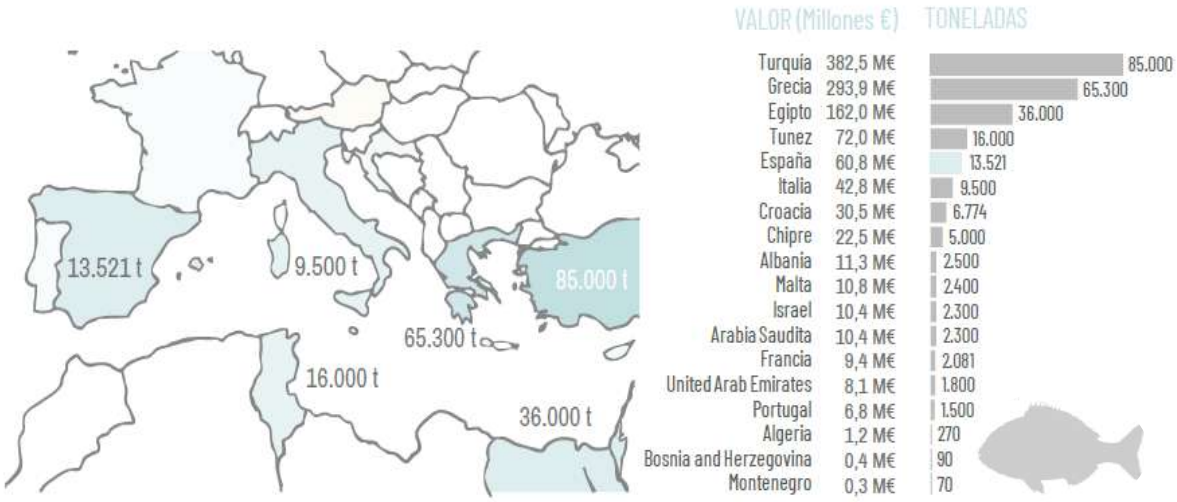


Figura 9. Distribución de la producción de dorada (*Sparus aurata*) de acuicultura en el área mediterránea en 2019 en volumen (toneladas) y valor (millones de euros). Adaptado de la FAO, 2020 (APROMAR, 2020).

6.1.2. Enfermedades predominantes que afectan a la dorada de piscifactoría

Según un estudio reciente realizado por Muniesa et al. (2020) en el que se realizó una encuesta en la que participaron un total de 50 unidades de producción (31 con criadero, 16 con *hatchery* o vivero y tres con plantas de procesado) de 27 empresas, situadas en 10 países mediterráneos (Croacia, Chipre, Egipto, Francia, Grecia, Italia, Portugal, España, Túnez y Turquía), las enfermedades más importantes que afectan al sector acuícola dedicado al cultivo de la dorada son:

- Una de las principales enfermedades que afecta la dorada durante su fase de engorde es la causada por el ectoparásito monogéneo de las branquias, *Sparicotyle chrysophrii*, causando mortalidades de hasta el 30% y con la mayor prevalencia ocurriendo a finales de la primavera y el verano. Los expertos han calificado la esparicotilosis como la enfermedad más importante que afecta específicamente a la dorada. La infección por *S. chrysophrii* provoca el letargo de los peces debido a la hipoxia y la anemia grave (Sitjà-Bobadilla et al., 2006). En los peces infectados por

S. chrysophrii también es frecuente encontrar infecciones secundarias por otros parásitos y bacterias (Padrós et al., 1995). En consecuencia, la esparicotilosis puede

llegar a reducir la tasa de crecimiento, aumentar el factor de conversión alimenticia total (FCA), incrementando la necesidad de alimento para > 50.000 toneladas en la producción mediterránea (Rigos et al., 2016). La enfermedad también hace que los peces sean más vulnerables a la manipulación y a los factores de estrés ambiental, y puede ser la causa de las mortalidades notificadas en los registros estadísticos en la categoría "otras causas".

- El "Síndrome de Invierno" o "Enfermedad de Invierno" es otra de las enfermedades frecuentemente reportadas en esta etapa de producción, afectando asiduamente a la dorada durante el periodo de bajas temperaturas del agua, periodo comprendido entre enero y mayo (Muniesa et al., 2020). Además de estas enfermedades, también se encuentra el patógeno *Pseudomonas anguilliseptica*, que es uno de los principales agentes responsables de los brotes infecciosos asociados a la "Enfermedad de Invierno", siendo considerado un patógeno más oportunista, cuyas infecciones suelen producirse cuando los peces están bajo estrés ambiental e inmunosupresión (Ibarz, et al., 2010), pudiendo alcanzar unas tasas de mortalidad entre el 10-15% en diferentes explotaciones de la Península Ibérica (con un pico del 30%) entre enero (12 °C) y abril (18-20 °C) (Cascarano et al., 2021).

- En cuanto a las primeras etapas de desarrollo, las infecciones bacterianas asociadas principalmente a las bacterias Gram negativas del género *Vibrio* spp. y la infección por betanodavirus son las más comunes, afectando larvas, alevines y juveniles de dorada, llegando a porcentajes de un 40% de mortalidad (Savoca et al., 2021).

- Por otro lado, otra enfermedad que cabe destacar en esta especie, y ésta siendo una de las más importantes que afectan al cultivo de dorada, es la conocida como pasteurelosis. Está ocasionada por la bacteria *Photobacterium damselae* subespecie *piscicida* (anteriormente clasificada como *Pasteurella piscicida*, de ahí el nombre de la enfermedad) (Magariños et al., 1994). Se produce con temperaturas del agua superiores a 18 ºC, y puede cursar como enfermedad aguda, con una mortalidad de

hasta un 40% (Cascarano et al., 2021), o como una enfermedad crónica con una mortalidad en goteo. Externamente no presenta síntomas diferenciales, y a nivel interno sólo se puede observar una inflamación del bazo, que a veces muestra gránulos blanquecinos. Así se diagnostica mediante aislamiento de la bacteria.

6.2. El cultivo del salmón del Atlántico (*Salmo salar*)

6.2.1. Relevancia de la especie

El salmón del Atlántico (*Figura 10*) es una especie carnívora, eurihalina anádroma, que se distribuye por el norte del océano Atlántico, tanto en la costa este de Norteamérica como en la costa de Europa, así como por el océano Ártico, el mar Báltico, el mar Mediterráneo y el mar Negro. Se le puede encontrar de forma natural en muchos de los países que baña el norte del océano Atlántico y todas las costas europeas, además de que ha sido introducido por el hombre en la República Checa, Suiza, Argentina, Australia, Chile y Nueva Zelanda.

Figura 10. Adulto de salmón del Atlántico (*Salmo salar*). Fuente: *Norwegian Seafood Council*.

En el medio natural, este pez anádromo desova en agua dulce, donde los alevines (~ 2 cm) emergen de los huevos, subsistiendo del saco vitelino adherido hasta que llegan a la etapa juvenil. Los juveniles, conocidos como "*parr*" permanecen en agua dulce de 2 a 5 años. Los salmones "*parr*" presentan 8 a 11 franjas pigmentadas a cada

lado del cuerpo que se alternan con una hilera única de manchas rojas a lo largo de la línea lateral. Antes de migrar al agua salada, los salmones sufren varios cambios fisiológicos y de comportamiento, en un proceso conocido como "esmoltificación" que los prepara para su vida en el mar. En la etapa "*smolt*", el color del cuerpo se vuelve plateado y negro con tintes verdes, azules y cafés. Ya en el mar, el salmón del Atlántico prefiere temperaturas de 4 a 12°C, aunque puede permanecer periodos breves a temperaturas más bajas o altas de -0.7 y 27.8°C, respectivamente (Bergheim & Fivelstad, 2014).

A nivel industrial, la salmonicultura se desarrolló en la década de 1980 en Noruega, con el uso de las jaulas marinas, lo que incitó el desarrollo del cultivo en Escocia y, posteriormente, en Irlanda, Canadá, la costa noreste de los EEUU, Chile y Australia (Tasmania), donde las bajas temperaturas del agua propician su cultivo. La industria acuícola del salmón ha crecido sustancialmente en los últimos 40 años a nivel mundial, debido al intenso avance en las investigaciones en acuicultura y a la fuerte disminución de las pesquerías naturales. Hoy en día aproximadamente el 80% de la producción salmonera del mundo es cultivada. Los principales países productores a nivel mundial de salmón del Atlántico se dividen en cinco países, siendo Noruega el más importante, con una cuota de producción anual de 1.3 millones de toneladas (representando el 55,3% del total de producción), junto con Escocia con 204,000 toneladas (7,6%) y las Islas Feroe con 73,000 toneladas (3,3%) en Europa. El segundo país productor, Chile con 701,000 toneladas anuales (25,4%), está en Sudamérica, y Canadá con 118,00 toneladas (6%) en Norteamérica (Iversen et al., 2020; FAO, 2021) (*Figura* 11).

Figura 11. Distribución de la producción del salmón del Atlántico (*Salmo salar*) de acuicultura alrededor del mundo en volumen (toneladas) y porcentaje. Adaptado de la FAO, 2021 (Iversen et al., 2020).

Con respecto al cultivo de esta especie, el salmón del Atlántico tiene una etapa inicial en agua dulce (*parr*) que se realiza en instalaciones en tierra. Cuando tienen entre 1 año y 18 meses, y alcanzan un peso de 50-90 g, se les traslada a jaulas en el mar. Allí se crían durante 12 a 18 meses, hasta alcanzar un peso en cosecha de 4 a 5 kg. Además, la cría de salmón también requiere una cierta cantidad de corriente para permitir el flujo de agua a través de la granja. Sin embargo, la corriente debe estar por debajo de un determinado nivel para que los peces puedan moverse libremente por los lugares. Estas condiciones suelen darse en aguas protegidas por archipiélagos y fiordos, lo que excluye muchas costas. Sin embargo, la cría en alta mar es un enfoque emergente. Las granjas en alta mar se sitúan en aguas más profundas y menos protegidas, donde las corrientes oceánicas son más fuertes que en la costa, por lo que requieren jaulas más robustas (Bergheim & Fivelstad, 2014; Iversen et al., 2020).

6.2.2. Enfermedades predominantes que afectan al salmón del Atlántico de piscifactoría

Siempre existe la preocupación por los posibles efectos de las enfermedades a lo largo del ciclo de producción. Aunque muchos de los mismos patógenos son preocupantes independientemente de la etapa de vida, algunas enfermedades son más frecuentes en las primeras etapas. Por ejemplo, en Noruega, las infecciones fúngicas (principalmente ocasionadas por *Saprolegnia* spp.) en los huevos y los alevines son la principal preocupación en las primeras fases de cultivo. En el caso de Canadá, las principales patologías asociadas al cultivo de esta especie se centran en el virus de la septicemia hemorrágica viral, la enfermedad entérica de la boca roja causada por *Yersinia ruckeri* y *Saprolegnia* spp., enfermedades que afectan directamente la producción de alevines (Noga, 2010).

Entre otras enfermedades importantes y recurrentes, se encuentra la enfermedad pancreática, ésta es una enfermedad de importancia económica en la acuicultura europea de salmónidos que afecta particularmente al salmón del Atlántico en las jaulas en el mar de Irlanda, Noruega y Escocia. Es causada por un virus perteneciente al género *Alphavirus* dentro de la familia *Togaviridae* (Reid et al., 2017), donde las tasas de mortalidad registradas acerca de esta enfermedad alcanzan hasta un 48% (McLoughlin & Graham, 2007). A nivel de parásitos en el salmón, se describen a una serie de crustáceos maxilópodos llamados copépodos pertenecientes a la familia Caligidae, comúnmente llamados piojos de mar y son los ectoparásitos más reportados en las especies de salmones silvestres y cultivables. La especie *Caligus royercresseyi*, fue descripta por primera vez en 1997 por su capacidad de infestar salmónidos, especialmente truchas y salmón del Atlántico comercialmente cultivados en Chile (Dresdner et al., 2019). Por otro lado, y también perteneciente a la familia Caligidae, se encuentra el *Lepeophtheirus salmonis*, igualmente perteneciente a la familia Caligidae, se encuentra el *Lepeophtheirus salmonis*, igualmente conocido como piojo de mar, la especie calígida predominante en el hemisferio norte (Torrissen et al., 2013). Ambos tienen una distribución amplia, estando presente en el Océano Pacífico y Atlántico, afectando tanto a especies

silvestres como al salmón de cultivo (Torrissen et al., 2013; Dresdner et al., 2019). Con el crecimiento mundial del cultivo intensivo de salmónidos durante la última década, el control del piojo de mar, ha llegado a ser una de las principales preocupaciones en la industria, debido a las elevadas pérdidas económicas y efectos medio ambientales que genera (Costello, 2009; Núñez-Acuña et al., 2015).

Finalmente, la bacteria Gram-negativa *Aeromonas salmonicida* subsp. *salmonicida* es el agente causante de la furunculosis, una enfermedad sistémica de los peces de la familia de los salmónidos. La furunculosis es una enfermedad omnipresente que afecta a las explotaciones acuícolas de todo el mundo y se caracteriza por una elevada mortalidad y morbilidad, alcanzando tasas de mortalidad de hasta el 50% (Dallaire-Dufresne et al., 2014; Taranger et al., 2015). La furunculosis debe su nombre a los forúnculos que aparecen en la piel y la musculatura de los peces afectados por la forma subaguda o crónica de la enfermedad. Esta enfermedad infecciosa se ve exacerbada por el estrés, los bajos niveles de oxígeno y las altas densidades. Los brotes se producen con mayor frecuencia a temperaturas superiores a 10 °C, la enfermedad es altamente infecciosa y puede causar infecciones agudas con una rápida aparición de la mortalidad (Vincent et al., 2019). La desinfección de los huevos fecundados es la intervención más importante contra la furunculosis en los criaderos, siendo este tratamiento obligatorio en Noruega. Hoy en día existen vacunas eficaces para prevenir esta enfermedad, no obstante, la vacunación implica una fuerte manipulación de los peces y un alto coste, por lo tanto, el uso de antibióticos era, y sigue siendo, el método preferido para tratar la furunculosis. Sin embargo, cada vez aparecen más casos de cepas de *A. salmonicida* subsp. *salmonicida* resistentes o incluso multirresistentes a los antibióticos (Vincent et al., 2014; Trudel et al., 2016; Bartkova et al., 2017). Por ende, está claro que se necesitan alternativas eficaces a los antibióticos para controlar esta enfermedad.

En resumen y con el afán de evaluar los dos aditivos funcionales probados durante los años de realización de esta tesis, podríamos contextualizarla de la siguiente manera, 1) la competencia inmunitaria de los esplenocitos expuestos a un PAMP, como el LPS, validación realizada mediante un ensayo *ex vivo* y la evaluación de la

expresión génica de un repertorio de marcadores de genes inmunitarios analizados vía PCR cuantitativa en doradas con una dieta funcional suplementada con un extracto de hojas de plantas medicinales de salvia y hierbaluisa (Capítulo I), 2) además, indicadores claves de rendimiento, como son el crecimiento somático, la supervivencia y el FCA, han sido también evaluados en la dorada alimentadas con el aditivo obtenido de la salvia y la hierbaluisa (Capítulo I, II y III); 3) un análisis transcriptómico basado en microarrays del intestino y el riñón cefálico en dorada (Capítulo II) y salmón del Atlántico (Capítulo III) alimentados con el extracto de salvia y hierbaluisa; 4) el mismo análisis transcriptómico basado en microarrays en salmones alimentados con una dieta suplementada con un extracto bioactivo procedente de la aceituna, además del crecimiento somático y la supervivencia (Capítulo IV); 5) un análisis de enriquecimiento funcional para identificar clases de genes sobrerrepresentados que pudieran tener una asociación con respuestas biológicas concretas; y 6) la aplicación de metodología complementaria para apoyar y validar el estudio molecular (Capítulos II, III y IV). En cuanto a las metodologías complementarias utilizadas, éstas variaron en función del tejido a ser evaluado. Por ejemplo, posibles propiedades inmunomoduladoras fueron evaluados en diferentes parámetros inmunitarios humorales en el plasma al final del estudio nutricional (Capítulo I). En segundo lugar, evaluamos las propiedades histoquímicas de las mucinas almacenadas en las células caliciformes del intestino de la dorada (Capítulo II). Por último, se llevó a cabo una prueba de validación mediante un reto bacteriano con un patógeno habitual de la especie evaluada, en este caso, en el salmón del Atlántico con *A. salmonicida* subsp. *salmonicida* (Capítulos III y IV).

HIPÓTESIS DE TRABAJO Y OBJETIVOS

Hipótesis de trabajo

La hipótesis sobre la que se articula la presente tesis doctoral es que mediante el uso de fitogénicos es posible modular la respuesta inmunológica de los peces de crianza y mejorar su respuesta frente a estresores bióticos como bacterias patogénicas, y, por lo tanto, promover la resistencia frente a enfermedades a través de una mejor respuesta inmunológica del animal.

OBJETIVOS

Objetivo general

- Estudiar y caracterizar los efectos de una combinación de aditivos fitogénicos para piensos obtenido de un extracto de dos plantas medicinales (salvia y hierbaluisa), y la de un fitogénico obtenido de la fruta del olivo (aceituna) a nivel local y sistémico - bazo, intestino y riñón cefálico – en la dorada y el salmón del Atlántico, así como su eficacia contra patógenos comunes, mediante enfoques de análisis complementarios de validación.

Objetivos específicos

- Evaluar el potencial en los indicadores clave de rendimiento (KPI, por sus siglas en inglés, *key performance indicator*), entre ellos el crecimiento e índice de conversión alimenticia de los aditivos alimentarios probados, en este caso con ambos fitogénicos en la dorada y el salmón.

- Evaluar los marcadores inmunes humorales y la capacidad inmunitaria celular, mediante un ensayo *ex vivo* con cultivo de células primarias de esplenocitos de dorada con el fitogénico obtenido de la salvia y la hierbaluisa.

- Describir la respuesta inmunitaria transcripcional del intestino de la dorada a la administración de una dieta suplementada con fitogénicos obtenidos de la salvia y la hierbaluisa;

- Describir las alteraciones histoquímicas del intestino a nivel celular promovidas por el aditivo funcional probado.

- Evaluar los posibles efectos inmunomoduladores en la respuesta transcripcional del riñón cefálico del salmón, mediante el análisis de microarray y los respectivos marcadores inmunes humorales con ambos fitogénicos;

- Validar en salmón sobre el potencial de protección de ambos fitogénicos probados contra la infección bacteriana, en este caso con el patógeno *Aeromonas salmonicida*.

CAPÍTULO I

The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*)

Aquaculture 524:735291

Aquaculture 524 (2020) 735291

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

The growth promoting and immunomodulatory effects of a medicinal plant leaf extract obtained from *Salvia officinalis* and *Lippia citriodora* in gilthead seabream (*Sparus aurata*)

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ARTICLE INFO

Keywords:
Additive
Functional diet
Immunity
Pathogen-associated molecular pattern (PAMP)
Ursolic acid
Verbascoside

ABSTRACT

In the present study, we evaluated the effects of a medicinal plant leaf extract (MPLE; 10%, ursolic acid, 3% other triterpenic compounds; 2% verbascoside and < 1% polyphenols) obtained from *Lippia citriodora* and *Salvia officinalis* on somatic growth and immune responses in juvenile gilthead seabream (*Sparus aurata*). Fish (initial body weight = 26.0 ± 0.1 g) were fed two isoproteic (48% crude protein, 7% fishmeal), isolipidic (17% crude fat) and isoenergetic diets (21.7 MJ/kg), one of them containing 0.1% MPLE. Both diets were tested using four replicate tanks during 92 days. At the end of the trial, a significant increase in growth was observed in fish fed the diet containing the additive in comparison to fish fed the control diet (189.6 ± 2.5 g *vs.* 173.8 ± 4.1 g, respectively; $P < 0.05$). Specific growth rates (SGR) in fish fed the feed supplemented with 0.1% MPLE were significantly higher than in fish fed the control diet ($SGR_{0–92\ days\ (0.1\%\ MPLE\ diet)} = 2.26 \pm 0.01\%\ day^{-1}$, $SGR_{0–92\ days\ (control\ diet)} = 2.16 \pm 0.02\%\ day^{-1}$; $P < 0.05$). Feed conversion ratio (FCR) values in fish fed the control diet were higher than those in fish fed the MPLE diet ($FCR_{control\ diet} = 1.23 \pm 0.02$ *vs.* $FCR_{0.1\%\ MPLE\ diet} = 1.10 \pm 0.02$; $P < 0.05$). When evaluating non-specific immune plasmatic parameters, no significant variations were registered at the level of bacteriolytic and complement activities, nor protein IgM levels ($P > 0.05$). In order to evaluate the cellular immune competence of fish, an *ex vivo* assay with splenocytes primary cell culture (SPCC) from both dietary groups was conducted. SPCC were incubated with lipopolysaccharide (LPS) for 24 h and the expression of genes associated to several immune processes was evaluated (humoral immune response, pro- and anti-inflammatory cytokines, cell surface markers, and antioxidant enzymes). Particularly at 4 h post-exposure, dietary supplementation with 0.1% MPLE enhanced SPCC immune response to LPS by the up-regulation of genes involved in humoral immunity (*lys, IgM*), pro- (*tnf-α, il-1β*) and anti-inflammatory (*tgf-β1, il10*) cytokines, the leucocyte cell surface marker *cd4*, and antioxidative stress enzymes (*mn-sod, cat*). Therefore, a medicinal plant leaf extract (MPLE) obtained from L. *citriodora* and *S. officinalis* may be considered as efficient additive to be used in aquafeed since it does not induce a significant immune reaction under basal conditions, but it provides immune protection after LPS treatment, together with increasing overall fish growth and improvement of feed efficiency values.

1. Introduction

Functional feeds are regarded as the future of the aquaculture industry. By preventive health management through feeding practices, aquatic animals can divert more energy to somatic growth and reduce biological energy reserves needed to fight disease or stress resistance.

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https://doi.org/10.1016/j.aquaculture.2020.735291
Received 30 January 2020; Received in revised form 24 March 2020; Accepted 27 March 2020
Available online 29 March 2020
0044-8486/

Nowadays, functional feeds include specific ingredients with specific functions or special product characteristics; therefore, providing solutions to recurrent problems in animal production cycles rather than only focusing on growth performance issues. A reality that affects the aquaculture industry and still unresolved, is the excessive use of antibiotics, regardless of the global strategy promoted by the Food and Agriculture Organization (FAO, 2016). In recent years, the increase in the use of antimicrobials has been reported due to intense worldwide fish farming and the spreading of several bacterial diseases (Defoirdt et al., 2011). However, antibiotic prophylaxis represents a high cost and leads to undesirable side effects such as bioaccumulation of drug residues, pollution, and increased antibiotic resistance. A suitable solution to replace the excessive administration of antibiotics in the aquaculture industry is the use of additives such as immunostimulants that may be used in functional feeds to improve resistance to diseases by strengthening the innate immune defense mechanisms in aquatic animals (Dawood et al., 2018; Fuchs et al., 2015; Vallejos-Vidal et al., 2016; Wang et al., 2017). Among them, the use of immunostimulants from plant materials has been recognized as an ecofriendly approach for the control of pathogens and regulation of host health, as they possess medicinal properties that have been reported to have a key role in enhancing fish immunity (Vaseeharan and Thaya, 2014). In this context, plant extracts or their by-products contain several active compounds, including phenols, polyphenols, alkaloids, terpenoids, lectines, and polypeptides, that have been shown to be effective alternatives to traditional prophylaxis and vaccines (Chakraborty and Hancz, 2011; Galina et al., 2009).

In this study, we evaluated the growth and immune response in juvenile gilthead sea bream (*Sparus aurata*) fed with a functional diet containing a medicinal plant leaf extract from sage (*Salvia officinalis*, Lamiaceae) and lemon verbena (*Lippia citriodora*, Verbenaceae). Extracts of sage are rich in phenolic compounds (*e.g.*, coumarins, flavonoids, tannins) (Ghorbani and Esmaeilizadeh, 2017) and triterpenes, which are natural components found in a variety of common European plants and fruits, which are gaining attention for their functional benefits (Babalola and Shode, 2013). In traditional medicine, this plant has been reputed for its potential antitumor and antioxidant activities, anti-inflammatory properties and antiseptic effects (Ghorbani and Esmaeilizadeh, 2017; Jedinák et al., 2006). The extracts from the aromatic and medicinal plant lemon verbena contain a large quantity polyphenolic and triterpenic compounds, as well as verbascoside and its derivates (Mauriz et al., 2015; Quirantes-Piné et al., 2009). The above-mentioned compounds have reported beneficial pharmacological activities, including antioxidant, anti-inflammatory and antineoplastic properties in addition to numerous wound-healing and neuroprotective properties (Alipieva et al., 2014; Caturla et al., 2011; Funes et al., 2009). In fish, the anti-inflammatory activity of a triterpenic compound like ursolic acid was reported in zebrafish (*Danio rerio*) (Ding et al., 2015). Furthermore, a strong antiviral activity both *in vitro* and *in vivo* has recently been reported in rainbow trout (*Oncorhynchus mykiss*) (Li et al., 2019). However, none of these studies has used the strategy of a dietary administration to evaluate its applicability in aquaculture. By contrast, to the best of our knowledge, there are no antecedents of the verbascoside effect upon fish health.

The aim of this study was to evaluate a medicinal plant leaf extract (10%, ursolic acid, 3% other triterpenic compounds; 2% verbascoside and < 1% polyphenols) from sage (*S. offticinalis*) and lemon verbena (*L. citriodora*) as a feed additive, using gilthead seabream as a model species for marine aquaculture. This extract contains several compounds that are reputed in traditional medicine for their immunomodulatory properties, which if they also function in fish, would be beneficial in functional aquafeeds. Thus, we decided to test this phytochemical extract on growth performance and the systemic immune response through the evaluation of humoral immune parameters. The beneficial effects of the dietary administration of the medicinal plant leaf extract (MPLE) to a bacterial challenge were evaluated at the gene expression level in splenocytes by a short-term *ex vivo* stimulation with LPS, a broadly recognized pathogen-associated molecular pattern (PAMP).

2. Material and methods

2.1. Fish and rearing conditions

A total of 300 gilthead seabream (body weight, BW = 5–8 g) were purchased from a commercial fish farm (Andromeda Group, Burriana, Spain) and transported by road (1 h) to IRTA facilities at Sant Carles de la Ràpita (Spain). Once there, fish were acclimatized for three weeks in 450 L tanks connected to a water recirculation system (IRTAmar™) at an initial density of 2 kg m^{-3}. Acclimation was conducted in the same experimental tanks (450 L) where the nutritional experiment was carried out. Water temperature (22–27 °C), oxygen (6.1 ± 0.2 mg L^{-1}) (OXI330, Crison Instruments), and pH (7.5 ± 0.01) (pHmeter 507, Crison Instruments, Barcelona, Spain), were daily controlled, whereas salinity (35‰) (MASTER-20 T; ATAGO Co. Ltd), as well as ammonia (0.13 ± 0.1 mg NH_4^+ L^{-1}) and nitrite (0.18 ± 0.1 mg NO_2^- L^{-1}) levels (HACH DR9000 Colorimeter, Hach®, Spain) were weekly monitored. Just before the start of the trial, all necessary animals (n = 280, 35 fish per tank) were individually measured in BW and standard length (SL) and distributed homogeneously among the eight experimental tanks.

2.2. Experimental diets and fish sampling

Experimental diets used in this trial were manufactured by SPAROS Lda (Portugal). Once received and during the entire trial (92 days), they were stored in a refrigeration chamber at 4 °C to avoid their oxidation. Two experimental diets with low fishmeal (FM) content (7% FM) were tested: a control diet (48% protein, 17% lipids and energy: 21.7 MJ/kg) and the same diet but supplemented with the MPLE additive obtained from *S. officinalis* and L. *citriodora* at 0.1% inclusion (Table 1). This inclusion level was chosen according to previous results using similar bioactive compounds (Gisbert et al., 2017). Sage and verbena leaf extracts (5 parts of sage: 1 part of verbena) were produced by NATAC Biotech SL (Madrid, Spain) using water/ethanol extraction (plant leaf extract ratio 5:1) and characterized as described in Arthur et al. (2011) and Wójciak-Kosior et al. (2013). The biochemical composition in terms

Table 1
List of ingredients and proximal composition of experimental diets.

Ingredients, %	Control diet	MPLE diet
Fishmeal LT70	7.0	7.0
Soy protein concentrate	21.0	21.0
Pea protein concentrate	12.0	12.0
Wheat gluten	12.0	12.0
Corn gluten	12.0	12.0
Soybean meal 48	5.0	5.0
Wheat meal	10.4	10.4
Fish oil (SAVINOR)	15.0	15.0
Vitamin and mineral Premix PV01	1.0	1.0
Soy lecithin - Powder	1.0	1.0
Binder (guar gum)	1.0	1.0
MCP	2.0	2.0
L-Lysine	0.3	0.3
L-Tryptophan	0.1	0.1
DL-Methionine	0.2	0.2
MPLE	–	0.1
Proximate composition		
Crude protein, %	48.37	48.37
Crude fat, %	17.19	17.21
Fiber, %	1.52	1.52
Ash, %	5.88	5.88
Gross Energy, MJ/kg	21.62	21.62

Abbreviation: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

of the tested extract contained 73% carbohydrates, 2% crude lipids, < 1% crude proteins, 5% salts, 4% water, 10% ursolic acid, 3% other triterpenic compounds, 2% verbascoside and < 1% polyphenols. Thus, the content in plant-derived bioactive compounds in the experimental diet was 0.01% ursolic acid, 0.003% other triterpenic compounds, 0.002% verbascoside and < 0.001% polyphenols.

The trial lasted 92 days and each diet was tested by means of four replicate tanks. Diets were distributed eight times per day by automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Finland) at the daily rate of 3.0% of the stocked biomass, which approached apparent satiation. One to four hours after feed administration, uneaten pellets were recovered from the bottom of the tank, dried in an oven (100 °C) and their dry weight used for estimating the amount of uneaten feed and calculate feed intake. Sampling to monitor fish growth took place monthly from the nutritional trial in order to adjust feeding rate and evaluate somatic growth performance. For that purpose, all fish in each tank were netted, gently anaesthetized (tricaine methanesulfonate, MS-222, 50 mg L^{-1}) and their BW (g) and standard length (SL, cm) determined. Fish growth was evaluated by means of the following indices: Fulton's condition factor (K) = (BW_f / SL_f^3) × 100; specific growth rate in BW (SGR_{BW}, %) = [(ln BW_f − ln BW_i) × 100] / time (d); where BW_f and BW_i correspond to final and initial BW, and SL_f corresponds to final SL. Feed utilization was evaluated by the following formula: feed conversion ratio (FCR) = feed intake (g) / increase of fish biomass (g).

Proximate composition of the extract and experimental diets was determined as follows: crude fat was quantified gravimetrically after extraction in chloroform/methanol (2:1) and evaporation of the solvent under a stream of N followed by vacuum desiccation overnight (Folch et al., 1957); crude protein content was determined according to Lowry et al. (1951); ash contents were determined by keeping the sample at 500 to 600 °C for 24 h in a muffle furnace (AOAC, 1990) and water content was estimated by sample drying at 120 °C for 24 h. All chemical analyses were performed by duplicate.

2.3. Humoral immune parameters

After fish were measured, blood (*ca.* 1 mL) was taken from anaesthetized fish (n = 5 fish per tank) by caudal puncture with lithium-heparinized syringes and immediately centrifuged (2000 ×g for 20 min at 4 °C) to separate plasma. Levels of immunoglobulin M (IgM) were measured by using the enzyme-linked immunosorbent assay (ELISA) (Wells et al., 1986). Aliquots of 100 μL of plasma (1/5 diluted with 50 mM carbonate-bicarbonate buffer, pH 9.6) were placed in flat-bottomed 96-well plates in triplicate and coated by overnight incubation at 4 °C. After three rinses with PBT buffer (20 mM Tris-HCl, 150 mM NaCl and 0.05% Tween 20, pH 7.3) the plates were blocked for 2 h at room temperature with blocking buffer containing 3% bovine serum albumin (BSA, Sigma) in PBT buffer, followed by three rinses with PBT buffer. The plates were then incubated for 1 h with 100 μL per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) (1/100 in blocking buffer), washed and incubated with the secondary antibody anti-mouse IgG-HRP (streptavidin horseradish-peroxidase) (1/1000 in blocking buffer, Sigma). After exhaustive rinsing with PBT buffer the plates were developed using 100 μL of a 0.42 mM solution of 3,3′,5,5′- tetramethylbenzidine hydrochloride (TMB, Sigma), which was prepared daily in a 100 mM citric acid/sodium acetate buffer (pH 5.4) containing 0.01% H_2O_2. The reaction was allowed to proceed for 10 min and stopped by the addition of 50 μL of 2 M H_2SO_4 before the plates were read at λ = 450 nm in a plate reader (FLUOstar Omega, BMG Labtech). Negative controls consisted of samples without plasma, whose optical density (OD) values were subtracted for each sample value.

Natural haemolytic complement activity was measured in plasma according to Guardiola et al. (2018). The buffers used were: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as the previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for natural haemolytic complement determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells mL^{-1}. Twenty μL of RaRBC suspension were then added to 40 μL of serially diluted plasma in Mg-EGTA-GVB buffer. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 40 μL of distilled water or Mg-EGTA-GVB buffer to 20 μL samples of RaRBC, respectively. Samples were incubated at room temperature for 100 min with regular shaking every 20 min. The reaction was stopped by adding 150 μL of cold EDTA-GVB. Samples were then centrifuged (400 xg for 5 min at 22 °C) and the extent of haemolysis was estimated by measuring the optical density of the supernatant at λ = 414 nm in a microplate reader (Synergy HT, Switzerland). The degree of haemolysis (Y) was calculated and the lysis curve for each specimen was obtained by plotting Y = (1-Y)-1 against the volume of plasma added (μL) on a log-log scaled graph. The volume of plasma producing 50% haemolysis (ACH_{50}) was determined and the number of ACH50 units/ mL obtained for each experimental fish sample.

The fish pathogen *Vibrio anguillarum* was used in the bactericidal assay. The strain was grown from 1 mL of stock culture that had been previously frozen at −80 °C. The bacteria cells were cultured for 48 h at 25 °C in Triptic Soy Agar (TSA, Difco Laboratories), and then inoculated in Triptic Soy Broth (TSB, Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (*w/v*). Bacteria in TSB medium were then cultured at the same temperature, with continuous shaking (100 rpm) for 24 h. Exponentially growing bacteria were resuspended in sterile PBS and adjusted to 10^8 colony forming units per mL (CFU mL^{-1}).

Bactericidal activity was determined following the method of Stevens and Kehrli (Stevens et al., 1991) with some modifications. Samples of 20 μL of plasma were added (in six replicates) to the wells of a flat-bottomed 96-well plate. PBS solution was added to some wells instead of the plasma (positive control). Aliquots of 20 μL of the previously cultured bacteria were added and the plates were incubated for 5 h at 25 °C. Then, 25 μL of MTT (1 mg mL^1) were added to each well and the plates were incubated again for 10 min at 25 °C to allow the formation of formazan. Plates were then centrifuged (2000 xg for 10 min) and the precipitates dissolved in 200 μL of DMSO were transferred to a new flat-bottom 96-well plate. The absorbance of the dissolved formazan was measured at λ = 570 nm. Bactericidal activity was expressed as percentage of non-viable bacteria, calculated as the difference between absorbance of surviving bacteria compared to the absorbance of bacteria from positive controls (100%).

2.4. Ex vivo immune stimulation of splenocytes with LPS

In order to evaluate the immunomodulatory effect of the tested additive when fish come in contact with a pathogenic organism, an *ex vivo* assay was conducted. For this purpose, the spleen was used because of its key role as a secondary lymphoid tissue and, therefore, its specific capacity to activate the immune response in face of a widely recognized pathogen-associated molecular pattern (PAMP) like lipopolysaccharide (LPS).

At the end of the nutritional trial, six specimens from each experimental group (biological replicates) were sacrificed with an overdose of anesthetic (> 150 mg L^{-1}, MS-222) and their spleens removed. The *ex vivo* protocol and the dose of LPS used was similar to that described by Campoverde et al. (Campoverde et al., 2017). In brief, the spleen of each fish was passed through a 100 μm nylon mesh cell strainer (SefarNytal PA-13xxx/100, Spain) in Leibovitz L15 medium (Gibco) supplemented with 1:1000 penicillin-streptomycin (Gibco, catalogue number 15140–122) and 2% foetal calf serum (Gibco, catalogue number 10270–098). The resulting cell suspension was collected and centrifuged (at 400 xg for 10 min at room temperature). Then, the supernatant was discarded and replaced with 10 mL of Leibovitz L15

medium. The cell suspension was again centrifuged and supernatants removed and replaced with 30 mL of media. Cells were distributed to 12-well microtiter plates in 5 mL aliquots (2 wells per fish; methodological replicates). The obtained splenocyte primary cell cultures (SPCC) were incubated with a bacterial-type PAMP, LPS (Sigma, #L3129–100 MG). For this purpose, LPS was dissolved in sterile PBS. A LPS dose (50 μg mL^{-1}; Campoverde et al., 2017) was added to evaluate its effect upon the SPCC from control diet ($SPCC_{CD}$ + LPS) and from 0.1% MPLE diet ($SPCC_{MPLE}$ + LPS). The assay was carried out on microtiter plates (Greiner Bio-One, Spain). LPS-free samples were obtained incubating the SPCC from control ($SPCC_{CD}$ + PBS) and 0.1% MPLE diets ($SPCC_{MPLE}$ + PBS) with 250 μL of PBS. In order to evaluate the expression profile of immune genes, splenocytes were harvested at 4, 12 and 24 h after LPS exposure, centrifuged at 400 xg for 10 min at room temperature, and the supernatant discarded. Splenocytes with no stimuli were harvested immediately prior to the beginning of the treatment (time zero). After cell centrifugation, the pellet was immediately suspended in 1.5 mL of RNAlater® (Sigma-Aldrich, Spain), incubated overnight at 4 °C, then stored at −80 °C for further gene expression analyses.

2.5. RNA extraction and cDNA synthesis

Spleen total RNA was extracted using the QIAGEN RNeasy® Mini Kit following the manufacturer's recommendations. The amount of isolated RNA was determined by spectrophotometry with an ND-2000 NanoDrop (Thermo Scientific™) and its quality was evaluated by means of agarose gel electrophoresis (2%) according to Masek et al. (2005). Once the quality of the extracted RNA was verified, single-stranded cDNA was synthesized in order to evaluate their expression profile. For cDNA synthesis, 1 μg of total RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (QuantiTect® Reverse Transcription Kit) in a final reaction volume of 20 μL according to the instructions provided by the manufacturer.

2.6. Gene expression analyses by real-time PCR (qPCR)

The gilthead sea bream SPCC treatments were analyzed by qRT-PCR in order to evaluate the modulation of a set of immune-related genes. The screening included the analysis of humoral response (lysozyme [*lys*]; immunoglobulin M [*IgM*]), pro-inflammatory (*interleukin 1 beta* [*il-1β*]; *tumor necrosis factor alpha* [*tnf-α*]) and anti-inflammatory cytokines (*il-10; transforming growth factor beta 1* [*tgfβ1*]), the surface cell marker *cd4*, and antioxidant enzymes (manganese superoxide dismutase [*mn-sod*]; catalase [*cat*]). Two different reference genes (*β-actin; 18S*) were assessed using the BestKeeper software (Pfaffl et al., 2004) to elucidate which one had less variation. Thus, *β-actin* was included as the reference gene for expression analyses. The specific primer set for each gene are detailed in Table 2.

The primer amplification efficiency (E) for all the genes included in this analysis was determined using a reference pool containing 1 μL of each sample included in this study. Based on the value of the slope of the regression line obtained, E was calculated according to the formula described in Pffaffl (Pfaffl, 2001) and E values reported in Table 2. Quantitative PCR reactions were performed with 2.5 μL iTaq universal SYBR green supermix (Bio-Rad Laboratories), 0.1 μL forward and reverse primers (final concentration of 500 nM at the reaction volume) and 1.3 μL of miliQ H_2O using 1:4 cDNA dilution from all the cDNA stock samples. The thermal conditions used were 3 min at 95 °C of preincubation followed by 40 cycles at 95 °C for 30 s and 60 °C for 30 s. An additional temperature ramping step from 65 to 95 °C was included to produce the melting curves and thus, verify the amplification of a unique single product on all samples. All the reactions were performed in duplicate using a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Quantification was done according to the Pfaffl method (Pfaffl, 2001) corrected for efficiency of each primer set obtained. The normalized relative expression (NRE) value for each diet (control diet; 0.1% MPLE diet) and experimental condition (PBS; LPS) was calculated using the time zero (calibrator) and normalized to the β-actin (reference gene) expression. The results were expressed as mean expression values obtained at 0, 4, 12, and 24 h of treatment ($n = 6$ fish per diet, experimental condition, and time-point assessed).

2.7. Statistical analysis

Differences in somatic growth and fish condition between both diets (control; 0.1% MPLE) were evaluated by means of a *t*-test. Two-way ANOVA test was used to determine differences in gene expression between dietary groups (factor 1) and sampling times (factor 2). Prior to ANOVA analyses, all data were checked for normality and homogeneity of variances. When statistical significances were found between groups ($P < 0.05$), a post-hoc Tukey test was conducted. Results in growth performance parameters and gene expression values are expressed as the mean ± SD (standard deviation). All statistical analyses were performed using Graph Pad Prism V.6.1. Software (GraphPad Software, San Diego, USA).

2.8. Ethics statement

The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013), and authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (Spain) for the use of laboratory animals.

3. Results

3.1. Somatic growth performance and feed utilization parameters

At the end of the 92-days trial, survival was similar among groups with values ranging between 98.0 and 99.0% ($P > 0.05$). Gilthead seabream fed the diet containing 0.1% MPLE were 8.3% heavier than those fed the control diet (189.6 ± 2.5 g *vs.* 173.8 ± 4.1 g, respectively; $P < 0.05$). Similarly, SGR values in fish fed the 0.1% MPLE diet were higher than those recorded in the control group (SGR = 2.26 ± 0.001% day^{-1} *vs.* 2.16 ± 0.018% day^{-1}, respectively; $P < 0.05$). No significant differences in SL_f and K and were found between both groups (Table 3; $P > 0.05$). Values of FCR were lower in fish fed the 0.1% MPLE diet than in those fed the control diet (Table 3; $P < 0.05$).

3.2. Non-specific humoral immune parameters

At the end of the feeding trial, there were no significant differences in the IgM levels, either bacteriolytic nor complement activities among gilthead seabream specimens fed both diets (Table 4; $P > 0.05$).

3.3. Gene expression in splenocytes incubated with LPS (ex vivo trial)

Normalized relative expression (NRE) for each gene from different experimental groups are presented in the Supplementary file 1. Regarding the humoral immune response, at 4 h post-exposure (hpe), *lys* in $SPCC_{MPLE}$ + LPS was significantly higher than in the $SPCC_{CD}$ + LPS (Fig. 1a; $P < 0.05$). At 12 hpe, *lys* expression levels in $SPCC_{MPLE}$ + LPS increased in comparison to the same treatment at 4 hpe, while these values were significantly higher than those observed from the same group, but just incubated with PBS ($SPCC_{MPLE}$ + PBS) ($P < 0.05$). The same effect, although at a lower magnitude, was observed between $SPCC_{CD}$ + LPS and $SPCC_{CD}$ + PBS. At 24 hpe, *lys* levels decreased in $SPCC_{CD}$ + LPS and $SPCG_{MPLE}$ + LPS compared to 12 hpe ($P < 0.05$); thus, decreasing to similar values recorded prior to LPS stimulation ($P < 0.05$).

Table 2
Sequence of primers used in real-time PCR analysis.

Gene name	Acronym	Accession no.	Sequence 5′→3′	Amplification efficiency (%)
β-Actin	*β-actin*	X89920	FW: TCCTGCGGAATCCATGAGA RV: GACGTCGCACTTCATGATGCT	1.99
Lysozyme	*lys*	AM749959.1	FW: TCATCGCTGCCATCATCTCC RV: TGTTCCTCACTGTCCCATGC	1.96
Immunoglobulin M	*igm*	JQ811851.1	FW: GATCGTGACATCGTCTGAGG RV: TGTTGGGTTGTGGTTGTAGG	2.01
Interleukin 1β	*il1β*	AJ277166.2	FW: TCAGCACCGCAGAAGAAAAC RV: TAACACTCTCCACCCTCCAC	1.99
Tumor necrosis factor alpha	*tnf-α*	AJ413189	FW: CAGGCGTCGTTCAGAGTCTC RV: CTGTGGCTGAGAGGTGTGTG	1.99
CD4 molecule	*cd4*	AM489485.1	FW: TAGCGGAAAGTGGAGGTGTG RV: GCCTGGGGTGTCTCATCTTC	2.00
Interleukin 10	*il10*	JX976621.1	FW: GAGCGTGGAGGAATCTTTCAA RV: GATCTGCTGGATGGACTGC	2.01
Transforming growth factor Beta 1	*tgfβ1*	AF424703.1	FW: AGACCCTTCAGAACTGGCTC RV: ACTGCTTTGTCTCCCCTACC	1.95
Manganese superoxide dismutase	*mn-sod*	JQ308833.1	FW: CCTGACCTGACCTACGACTATGG RV: AGTGCCTCCTGATATTTCTCCTCTG	1.97
Catalase	*cat*	JQ308823	FW: TGGTCGAGAACTTGAAGGCTGTC RV: AGGACGCAGAAATGGCAGAGG	2.01

Table 3
Survival, growth performance and feed efficiency parameters in gilthead seabream (*Sparus aurata*) fed experimental diets. Values are expressed as the mean ± SD (n = 4 tanks).

	Control diet	Control diet + 0.1% MPLE
Survival (%)	98.0 ± 1.0	99.0 ± 0.8
BW_i (g)	26.0 ± 0.2	26.0 ± 0.2
BW_f (g)	173.8 ± 8.2 a	189.6 ± 5.0 b
SL_f (cm)	18.8 ± 0.32	19.2 ± 0.20
Fulton's condition factor (K)	2.65 ± 0.04	2.68 ± 0.03
SGR (% day^{-1})	2.16 ± 0.004 a	2.26 ± 0.002 b
FCR	1.23 ± 0.04 b	1.10 ± 0.04 a

Abbreviation: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).
Different letters denote statistical significant differences among groups (t-test, $P < 0.05$).

Regarding *IgM*, $SPCC_{CD}$ + LPS remained stable throughout the study (from time zero to 24 hpe) ($P > 0.05$). However, the $SPCC_{MPLE}$ + LPS showed higher *IgM* levels compared to $SPCC_{CD}$ + LPS (Fig. 1b, $P < 0.05$). On the other hand, at 12 and 24 hpe no differences in *IgM levels* were detected in none of the diets and treatments evaluated ($P > 0.05$). Collectively, the expression of *lys* and *IgM* suggest that the activation of the humoral immune response in $SPCC_{MPLE}$ + LPS is perceived at 4 hpe, while in fish fed the control diet the response was characterized by a delayed activation of response (*lys*) or even no effect (*IgM*).

The expression of the pro-inflammatory cytokines *il-1β* and *tnfα* was also determined. A significant ten-fold increase of *il-1β* was registered in $SPCC_{MPLE}$ + LPS at 4 hpe compared to $SPCC_{MPLE}$ + PBS (Fig. 1c; $P < 0.05$). In fish fed the control diet, a significant increase was also observed in the expression of *il-1β* in $SPCC_{CD}$ + LPS at 4 hpe compared to $SPCC_{CD}$ + PBS. Importantly, at 4 hpe the *il-1β* expression value was also higher in $SPCC_{MPLE}$ + LPS when it was compared to $SPCC_{CD}$ + LPS ($P < 0.05$). The expression of *il-1β* diminished at 12 hpe in all the treatments evaluated, although it was still significantly higher in $SPCC_{MPLE}$ + LPS in comparison to $SPCC_{MPLE}$ + PBS. By contrast, *il-1β* levels in SPCC from fish fed the control diet were similar at 12 hpe when comparing the LPS and PBS treatments. No differences were registered at 24 hpe between both evaluated treatments ($P < 0.05$).

The pro-inflammatory cytokine *tnf-α* showed increased expression at 4 hpe in $SPCC_{MPLE}$ + LPS, as well as in $SPCC_{CD}$ + LPS, though the magnitude of increase was higher in fish fed the additive ($P < 0.05$; Fig. 1d). At 12 hpe, in both dietary groups *tnf-α* expression decreased with regard to 4 hpe. In particular, *tnf-α* levels in $SPCC_{CD}$ + LPS were similar to those recorded at basal level. By contrast, *tnf-α* levels in $SPCC_{MPLE}$ + LPS were still significantly higher than those recorded at the beginning of the LPS exposure ($P < 0.05$). At 24 hpe, *tnf-α* expression returned to basal expression levels ($P > 0.05$). The pro-inflammatory *il-1β* and *tnf-α* data provided more evidence of activation and significantly higher immune response occurring in $SPCC_{MPLE}$ + LPS.

The leukocyte membrane marker *cd4* showed a significant increase only at 4 hpe in $SPCC_{MPLE}$ + LPS compared to $SPCC_{MPLE}$ + PBS ($P < 0.05$; Fig. 1e), but also compared to $SPCC_{CD}$ + LPS evaluated at the same time-point. This increase in $SPCC_{MPLE}$ + LPS at 4 hpe was also observed in a time-dependent manner compared to 0 hpe. No differences were observed for the other time-points and treatments assessed ($P > 0.05$). This data suggested a correlation between the activation of the pro-inflammatory response and the CD4+ immune cell populations

Table 4
Levels of protein immunoglobulin M (IgM) and complement and bacteriolytic activities measured in gilthead seabream (*Sparus aurata*) plasma fed experimental diets.

	Control diet	Control diet + 0.1% MPLE
Protein IgM (Δ O.D. at λ = 450 nm)	0.55 ± 0.06	0.66 ± 0.06
Complement activity (Δ O.D. at λ = 540 nm)	94.85 ± 50.8	122.5 ± 50.0
Bacteriolytic activity (ACH_{50} U mL^{-1})	81.16 ± 0.66	80.39 ± 0.32

Abbreviation: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*); O.D. = optical density; ACH_{50} = volume of plasma producing 50% haemolysis ($P > 0.05$).
Data are expressed as mean ± SEM [n = 4; calculated from the mean of each tank (n = 5 fish per tank)]. No significant differences were registered (t-test; $P > 0.05$).

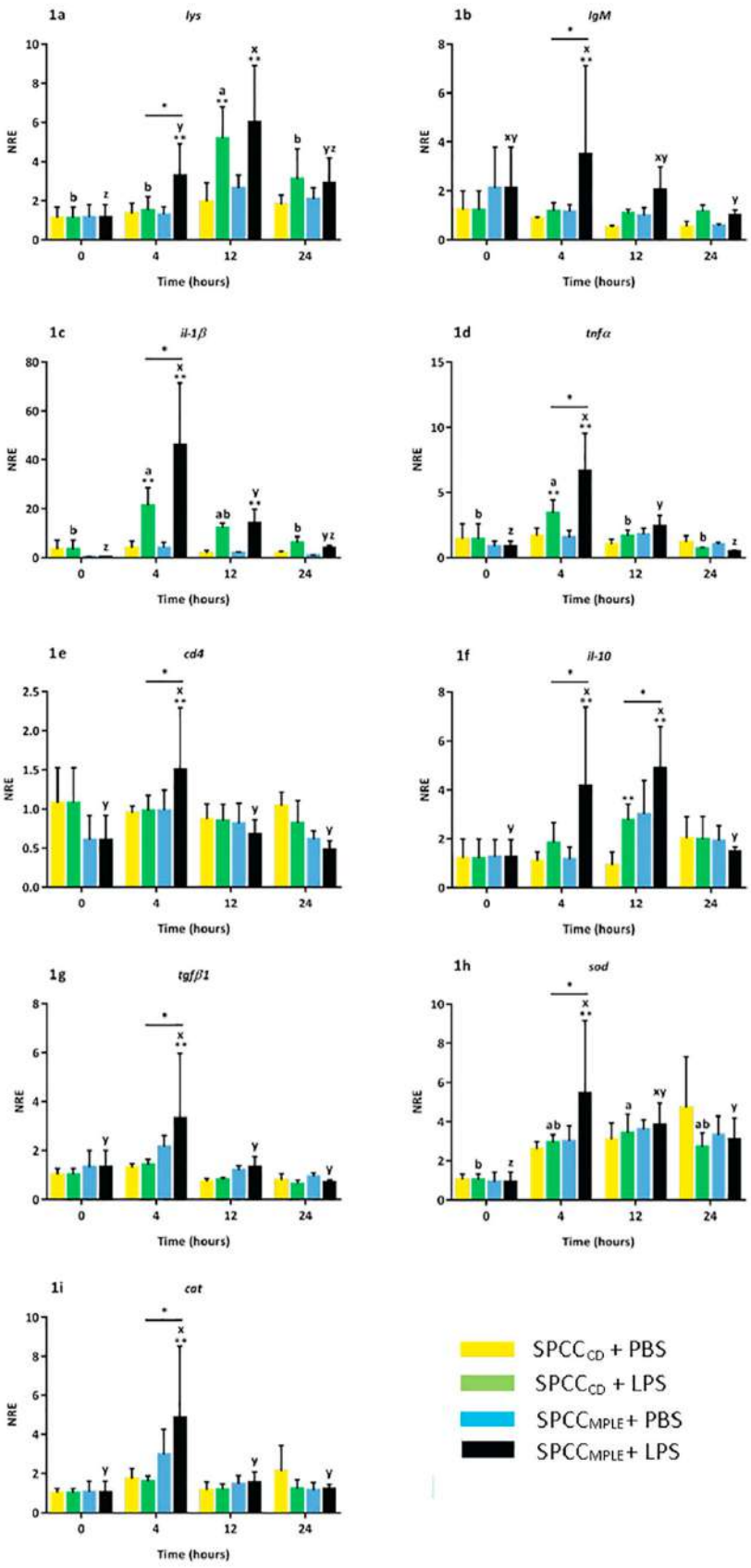

Fig. 1. Normalized relative expression (NRE) of immune-related genes on gilthead seabream (*Sparus aurata*) fed with the 0.1% MPLE diet after 92 days of feeding. The expression of *lys*, *cd4*, *IgM*, *il-1β*, *tnf-α*, *il-10*, *tgfβ1*, *sod* and *cat* was evaluated in splenocytes primary cell culture (SPCC) isolated from gilthead sea breams at 4, 12 and 24 h after exposure to PBS or LPS. Yellow bar: PBS-treated splenocytes from gilthead sea bream fed with control diet (SPCC$_{CD}$ + PBS). Green bars: LPS-treated splenocytes from gilthead sea bream fed with control diet (SPCC$_{CD}$ + LPS). Blue bars: PBS-treated splenocytes from gilthead sea bream fed with 0.1% UA-VB diet (SPCC$_{MPLE}$ + PBS). Black bars: LPS-treated splenocytes from gilthead sea bream fed with 0.1% UA-VB diet (SPCC$_{MPLE}$ + LPS). The time 0 h corresponds to the basal state prior to the beginning of the treatment. Statistical analysis: Two-way ANOVA with Tukey's *post hoc* test. Asterisk (*) represents significant differences between LPS treatments at the same time-point evaluated; (**) represents significant differences between cells treated with PBS and LPS within the same diet and time-point evaluated; different letters (a, b and c) represent significant differences between the control diet different post-exposure times with LPS ($P < 0.05$). Different letters (x, y and z) represent significant differences between the 0.1% UA-VB diet at different post-exposure times with LPS ($P < 0.05$). Abbreviations: MPLE, medicinal plant leaf extract obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*); *il-1β*, *interleukin 1 beta*; *tnf-α*, *tumor necrosis factor alpha*; *il-10*, *interleukin 10*; *tgfβ1*, *transforming growth factor beta 1*; *cd4*, *cluster of differentiation 4*; *mn-sod*, *manganese superoxide dismutase*; *cat*: *catalase*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated to the MPLE dietary additive.

Expression analysis of anti-inflammatory cytokines (*il-10*; *tgfβ1*) demonstrated levels for *il-10* remained stable throughout the 24 h-study and they were not affected by the exposure of SPCC to LPS in fish group fed the control diet ($P > 0.05$; Fig. 1f). However, at 4 hpe a significant increase was recorded in $SPCC_{MPLE}$ + LPS compared to $SPCC_{CD}$ + LPS ($P < 0.05$). Similarly, this up-regulation of the $SPCC_{MPLE}$ + LPS was also registered concerning the $SPCC_{MPLE}$ + LPS. At 12 hpe, *il-10* levels in $SPCC_{MPLE}$ + LPS was still higher compared to $SPCC_{MPLE}$ + PBS and $SPCC_{CD}$ + LPS. At 24 hpe, no differences on the expression of *il-10* were detected ($P > 0.05$). The expression of *tgfβ1* in $SPCC_{MPLE}$ + LPS increased compared to both $SPCC_{MPLE}$ + PBS and $SPCC_{CD}$ + LPS, whereas expression levels reached basal values at 12 hpe in $SPCC_{MPLE}$ + LPS ($P > 0.05$). Thus, the same expression pattern observed at 4 hpe of anti-inflammatory (*il*-10 and *tgfβ1*) *and* pro-inflammatory actors of the humoral and cytokine responses, suggests that a coordinated and also intimate control of immune response takes place in $SPCC_{MPLE}$ + LPS and whose response was not perceived in $SPCC_{CD}$ + LPS.

The expression of anti-oxidative stress enzymes (*mn-sod*; *catalase*) was also evaluated. The level of *mn-sod* was significantly up-regulated at 4 hpe in $SPCC_{MPLE}$ + LPS compared to $SPCC_{MPLE}$ + PBS (Fig. 1h; $P < 0.05$). Importantly, the expression in $SPCC_{MPLE}$ + LPS was also higher than in $SPCC_{CD}$ + LPS ($P < 0.05$). After 4 hpe, the expression values in $SPCC_{MPLE}$ + LPS progressively decreased at 12 hpe and 24 hpe. However, *mn-sod* levels in $SPCC_{MPLE}$ + LPS at 24 hpe were still higher than those recorded at 0 h ($P < 0.05$). A similar trend was observed in $SPCC_{CD}$ + LPS. However, the highest significant expression peak of *mn-sod* in $SPCC_{CD}$ + LPS was only registered at 12 hpe ($P < 0.05$), then returned to basal expression levels at 24 hpe ($P > 0.05$).

On the other hand, the expression profile of catalase (*cat*) had a similar trend as was observed for mn-*sod*. Levels of *cat* in SPCC from fish fed the control diet ($SPCC_{CD}$ + LPS; $SPCC_{CD}$ + PBS) remained stable throughout the 24 h-study ($P > 0.05$; Fig. 1i). The highest expression in *cat* was registered in $SPCC_{MPLE}$ + LPS at 4 hpe, values that were significantly higher than those recorded in $SPCC_{MPLE}$ + PBS, $SPCC_{CD}$ + LPS and $SPCC_{CD}$ + PBS ($P < 0.05$). In $SPCC_{MPLE}$ + LPS, *cat* expression decreased at 12 hpe ($P < 0.05$) and remained constant at 24 hpe ($P > 0.05$). In sum, the antioxidant gene expression profile suggests that a tight control of the oxidative process is produced in $SPCC_{MPLE}$ + LPS at the same time that the peak in immune response activation (4 hpe) was registered.

Altogether, our results suggested that SPCC from gilthead seabream fed the 0.1% MPLE ($SPCC_{MPLE}$ + LPS) showed an earlier activation and higher magnitude immune response than the observed response of the fish fed the control diet. This response seemed to be intimately regulated by both anti-inflammatory and anti-oxidant mechanisms.

4. Discussion

In this study, the effect of a functional diet formulated with low fishmeal levels (7%) and supplemented with 0.1% medicinal plant leaf extract from sage (*S. officinalis*) and lemon verbena (*L. citriodora*) was evaluated in terms of growth performance, non-specific humoral immune response parameters, and the expression profile of genes related to several immune processes including humoral response, pro- and anti-inflammation, and antioxidant enzymes in an *ex vivo* assay using SPCC. Our results showed that 0.1% MPLE increased the body weight and improved feed utilization (FCR) with no effects on the plasma immune parameters in gilthead seabream. Importantly, when isolated splenocytes were incubated with LPS (*ex vivo* conditions) their immune response was activated earlier in those fish fed the 0.1% MPLE, and accompanied by regulatory mechanisms at both anti-inflammatory and anti-oxidant levels.

Although functional diets in aquaculture are not considered as a primary strategy for promoting somatic growth, several studies have shown an improvement in growth performance when fish are fed these kinds of diets (Vallejos-Vidal et al., 2016; Wang et al., 2017). Under present experimental conditions, the supplementation of a basal diet with low FM levels with 0.1% MPLE increased growth performance compared to the control diet. In particular, fish fed the diet containing the plant extracts were 8.3% heavier than the control group. Similar results were observed in rainbow trout (*O. mykiss*) fed with dietary inclusion of sage oils (Sönmez et al., 2014) and post-weaned piglets fed with a lemon verbena additive (Pastorelli et al., 2012). These results might be partially attributed to the potential growth-promoting effects of polyphenolic compounds like verbascoside (Chakraborty and Hancz, 2011). However, these results may also be attributed to triterpenoid compounds, among which the ursolic acid, which has been reported to promote muscular growth by hypertrophy of skeletal muscular fibers in mice (Kunkel et al., 2012) and rainbow trout (Fernández-Navarro et al., 2006). These results in terms of growth are of special relevance due to the low content of FM (7%), representing 75% of FM replacement in tested diets; thus, supporting the change of the aquaculture industry towards compound diets less dependent on wild fishery-derived raw materials (Froehlich et al., 2018).

In addition to evaluating the potential growth-promoting effects of the tested plant extract used in this study, the authors wanted to screen their potential immunomodulatory effects (Vallejos-Vidal et al., 2016). For this purpose, different humoral immune parameters were evaluated in plasma at the end of the nutritional study, as well as the immune competence of splenocytes when exposed to a PAMP, like bacterial LPS, by means of an *ex vivo* assay. The evaluation of plasmatic immune parameters (bacteriolytic and complement activities, and IgM levels) revealed no significant immunostimulant effect of the tested additive, although other studies on feed additives derived from medicinal plants have reported increases in the activities of the above-mentioned parameters (Awad and Awaad, 2017; Harikrishnan et al., 2011; Vaseeharan and Thaya, 2014). Some authors have shown that the use of additives does not always have the expected immunological response, since the administration of natural additives showed counter-productive results (distress situation) due to the bio-energetic cost of prolonged and enhanced immune responses (Álvarez-Rodríguez et al., 2018). Furthermore, the lack of transversal standardized experimental dietary evaluation procedures impedes any comparison between the obtained results and those from the literature (Vallejos-Vidal et al., 2016). At first sight, it may seem that the tested compounds did not modulate the immunity in gilthead seabream. Thus, from these results it could be presumed that 0.1% MPLE had no effect upon the immunity. On the other hand, the results of the *ex vivo* study using SPCC stimulated with LPS, as described below, showed a stimulatory effect. These results may not be surprising taking into consideration that the activation of the immune response represents an important increase in energy expenditure; thus, affecting the energy budget of the organism (Aída et al., 2016). Based on these antecedents, our results suggested that the tested additive from MPLE administered at 0.1% during 92 days did not modify the status of immune homeostasis. One possible reason is that systemic humoral immune of the fish could adapt to the supplemented feed without major energetic consequences, because 92 days can be considered a long time for determining immunostimulation. Nevertheless, this basal conditioning was modified and apparently potentiated in the presence of a pathogenic *stimulus*, whereas under normal conditions humoral non-specific immune systems were not enhanced. Thus, new studies using other additive concentrations or shorter administration times could bring some additional light to this complex issue.

The evaluation of the systemic immune response of gilthead seabream using an *ex vivo* trial with SPCC exposed to LPS assessed changes in gene expression of a repertoire of classical immune gene markers (Vallejos-Vidal et al., 2016). In particular, the expression of *lys* and *IgM* were up-regulated at 4 hpe in SPCC from gilthead seabream fed the 0.1% MPLE diet and remained stable until 12 hpe, whereas they

returned to basal levels (0 h) at 24 hpe. A similar trend in *lys* expression patterns were found in SPCC from when compared to the control group, although the magnitude of increase in gene expression after LPS exposure in SPCC over the control group was significantly lower than SPCC fed the diet containing the medicinal plant extract. Lysozyme and IgM play an important role as defense molecules of the immune response. In particular, lysozyme is important in mediating protection against microbial invasion (Saurabh and Sahoo, 2008). IgM is the most common immunoglobulin in plasma and mucus and the key player in the orchestration of the systemic immune memory responses in teleosts (Parra et al., 2015). Several authors have reported increased values in the plasmatic non-specific immune response after the activation of the immune system with plant-derived immunostimulants. For instance, tilapia (*Oreochromis niloticus*) fed a diet supplemented with the Chinese herb *Astragalus radix,* which is rich in polyphenols, showed a significant increase of lysozyme in serum (Yin et al., 2006), whereas Akrami et al. (Akrami et al., 2015) found an increase in serum lysozyme activity in beluga sturgeon (*Huso huso*) fed a diet supplemented with garlic. On the other hand, lower levels of liver lysozyme were found in gilthead sea bream fed the diets supplemented with maslinic acid, a triterpenic olive-derived (Reyes-Cerpa et al., 2018). These results were not in agreement with our findings, since even though we tried to analyze lysozyme in our plasma samples, values were below detection levels in both groups (data not presented), which supported the above-mentioned hypothesis that the tested additives had an immune homeostatic effect.

Regarding IgM, there was an increase in IgM levels in the spleen of mice when polyphenolic compounds were administered (Oršolić et al., 2005), whereas triterpenes were found to act similarly (Jie et al., 1984). Regarding fish, Reyes-Cerpa et al. (2018) reported that Atlantic salmon (*Salmo salar*) fed functional diets, containing different medicinal plants rich in phenolic compounds, demonstrated an up-regulation of *IgM* in the spleen that was confirmed by increases in B lymphocyte-produced antibodies in the serum. The above-mentioned results are in agreement with those obtained in our study, suggesting a potential adjuvant effect of the MPLE that may be responsible for antibody production when SPCC were stimulated with LPS (Reyes-Cerpa et al., 2018). As it was previously mentioned, the gene expression patterns observed for *lys* and *IgM* in SPCC after their exposure to LPS did not match with the plasmatic levels of these proteins in fish fed the 0.1% MPLE. These differences could be related to the absence (because of the end of nutritional trial) or presence of LPS (*ex vivo*) and its intrinsic capacity to activate the expression of immune-related genes (Shepherd et al., 2018). The present results suggested that the administration of 0.1% MPLE potentiates the splenocytes humoral immune response in a time and magnitude-dependent manner.

The pro-inflammatory response plays a key role in the success of control and eradication of pathogens. The current study revealed that MPLE increased the expression levels of both *il-1β* and *tnf-α*. IL-1 is an early secreted pro-inflammatory cytokine responsible for a cascade of effects on different members of this citokyne family that leads to signal transduction and activation of the nuclear factor (NF)-kB pathway (Engelsma et al., 2002). In addition, *tnf-α* is one of the immune genes initially expressed at an early stage of infection in fish having a key role in the activation of macrophages/phagocytes and enhancing their microbial killing activity; thus, promoting leucocyte proliferation and migration (Hayden and Ghosh, 2014; Zou and Secombes, 2016). Two major classes of leukocytes are the $CD4^+$ and $CD8^+$ leukocytes; so named because of the presence on their respective cell surface of these specific markers. Among these, the $CD4^+$ leukocytes are referred to in some literature as "helper" T-lymphocytes because they aid in the regulation/activation of response by $CD8^+$ cells, or "natural killer" T-lymphocytes, through their secretion of many types of cytokines; among them IL1-β. Accordingly, our finding of an increase in the expression of *cd4* suggested that the pro-inflammatory response is promoting the proliferation of CD4+ leukocyte cells in 0.1% MPLE-fed fish. The immuno-stimulatory activity of *il-1β* and *tnf-α* in response to a bacterial challenge was previously shown in carp (*Cyprinus carpio*) injected with recombinant *il-1*, resulting in an enhancement of agglutinating antibody titers against *Aeromonas hydrophila* (Yin and Kwang, 2000). Similarly to our results, *il-1β* was up-regulated in trout (*O. mykiss*) in a dose-dependent manner in phagocytes from head kidney exposed to LPS (Zou et al., 2000) and carp (Engelsma et al., 2006) confirming its role in the regulation of the inflammatory response, as well as modulating the expression of *il-17* family members, which are important for antibacterial defense (Zou and Secombes, 2016). Under the present *ex vivo* experimental conditions, the increase of *il-1β* in SPCC of gilthead sea bream fed with the tested additive and exposed to LPS may also be attributed to the coordinated response with the up-regulation of *tnf-α*. It has been reported that in rainbow trout head kidney leukocytes and monocytes/macrophages treated with recombinant TNF-α triggered the expression of a number of immune genes associated with inflammation, including *il-1β*, *il-8*, *il-17C* and *cox-2*, and genes involved in antimicrobial responses (Zou et al., 2003). Thus, the up-regulated expression of *il-1β* and *tnf-α* could be the result of a coordinated immune response mechanism favored by the administration of the 0.1% MPLE as a dietary additive. It is worth noting that our results showed a differential response in the up-regulation of *il-1β* and *tnf-α* between the MPLE and control diets. Collectively, these results suggested that splenocytes from gilthead seabream fed the 0.1% MPLE had an increased pro-inflammatory immune activity that could likely be mediated by the proliferation of $CD4^+$ leucocyte cells.

When assessing the immune condition, the evaluation of genes associated to the anti-inflammatory response is important since they regulate and reduce the expression of pro-inflammatory cytokines (Reyes-Cerpa et al., 2013) when necessary, to prevent collateral damage to host tissues and avoid wasting bioenergetic resources (Moore et al., 2001). IL-10 is an anti-inflammatory cytokine and suppresses immune responses (Zou and Secombes, 2016) through its regulatory effect upon pro-inflammatory cytokines, as it has been shown in *in vitro* studies with goldfish (*Carassius auratus*) monocytes activated with heat-killed *Aeromonas salmonicida* then incubated with IL-10 (Grayfer et al., 2011). The regulatory role of IL-10 has also been reported in LPS-activated immune cell populations (neutrophils and macrophages) in carp (Piazzon et al., 2015). Additionally, we found an up-regulation of *tgf-β1*. Previous antecedents in teleost fish have proposed an important role for this cytokine in the control of the pro-inflammatory process and the resolution of pathogenic infective processes (Reyes-Cerpa et al., 2014; Reyes-López et al., 2015). The augmentation of expression of *tgf-β1* could be mediated by IL-1, as it has been reported in primary head kidney-derived macrophages (Castro et al., 2011), and appears to be mediated *via* the NF-B and MAPK signaling pathways (Yang et al., 2014). The regulation by *tgf-β1* of the LPS-induced pro-inflammatory response in grass carp (*Ctenopharyngodon idella*), has been previously reported (Wei et al., 2015). Present results were in agreement with those obtained by Zhan et al. (2015) where *tgf-β1* expression increased in the head kidney and spleen of tilapia challenged with *Streptococcus agalactiae* and stimulated by LPS. Thus, the up-regulation of both anti-inflammatory cytokines measured in this study, *il-10* and *tgfβ1*, confirmed the anti-inflammatory properties of verbascoside (Alipieva et al., 2014) and ursolic acid (Baricevic et al., 2001), while at the same time stimulating some pro-inflammatory responses, such that it is likely that a balanced immune response was maintained. These data suggested that the splenocytes from fish fed the 0.1% MPLE diet exerted a tight control of the immune response to LPS by means of the up-regulation of anti-inflammatory cytokines at the same time-point (4 hpe) where the pro-inflammatory response peaked. Overall, immune protection was thereby established, and potentially improved, with a general homoeostasis being maintained.

Reactive oxygen species (ROS) compose an important defense mechanism involved in the activation of the immune response including the activation of T cells (Chen et al., 2018). However, the imbalance of

ROS, which can be a cause of oxidative stress, has been associated to aberrant immunity (Chen et al., 2018). Thus, several cellular self-protective mechanisms against this potential damage should also be tightly regulated during an immune response to prevent collateral damage. In this way, *mn-sod* and *cat* are two enzymes involved in the cellular defense against uncontrolled oxidative processes and catalyze the reduction of superoxide radicals and H_2O_2 (Otto and Moon, 1996). To minimize the damaging effects of ROS, these two antioxidant enzymes have related functions and are considered as the first line of defense against oxygen toxicity due to their inhibitory effects on oxygen radical formation (Li et al., 2009; Pandey et al., 2003). Furthermore, the presence of phenolic compounds in sage and lemon verbena have been reported to be responsible for the high antioxidant and antibacterial capacity of these medicinal plants (Bulfon et al., 2014; Funes et al., 2009). Results from the current study were in agreement with the above-mentioned findings, as changes in levels of *mn-sod* and *cat* expression by SPCC exposed to LPS occurred as a response by fish fed the 0.1% MPLE diet. These data suggested that fish fed with 0.1% MPLE had an increased *redox* capacity related to the presence of triterpenic (Rufino-Palomares et al., 2011) and polyphenolic (Sönmez et al., 2014) compounds, protecting against reactive oxygen species and stimulation of the antioxidant defenses of the organism (John et al., 2001).

Despite the potential benefits of the tested MPLE obtained from sage and lemon verbena in terms of growth performance and immunostimulatory properties reported in the current study, verbascoside extracted from *Kigelia africana* has been reported to promote genotoxicity in human lymphocytes (Santoro et al., 2008). However, our study demonstrated that the long administration of a feed additive containing verbascoside at low levels (0.002%) had no toxic effects in gilthead sea bream. These results were in agreement to other studies in different animal models that reported that this compound posed no risk on animal health (Etemad et al., 2015, 2016; Perucatti et al., 2018 among others).

5. Conclusions

In summary, present data suggest that the inclusion of a medicinal plant leaf extract obtained from sage and lemon verbena at 0.1% in diets with low FM levels not only promoted somatic growth and reduced FCR values in gilthead seabream, but also enhanced their systemic immune response as indicated by changes in gene expression of a repertoire of markers in an *ex vivo* trial using SPCC exposed to LPS. However, the above-mentioned effects were not seen in bacteriolityc, complement activities, and/or IgM levels in plasma, which may indicate, in comparison to other immunostimulants, a very tight control of the immune status mediated by the tested compounds (immune homeostasis), that functions well with the host strategy to save energy for metabolic purposes when no real immune response is needed. Altogether, the up-regulation of genes involved in non-specific immune response (*lys*, *IgM*), as well as pro- (*tnf-α*, *il-1β*) and anti-inflammatory (*tgf-β1*, *il10*) cytokines, surface T-cell marker *cd4*, and antioxidative stress enzymes (*mn-sod*, *cat*) indicated that the tested feed additive, rich in triterpenic and polyphenolic compounds, mainly ursolic acid and verbascoside, has immunomodulatory properties that can be useful for incorporation in aquafeeds.

Declaration of Competing Interest

Authors declare when submitting this article that we do not have any conflict of interests.

Acknowledgements

This work has been financed through the DIETAplus project of the JACUMAR (Junta de Cultivos Marinos, MAPAMA; Spanish government), which is co-funded with FEMP funds (EU), as well as by the ERC (European Research Council) in MedAID project (Mediterranean Aquaculture Integrated Development; Grant Agreement nb. 727315) and Fundación Seneca de la Región de Murcia (Grupo de Excelencia grant no. 19883/GERM/15). Héctor R. Salomón Figueredo is supported by a PhD grant from the government of Paraguay (BECAL). Joana P. Firmino has been subsidized by the Industrial PhD program of the Generalitat de Catalunya and TECNOVIT-FARMFAES. Eva Vallejos-Vidal was granted with DICYT-USACH Postdoctoral fellowship (Nb. 022043IB).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735291.

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CAPÍTULO II

Medicinal plant leaf extract from sage and lemon verbena promotes intestinal immunity and barrier function in gilthead seabream (*Sparus aurata*)

Frontiers in Immunology 12:670279

frontiers in Immunology

ORIGINAL RESEARCH
published: 14 May 2021
doi: 10.3389/fimmu.2021.670279

Medicinal Plant Leaf Extract From Sage and Lemon Verbena Promotes Intestinal Immunity and Barrier Function in Gilthead Seabream (*Sparus aurata*)

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The inclusion of a medicinal plant leaf extract (MPLE) from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*), rich in verbascoside and triterpenic compounds like ursolic acid, was evaluated in gilthead seabream (*Sparus aurata*) fed a low fishmeal-based diet (48% crude protein, 17% crude fat, 21.7 MJ kg^{-1}, 7% fishmeal, 15% fish oil) for 92 days. In particular, the study focused on the effect of these phytogenic compounds on the gut condition by analyzing the transcriptomic profiling (microarray analysis) and histological structure of the intestinal mucosa, as well as the histochemical properties of mucins stored in goblet cells. A total number of 506 differentially expressed genes (285 up- and 221 down-regulated) were found when comparing the transcriptomic profiling of the intestine from fish fed the control and MPLE diets. The gut transcripteractome revealed an expression profile that favored biological mechanisms associated to the 1) immune system, particularly involving T cell activation and differentiation, 2) gut integrity (i.e., adherens and tight junctions) and cellular proliferation, and 3) cellular proteolytic pathways. The histological analysis showed that the MPLE dietary supplementation promoted an increase in the number of intestinal goblet cells and modified the composition of mucins' glycoproteins stored in goblet cells, with an increase in the staining intensity of neutral mucins, as well as in mucins rich in carboxylated and weakly sulfated glycoconjugates, particularly those rich in sialic acid residues. The integration of transcriptomic and histological results showed that the evaluated MPLE from sage and lemon verbena is responsible for the maintenance of intestinal health, supporting gut

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Edited by:
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University of Aberdeen,
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Reviewed by:
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Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: 20 February 2021
Accepted: 26 April 2021
Published: 14 May 2021

Citation:
Salomón R, Reyes-López FE, Tort L, Firmino JP, Sarasquete C, Ortiz-Delgado JB, Quintela JC, Pinilla-Rosas JM, Vallejos-Vidal E and Gisbert E (2021) Medicinal Plant Leaf Extract From Sage and Lemon Verbena Promotes Intestinal Immunity and Barrier Function in Gilthead Seabream (Sparus aurata). Front. Immunol. 12:670279. doi: 10.3389/fimmu.2021.670279

homeostasis and increasing the integrity of the intestinal epithelium, which suggests that this phytogenic may be considered as a promising sustainable functional additive for aquafeeds.

Keywords: cell proliferation, feed additive, gut health, innate immunity, lectin histochemistry, ursolic acid, verbascoside acid, GALT

INTRODUCTION

Aquaculture will supply the majority of aquatic dietary protein by 2050 (1), playing a relevant role in food security and supply, and poverty alleviation (2). The sustained growth of aquaculture is highly dependent on the intensification of production (3), sustainable feed formulations (4) and generating farming conditions supporting fish health and welfare (5). Among the former concepts, disease is considered a main persistent threat to intensive fish farming, which represents an estimated US$6 billion loss per annum at a global scale (5). Under this scenario, aquaculture depends on the use of antibiotics to fight against infectious diseases that threatens production (6), with emerging infectious diseases forecast to increase with warmer temperatures (7). However, their use tend to result in the emergence of antimicrobial resistant bacteria, which may not represent a direct threat in terms of aquatic food consumption, but they could directly impact production itself by lowering drug efficacy, decreasing the animal's immune system and selecting more virulent strains of pathogens (7). Considering the above-mentioned reasons, along with the increasing public awareness regarding food safety issues and the environmental impact linked to antibiotics' use and animal welfare (7, 8), the development of functional feeds focused on promoting and modulating the host's immune response has been encouraged during the last decade (9–12).

Functional feeds are recognized for promoting the growth, welfare and health of farmed animals coupled with an improvement and/or modulation of their immune system, as well as inducing physiological benefits beyond traditional feeding practices (13). In this sense, by preventive health management through the diet, fish can divert more energy to somatic growth and reduce biological energy reserves needed to fight disease or stress resistance (14). Furthermore, they can be used in addition to chemotherapeutic agents and vaccines (15). Among the long list of feed additives used in animal production (9, 16), phytogenics derived from herbs, spices, medicinal or aromatic plants are residue-free, unlike synthetic antibiotics, and are safe ingredients for sustainable feeds (11, 17, 18). Although the mode of action of most phytogenic feed additives has not yet been fully elucidated, they are well-known for their antimicrobial, immunomodulatory, antioxidative, and growth-promoting effects in livestock (17) and aquatic animals (9, 18).

In a recent study from our research group, we showed that a phytogenic feed additive from sage (*Salvia officinalis*, Lamiaceae) and lemon verbena (*Lippia citriodora*, Verbenaceae) is an effective additive for aquafeeds since its inclusion at 0.1% in diets with low fishmeal (FM) content not only improved some key performance indicators (i.e., growth and feed efficiency performances), but also promoted fish systemic immunity. In particular, an *ex vivo* study with splenocytes from fish fed this phytogenic exposed to lipopolysaccharide (LPS) showed an up-regulation of genes involved in non-specific immune response, as well as pro- and anti-inflammatory cytokines, surface T-cell marker cd4, and antioxidative stress enzymes (14). However, the effects of this phytogenic feed additive still needs to be explored in terms of local immune response, especially at the intestinal level, since optimal health and functionality of the intestinal mucosa is essential for sustainable animal production (19, 20). This is of special relevance under the current scenario in which aquafeeds are formulated with low levels of fishmeal (FM) (21), since several studies have indicated that low FM diets compromised systemic immunity (22–25), as well as gut immune response (26, 27). In this context, intestinal immunity is a key factor in maintaining the general health of aquatic animals (20). The intestinal mucosa is a complex organ composed of the digestive epithelium with its specific structure, the gut-associated lymphoid tissue (GALT), and the mucus overlying the epithelium with its commensal microbiota (19). Furthermore, the intestinal epithelium acts as a selectively permeable barrier for dietary nutrients, electrolytes and water, while maintaining an effective defense against pathogens and tolerance towards dietary antigens. Thus, the GALT is reputed for mediating mucosal innate and adaptive immune responses, as well as being a key element for proper distinction between pathogens and commensal microbiota inhabiting the intestine (28–30).

Considering the importance of interaction between the diet and the gut, in the current study we evaluated the transcriptomic profile and histochemical properties of mucins stored in goblet cells of the intestine in juvenile gilthead seabream (*Sparus aurata*) fed a functional diet containing a medicinal plant leaf extract (MPLE) from sage and lemon verbena. This species is recognized as the most important Mediterranean aquaculture fish species in terms of volume and economic value (31). For this purpose, we focused on the modulation of the intestinal mucosa functionality and health by the above-mentioned phytogenics when included in a diet with low fishmeal levels.

MATERIAL AND METHODS

Diets, Fish and Rearing Conditions

Two isoproteic (48% crude protein), isolipidic (17% crude fat) and isoenergetic (21.7 MJ kg^{-1}) experimental diets were formulated with a low FM content (7% FM) as described in Salomón et al. (14). Diets, named as control and MPLE, only

differed in their content of the feed additive evaluated, the MPLE obtained from sage and verbena leaves, which was included in the MPLE diet at 0.1% (**Table 1**). The MPLE was obtained by NATAC Biotech SL using water/ethanol extraction (plant leaf extract ratio 5:1) and characterized as described in Arthur et al. (32) and Wójciak-Kosior et al. (33). The tested extract (proximate composition: 73% carbohydrates, 2% crude lipids, <1% crude proteins, 5% salts and 4% water) contained 10%, ursolic acid, 3% other triterpenic compounds, 2% verbascoside and <1% polyphenols. Thus, the content in plant-derived bioactive compounds in the MPLE diet was 100 ppm ursolic acid, 30 ppm other triterpenic compounds, 60 ppm verbascoside and <10 ppm polyphenols. This phytogenic was incorporated in the mixture prior to extrusion. In brief, all powder ingredients were mixed in a double-paddle mixer (model RM90L, Mainca, Spain) and ground (below 250 µm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). Oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Immediately after coating, diets were packed in sealed plastic buckets and shipped by road to the facilities at IRTA Sant Carles de la Ràpita, Spain. Both extruded diets (pellet size: 2 mm) used in this trial were manufactured by SPAROS Lda. (Portugal) and kept at 4°C until their administration. Proximate composition of the extract and experimental diets have been described in Salomón et al. (14).

TABLE 1 | List of ingredients and proximal composition of experimental diets; control and a basal diet supplemented with a medicinal plant leaf extract (MPLE) obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

Ingredients, %	Control diet	MPLE diet
Fishmeal LT70	7.0	7.0
Soy protein concentrate	21.0	21.0
Pea protein concentrate	12.0	12.0
Wheat gluten	12.0	12.0
Corn gluten	12.0	12.0
Soybean meal 48	5.0	5.0
Wheat meal	10.4	10.4
Fish oil (SAVINOR)	15.0	15.0
Vitamin and mineral Premix PV01	1.0	1.0
Soy lecithin – Powder	1.0	1.0
Binder (guar gum)	1.0	1.0
MCP	2.0	2.0
L-Lysine	0.3	0.3
L-Tryptophan	0.1	0.1
DL-Methionine	0.2	0.2
MPLE	–	0.1
Proximate composition		
Crude protein, %	48.37	48.37
Crude fat, %	17.19	17.21
Fiber, %	1.52	1.52
Ash, %	5.88	5.88
Gross Energy, MJ/kg	21.62	21.62

MPLE, medicinal plant leaf extract obtained from sage (Salvia officinalis) and lemon verbena (Lippia citriodora).
Proximal compositions of the diets were according to Salomon et al. (14), following the AOAC guidelines.

A total of 300 gilthead seabream (body weight, BW = 5.0 ± 0.2 g; mean ± standard deviation) were obtained from a commercial fish farm, Piscicultura Marina Mediterránea SL (Andromeda Group, Burriana, Spain) and transported to the experimental facilities of IRTA in Sant Carles de la Ràpita (Tarragona, Spain). Then, fish were acclimatized for three weeks in 450 L tanks connected to a water recirculation system (IRTAmar™) at an initial density of 2 kg m^{-3}. Acclimation was conducted in the same experimental tanks (450 L) where the nutritional experiment was carried out. Just before the start of the trial, all animals were gently anesthetized (50 mg l^{-1} MS-222, Sigma-Aldrich, Madrid, Spain), individually measured in BW (26.0 ± 0.2 g) and distributed homogeneously among the eight experimental tanks (n = 35 fish per tank; 4 replicate tanks per experimental diet). During the trial that lasted 92 days, fish were fed at the daily rate of 3.0% of the stocked biomass, which approached apparent satiation as described in Salomón et al. (14). Feed ration was regularly adjusted by means of intermediate samplings along the trial. At the end of the trial, all fish were anesthetized as previously indicated and measured for individual BW. Fish performance in terms of survival ($S_{control\ diet}$ = 98.0 ± 1.0%; $S_{MPLE\ diet}$ = 99.0 ± 0.8%), growth ($BW_{control\ diet}$ = 173.8 ± 8.2 g; $BW_{MPLE\ diet}$ = 189.6 ± 5.0g) and feed conversion ratio ($FCR_{control\ diet}$ = 1.25 ± 0.04; $FCR_{MPLE\ diet}$ = 1.10 ± 0.04) was already published in Salomón et al. (14). Twelve fish from each experimental diet (n total= 3 fish per tank replicate) were randomly selected and sacrificed with an overdose of anesthetic (300 mg l^{-1} MS-222) for gut transcriptomic and histological analyses. Sacrificed animals were eviscerated and mid-anterior intestine samples (1-1.5 cm length per specimen) were put in RNAlater™ (Invitrogen, Thermo Fisher Scientific, Lithuania), incubated overnight at 4°C, and stored at -80°C until RNA extraction. This region of the intestine (mid-anterior section) was chosen due to its specialized immunological functionality when compared with other intestinal sections (34). In addition, a similar piece of tissue was also dissected and fixed in 10% v/v neutral formaldehyde (pH: 7.2 ± 0.01) buffered with sodium phosphate (0.1M) for histological and histochemical purposes.

Water temperature (25.1 ± 1.5°C, range: 22-27°C), oxygen (6.1 ± 0.2 mg l^{-1}) (OXI330, Crison Instruments), and pH (7.5 ± 0.01) (pHmeter 507, Crison Instruments, Barcelona, Spain) were daily controlled. Salinity (35‰) (MASTER-20 T; ATAGO Co. Ltd), ammonia (0.13 ± 0.1 mg NH_4^+ l^{-1}) and nitrite (0.18 ± 0.1 mg NO^- l^{-1}) levels (HACH DR9000 Colorimeter, Hach®, Spain) were weekly monitored. The trial was run under natural photoperiod according to the season of the year (August to November; 40°37'41" N).

Transcriptional Analysis

RNA Isolation and Quality Control

Total RNA was extracted from mid-anterior intestine of individual fish (n = 12 fish per dietary treatment) using the RNeasy® Mini Kit (Qiagen, Germany). Total RNA from each individual sample was eluted in a final volume of 35 µL nuclease-free water and treated with DNAse using the DNA-free™ DNA Removal Kit according to manufacturer's instructions

(Invitrogen, Thermo Fisher Scientific, Lithuania). Total RNA concentration and purity were quantified using a Nanodrop-2000® spectrophotometer (Thermo Scientific, USA) and stored at -80°C until analysis. Prior to hybridization with microarrays (3 pooled RNA per dietary condition), four individual RNA samples were pooled by mixing a volume of 1.5 µL [(RNA) = 133.33 ng/µL] per individual sample [final volume = 6 µL; (RNA) = 133.33 ng/µL] and checked for integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Spain). Pooled RNA analyzed in this study were selected by the criteria of a RIN value > 8.5 (**Supplementary Figure 1**). This methodological approach of pooling RNA from four different specimens in each replicate (n = 3 microarray replicates) allowed authors evaluating population's variability (n = 4 replicate tanks; 1 fish per tank in each pooled RNA; N = 12 animals); however, the information regarding individual variability was lost with this choice.

Microarray Hybridization and Analysis

Transcriptional analysis was carried out using the Aquagenomics *Sparus aurata* oligonucleotide microarray v2.0 (4 x 44 K) (SAQ) platform. The detailed information about the platform and the transcriptomic raw data for all samples included in this current analysis is available through the public repository Gene Expression Omnibus (GEO) at the US National Centre for Biotechnology Information (NCBI) (accession number GPL13442 and GSE166558, respectively).

Transcriptomic analysis from both experimental groups were conducted as described by Reyes-López et al. (35). Briefly, 200 ng of total RNA from each sample pool was reverse transcribed along with Agilent One-Color RNA spike-in kit (Agilent Technologies, USA). Then, total RNA was used as template for Cyanine-3 (Cy3) labelled cRNA synthesis and amplification with the Quick Amp Labelling kit (Agilent Technologies). cRNA samples were purified using the RNeasy micro kit (Qiagen). Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000® spectrophotometer. Then, 1.5 mg of Cy3-labeled cRNA with specific activity > 6.0 pmol Cy3/mg cRNA were fragmented at 60°C for 30 min, and then the samples were mixed with hybridization buffer and hybridized to the array (ID 025603, Agilent Technologies) at 65°C for 17 h using the Gene expression hybridization kit (Agilent Technologies). The microarray washes were conducted as recommended by the manufacturer using Gene expression wash buffers (Agilent Technologies) and stabilization and drying solution (Agilent Technologies). Microarrays slides were scanned with an Agilent Technologies Scanner (model G2505B); spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0 (Agilent Technologies). Quality reports were checked for each array. Although validation by mean of qPCR is required when there is a high risk of obtaining paralog genes and unspecific hybridization; in our study, we used an oligonucleotide-based microarray, which has probes with a reduced number of bases and high affinity, which avoids the necessity of conducting the validation of gene expression results by qPCR validation.

Transcripteractome

The Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 11.0 (https://string-db.org) was used in order to obtain the gut transcripteractome for those differentially expressed genes (DEGs) from fish fed the MPLE diet in comparison to the control group (P- value < 0.05) (36). This functional network analysis has gained increasing attention because of the association between different genes sorted in different clusters that may have complementary functions into a biological context of response (35). A protein-protein interaction (PPI) network of DEGs was conducted with a high-confidence interaction score (0.9) using *Homo sapiens* as model organism. Furthermore, to confirm matches of the genes acronyms tag between both *H. sapiens* and gilthead seabream species Genecards (37) and Uniprot (38) databases were used. In order to confirm match of gene acronyms between both *H. sapiens* and gilthead seabream species, human orthology identification based on gene/protein name was accessed through the Genecards (www.genecards.org) (37) and Uniprot (www.uniprot.org) (UniProt, 2019) databases. Additionally, protein-protein BLAST (BLASTp) were analyzed (E-value < 10^{-7}; query cover > 95%). Gene ontology (GO) enrichment analysis for the DEGs were also performed by STRING (P < 0.05).

Histological and Histochemical Analyses

Samples (n = 12 fish per experimental diet) were embedded in paraffin and sagitally sectioned (5-6 µm). A total of 576 sections (2 per each sample x 24 samples x 12 techniques) were used for histological and histochemical purposes. Two sections per each sample were stained with hematoxylin–VOF for descriptive purposes; the rest were used for evaluating the histochemical properties of epithelial and mucous cells. In brief, Schiff, Periodic Acid Schiff (PAS), diastase-PAS and Alcian Blue (AB) pH 2.5, 1 and 0.5 (carboxylated and sulphated glycoconjugates/glycoproteins) techniques were used for studying carbohydrate distribution. Furthermore, several horseradish peroxidase (HRP) conjugated lectins (Sigma-Aldrich, Spain) were used for proper characterization of different glucidic residues bound to the glycoconjugates; in particular, *Canavalia ensiformes*/ConA (Mannose and/or Glucose), *Ulex europeus*/UEA-I (L-Fucose), *Triticum vulgaris*/WGA (N-acetyl-D-glucosamine and/or N-acetylneuraminic acid, NeuNAc/sialic acid/NANA), Glycine max/SBA (α-N-acetyl-D-galactosamine) and *Sambucus nigra*/SNA (NeuNAc/sialic acid/NANA). Lectin concentrations ranged between 15 µg ml^{-1} to 30 µg ml^{-1}. Regarding negative controls, omission of the respective lectin, substitution of lectin-HPR conjugates by TBS and treatments with different enzymes were performed according to Sarasquete et al. (39). The peroxidase activity was visualized with 3,3-diaminobenzidine tetra hydrochloride/DAB and hydrogen peroxide (0.05%). All the techniques were performed according to Pearse (40) and following proper standardized techniques and protocols (41). All reagents were purchased from Sigma Chemical Co. St Louis, MO, USA.

Histological images were taken with a Leitz Wetzlar microscope with a built-in SPOT Insight Color camera (Ernst Leitz Wetzlar GmbH, Germany). Results were manually

registered using a semi-quantitative assessment scoring based on color intensity scores (0, negative; 1, weak; 2, moderate; 3, intense; 4, very intense) from four independent observers, comparing the sections of the control with the experimental diet. The mucous cell count was determined in four different sites of each histological section, and the number of cells expressed per length unit of the basal lamina of the mucosal epithelium (1 mm) according to Yamamoto et al. (42).

Ethics Statement

All animal experimental procedures were complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015), and authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain) for the use of laboratory animals.

Statistics

Extracted raw data from microarrays were imported and analyzed with Genespring version 14.5 GX software (Agilent Technologies). The 75% percentile normalization was used to standardize arrays for comparisons and data were filtered by expression. An unpaired t-test was conducted without correction to identify those DEGs between fish fed control and MPLE diets. The Principal Component Analysis (PCA) was carried out using GeneSpring software, four eigenvectors were calculated to describe the aggrupation of the MPLE and control groups in a 3D plot. Venn diagram and the hierarchical heatmap were all obtained also with Genespring (version 14.5 GX software, Agilent). Changes in the number of mucous cells between experimental diets were analyzed by means of an unpaired t-test assuming data homoscedasticity (Barlett's test). All the analysis were performed using GraphPad PRISM 7.00. The level of significance was set at $P < 0.05$ for all statistical tests.

RESULTS

Organization of the Intestine and Mucins' Histochemistry Produced by Goblet Cells

In both experimental groups, the intestinal mucosa was lined by a simple columnar epithelium with basal nuclei, basophilic cytoplasm and prominent brush border with scattered goblet cells. The organization of the lamina propria-submucosa and muscular layers was normal. No signals of histological alterations associated to inflammatory processes in the intestine were observed in fish fed the MPLE diet compared to the control group (**Figures 1A, B**). Fish fed the MPLE diet showed a higher density of goblet cells along the intestinal epithelium compared to fish fed the control diet (**Figure 2**).

Regarding the histochemical properties of mucins in goblet cells from the anterior intestine, results showed a variable richness of neutral glycoproteins (PAS and diastase-PAS positive) (**Table 2**). In addition, mucins from goblet cells showed a mixture of carboxylated (AB pH = 2.5) and sulphated acidic groups (weak and strongly ionized; AB pH = 1.0 and pH 0.5, respectively) (**Table 2** and **Figure 1C**). Furthermore, a specific affinity for WGA, SNA and SBA lectins was detected in the mucinous content of goblet cells (**Table 2** and **Figures 3A–L**). Moreover, no variations were detected in the distribution of mucosal cell glycoconjugates between the upper and the bottom areas of the intestinal folds. When comparing both dietary groups, the dietary administration of the MPLE modified the composition of glycoproteins of mucins produced by goblet cells, with an increase in the staining intensity of neutral mucins, as well as in mucins rich in carboxylated and weakly sulphated glycoconjugates (**Table 2** and **Figures 1C, D**). In addition, an increase in affinity for WGA and SBA lectins and a decrease in the affinity for the SNA lectin was found in the mucinous content of goblet cells, whereas no changes were detected regarding ConA and UEA-I lectins (**Table 2** and **Figures 3A–L**).

Microarrays and Gut Transcripteractome

A total number of 506 DEGs were found when comparing the transcriptomic profiling of the intestine from gilthead seabream fed the control and MPLE diets ($P < 0.05$; **Figure 4A** and **Supplementary Table 1**). Common segregation among the pool samples within the same dietary treatment was observed in the hierarchical clustering for the gut transcriptomic response based in similitude patterns of the DEGs response ($P < 0.05$; **Figure 4B**). The observed segregation among dietary treatments is supported by the PCA analysis for the analyzed samples (**Figure 4C**); in particular, four eigenvectors were calculated and three principal components were plotted, explaining the 83.2% of the total variability [component 1 (X-axis): 45.5%; component 2 (y-axis): 24.4%; component 3 (z-axis): 13.3%]. The detailed analysis of gene absolute fold-change (AFC) revealed that genes were mostly up-regulated in fish fed the MPLE diet (56.3% of DEGs), while its modulation was moderate in terms of AFC intensity (**Figure 4**). In particular, 285 of the above-mentioned DEGs were up-regulated with 219 of them within the $1.0 \leq AFC \leq 1.5$ interval, 58 DEGs were grouped within the $1.5 \leq AFC \leq 2.0$, and the last 9 DEGs up-regulated were grouped $2.0 \leq AFC \leq 3.0$. In contrast, 221 genes were significantly down-regulated and grouped in the range $-1.0 \leq AFC \leq -3.0$. ($P < 0.05$). In particular, 164 DEGs were mainly concentrated in the $-1.0 \leq AFC \leq -1.5$ range. Among them, 42 DEGs were grouped within the $-1.5 \leq AFC \leq -2.0$ interval, 10 DEGs were felt within the $-2.0 \leq AFC \leq -3.0$ category, while only 5 were found to have an AFC higher than -3 (**Figure 4**).

From the whole set of DEGs, a functional network analysis was performed. The transcripteractome showed 244 genes with 552 interactions (edges). The remaining 264 DEGs were classified as unknown genes; thus, they were excluded from the analysis. According to GO results and their respective annotation hierarchy, three main representative groups of genes were identified in the transcripteractome among the totality of biological processes obtained from the enrichment analysis (**Supplementary Table 2**): (1) immune system processes, (2) cellular development and organization, and (3) cellular catabolism (**Supplementary Tables 3–5**). **Table 3** summarizes the most relevant DEGs in fish fed the MPLE diet in relation to the above-mentioned biological processes.

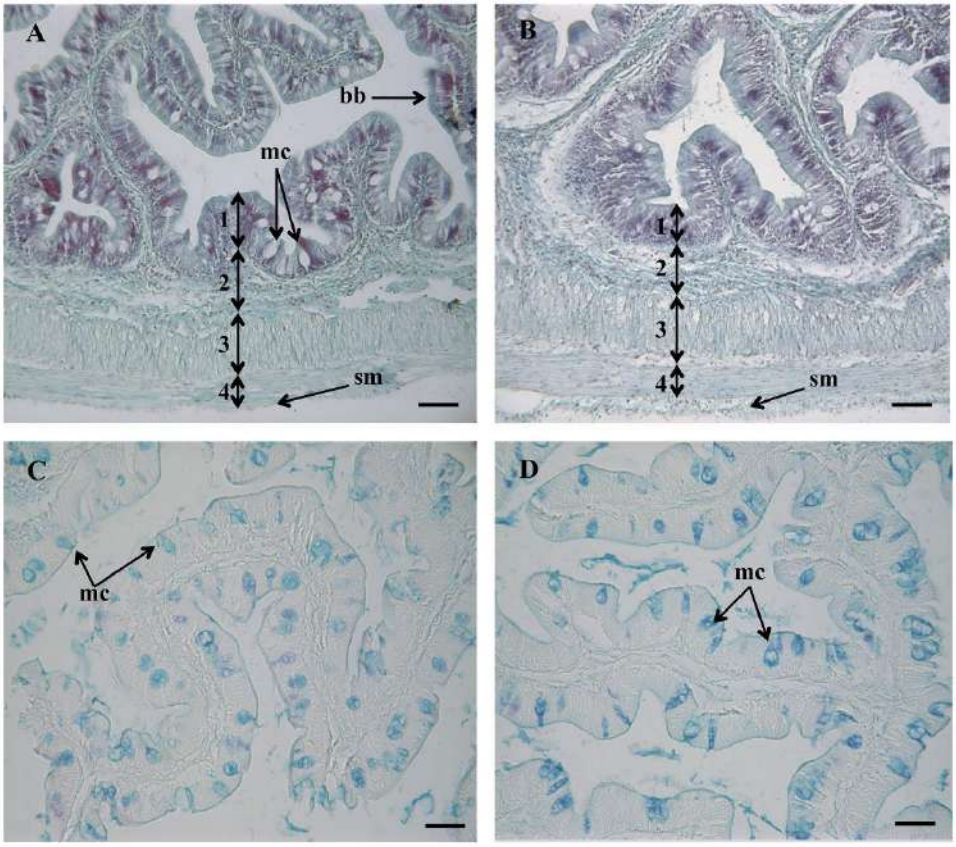


FIGURE 1 | Histological organization in the intestine of gilthead seabream (*Sparus aurata*) fed a control diet **(A)**, and medicinal plant leaf extract (MPLE)-supplemented diet **(B)**. Numbers indicate the different intestinal layers: (1) mucosa; (2) lamina propria-submucosa; (3) circular muscle layer; (4) longitudinal muscle layer. bb: brush border; mc: mucous cells; sm: serous membrane. Histochemical properties of mucins secreted by intestinal goblet cells with regard to their content on carboxylated and/or sulphated acidic groups (Alcian Blue pH = 2.5) from fish the control diet **(C)** and the MPLE-supplemented diet **(D)**. mc, mucous cells. The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*). Staining: hematoxylin-VOF **(A, B)**, Alcian Blue pH = 2.5/PAS **(C, D)**. Scale bar = 50 μm.

Regarding the dietary regulation of biological processes related to gut immunity, 18 genes were up-regulated and 14 genes down-regulated in fish fed the MPLE diet. In particular, several relevant GOs related to immunity were obtained such as "T cell activation" (GO:0042110; 7 up-regulated genes; 7 down-regulated genes), "T cell differentiation" (GO:0030217; 6 up-regulated genes; 5 down-regulated genes), "T cell lineage commitment" (GO:0002360; 3 up-regulated genes; 2 down-regulated genes), "leukocyte differentiation" (GO:0002521; 8 up-regulated genes; 7 down-regulated genes), "lymphocyte activation" (GO:0046649; 7 up-regulated genes; 9 down-regulated genes), "leukocyte activation" (GO:0045321; 15 up-regulated genes; 12 down-regulated genes), "lymphocyte differentiation" (GO:0030098; 6 up-regulated genes; 6 down-regulated genes), "CD4+ or CD8+, α-β T cell lineage commitment" (GO:0043369; 2 up-regulated genes; 2 down-regulated genes), "T cell selection" (GO:0045058; 3 up-regulated genes; 2 down-regulated genes) and "intracellular receptor signaling pathway" (GO:0030522; 7 up-regulated genes; 2 down-regulated genes) (**Figure 5** and **Supplementary Table 3**).

Furthermore, the MPLE diet promoted the regulation of biological processes associated with cellular development and organization with 19 up-regulated and 14 down-regulated genes. Among them, we found the terms "regulation of adherens junction organization" (GO:1903391; 3 up-regulated genes; 3 down-regulated genes), "regulation of anatomical structure morphogenesis" (GO:0022603; 14 up-regulated genes; 12 down-regulated genes), "negative regulation of cell size" (GO:0045792; 2 up-regulated genes; 1 down-regulated gene), "establishment of endothelial barrier" (GO:0061028; 3 up-regulated genes; 1 down-regulated gene), "regulation of cell junction assembly" (GO:1901888; 4 up-regulated genes; 2 down-regulated genes), "regulation of cell-substrate adhesion" (GO:0010810; 4 up-regulated genes; 5 down-regulated genes), "negative regulation of cell-substrate adhesion" (GO:0010812; 3 up-regulated genes; 2 down-regulated genes), "regulation of focal adhesion

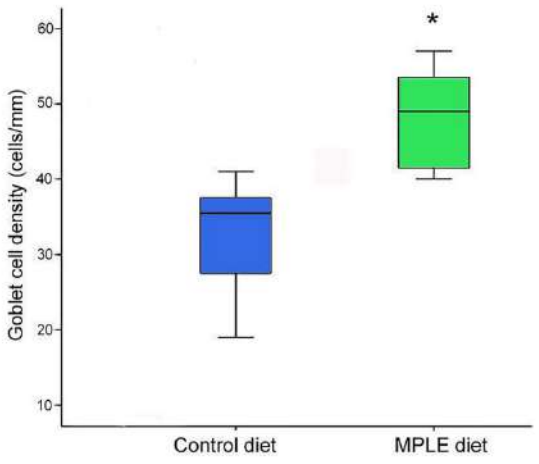


FIGURE 2 | Goblet cell density in the intestine of gilthead seabream (*Sparus aurata*) fed a control or a medicinal plant leaf extract (MPLE)-supplemented diet. The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*). The asterisk indicates statistically significant differences among dietary treatments (*t*-test; $P < 0.05$).

TABLE 2 | Histochemical characteristics and lectin affinity of mucins produced by goblet cells from anterior intestine of gilthead seabream (*Sparus aurata*) fed the control and this basal diet supplemented with a medicinal plant leaf extract (MPLE) obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

	Control diet	MPLE diet
General histochemistry		
Neutral glycoproteins	1 - 2	2 - 3
Carboxylated glycoproteins	1 - 3	3
Weakly inonised sulphated glycoconjugates	2 - 3	3
Strongly ionised sulphated glycoconjugates	2 - 3	2 - 3
Lectin histochemistry		
ConA (Man/Glu)	0	0
WGA (βGlcNAc>>NeuNAc/sialic acids/NANA)	2 - 3	3
SNA (Neur5Acα2; sialic acids/NANA)	1 - 3	0
SBA (α/β GalNAc)	0 - 3	1 - 3
UEA-I (Fuc)	0	0

Semi-quantitative assessment scoring based on color intensity scores: 0, negative (non detected); 1, weak; 2, moderate; 3, intense; 4, very intense.

assembly" (GO:0051893; 3 up-regulated genes; 2 down-regulated genes), "negative regulation of adherens junction organization" (GO:1903392; 1 up-regulated genes; 1 down-regulated genes), and "cell aging" (GO:0007569; 3 up-regulated genes; 1 down-regulated gene) (**Figure 6** and **Supplementary Table 4**).

The tested functional feed additive resulted in the positive regulation of biological processes related to cellular proteolytic processes, showing 35 up-regulated and 11 down-regulated genes. In particular, "cellular macromolecule catabolic process" (GO: 0044265; 23 up-regulated genes; 6 down-regulated genes), "proteolysis" (GO:0006508; 27 up-regulated genes; 8 down-regulated genes), "protein catabolic process" (GO:0030163; 20 up-regulated genes; 3 down-regulated genes), "proteolysis involved in cellular protein catabolic process" (GO:0051603; 17 up-regulated genes; 3 down-regulated genes) and "ubiquitin-dependent protein catabolic process" (GO:0006511; 14 up-regulated genes; 3 down-regulated genes) were obtained (**Figure 7** and **Supplementary Table 5**).

DISCUSSION

Considering the close relationship between diet and gut condition and the consequences on the organism and overall health, evaluating the interactions between dietary ingredients and the intestine is of special relevance due to the wide array of functions that have been associated to the gastrointestinal tract (34, 43). This is of special relevance when evaluating functional feed additives that are supposed to promote health and nutrition in farmed animals (9, 44). In this context, the present study aimed to evaluate the effects of a MPLE from sage and lemon verbena on the transcriptomic profiling, histological organization and lectin histochemistry of the intestine. Extracts from these medicinal plants are reputed for their beneficial pharmacological activities, including antiseptic, antioxidant, and anti-inflammatory properties among others (45–47). Moreover, in gilthead seabream we demonstrated that in addition to act as growth promoters, they modulated systemic immunity when splenocytes were incubated with a bacterial-type pathogen-associated molecular pattern (PAMP) like LPS (14).

MPLE From Sage and Lemon Verbena Exert Controlled Immune and Pro-Inflammatory Responses in GALT

Fish possess innate and adaptive immune defense systems. The innate parameters are at the forefront of immune defense and are a crucial factor in disease resistance. The adaptive response of fish is essential for long-lasting immunity and is considered as a key factor in improving prophylactic strategies, and the use of functional feeds with immunomodulatory properties (20, 48). T cells are one of the main players of the adaptive immune response, being the intestine of teleosts an important site of T cells production among mucosal tissues (49, 50). In particular, T cells represent the major leucocyte population within the teleost gut (51) with relevant cytotoxic activity (52), as well as playing an important role in foreign antigen recognition and gut homeostasis (53, 54). Under the current experimental conditions, MPLE from sage and lemon verbena promoted T cell activation, differentiation and selection in gilthead seabream gut. Thus, the MPLE-supplemented diet induced up-regulation of the mammalian target of rapamycin (*mtor*) gene in the gut of gilthead seabream. The mTOR signaling pathway has been reported to promote the production of anti-inflammatory cytokines by myeloid immune cells with an important ability to limit the pro-inflammatory mediators (55) thus, playing a crucial role in intestine inflammation, epithelial morphogenesis (56, 57), as well as being an important central regulator of immune responses (58, 59). The increase in *mtor* expression may be associated to the down-regulation of *nlrc3*, which is a negative regulator of the PI3K/AKT/mTOR pathway (60),

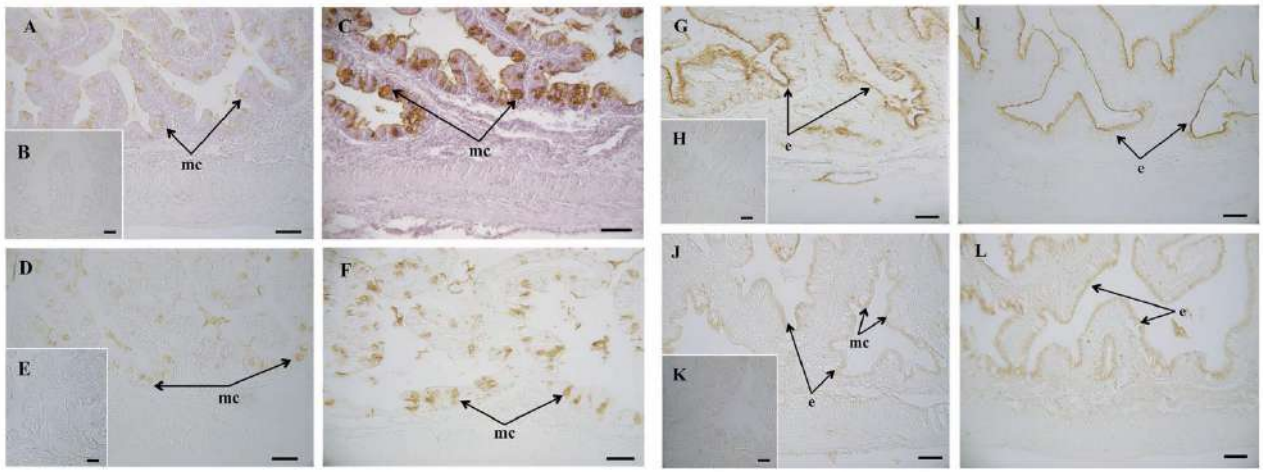


FIGURE 3 | Histochemical localization of glycoconjugates containing sugar residues in the intestine of gilthead seabream (*Sparus aurata*) fed a control or a medicinal plant leaf extract (MPLE)-supplemented diet. Presence of glycoconjugates containing N-acetyl-D-glucosamine and/or N-acetylneuraminic acid residues in mucous cells of *S. aurata* fed a control **(A)** (**B**: negative control) or a MPLE-supplemented diet **(C)**. Note the increase in affinity for WGA lectin in the mucinous content of goblet cells from MPLE diet. Glycoconjugates containing α-N-acetyl-D-galactosamine residues in mucous cells of *S. aurata* fed a control **(D)** (**E**: negative control) or a MPLE-supplemented diet **(F)**. Results denote a moderate increase in affinity for the SBA lectin in the mucous cells from MPLE diet. Histochemical detection of glycoconjugates containing N-acetylneuraminic acid/sialic acid residues in the intestine from control **(G)** (**H**: negative control) or MPLE group **(I)**. Note the decrease in affinity for the SNA lectin in the intestinal epithelium of *S. aurata* fed a MPLE-supplemented diet. Glycoconjugates containing α-mannose/α-glucose residues in intestine from *S. aurata* fed a control **(J)** (**K**: negative control) or a MPLE-supplemented diet **(L)**. Observe the increase in affinity for the ConA lectin in the intestinal epithelium of *S. aurata* fed a MPLE-supplemented diet. Mucous cells were negative for ConA lectin in both control and MPLE groups. e: epithelium; mc: mucous cells. The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*). Scale bar = 50 μm.

thus suggesting a balanced inflammatory dietary-induced response in the gut of fish fed the MPLE diet.

The NF-kB pathway is involved in the transcriptional regulation of several cytokines, chemokines, transcription factors, antimicrobial peptides, and interferon-stimulated genes, playing a critical role in regulating the survival, activation and differentiation of innate and adaptive immune cells (61); thus, playing a key role in regulating gut inflammation (62). In this way, several genes associated with the NF-κB pathway (*cyld, clec4e, nlrc3, chi3l1, bcl2*) were differentially regulated by the dietary supplementation of MPLE. Although scarce information is available for teleost fish, CYLD identified in rainbow trout (*Oncorhynchus mykiss*) would have a similar function as in mammals (63). In our study, *cyld* was up-regulated, which may indicate that in fish fed the MPLE diet this gene acted as an inhibitor of the NF-κB signaling pathway, thus acting as a negative regulator of gut inflammation (63, 64). Accordingly, the up-regulation of ubiquitin specific peptidase 4 (*usp4*) and down-regulation of Ubiquitin specific peptidase 7 (*usp7*) may be also associated to the inhibition of the activation of NF-κB and mitogen-activated protein kinases stimulated by innate immune receptor (65–67). Additionally, *chi3l1* was also up-regulated in fish fed the MPLE diet, promoting cell survival and proliferation, but not acting as a first inflammation-responsive factor. Thus, the differential regulation of the above-mentioned genes provide evidence that MPLE might induced inflammatory mechanisms related to the NF-κB signaling pathway (68, 69), which involved the production of pro-inflammatory cytokines (70). In this sense, histological data allow discarding the hypothesis that the administration of the MPLE caused an exacerbated gut inflammation, which would have been potentially detrimental for the health and condition of the intestinal mucosa of the gilthead seabream.

The Caspase-8 is a crucial executor for apoptotic initiation in fish (71). In our study, *casp8* was significantly up-regulated in the intestine of fish fed the MPLE-supplemented diet. Several studies have reported the advantageous outcomes of having the *casp8* expressed in a controlled way, demonstrating the essential role of this gene in maintaining the gut barrier in response to mucosal pathogens by permitting inflammatory shedding and preventing necroptosis of infected cells (72, 73). In addition, *stat3* (Signal transducer and activator of transcription 3) was down-regulated, which suggests a healthy intestinal mucosa as this gene is positively regulated by pro-inflammatory cytokines under mucosal damage (74). Furthermore, our enrichment analysis showed regulation of biological processes associated with cellular proteolysis, in particular several genes coding for de- and ubiquitination (*cyld, uba1, ube2a, ube2g1, ube2d2, usp4, usp7*) and proteasome (*cops4, psmb2, psmd1, psmb5, psmd5, sqstm1*) that may be indirectly involved in NF-kB pathway. In addition, degradation of a protein *via* the ubiquitin-proteasome pathway and subsequent proteolysis are important mechanisms in the

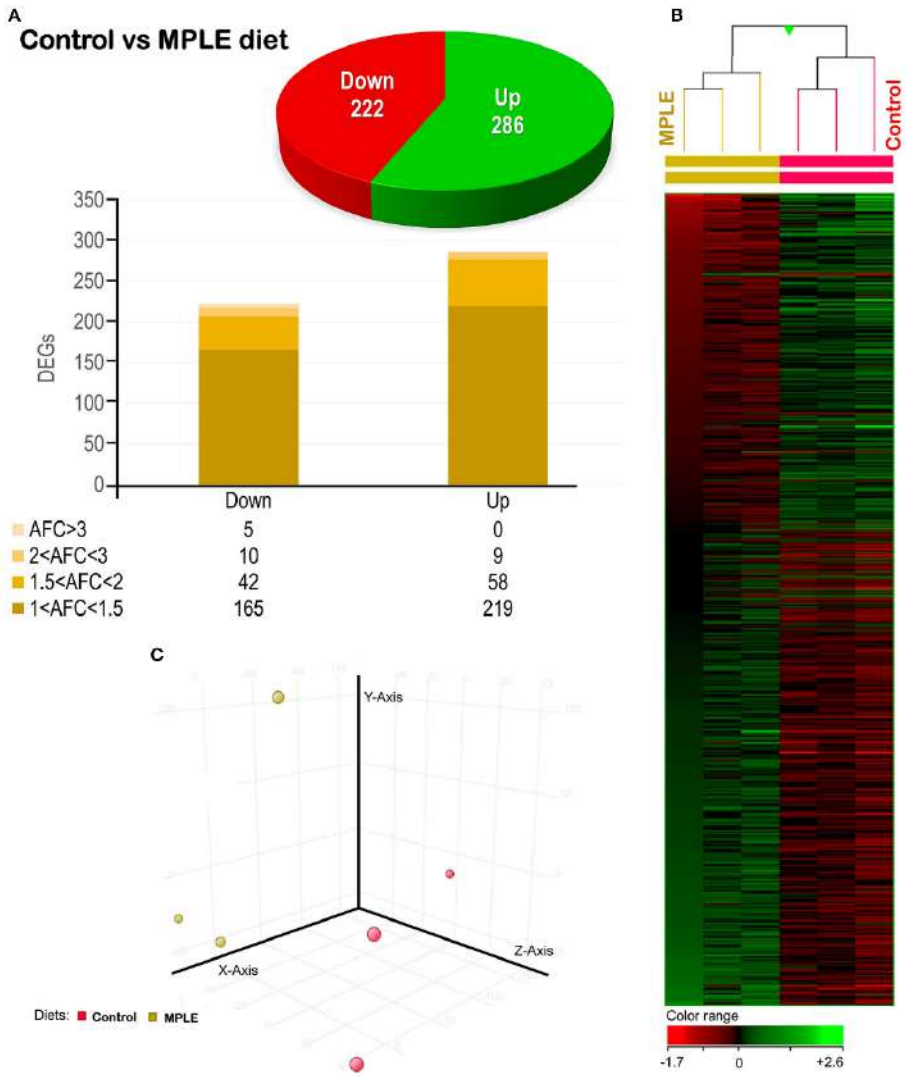


FIGURE 4 | Differential expression analysis of the gilthead seabream (*Sparus aurata*) mid-anterior intestine transcriptomic response to MPLE diet. **(A)** Distribution of the differential expressed genes (DEGs) obtained from the microarray-based transcriptomic analysis. The absolute fold change (AFC) indicates the fold-change magnitude interval of response. **(B)** Hierarchical clustering of the gilthead seabream mid-anterior intestine transcriptomic response for the control and MPLE diet, based in similitude patterns of the differentially expressed genes (DEGs) detected from three sample pools per dietary group. Data of the six microarrays are depicted, one for each representing the pooled RNA. Genes from each replicate are ordered from lower to higher AFC intensities using the left sample (randomly chosen) as a reference for ordering the other five samples (GeneSpring version 14.5 GX software; Agilent Technologies). Both increased and decreased gene expression pattern is shown in green and red, respectively, according to the color range (bottom). All transcripts represented are statistically significant ($P < 0.05$). **(C)** Principal component analysis (PCA) of the DEGs for the gilthead seabream intestine in response to the control (yellow node) and MPLE-supplemented diet (red node). The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

regulation of cell cycle, cell growth, tissue regeneration, signal transduction, and gene transcription (75).

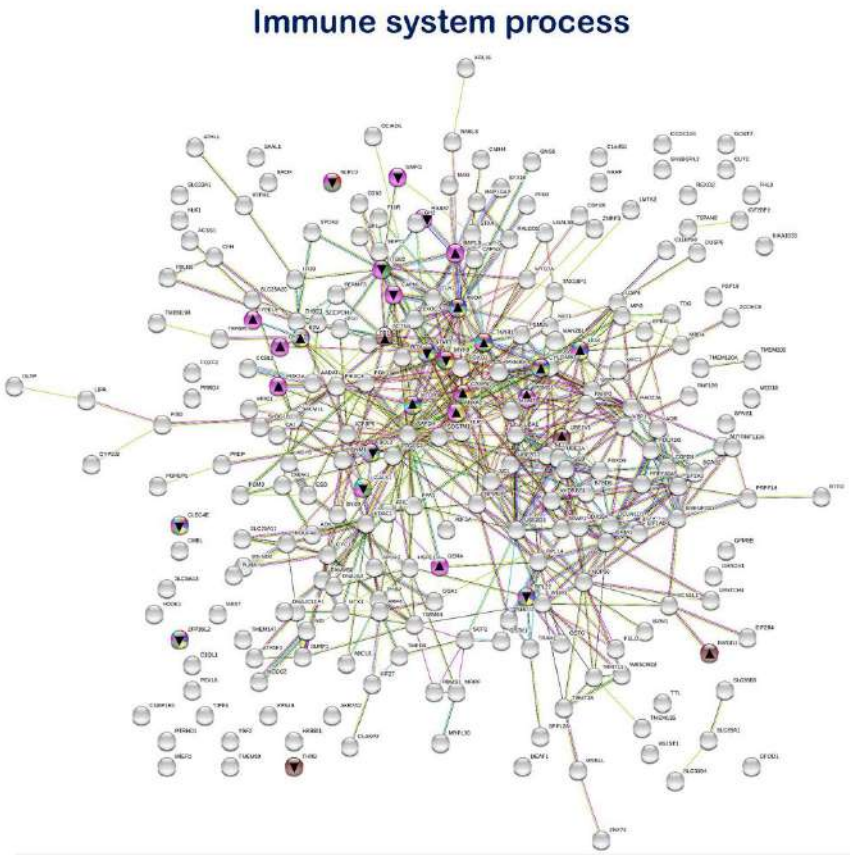
A successful immune response involves the tight control of a wide repertoire of processes including activators and regulators at cellular and molecular level. In this context, another up-regulated gene that deserves attention is the Chitinase 3 like 1 (*chi3l1*). This gene is reputed for regulating the AKT1 signaling pathway (76), which controls innate immune cell development and function (77). Additionally, we found down-regulation of the NOD-like family CARD domain containing 3 (*nlrc3*), which plays an important role in modulating T cell responsiveness and inhibition of the pro-inflammatory mechanism (78). Besides, galectin 1 (*lgals1*) was also down-regulated in the intestine of fish fed the MPLE diet. LGALS1 is considered a master regulator of homeostatic signals to shut off T-cell effector functions (79), acting as an immunoregulator and in turn modulating the

TABLE 3 | List of the most relevant DEGs related to three main representative biological processes identified by the transcripteractome (1, immune system processes; 2, cellular development and organization; 3, cellular catabolism) in fish fed the MPLE diet.

Gene description	Gene acronym	Biological processes	AFC	*P*-value
CYLD lysine 63 deubiquitinase	*cyld*	1	2,374	0,027
Actinin Alpha 4	*actn4*	2	1,631	0,022
Proteasome 26S subunit, non-ATPase 1	*psmd1*	1, 3	1,582	0,015
Ubiquitin like modifier activating enzyme 1	*uba1*	1, 3	1,570	0,020
Chitinase 3 Like 1	*chi3l1*	1	1,549	0,046
Proteasome 26S subunit, non-ATPase 5	*psmd5*	1, 3	1,537	0,041
Erythrocyte membrane protein band 4.1	*epb41*	2	1,476	0,032
Caspase 8	*casp8*	1	1,472	0,004
Sequestosome 1	*sqstm1*	1, 3	1,413	0,031
Proteasome subunit beta 5	*psmb5*	1, 3	1,408	0,020
Zinc and ring finger 3	*znrf3*	3	1,336	0,038
RAP1B, member of RAS oncogene family	*rap1b*	2, 3	1,321	0,004
LIM domain binding 1	*ldb1*	2, 3	1,311	0,019
COP9 signalosome subunit 4	*cops4*	1, 3	1,255	0,001
Ubiquitin specific peptidase 4	*usp4*	1, 3	1,231	0,037
Ras homolog family member A	*rhoa*	2	1,222	0,047
Fibroblast growth factor 18	*fgf18*	3	1,206	0,041
Mechanistic target of rapamycin kinase	*mtor*	1, 3	1,176	0,021
Proteasome subunit beta 2	*psmb2*	1, 3	1,167	0,023
Phosphoglycerate kinase 1	*pgk1*	3	1,164	0,046
Ubiquitin conjugating enzyme E2 A	*ube2a*	1, 3	1,102	0,039
Ubiquitin conjugating enzyme E2 G1	*ube2g1*	1, 3	-1,100	0,001
C-Type lectin domain family 4 member e	*clec4e*	1	-1,154	0,013
NLR family card domain containing 3	*nlrc3*	1	-1,315	0,020
Signal transducer and activator of transcription 3	*stat3*	1, 3	-1,367	0,0005
BCL2, apoptosis regulator	*bcl2*	1	-1,378	0,038
Ubiquitin specific peptidase 7	*ups7*	1, 3	-1,385	0,044
Galectin 1	*lgals1*	1, 3	-1,452	0,020
Ubiquitin conjugating enzyme E2 D2	*ube2d2*	1, 3	-1,457	0,021

pro-inflammatory cytokines secretion (80). At receptor level, the C-type lectin domain family 4 member e (*clec4e*, also known as mincle) was down-regulated in the intestine of fish from the MPLE group. Mincle is a transmembrane germline-encoded pattern recognition receptors (PRRs) with a pivotal role in the activation of the immune response (81, 82). Thus, when considering the overall expression patterns of DEGs, the dietary-induced activation of the immune function by the tested MPLE seems to be a well-controlled process, according to the observed balance between the up- and down-regulated DEGs (18 and 14 genes, respectively) and also the functional role of the DEGs related to T cells. As mentioned above, these results are in agreement with those recently published by Salomón et al. (14) where the same feed additive showed a tightly controlled systemic immune response in an *ex vivo* assay using splenocytes stimulated by LPS.

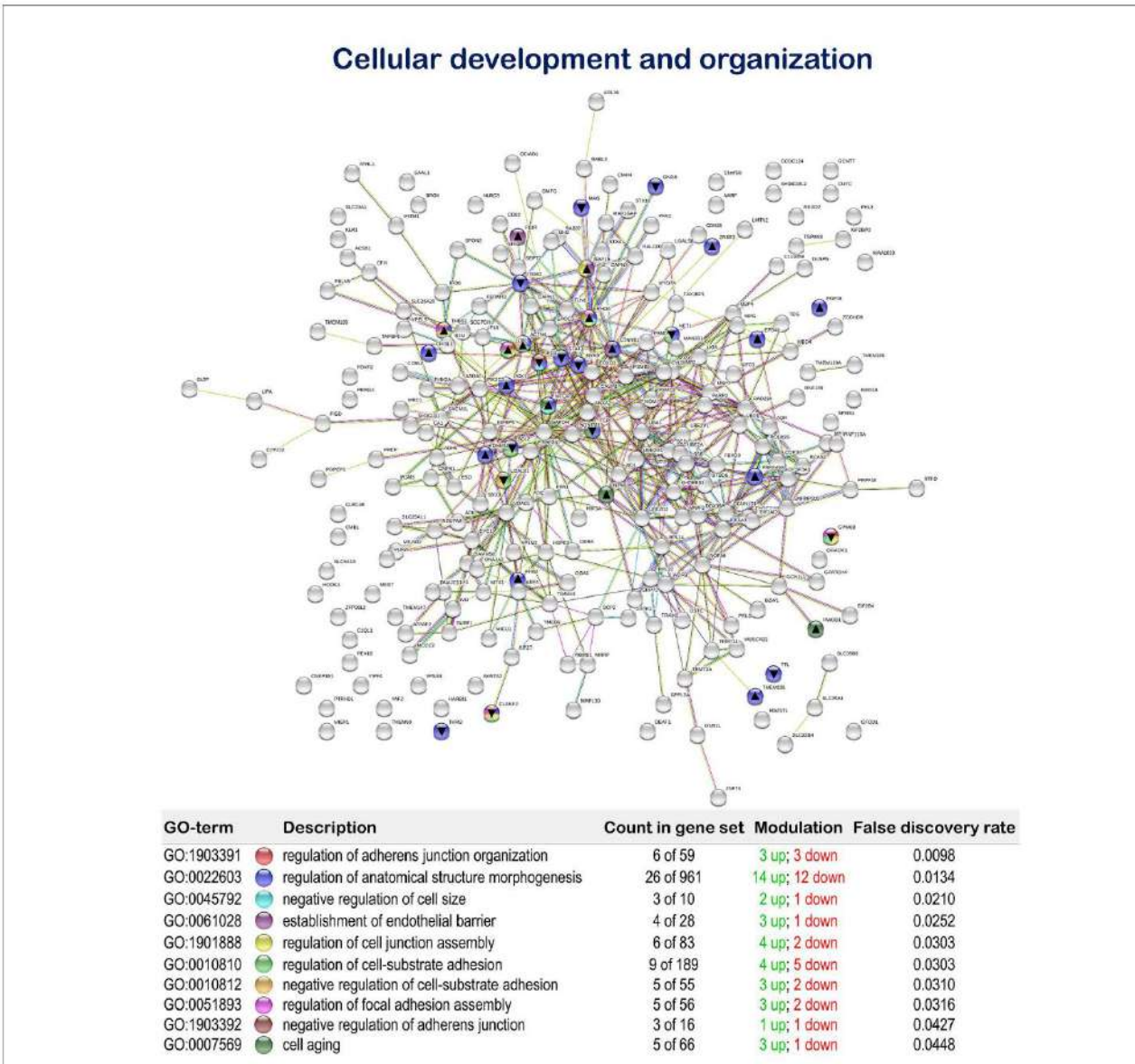
It is also of interest to illustrate that fish fed the supplemented-MPLE diet resulted in an increase of goblet cell population in the intestinal epithelium. These secretory cells are responsible for producing a thick layer of mucus that forms the front line of innate host defense, among other functions (83). The increase in intestinal goblet cell number in fish fed MPLE-supplemented diet would benefit fish by providing an effective immune barrier against potentially pathogenic gut bacteria. In addition to changes in their number, goblet cells also changed their histochemical composition. Particularly, we observed an increment in staining intensity of neutral and acid carboxylated and sulphated glycoproteins, results that are of special relevance since O-glycosylation pattern of mucin glycoproteins and their polypeptide backbone structure in combination with the depth and viscosity of the mucus layer, are important factors that regulate gut health and condition (84). In this sense, the increase in acid carboxylated (AB pH = 2.5) and sulphated (weak and strongly ionized; AB pH = 1.0 and pH 0.5, respectively) mucin glycoproteins produced by goblet cells from fish fed the MPLE-supplemented diet would be associated to an increment in the viscous properties of the mucus (85, 86). Our results are in agreement with those reported in poultry where the



GO-term	Description	Count in gene set	Modulation	False discovery rate
GO:0042110	T cell activation	14 of 225	7 up; 7 down	0.00043
GO:0030217	T cell differentiation	11 of 131	6 up; 5 down	0.00043
GO:0002360	T cell lineage commitment	5 of 19	3 up; 2 down	0.0014
GO:0002521	leukocyte differentiation	15 of 313	8 up; 7 down	0.0017
GO:0046649	lymphocyte activation	16 of 358	7 up; 9 down	0.0018
GO:0045321	leukocyte activation	26 of 894	14 up; 12 down	0.0024
GO:0030098	lymphocyte differentiation	12 of 226	6 up; 6 down	0.0033
GO:0043369	CD4-positive or CD8-positive, alpha-beta T cell	4 of 14	2 up; 2 down	0.0046
GO:0045058	T cell selection	5 of 37	3 up; 2 down	0.0103
GO:0030522	intracellular receptor signaling pathway	9 of 173	7 up; 2 down	0.0197

FIGURE 5 | Immune system process related Protein-Protein Interactions Network (PPI) network of the differentially expressed genes (DEGs) in the mid-anterior intestine of juvenile gilthead seabream (*Sparus aurata*) fed the mixture of MPLE obtained from sage (*S. officinalis*) and lemon verbena (*L. citriodora*) (see also **Supplementary Table 3**). Nodes colors indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. ▲ nodes represent up-regulated genes and ▼ nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend. Network Stats: number of nodes: 243; number of edges: 550; average node degree: 4.53; avg. local clustering coefficient: 0.416; expected number of edges: 423; PPI enrichment p-value: 1.98e-09. The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

administration of phytogenics from sage promoted the secretion of neutral and acidic mucins in gut as well as mucus layer thickness in the ileum (87). Regarding lectin histochemistry, mucins from fish fed the MPLE-supplemented diet showed an increase in affinity for the WGA and SBA lectins, while for SNA lectin affinity was diminished. These results indicated that the tested feed additive changed the glycosylation patterns of intestinal mucins, being of special relevance the increase in WGA lectin affinity, as they indicate an increase in the presence of sialic acid residues. Sialic acids are involved in recognition processes, and they participate in defense mechanisms against microorganisms (88). These results from lectin histochemistry reinforce the hypothesis that the MPLE-supplementation varied the composition of mucus produced by goblet cells; thus, modulating an effective immune barrier against potentially pathogenic gut bacteria.

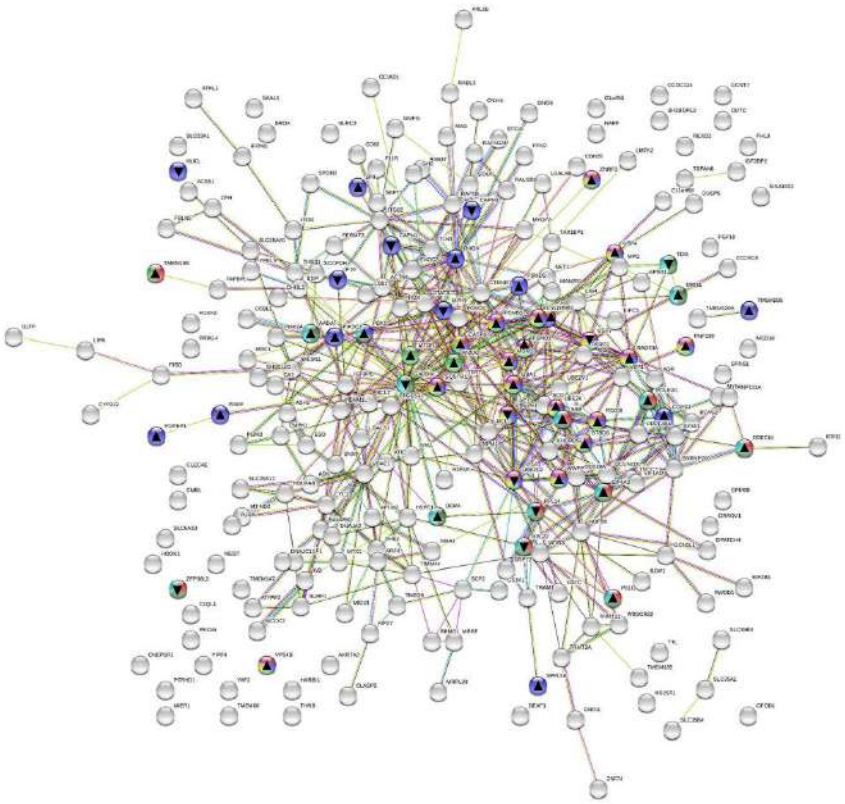


GO-term	Description	Count in gene set	Modulation	False discovery rate
GO:1903391	regulation of adherens junction organization	6 of 59	3 up; 3 down	0.0098
GO:0022603	regulation of anatomical structure morphogenesis	26 of 961	14 up; 12 down	0.0134
GO:0045792	negative regulation of cell size	3 of 10	2 up; 1 down	0.0210
GO:0061028	establishment of endothelial barrier	4 of 28	3 up; 1 down	0.0252
GO:1901888	regulation of cell junction assembly	6 of 83	4 up; 2 down	0.0303
GO:0010810	regulation of cell-substrate adhesion	9 of 189	4 up; 5 down	0.0303
GO:0010812	negative regulation of cell-substrate adhesion	5 of 55	3 up; 2 down	0.0310
GO:0051893	regulation of focal adhesion assembly	5 of 56	3 up; 2 down	0.0316
GO:1903392	negative regulation of adherens junction	3 of 16	1 up; 1 down	0.0427
GO:0007569	cell aging	5 of 66	3 up; 1 down	0.0448

FIGURE 6 | Cellular development and organization related Protein-Protein Interactions Network (PPI) network of the differentially expressed genes (DEGs) in the mid-anterior intestine of juvenile gilthead seabream (*Sparus aurata*) fed the mixture of MPLE obtained from sage (*S. officinalis*) and lemon verbena (*L. citriodora*) (see also **Supplementary Table 4**). Nodes colors indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. ▲ nodes represent up-regulated genes and ▼ nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend. Network Stats: number of nodes: 243; number of edges: 550; average node degree: 4.53; avg. local clustering coefficient: 0.416; expected number of edges: 423; PPI enrichment p-value: 1.98e-09. The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

MPLE From Sage and Lemon Verbena Promote Gut Integrity and Cell Proliferation

The homeostatic balance between epithelial cell proliferation and apoptosis is essential for the maintenance of the epithelial function, including regulation of epithelial permeability, the inflammatory response and the absorption of nutrients (89). An imbalance in the intestinal barrier structure can trigger an uncontrollable immune reaction in the intestinal micro environment, increasing the translocation of bacterial antigens and stimulating inflammation in the intestine (90). Thus, the maintenance of the integrity of the gut barrier is essential to counteract such imbalances and guarantee fish growth, health and welfare (91). Dietary MPLE from sage and lemon verbena modulated the expression of genes involved in biological processes related to cellular development and organization. In particular, Ras homolog family member A (*rhoa*) was



GO-term	Description	Count in gene set	Modulation	False discovery rate
GO:0044265	cellular macromolecule catabolic process	29 of 842	23 up; 6 down	0.00037
GO:0006508	proteolysis	35 of 1203	27 up; 8 down	0.00065
GO:0030163	protein catabolic process	23 of 615	20 up; 3 down	0.00076
GO:0051603	proteolysis in cellular protein catabolic process	20 of 532	17 up; 3 down	0.0018
GO:0006511	ubiquitin-dependent protein catabolic process	18 of 461	14 up; 3 down	0.0025

FIGURE 7 | Proteolysis related Protein-Protein Interactions Network (PPI) network of the differentially expressed genes (DEGs) in the mid-anterior intestine of juvenile gilthead seabream (*Sparus aurata*) fed the mixture of MPLE obtained from sage (*S. officinalis*) and lemon verbena (*L. citriodora*) (see also **Supplementary Table 5**). Nodes colors indicate the biological processes for each DEG represented. Gene Ontology (GO) definitions, count of DEGs within each biological processes and respective false discovery rate are described in the graphical figure legend. ▲ nodes represent up-regulated genes and ▼ nodes represent down-regulated genes. Graphic keys and network stats are indicated in the graphical figure legend. Network Stats: number of nodes: 243; number of edges: 550; average node degree: 4.53; avg. local clustering coefficient: 0.416; expected number of edges: 423; PPI enrichment p-value: 1.98e-09. The MPLE included in the basal diet was obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*).

up-regulated in the intestine of fish fed the MPLE-diet. RhoA belongs the small GTPase protein family, thus having a functional role in regulating many cellular events, including cell migration, organization of the cytoskeleton, cell cycle progression and cell adhesion (92). In this sense, *rhoa* expression has been associated to the assembly, regulation and maintenance of adherens (AJs) and tight (TJs) junctions (93, 94). Adherens junctions are cadherin/catenin-containing adhesive structures located below the tight junctions on the bilateral membrane (95). In particular, the Erythrocyte membrane protein band 4.1 (*epb41*) was another up-regulated gene in the intestine of fish fed the MPLE-diet. EPB41 regulates cell proliferation and adhesion (96), as well as the integrity of the AJ (95) and plays an important role in the organization and function of the TJs and AJs by establishing a link between the TJ and actin in the cytoskeleton (97). Furthermore, other DEGs related to the formation and stabilization of AJs and TJs (*f11r, actn4, rap1b*) were also up-regulated in the gut of fish fed the

MPLE-supplemented diet. The F11 receptor (F11R) also known as the Junctional adhesion molecule (JAM)-A is a cell-cell adhesion molecule of the immunoglobulin superfamily, which is expressed by a variety of tissues, regulating diverse processes such as epithelial and endothelial barrier formation, among others (98). Our results are in agreement with those found in gilthead seabream fed a phytogenic feed additive derived from olive oil (89). Alpha-actinin-4 (ACTN4) is a member of the superfamily of actin-binding, which is localized at AJs and more specifically as a component of the *zonula occludens* of the TJ (99) and/or belt desmosomes in the *zonula adherens* (100) in connection with α-catenin, regulating the actin cytoskeleton and increasing cellular motility (101, 102). Another gene that was up-regulated by the dietary administration of MPLE from sage and lemon verbena was the *rap1b*. This gene is a member of RAS oncogene family being necessary for normal human endothelial cell function (103), as well as having a crucial role for T cell homeostasis in the intestine (104). Regarding the gut integrity barrier, *rap1b* controls epithelial permeability probably by regulation of PI3K/Akt and correct nectin localization (94).

The homoeostasis of the constantly renewing intestinal epithelium relies on an integrated control of proliferation, differentiation and apoptosis, as well as on the functional architecture of the epithelial cells. In this sense, different DEGs indicated that the MPLE-supplemented diet regulated mostly processes involved cell proliferation (*fgf18*, *pgk1*, *ldb1*, *znrf3*, *mtor*, *lgals1*, *stat3*) and de- and ubiquitination (*cyld*, *uba1*, *ube2a*, *ube2g1*, *ube2d2*, *usp4*, *usp7*) and proteasome (*cops4*, *psmb2*, *psmd1*, *psmb5*, *psmd5*, *sqstm1*) pathways. The Fibroblast growth factor 18 (FGF18) is a pleiotropic growth factor which stimulates the proliferation of mesenchymal and epithelial cells and tissues (105); thus, its up-regulation in the tissue of gilthead seabream fed the MPLE-supplemented diet suggests that the tested phytogenic feed additive might promote cell proliferation. Similar conclusions may be drawn when considering the up-regulation *ldb1* (LIM domain binding 1) and *znrf3* (Zinc and ring finger 3), since these genes regulate cell proliferation in intestinal crypts by means of the regulation of the Wnt/ß-catenin signaling (106, 107). In addition, the up-regulation of *mtor* (mammalian Target of Rapamycin) and *pgk1* (Phosphoglycerate kinase 1) indicate the regulation of intestinal cell proliferation by means of controlling cell metabolism (108, 109). These results are supported by the down-regulation of *lgals1* (galectin 1), since this gene product may act as a negative growth factor that regulates cell proliferation (110). Differential expression of genes involved in de- and ubiquitination and proteasome pathways supports the above-mentioned idea that the tested MPLE-supplemented diet promotes and regulates cell cycle associated to intestinal cell proliferation (75) as well in cell junctions (111). All these results together suggested that the MPLE from sage and lemon verbena might promote a positive effect on intestinal cell proliferation, a process that is essential for the maintenance of the integrity and health of the gastrointestinal tract (112). In line with this idea, no signs of tissue damage were observed at histological level either pathways involved in cell apoptosis were found in these animals.

CONCLUSIONS

The present study shows that phytogenics obtained from sage and lemon verbena included at 0.1% in diets with a low FM content (7%), promoted transcriptional innate and adaptive immune responses in gut, especially through the modulation of those processes involved in T cell activation, differentiation and selection. Furthermore, the evaluated feed additive increased the number of intestinal goblet cells and modified the glycosylation properties of lectins from mucins. These changes resulted in a moderate increase in sialic acid residues, which also supports the idea that phytogenics from sage and common verbena might enhance gut immunity. Overall, this study shows that the evaluated phytogenic can be used as a safe feed additive for gilthead seabream, since its immunomodulatory properties were observed without compromising gut homeostasis and integrity of the intestinal epithelium.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015), and authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain).

AUTHOR CONTRIBUTIONS

Conceptualization, EG. Methodology, EV-V, FER-L, CS, JO-D, RS. Formal analysis, RS, FER-L, JF, EV-V. Resources, EG, CS. Writing original draft, RS. Writing review and editing, EV-V, RS, FER-L, EG, JF, LT. Visualization, RS, FER-L, EV-V, JO-D, EG. Supervision, EG, EV-V. Project administration, EG. Funding acquisition, EG. All authors contributed to the article and approved the submitted version.

FUNDING

This work has been financially supported by the project "Nutritional strategies for the improvement of productive performance: the use of functional feeds and health diets in aquaculture (DIETAplus)", funded by JACUMAR (Ministry of Agriculture, Fisheries and Environment of Spain, MAPAMA) and FEMP (EU), as well as by the ERC (European Research Council) in MedAID project (Mediterranean Aquaculture

Integrated Development; Grant Agreement Nb. 727315). FR-L thanks the support of Fondecyt Regular grant (Nb. 1211841; ANID; Government of Chile). RS is supported by a PhD grant from the government of Paraguay (BECAL). JF have been subsidized by the Industrial PhD program of Generalitat de Catalunya and TECNOVIT-FARMFAES S.L. (Nb. 2017 DI 017). EV-V was financially granted with DICYT-USACH Postdoctoral fellowship (Nb. 022043IB). Collaboration between Ibero-American researchers has been done under the framework of the network LARVAplus "Strategies for the development and improvement of fish larvae production in Ibero-America" (117RT0521) funded by the Ibero-American Program of Science and Technology for Development (CYTED, Spain).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.670279/full#supplementary-material

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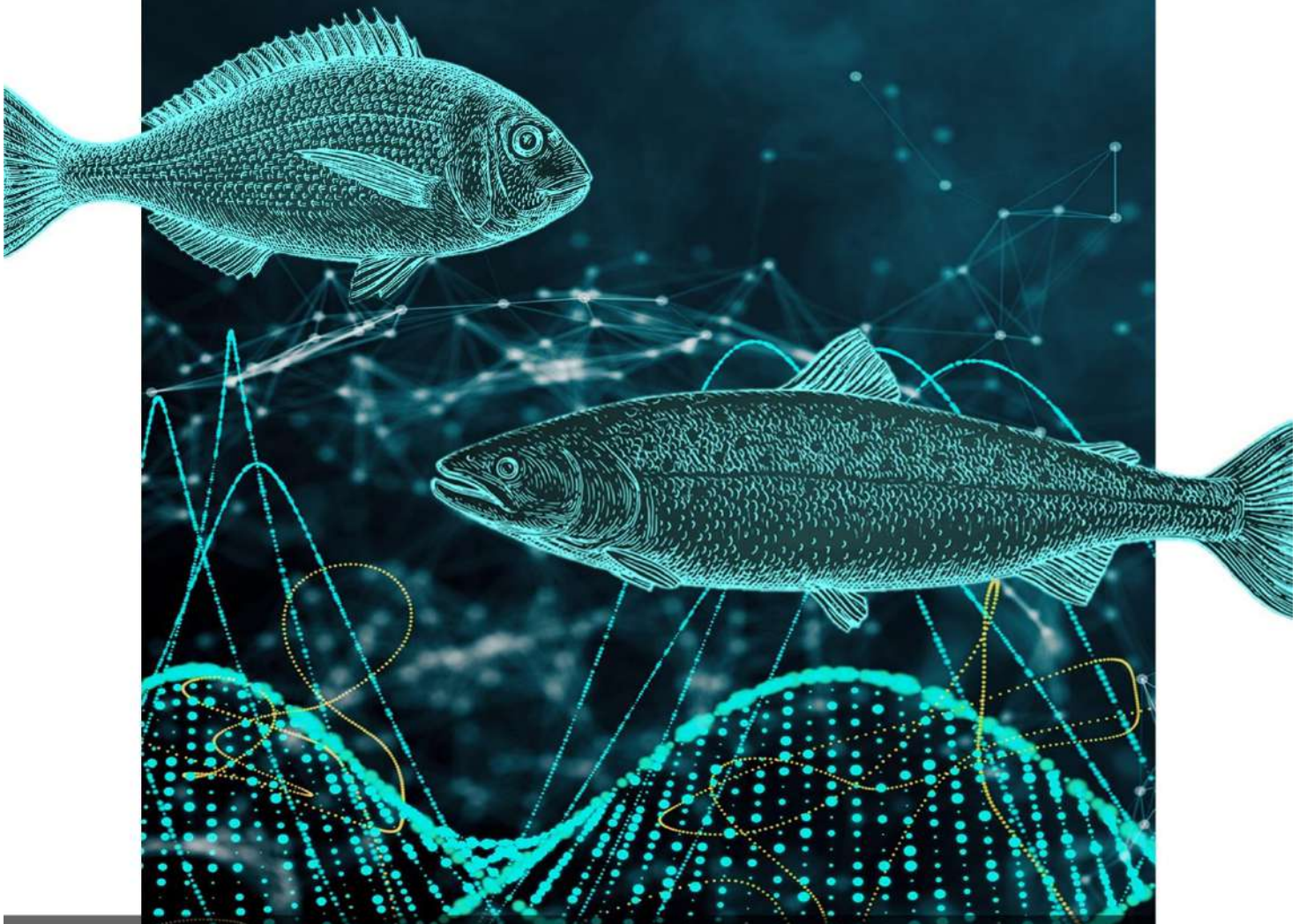
Conflict of Interest: JQ and JP are current NATAC BIOTECH S.L. employers. FER-L was Senior research associate by Consorcio Tecnológico de Sanidad Acuícola, Ictio Biotechnologies S.A.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CAPÍTULO III



Phytogenics from sage and lemon verbena promote growth, systemic immunity and disease resistance in Atlantic salmon (*Salmo salar*)

Frontiers in Marine Science 9:828497

frontiers in Marine Science

ORIGINAL RESEARCH
published: 08 February 2022
doi: 10.3389/fmars.2022.828497

Phytogenics From Sage and Lemon Verbena Promote Growth, Systemic Immunity and Disease Resistance in Atlantic Salmon (*Salmo salar*)

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The transcriptomic response of the head kidney, the main lymphohematopoietic tissue of the body, was evaluated in Atlantic salmon (*Salmo salar*) smolts fed a functional feed containing a phytogenic rich in verbascoside and triterpenic compounds like ursolic acid. Fish (initial body weight = 55.0 ± 0.1 g) were fed two experimental diets (40% crude protein, 22% crude fat; 21.6 MJ/kg gross energy) that only differed in the phytogenic content (0.1% inclusion). Each diet has six replicates and was tested over a period of 133 days. The tested zootechnical feed additive a medicinal plant leaf extract (MPLE) obtained from sage (*Salvia officinalis*) and lemon verbena (*Lippia citriodora*). At the end of the trial, smolts fed the MPLE diet were heavier than their congeners from the control group (271.5 ± 7.9 g vs. 240.2 ± 19.3 g, respectively; $P < 0.05$). Feed conversion ratio (FCR) values in fish fed the control diet were higher than those in fish fed the MPLE diet ($FCR_{controldiet}$ = 1.27 ± 0.08 vs. $FCR_{0.1\%MPLEdiet}$ = 1.08 ± 0.05; $P < 0.05$). The immunomodulatory properties of the functional diet were evaluated by means of an *in vivo* challenge with *Aeromonas salmonicida* subsp. *salmonicida* (1×10^7 CFU mL^{-1}). The microarray analysis of head kidney samples from both dietary groups revealed 1,178 differentially expressed genes (802 upregulated and 376 downregulated). Among them, several biological processes related to immunity were identified in fish fed the MPLE diet (*i.e.*, interferon-gamma-mediated signaling pathway, antigen processing and presentation of peptide antigen *via* MHC class II, autophagy, regulation of i-kappaB kinase/NF-kappaB signaling, and leukocyte activation). Results from the bacterial challenge showed that survival rates were higher in smolts from the MPLE group (90.6 ± 6.4%) in comparison to the control group (60.7 ± 13.5%), confirming the functional benefits of the phytogenic in terms of host's immunity and disease resistance. Biological processes such as cytoskeleton organization and regulation of cellular protein metabolic process detected in fish fed the MPLE diet supported the metabolic changes related to increased somatic growth promoted. The present findings showed that the

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Edited by:
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Jimei University, China

Reviewed by:
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Specialty section:
This article was submitted to Marine Fisheries, Aquaculture and Living Resources, a section of the journal Frontiers in Marine Science

Received: 03 December 2021
Accepted: 04 January 2022
Published: 08 February 2022

Citation:
Salomón R, Furones MD, Reyes-López FE, Tort L, Firmino JP, Quintela JC, Pinilla-Rosas JM, Vallejos-Vidal E and Gisbert E (2022) Phytogenics From Sage and Lemon Verbena Promote Growth, Systemic Immunity and Disease Resistance in Atlantic Salmon (Salmo salar). Front. Mar. Sci. 9:828497. doi: 10.3389/fmars.2022.828497

inclusion at 0.1% of the tested MPLE obtained from sage and lemon verbena in diets for Atlantic salmon smolts promoted somatic growth, and enhanced their systemic immune response and reduced mortality when fish were challenged with *A. salmonicida* cumulative, the causative agent of furunculosis in salmonids.

Keywords: feed additive, aquaculture, systemic immunity, Atlantic salmon, *Aeromonas salmonicida*, phytogenics

INTRODUCTION

Aquaculture is predicted to be the main source of aquatic dietary protein sources by 2050, playing a relevant role in food security and supply, as well as in poverty alleviation (Stentiford et al., 2020). The Atlantic salmon (*Salmo salar*) is the most important fish species consumed in the countries of the first world, whose production has strongly increased due to the development of this industry in the northern Europe and in North and South America, with Norway and Chile as the main world producers (FAO, 2021). Despite the sector's efforts focused on competitiveness and sustainable development to build this thriving sector, this rapid and continuous growth of the salmon farming has some side effects. In this sense, under intensive farming, fish can be influenced by various environment-related biotic and abiotic factors that can have potentially harmful or stressful effects (Taranger et al., 2015). All these factors have a negative impact on fish welfare and overall rearing performance, increasing susceptibility to disease; thus, negatively impacting the industry by causing health crises and economic losses (Tort, 2011; Taranger et al., 2015). This makes aquatic animal diseases one of the main factors limiting the growth of aquaculture and its sustainability (Reverter et al., 2020; Naylor et al., 2021).

Despite the fact that in 2022 several countries, including the EU, will ban the regular administration of antimicrobial agents in farming, including preventive group treatments (More, 2020), the general use of antibiotics for prophylactic purposes linked to intensive aquaculture activities can still be detected in some of the major aquaculture producing countries (Lulijwa et al., 2020; Schar et al., 2020). Thus, the need to develop health preventive treatments is becoming more of a necessity than an option. Thus, among the repertoire of tested strategies related to health management (Barrett et al., 2020; Miccoli et al., 2021), functional feeds are reputed as one of the most affordable solutions in terms of their prophylactic application. These diets are formulated for supporting the nutritional and physiological requirements of fish, as well as providing protection in front of biotic and abiotic stressors that are intrinsic to aquaculture rearing conditions (Waagbø and Remø, 2020). In this regard, the development and application of functional feeds represent a sound strategy for the aquaculture industry, as they provide functional benefits for animal health beyond their nutritional value, taking into account the purpose of their use either as nutritional, sensory, or functional additives (Vallejos-Vidal et al., 2016; Dawood et al., 2018). Thus, feed additives promoting immunity and enhancing stress and disease resistance in farmed fish have received notorious attention by the industry and academia as environmentally-friendly health management strategies. Therefore, phytogenics are among others, one of the most widely evaluated and recognized zootechnical feed additives with immunomodulatory properties (Encarnação, 2016). Phytogenics are defined as environmentally friendly plant-derived bioactive compounds that show positive effects on animal growth and health-promoting, antimicrobial, antiparasitic, immunostimulant, antioxidant, and anti-inflammatory properties (Firmino et al., 2020; Dawood, 2021; Reverter et al., 2021).

Functional feeds based on phytogenics have been the focus of attention for the industry during the last decade due to their antimicrobial, immunostimulant, antioxidant, anti-stress, and growth-promoting functions (Awad and Awaad, 2017; Sutili et al., 2018; Hernández-Contreras and Hernández, 2020). Furthermore, phytogenics have shown to enhance both humoral and cellular immune response in teleosts (Elumalai et al., 2020; Firmino et al., 2021a,b), whereas other studies have also demonstrated their antimicrobial activity against a wide range of pathogenic organisms (Vaseeharan and Thaya, 2014; Firmino et al., 2021a). These functional properties make them very attractive for the industry as potential prophylactic dietary treatments. In particular, the current investigation endeavors to explore the dietary effects of phytogenics derived from a mixture of medicinal plants, the sage (*Salvia officinalis*, Lamiaceae) and the lemon verbena (*Lippia citriodora*, Verbenaceae), both recognized for their health and growth-promoting properties for aquatic species (Elumalai et al., 2020; Salomón et al., 2020, 2021a). In particular, in a previous study from our group, we showed that a medicinal plant leaf extract (MPLE) from sage and lemon verbena promoted an improvement in the classical key performance indicators (KPIs) linked to somatic growth and feed efficiency. These effects were coupled with a tightly controlled systemic immune response in an *ex vivo* assay using gilthead seabream (*Sparus aurata*) splenocytes stimulated by lipopolysaccharide (LPS; Salomón et al., 2020). In addition, we have recently reported that this MPLE obtained from sage and lemon verbena promoted gut integrity and immunity; particularly, T cell activation and differentiation (Salomón et al., 2021a).

In traditional medicine, *S. officinalis* has long enjoyed a reputation for its health benefits and for treating all kinds of ailments. Sage is a common herbal plant widely cultivated in various parts around the world, but it is native to the Mediterranean region. In addition, is known to be rich in phenolic compounds such as flavonoids, tannins, coumarins, and triterpenes (Ghorbani and Esmaeilizadeh, 2017), which are a highly diverse group of natural components widely found in a variety of common European plants and fruits (Vincken et al., 2007; Babalola and Shode, 2013).

Thus, its content in functional compounds has attracted the attention within livestock and aquaculture industry. For instance, Simonová et al. (2010) reported that diets containing sage increased the energy content and amino acid profile in rabbit meat, in addition to promoting a good health condition of the animals. Similarly, Placha et al. (2015) demonstrated that sage promoted the integrity of the duodenal wall in laying hens. Regarding aquatic species, Sönmez et al. (2015) reported a positive effect of sage on growth performance and antioxidant enzyme activities in juvenile rainbow trout (*Oncorhynchus mykiss*). In this sense, several bioactive compounds have been identified in plants from the genus *Salvia*, such as flavonoids (Lu and Foo, 2000), phenolic acids (Wang et al., 1999), and pentacyclic triterpenes (Mašterová et al., 1989), among others. For instance, one of these triterpenic acids is ursolic acid, which is a pentacyclic terpenoid that has shown many beneficial properties effects on human health (Woźniak et al., 2015), and even in teleosts (Ding et al., 2015; Li et al., 2019). In zebrafish (*Danio rerio*), ursolic acid was reported to have anti-inflammatory activity (Ding et al., 2015), whereas, in rainbow trout, a strong antiviral activity was reported both *in vitro* and *in vivo* (Li et al., 2019).

Lippia citriodora, colloquially known as lemon verbena, is a plant species of the *Verbenaceae* family that mostly grows in South America and is cultivated in northern Africa and southern Europe. Lemon verbena leaf extract contains polyphenols, including phenylpropanoids such as verbascoside, iridoids like gardoside, and flavonoids such as luteolin-7-diglucoronide, among which verbascoside acid is the most abundant compound in lemon verbena leaves, so most of its beneficial effects are attributed to this phytochemical (Sánchez-Marzo et al., 2019). Several studies have indicated that verbascoside acid is responsible for multiple beneficial properties of lemon verbena like its antioxidant (Mosca et al., 2014; Martino et al., 2016), anti-inflammatory, and antineoplastic properties in addition to numerous wound-healing and neuroprotective properties (Funes et al., 2009; Caturla et al., 2011; Alipieva et al., 2014). The use of lemon verbena in juvenile sheep has been reported to promote embryo development by protecting the oocyte against oxidative stress (Martino et al., 2016). In addition, another study showed that pigs fed with a diet enriched with verbascoside rich showed an improvement in their growth and feed efficiency performances (Corino et al., 2007; Pastorelli et al., 2012). Despite the existing literature, information on the function of these bioactive compounds of plant origin, such as sage and lemon verbena, is still scarce regarding their applications in animal production, especially their immunomodulatory effects on the systemic immune response and their potential use as a functional feed additive to promote disease resistance in fish.

Under this context, the present study aimed to evaluate the transcriptional responses of the head kidney in Atlantic salmon smolts fed a functional feed containing a mixture of MPLE obtained from sage and lemon verbena. At the end of the nutritional trial, disease resistance in smolts was evaluated by means of a bacterial challenge with the causative agent of furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*).

MATERIALS AND METHODS

This study was divided into two different stages. Firstly, a nutritional trial during 133 days was conducted in order to evaluate the effects of the phytogenic on growth performance and transcriptomic analysis in head kidney of Atlantic salmon. The nutritional trial encompassed different periods, the parr phase (47 days; 19th December – 04th February); the smoltification phase, which started on the 5th of February and lasted 10 days; and the full seawater transfer stage that started on the 14th February until the end of the nutritional assay. In the second stage, fish from the nutritional trial were used in a bacterial challenge in order to test whether the tested MPLE diet provided protection to the host in front of a pathogenic bacteria responsible for furunculosis in salmonid fish.

Diets

Table 1 describes the ingredient list and proximate composition of the two experimental diets used in the current study. Diets were named as control and MPLE, and only differed in the level of inclusion of the MPLE obtained from *S. officinalis* and *L. citriodora*, which was 0.1% in the MPLE diet. The tested phytogenic was provided by NATAC Biotech SL and obtained as described in Salomón et al. (2020). The proximate composition of tested MPLE was: 73% carbohydrates, 2% crude lipids, <1% crude proteins, 5% salts and 4% water. In terms of phytogenic bioactive compounds, the MPLE contained: 10% ursolic acid

TABLE 1 | List of ingredients and proximal composition of experimental diets; control and a basal diet supplemented with MPLE tested in Atlantic salmon (*Salmo salar*).

Ingredients, %	Control diet	MPLE diet
Fishmeal LT70	17.5	17.5
Soy protein concentrate	20.0	20.0
Fish protein concentrate	2.5	2.5
Wheat gluten	9.0	9.0
Corn gluten	5.0	5.0
Faba beans	5.0	5.0
Wheat meal	16.23	16.13
Fish oil	12.0	12.0
Vitamin and mineral premix	1.0	1.0
Soy lecithin	0.5	0.5
Vitamin C35%	0.07	0.07
Monocalcium phosphate	3.0	3.0
Rapeseed oil	7.0	7.0
Betaine HCl	1.0	1.0
DL-Methionine	0.2	0.2
MPLE	–	0.10
Proximate composition		
Crude protein, %	40.03	40.02
Crude fat, %	22.15	22.15
Fiber, %	1.75	1.74
Starch, %	13.02	12.93
Ash, %	8.74	8.89
Gross energy, MJ/kg	21.60	21.58

(100 ppm), 3% other triterpenic compounds (30 ppm), 2% verbascoside (60 ppm) and <1% polyphenols (<10 ppm). Diets were manufactured by Sparos Lda (Olhão, Portugal). All powder ingredients were mixed accordingly to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 μm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size: 2 and 3 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 ml/min), temperature barrel 1 (32–34°C), temperature in barrel 2 (59–62), and temperature barrel 3 (111–114°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, Netherlands). Coating conditions were: pressure (700 mbar); spraying time under vacuum (approximately 90 s), return to atmospheric pressure (120 s). Feeds were stored at 4°C during the experimental period (133 days) in order to prevent their oxidation.

Fish and Experimental Design

Unvaccinated Atlantic salmon parrs (n = 1,550) were purchased from SARL SALMO (Gonneville-Le Thiel, France) and transported by road to IRTA-Sant Carles de la Ràpita research facilities (Sant Carles de la Ràpita, Spain). Once there, parrs were acclimated in two 2,000-L tanks connected to an open-flow system (12.0 ± 1.5°C) for 2 weeks under natural photoperiod and fed *ad libitum* a commercial feed (T2-2 Royal Optime, Skretting; proximate composition: 44% crude protein; 21% crude fat; 6.9% crude ash; 2.9% crude fiber).

Before the onset of the nutritional trial, parrs (n = 696) were gently anesthetized (50 mg L^{-1} tricaine methane sulfonate. MS-222, Sigma-Aldrich, Madrid, Spain) and individually measured in body weight (BW) and standard length (SL) to the nearest 0.1 g and 1 mm, respectively. Fish (55.0 ± 0.1 g and 16.2 ± 0.2 mm in BW and SL, respectively) were distributed among twelve experimental tanks (n = 58 fish per tank; 6 replicate tanks per experimental diet). Both experimental diets were offered to parrs at a daily feeding rate of 3.0% of the stocked biomass as described in Salomón et al. (2021b). In addition, feed utilization was evaluated by the following formula: feed conversion ratio (FCR) = feed intake (g)/increase of fish biomass (g).

During the parr phase that lasted 47 days, rearing conditions were as follows: water temperature and pH (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 12.2 ± 1.0°C, 7.4 ± 0.3 and 9.4 ± 0.8 mg L^{-1} (mean ± SD), respectively. The water flow rate in experimental tanks was maintained at approximately 9.0 L min^{-1} (open-flow system), which guaranteed two full tank's water renewal per hour. Photoperiod was 8 h light: 16 h darkness.

Smoltification started on the 5th of February and lasted 10 days. During this period, water salinity was increased progressively at *ca.* 3 ppt per day until reaching 35 ppt using filtered seawater according to SARL SALMO recommendations. Water temperature, pH, and oxygen levels during this period were 12 ± 0.1°C, 7.4 ± 0.3 and 9.6 ± 0.2 mg L^{-1}. The photoperiod during the smoltification period was 24 h light, 0 h darkness. Once fish were transferred to seawater 14th February, water quality and temperature were maintained by means of a water recirculation system (IRTAmar®; Spain) that maintained adequate water quality through UV, biological, and mechanical filtration. Water quality parameters during the rest of the trial were 12.1 ± 0.2°C, 7.4 ± 0.3 and 9.5 ± 0.2 mg L^{-1}. Ammonia and nitrite were ≤0.07 and 0.14 mg L^{-1}, respectively. Ammonia and nitrites were measured twice per week by means of a portable spectrophotometer (LOVIBOND MD600, Tintometer GmbH, Germany) using the Vario Ammonia Salicyklate F 10 mL (Tintometer GmbH, Germany) and Nitriver® 3 Nitrite reagent (Permachem® Reagent, HACH Lange, GmbH) assays. The photoperiod during the smolt stage was 24 h light: 0 h darkness. The illumination system for the smolt phase consisted of a led illumination system (Celer, Spain) with a light temperature of 4.000 K and light intensity of 1.540 lumens. At the end of the trial, all fish were netted, anesthetized with MS-222 as previously described and individually weighted.

Pathogenic Bacterial Challenge

At the end of the nutritional trial, smolts fed both diets were exposed to a bacterial challenge with the causative agent of furunculosis (*A. salmonicida* subsp. *salmonicida*). The internal coding for this pathogenic bacterial strain is IRTA-17-44, a strain available for courtesy of HIPRA (Amer, Spain). In brief, the bacterial inoculum was grown on TSA at 23.0 ± 1.0°C for 48 h. The inoculum was prepared to an optical density (OD) = 1.2 measured at λ = 550 nm, which corresponded to 1 × 10^8 CFU mL^{-1}. The bacterial suspension was ten-fold serially diluted in sterile PBS to prepare the desired inoculum density, which was confirmed by CFU's plate counting. Prior to the challenge trial, the lethal dose of 50% (LD_{50}) for *A. salmonicida* was determined for the experimental conditions established. For this purpose, thirty smolts (n = 10 per dose) were intraperitoneally injected (0.2 mL) at three different concentrations of the pathogenic bacteria (1 × 10^6, 1 × 10^7 and 1 × 10^8 CFU mL^{-1}). Ten additional fish were injected with PBS as methodological control. The LD_{50} was established at 1 × 10^7 CFU mL^{-1} (data not shown). For the challenge trial, 32 Atlantic salmon smolts (BW = 194.0 ± 29.1 g) per each dietary treatment were randomly distributed[1] into quadruplicate tanks (4 tanks per dietary treatment), with eight fish per tank (stocking density = 14–16 kg m^{-3}). During the acclimation period (5 days), fish were fed *ad libitum* with the same experimental diets used in the nutritional assay. After acclimation, fish were anesthetized and IP injected with 0.2 mL of 10^7 CFU/mL of *A. salmonicida* (IRTA-17-44).

Both the establishment of the *A. salmonicida* LD_{50} and the challenge trial were performed at IRTA's biosafety challenge room, in 32 cylindrical tanks (100 L) connected to a RAS unit (IRTAmar®) equipped with real-time control of oxygen and temperature, mechanical filtration, biofiltration, and ultraviolet disinfection of the water. The outflow water was chlorinated,

[1]https://www.randomizer.org

followed by ozone treatment before being discharged. Water quality conditions in terms of temperature and salinity were 13.1 ± 1.1°C and 32.3 ± 0.4 ppt, respectively. Mortality occurring after the first 12 h post-injection (hpi) was considered to be induced *A. salmonicida* rather than handling stress, since no mortality was found in the control group injected with PBS.

During the duration of the challenge (12 days), smolts were supervised every 2 h, six times per day, including weekends. Following the ethical guidelines for the use of animals in research, when fish became moribund, they were euthanized with an overdose of MS-222 (>150 mg L^{-1}). At the end of the challenge, all fish were sacrificed following the same procedure. A species-specific PCR (Beaz-Hidalgo et al., 2008) was performed from DNA of bacterial colonies recovered from head kidney smears of all moribund fish in order to confirm the cause of death. For this purpose, animals were aseptically dissected and a sample from the head kidney was taken and plated on TSA, incubated at 23°C for 72 h. Confluent pure bacterial growth was found from all samples, from which *A. salmonicida* was confirmed by means of PCR as described in Salomón et al. (2021b).

Transcriptional Analysis

RNA Isolation and Quality Control

At the end of the nutritional assay, three fish from each tank (n = 18 fish per diet) were sacrificed with an overdose of MS-222 (>150 mg L^{-1}). Then, head kidney was removed and fixed in RNAlater® (Sigma-Aldrich, Sant Louis, MO, United States), incubated overnight (4°C), and stored at −80°C. Total RNA from the head kidney of individual fish was extracted using TRI reagent (Sigma-Aldrich, Sant Louis, MO, United States) following the guidelines provided by the manufacturer. Total RNA concentration and purity were quantified using a Nanodrop-2000® spectrophotometer (Thermo Scientific™, United States) and stored at −80°C for further analysis. To check RNA integrity, samples were diluted (133.33 ng μL^{-1}) and the RNA Integrity Number (RIN) determined by means of an Agilent 2100 Bioanalyzer (Agilent Technologies, Spain). Only samples with a RIN value higher than 8.5 were selected for further microarray analysis. For each dietary group, we used for microarray analysis three head kidney pooled samples. Each pool consisted of one fish from each tank replicate (n = 18 fish per dietary group); thus, data regarding individual variability was lost with this analysis.

Microarray Design and Analysis

Gene expression analysis from head kidney samples was performed using the custom-commercial *Salmo salar* oligonucleotide microarray platform (AMADID 084881; Gene Expression Omnibus (GEO) access number: GPL28080; Agilent Technologies, United States). Data from this study are available in the GEO accession number GSE184485.

RNA handling and the microarray analysis of samples were conducted as described by Salomón et al. (2021b). Total RNA (200 ng) was reverse transcribed (Agilent One-Color RNA spike-in kit; Agilent Technologies) and used as a template for Cyanine-3 (Cy3) labeled cRNA synthesis and amplification (Quick Amp Labeling kit, Agilent Technologies). The RNeasy micro kit (Qiagen) was used for cRNA purification. Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000® spectrophotometer. Then, Cy3-labeled cRNA (1.5 mg) with specific activity >6.0 pmol Cy3/mg cRNA was fragmented (60°C, 30 min), and then mixed with the hybridization buffer and hybridized to the array (ID 084881, Agilent Technologies) at 65°C for 17 h (Gene expression hybridization kit; Agilent Technologies). The microarray was washed as indicated by the manufacturer (Gene expression wash buffers; Agilent Technologies), followed by the application of stabilizing and drying solutions (Agilent Technologies). Microarray slides were scanned (Agilent Technologies Scanner, model G2505B) and spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0 (Agilent Technologies). Quality reports were checked for each array. The identification of differentially expressed genes was done as described by Reyes-López et al. (2015). Data processing and mining were performed by means of the package STARS (NOFIMA, Norway) (Krasnov et al., 2011). Lowess normalization of log2-expression ratios (ER) was performed after removing the low-quality spots formerly identified. The selection of differentially expressed genes (DEGs) was done considering the difference between both diets following an unpaired t-test ($P < 0.05$).

Functional Network Analyses: Transcripteractomes

The transcripteractome analysis was conducted according to Reyes-López et al. (2021) using the Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 10.0[2] (Szklarczyk et al., 2019). A protein-protein interaction (PPI) network for DEGs was done with a high-confidence interaction score (value = 0.4). The mechanisms of response in which DEGs are involved were obtained from a comparative analysis using *Homo sapiens* as a reference organism. Thus, an *H. sapiens* acronym was assigned based on *S. salar* transcript annotation using Uniprot (2019) and Genecards (Stelzer et al., 2016) databases. When genes with no annotation match were found for Atlantic salmon, we assigned an orthologue *H. sapiens* Entrez Gene based on the homology between sequences using the best tBlastX (NCBI) hit. Matches with at least E value $\leq 1e^{-10}$ were only considered, whereas the Uniprot and Genecards databases were used to confirm the match of the gene acronym tag between both species. Gene ontology (GO) pathway enrichment analysis for biological processes (GO_BiologicalProcess-EBI-UniProt-GOA-ACAP-ARAP_10.11.2020_00h00) was obtained using ClueGO v2.5.7 (Bindea et al., 2009) through Citoscape 3.8.2 (Shannon et al., 2003). The enrichment and depletion of GO categories (two-sided hypergeometric test; $P < 0.05$) using the Benjamini-Hochberg correction. Furthermore, a GO Fusion was run in order to avoid redundant terms with a Kappa Score Threshold of 0.4 in order to propose more stringent GO terms associated to the mechanism of response for the MPLE diet. GO terms grouping was performed when the sharing group's percentage was above 50 ($P < 0.05$). The statistically significant GOs obtained from the enrichment analysis were assigned to each one of the nodes represented in the functional

[2]https://string-db.org

network. The ClueGo v2.5.7 a Cytoscape plug-in was used for visualizing nodes classified in different clusters based on their functionality. Hub genes of PPI networks were calculated by Cytoscape plug-in, *cytoHubba* (version 0.1), predicted the top 10 nodes using analysis algorithms including Maximum Clique Centrality (MCC; Chin et al., 2014).

Ethics Statement

Experimental procedures were conducted following the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and authorized by the Ethical Committee of IRTA (FUE-2020-01314717).

Statistics

Data in terms of somatic growth performance was compared between the control and the MPLE diets by means of a *t*-test ($P < 0.05$). Regarding the bacterial challenge, mortality rates were registered in both groups and data were depicted using Kaplan–Meier survival curves (Kaplan and Meier, 1958). Survival rates were calculated using the Mantel–Cox log-rank test. All the statistical analyses were conducted using SPSS for Windows® (version 15.0, SPSS Inc., Chicago, IL, United States). The Heatmapper server was used for constructing the hierarchical heatmap of DEGs (Babicki et al., 2016).

RESULTS

Survival and Growth Performance

At the end of the study, no significant differences in survival were found between Atlantic salmon smolts fed the control (98.9 ± 1.9%) and MPLE (99.4 ± 1.3%) diets ($P > 0.05$). Smolts fed the MPLE diet (271.5 ± 7.9 g) were 11.5% heavier than those fed the control diet (240.2 ± 19.3 g) ($P < 0.05$). Values of FCR were lower in fish fed the 0.1% MPLE diet (1.08 ± 0.05) than in those fed the control diet (1.27 ± 0.08) ($P < 0.05$).

Head Kidney Transcriptomic Results

A total of 1,178 DEGs were found in the head kidney of smolts fed the MPLE diet compared to the control group (**Figure 1**). The complete list of DEGs may be found in **Supplementary Table 1**. Most of the upregulated genes ($n = 523$) were found within the 0.8 < log2 absolute fold-change (| log2 FC|) < 1.4 interval. In addition, 243 genes were identified in the 1.4 < | log2 FC| < 2.5 interval, 35 transcripts in the 2.5 < | log2 FC| < 5.0, and only one single gene in the | log2 FC| > 5.0. Regarding downregulated genes, 289 transcripts were found in the 0.8 < | log2 FC| < 1.4 interval; other 76 transcripts were grouped in the 1.4 < | log2 FC| < 2.5 interval, whereas only 11 DEGs were included in the 2.5 < | log2 FC| < 5.0 expression interval. The detailed analysis of gene absolute log2 fold-change (| log2 FC|) revealed that genes were mostly upregulated in fish fed the MPLE diet (68.1% of DEGs), while its gene modulation was moderate in terms of FC intensity (**Figure 1A**). Results from the PCA are shown in **Figure 1B**, whereas those related to the hierarchical clustering heatmap of DGEs from both diets are depicted in **Figure 1C**.

Enrichment Analyses and Transcripteractome Results

The analysis of the transcripteractome showed the presence of thirty-four clusters (**Table 2**). The complete list of them is detailed in **Supplementary Table 2**. Among them, eleven clusters were identified with only one-single node, four clusters were constituted by two nodes, four clusters by three nodes, and four clusters by four nodes (**Figure 2**). Only one cluster was identified composed by five (GO:0042770: "signal transduction in response to DNA damage"), six (GO:0007492: "endoderm development"), seven (GO:0071901: "negative regulation of protein serine/threonine kinase activity"), and nine nodes (GO:0043122: "regulation of I-kappaB kinase/NF-kappaB signaling"). Other seven clusters contained more than ten nodes, including the "activation of cysteine-type endopeptidase activity involved in apoptotic process" (GO:0006919; eleven nodes), "response to organonitrogen compound" (GO:0010243; eleven nodes), "leukocyte activation involved in immune response" (GO:0002366; thirteen nodes), "intracellular signal transduction" (GO:0035556; thirteen nodes), "Autophagy" (GO:0006914; sixteen nodes). Importantly, two clusters registered more than thirty nodes including the "regulation of DNA-binding transcription factor activity" (GO:0051090; thirty-seven nodes), and the "actin filament organization" (GO:0007015; forty-one nodes) (**Figure 2**).

Through plugin *cytoHubba* in Cytoscape software, we evaluated the degree and betweenness centrality in the PPI network and screening the hub genes. Thus, the top ten hub genes with a high level of correlation for the selected clusters related to immunity and obtained from the enriched biological functions were selected for further consideration. From the "Antigen processing and presentation of peptide antigen *via* MHC class II", we identified six upregulated transcripts (*hla-dqa1*, *cd74*, *ctsl*, *ctsd*, *kif23*, *dync1li2*) and four downregulated hub genes (*klc1*, *hla-dmb*, *kif2a*, *sptbn2*) (**Figure 3A**). Considering the cluster "Interferon-gamma-mediated signaling pathway", among the top 10 hub genes, six transcripts were upregulated (*camk2α*, *hla-dqa1*, *trim21*, *trim22*, *med1*, *trim68*), and four of them downregulated (*jak1*, *jak2*, *tp53bp1*, *ncam1*) (**Figure 3B**). For the cluster "Regulation of I-kappaB kinase/NF-kappaB signaling", we identified five upregulated transcripts (*notch1*, *cebpb*, *smad3*, *sirt1*, *cd40*), and five others were downregulated (*gapdh*, *jak2*, *spi1*, *smad4*, *brd4*) (**Figure 3C**). Considering the cluster "Leucocyte activation involved in immune response", most of the hub genes were upregulated (*fn1*, *notch1*, *grb2*, *rac2*, *rdx*, *ezr*, *smad3*, *abl1*) compared to the two downregulated hub genes (*actb*, *jak2*) (**Figure 3D**). Regarding the cluster "Cytoskeleton organization", seven hub genes were upregulated (*fn1*, *notch1*, *itgb4*, *itgb5*, *itga11*, *col2a1*, *col4a5*), and three downregulated (*actb*, *gapdh*, *itga10*) (**Figure 3E**). Considering the cluster "Regulation of cellular protein metabolic process", just three genes were upregulated (*fn1*, *irs1*, *sirt1*), whereas seven genes showed a downregulation (*actb*, *gapdh*, *jak2*, *ptpn1*,

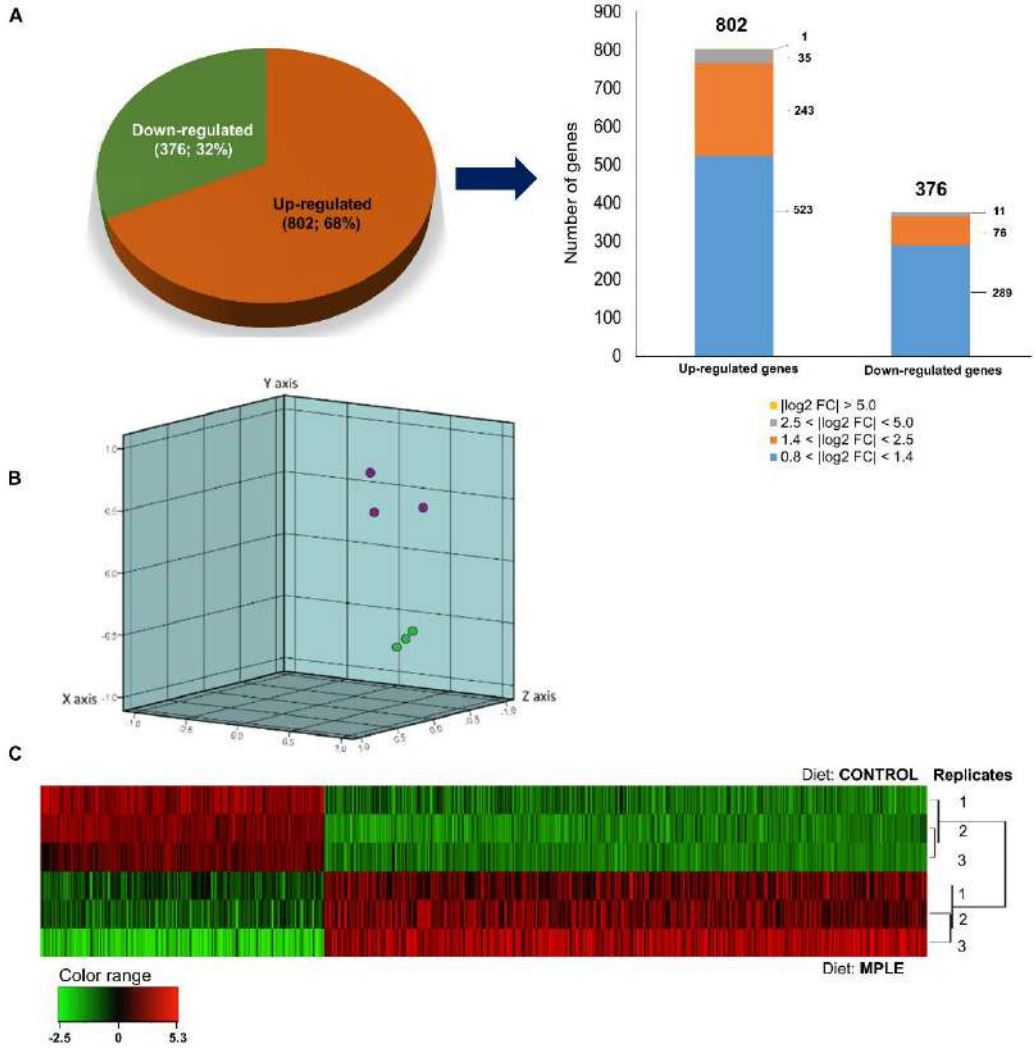

FIGURE 1 | Differential expression analysis of the Atlantic salmon (*S. salar*) head kidney transcriptomic response to MPLE diet. **(A)** Distribution (pie-chart) of the differential expressed genes (DEGs) obtained from the microarray-based transcriptomic analysis response fed a diet supplemented with phytochemicals from sage and lemon verbena. Absolute log2 fold-change (log2 FC) indicates the magnitude interval of response. **(B)** Principal component analysis (PCA) of the DEGs for the Atlantic salmon head kidney in response to the control (purple node) and phytogenic-supplemented diet (green node). **(C)** Hierarchical clustering of the Atlantic salmon head kidney transcriptomic response for the control and MPLE diet, based on similitude patterns of the DEGs detected from three sample pools per dietary group. Data of the six microarrays are depicted. The normalized intensity values (log2) obtained for each microarray analyzed for control (replicate 1, 2, and 3) and MPLE group (replicate 1, 2, and 3) are shown.

jak1, *smad4*, *insr*) (**Figure 3F**). The "Cellular biosynthetic process" cluster showed also ten hub genes. From them, seven genes were upregulated (*rps6*, *rpl19*, *rpl26*, *rpl12*, *rps7*, *rps17*, *dock4*), and three of them were downregulated (*rps10*, *rpl3l*, *eef1d*) (**Figure 3G**). For the GO "Autophagy," five genes were upregulated (*sirt1*, *prkaa2*, *slc38a9*, *trim21*, *abl1*), and other five genes were downregulated (*gapdh*, *ube2v1*, *ube2n*, *tp53bp1*, *gba*) (**Figure 3H**).

Bacterial Challenge Test

Results regarding the Kaplan-Meier survival rates curves of Atlantic salmon smolts injected intraperitoneally with *A. salmonicida* (1×10^7 UFC mL^{-1}) showed significant differences between the control and MPLE diets (**Figure 4**; $P < 0.05$). Smolts fed the MPLE diet showed higher survival rates (90.6 ± 6.4%, mean ± standard deviation) compared to those smolts fed the control diet (60.7 ± 13.5%).

DISCUSSION

Nowadays, transcriptome-based functional network analyses on teleost fish fed functional feeds have gained attention, since they provide further insight into the mode of action

TABLE 2 | List of the 34 total clusters related to representative biological processes identified by the transcripteractome in Atlantic salmon smolts fed the MPLE diet.

Cluster term	% Terms per group	No. GO	No. Genes
Actin cytoskeleton organization	18.98	41	300
Cellular biosynthetic process	17.13	37	556
Autophagy	7.41	16	230
Regulation of cellular metabolic process	6.02	13	407
Leukocyte activation involved in immune response	6.02	13	171
Response to organic substance	5.09	11	184
Activation of cysteine-type endopeptidase activity involved in apoptotic process	5.09	11	155
Regulation of I-kappaB kinase/NF-kappaB signaling	4.17	9	130
Negative regulation of protein serine/threonine kinase activity	3.24	7	178
Endoderm development	2.78	6	49
Signal transduction in response to DNA damage	2.31	5	38
Intracellular signal transduction	1.85	4	215
Regulation of histone methylation	1.85	4	32
Cytoskeleton organization	1.85	4	307
Regulation of intracellular steroid hormone receptor signaling pathway	1.85	4	79
Regulation of viral life cycle	1.39	3	24
Positive regulation of cell death	1.39	3	93
Regulation of cellular protein metabolic process	1.39	3	373
Positive regulation of neuron death	1.39	3	26
Sphingolipid metabolic process	0.93	2	18
Regulation of cellular protein localization	0.93	2	40
Carbohydrate derivative metabolic process	0.93	2	50
Chondrocyte differentiation	0.93	2	21
Protein localization to nucleus	0.46	1	22
Maintenance of protein location	0.46	1	11
Actin cytoskeleton reorganization	0.46	1	11
Regulation of cell shape	0.46	1	14
Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	0.46	1	12
Antigen processing and presentation of peptide antigen *via* MHC class II	0.46	1	11
Cell-cell adhesion	0.46	1	56
Hepaticobiliary system development	0.46	1	15
Stem cell differentiation	0.46	1	21
Interferon-gamma-mediated signaling pathway	0.46	1	10
Negative regulation of transcription by RNA polymerase II	0.46	1	52

of zootechnical additives with immunomodulatory properties on the host (Firmino et al., 2021a; Reyes-López et al., 2021; Salomón et al., 2021a,b). In this sense, this study was performed to gain insight into the potential immunomodulatory, disease resistance, and other biological effects of a phytogenic feed additive obtained from sage and lemon verbena in Atlantic salmon smolts. These bioactive compounds were chosen due to their health and growth-promoting properties in aquatic species (Elumalai et al., 2020; Salomón et al., 2020, 2021a) and screened as a potential additive for a functional feed in order to promote host's immunity and enhance disease resistance. In this context, we found that the inclusion of MPLE at 0.1% in Atlantic salmon smolts exerted a positive effect on somatic growth, being fish fed the diet containing the MPLE 11.5% heavier than the control group. Similar results have been observed in higher vertebrates (Corino et al., 2007; Pastorelli et al., 2012; Casamassima et al., 2013) and in fish species like gilthead sea bream fed the same feed additive (Salomón et al., 2020) and other species like rainbow trout or beluga fed functional diets containing sage and/or lemon verbena phytogenics (Sönmez et al., 2015; Dadras et al., 2020). These findings might be partially attributed to the potential effects of triterpenoid compounds, such the ursolic acid, which has been reported to promote muscular growth by the hypertrophy of muscular fibers in mice (Kunkel et al., 2012) and rainbow trout (Fernández-Navarro et al., 2006). Furthermore, the growth-promoting effects of polyphenolic compounds like verbascoside present in *L. citriodora* have also been observed in several studies (Corino et al., 2007; Pastorelli et al., 2012; Casamassima et al., 2013).

Regulation of Cellular Protein Metabolic and Cytoskeleton Organization

The transcripteractome results showed the enrichment of the biological processes related to "Regulation of cellular protein metabolic process (GO:0031323)", "Positive regulation

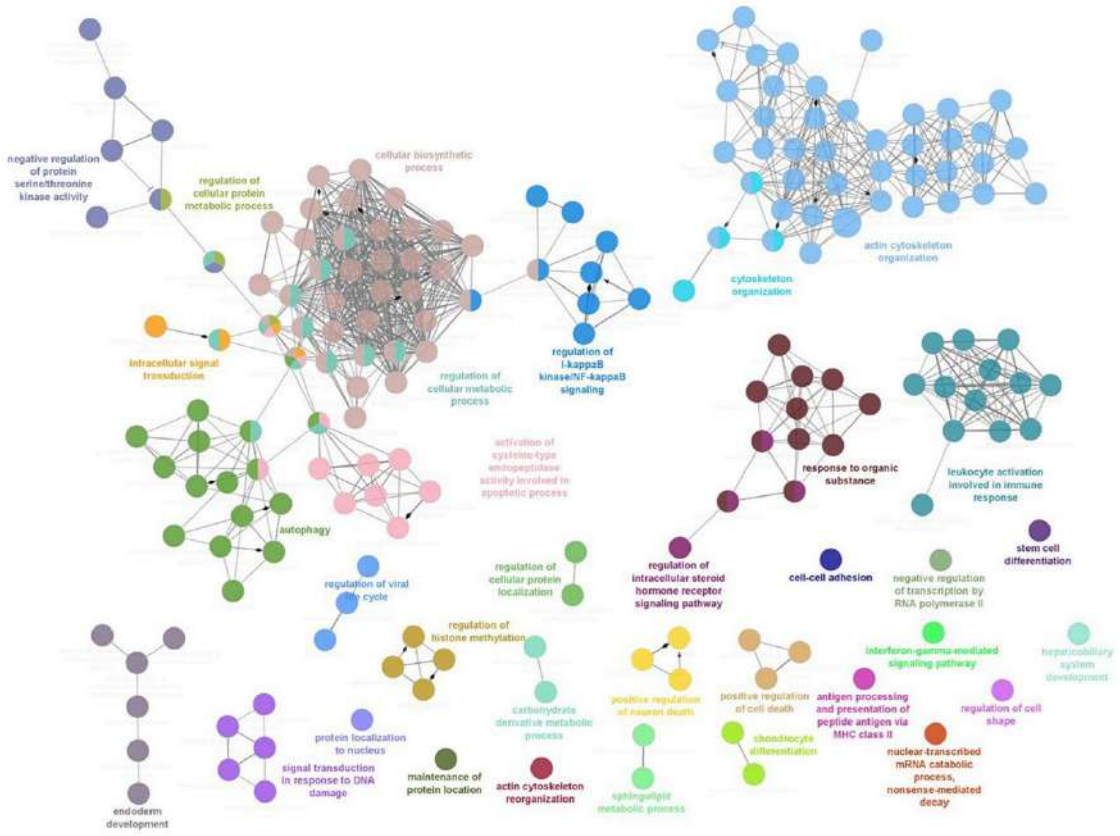

FIGURE 2 | Functional enrichment network analysis for biological processes based on the total number of differential expressed genes (DEGs) in head kidney of Atlantic salmon smolts fed the MPLE-supplemented diet. Each node represents a specific enriched biological process from the total number of DEGs. Each color indicates a cluster of closely related biological processes. The lines into the cluster indicated a closed relationship between biological processes. The details of each node are shown in **Supplementary Table 2**.

of metabolic process (GO:0009893)", and "Regulation of catabolic process (GO:0009894)". The identification of the above-mentioned biological processes in the head kidney of smolts fed the functional diet may be attributed to their higher somatic growth performance, which in turn may increase the metabolism of the body, and in particular, that of this lymphoid tissue (Shved et al., 2011; Khansari et al., 2017), as different gene expression patterns between both dietary groups indicated. For instance, among several hub genes identified by the *cytoHubba* analysis tool (Chin et al., 2014), *sirt1* was one of the hub genes that showed upregulation in the cluster named "Regulation of cellular protein metabolic". In mammals, its protein product (Sirtuin 1) play a vital role in metabolism as a mediator of endocrine function of several hormones modulating energy balance (Quiñones et al., 2014). Thus, our results are also in accordance with those of Lagouge et al. (2006) and Milne et al. (2007), who reported an upregulation of *sirt1* due to the dietary administration of resveratrol, a natural polyphenol found in several plants; results that confirmed the role of sirtuin 1 as key regulator of energy and metabolic homeostasis. Other hub genes related to "Regulation of cellular protein metabolic" process were the insulin receptor substrate 1 (*irs1*) and insulin receptor (*insr*), both transcripts were upregulated in the head kidney of smolts fed the MPLE diet. Their protein products are known as insulin receptor substrates, which are mediators of insulin signaling, and have a significant role in maintaining growth and metabolic cell functions (Caruso and Sheridan, 2011; Shved et al., 2011). This is relevant since insulin plays a fundamental role in the regulation of somatic growth and metabolism of all vertebrates (Hernández-Sánchez et al., 2006). Thus, the upregulation of genes associated to insulin receptors reinforces the hypothesis of a growth-promoting effect of the tested MPLE in Atlantic salmon smolts.

The cytoskeleton is the cellular structure that helps cells maintain their shape and internal organization; in particular, it spatially organizes the contents of the cell; it connects the cell physically and biochemically to the external environment; and it generates coordinated forces that enable the cell to move and change shape while providing cellular homeostasis and survival (Fletcher and Mullins, 2010). In this sense, we found the modulation of transcripts associated to the biological process "Cytoskeleton organization (GO:0007010)". Interestingly, several genes related to cell structure and morphogenesis (extracellular

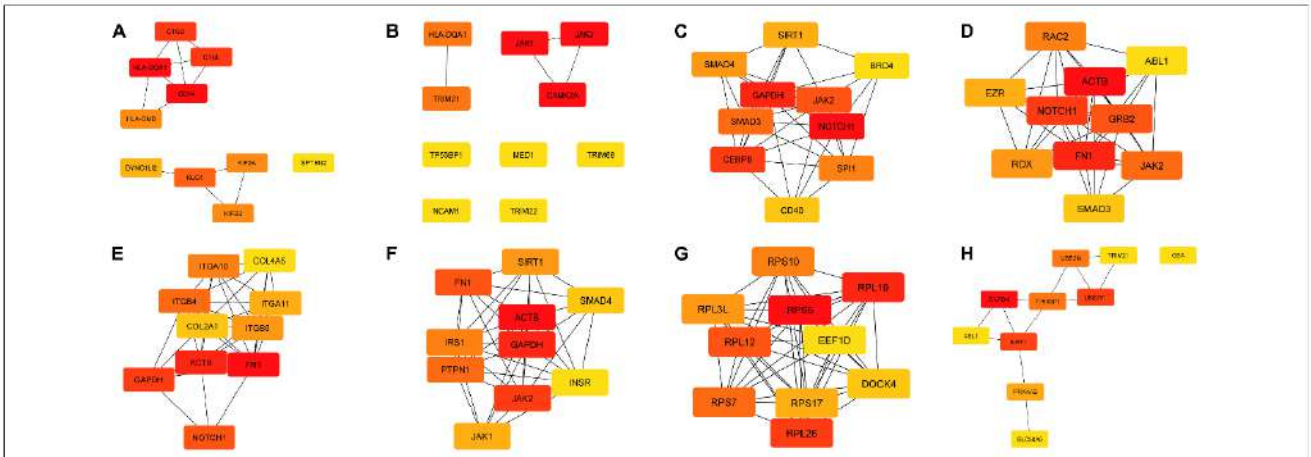

FIGURE 3 | Hub genes in each of the cluster terms identified from the enriched biological functions in the head kidney of Atlantic salmon smolts fed the MPLE supplemented diet. The principal cluster shows the ten-hub genes and the closest neighbor's genes for each network. The color intensity from yellow to red (i.e., yellow, orange, red) represents the degree of the hub gene (i.e., the relevance degree) in each network, being red those most relevant hub genes. **(A)** Antigen processing and presentation of peptide antigen *via* MHC class II. **(B)** Interferon-gamma-mediated signaling pathway. **(C)** Regulation of I-kappaB kinase/NF-kappaB signaling. **(D)** Leucocyte activation involved in immune response. **(E)** Cytoskeleton organization. **(F)** Regulation of cellular protein metabolic process. **(G)** Cellular biosynthetic process. **(H)** Autophagy.

matrix and cytoskeleton) presented a higher expression level in fish fed the MPLE-supplemented diet. Among the structural genes, several components of the extracellular matrix and cytoskeleton organization were upregulated in fish fed the MPLE diet, including genes of the integrin family (*itgb4*, *itgb5*, *itga10*, *itga11*) and fibronectin (*fn1*). In this context, *itgb4*, *itgb5*, *itga10* and *itga11* were identified as hub genes. Integrins link the extracellular matrix to the cytoskeleton, regulating signal transduction pathways intracellularly (Hynes, 2002). Furthermore, integrins also participate in the immune response (Han et al., 2016). Particularly, the upregulation of *fn1*, whose protein product (Fibronectin 1) is an important acute phase protein required for the protection and repair of the extracellular matrix (Jessen, 2015). Even more interesting, it has been shown that there is an interaction between fibronectin and integrins, which may induce cytoskeleton reorganization, focal adhesion formation, and importantly, cell-generated tension to unfold cryptic fibronectin, which is critical for fibronectin matrix assembly (Xu and Mosher, 2011).

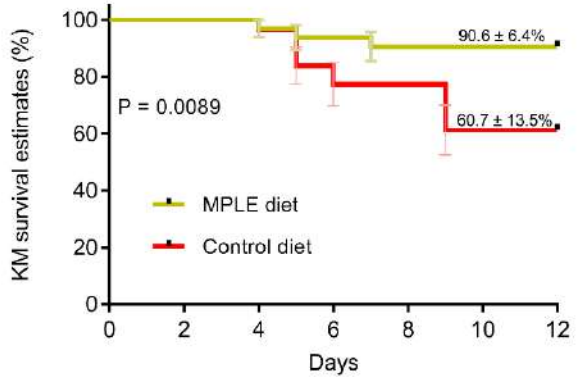

FIGURE 4 | Results of the bacterial challenge conducted in Atlantic salmon smolts intraperitoneally injected with 10^7 CFU mL^{-1} of *A. salmonicida*. Kaplan-Meier (KM) survival curves (%) for Atlantic salmon smolts intraperitoneally injected with *A. salmonicida* subsp. *salmonicida* (10^7 CFU mL^{-1}) during the 12 days challenge trial period. Data correspond to the mean ± standard error (4 replicates tanks per experimental diet; n = 8 fish per tank).

Immunomodulatory Effects of the Dietary Medicinal Plant Leaf Extract

The GO enrichment analysis of all DEGs indicated that the biological processes related to immunity in smolts were significantly modulated by the tested phytogenics in the head kidney, which were consistent with previous results on this additive at systemic and local levels (Salomón et al., 2020, 2021a). This organ undertakes immune functions similarly to the mammalian bone marrow, *i.e.*, hematopoiesis (Tort, 2011). In addition, the head kidney in fish is a basic organ forming blood elements; thus, it is potentially useful for identifying new immune-related genes (Gerdol et al., 2015). The immunomodulatory action at cellular and humoral levels of the tested phytogenic in the head kidney of Atlantic salmon smolts is further discussed as follows.

Antigen Processing and Presentation of Peptide Antigen *via* MHC Class II

Diet supplemented with MPLE modulated several biological processes related to immune effector cells in Atlantic salmon smolts. One of them was the cluster "Antigen processing and presentation of peptide antigen *via* MHC class II (GO:0002495)". Antigen processing and presentation are essential for triggering cellular and humoral immune responses, which are mediated

by T and B lymphocytes (Vyas et al., 2008). Thus, one of the major functions of MHC Class II molecules is presenting antigens derived from extracellular proteins for their recognition by $CD4^+$ T cells, being critical for the initiation of the antigen-specific immune response (Yagamuchi and Dijkstra, 2019). Furthermore, the α-and β-chain of MHC class II molecules are synthesized in the endoplasmic reticulum and associated with the class II invariant chain (also known as CD74) for proper folding, trafficking, and providing protection of the antigen-binding groove (Bryant and Ploegh, 2004). In our study, *cd74* was upregulated in fish fed the MPLE-supplemented diet. This gene is a cell-surface receptor for the cytokine known as macrophage migration inhibitory factor (MIF), which plays a specific role as an important component in the functional presentation of MHC class II restricted antigens (Gil-Yarom et al., 2017; Wang et al., 2017). Similar results regarding *cd74* expression were observed in virus-challenged Atlantic salmon, results that were correlated to an increased resistance to pancreas disease caused by salmonid alphavirus (Hillestad et al., 2020). In addition, several other genes like *hla-dqa1*, *ctsl*, *ctsd*, *kif23*, and *dync1li2* were upregulated in the cluster associated to this biological process. The major histocompatibility complex, class II, DQ alpha 1 (*hla-dqa1*), which is one of the MHC Class II family members was one of the upregulated hub genes involved in the above-mentioned cluster. This gene plays a central role helping the immune system to distinguish the host's own proteins from proteins made by viruses and bacteria (Lipton et al., 2011). This is of special relevance since proteins produced by the MHC class II are presented to the immune system, and if the immune system recognizes these peptides as foreign, it triggers a response to attack the invading viruses or bacteria (Mack et al., 1999). Furthermore, other hub genes like those belonging to the cathepsin family were also differentially transcribed between both experimental groups. This family of proteins are known to play important roles in antigen processing and presentation through the MHC II complex, being involved in adaptive immune responses (Conus and Simon, 2010). Under present experimental conditions, two cathepsins (*ctsd*, *ctsl*) were upregulated in smolts fed the phytogenic-supplemented diet. Cathepsin D (*ctsd*) is a lysosomal endoproteolytic aspartic proteinase that is involved in the presentation of antigenic peptides (Deussing et al., 1998), among other functions (Benes et al., 2008). It has also been shown that deficiency in cathepsin D may cause a delay in the innate immune response during both bacterial infection and septic shock (Cha et al., 2012). Similar to cathepsin D, cathepsin L is also described to be important in the innate response of teleost, playing key roles in host immune defense *via* the antigen processing and presentation, through the MHC II-associated presentation and regulation of $CD4^+$ T lymphocyte (Chen et al., 2020). Therefore, our data indicated that the regulation of several genes related to the antigen processing and presentation of peptide antigen *via* MHC class II pathway suggested that the tested phytogenics might be involved in the regulation of lymphocytes activity trough above-mentioned hub genes; thus, suggesting the stimulation of both innate and adaptive immune responses.

Interferon Gamma Mediated Signaling Pathway and Autophagy

As it was previously discussed, the dietary administration of MPLE modulated the biological process linked to "Antigen processing and presentation of peptide antigen *via* MHC class II". Interestingly, interferon gamma (IFN-γ) signaling has been shown to influence the entire process of antigen processing and presentation by inducing MHC class II; thus, contributing to immunity through the enhancement of pathogen-specific T cell responses (Decker et al., 2005). IFN-γ is mainly produced by activated T cells, natural killer cells, and antigen-presenting cells and it acts on many types of immune cells, regulating both innate and cell-mediated immune responses (Araki et al., 2013). Thus, IFN-γ plays critical roles not only in orchestrating both innate and adaptive immune responses against viruses and bacteria, but also in promoting inflammation (Zou and Secombes, 2011). Although, the activities mediated by this molecule are well known in mammals, several aspects of the IFN-γ system in teleosts remain a riddle to scientists (Pereiro et al., 2019). Recently, Hu et al. (2021) have demonstrated that the number of IFN-γ producing cells increased in rainbow trout challenged with *A. salmonicida*, results that were associated to an enhanced immune protection. These results may be of particular relevance under the present experimental condition, since Atlantic salmon smolts fed the MPLE diet showed higher survival rates (90.6 ± 6.4%) in comparison to those fed the control diet (60.7 ± 13.5%). Furthermore, another hub gene of relevance found in this biological process is the calcium/calmodulin-dependent protein kinase II alpha (*camk2α*). This gene is involved in the production of cytokines such interleukin-6, tumor necrosis factor-α and interferons in macrophages (Liu et al., 2008). In addition, the interaction between two other hub genes such janus kinase 1 (*jak1*) and janus kinase 2 (*jak2*) are required for association with the IFN-γ receptor chains and downstream signaling. Jak kinase function encompassed components of diverse signal transduction pathways that govern cellular survival, proliferation, differentiation, and apoptosis (Rane and Reddy, 2000; Yamaoka et al., 2004), being involved from disease resistance to maintaining immune tolerance (Villarino et al., 2015).

Another biological process that was modulated by the tested feed additive is "Autophagy" (GO:0006914), which is also modulated by IFN-γ (Pereiro et al., 2019). Autophagy is a highly conserved pathway that plays an important role in cellular physiology, adaptive responses to stress, and the immune response (Kuballa et al., 2012). Autophagy as a defense mechanism in teleost in front of intracellular bacterial and viral infections has been well documented (Meijer and van der Vaart, 2014; Pereiro et al., 2017, Yin et al., 2021). In this way, our results showed that "Autophagy (GO:0006914)" was regulated in the head kidney of the Atlantic salmon fed with the functional diet. According to the existing literature, studies have reported that autophagy as well as IFN-γ play a specific role against opportunistic pathogens such as *Aeromonas* spp. in farmed fish (Pereiro et al., 2016; Yin et al., 2021); thus, opening the possibility to understanding and relating our results to the increase in

disease resistance obtained from the bacterial challenge. For instance, Pereiro et al. (2016) reported a reduction in mortality in turbot (*Scophthalmus maximus*) when they were challenged with *A. salmonicida*. These results were attributed to the effect of IFN-γ in bacterial infections, and the participation of this protein in the inflammatory response (Pereiro et al., 2016). On the other hand, Yin et al. (2021) was able to demonstrate the role of autophagy in grass carp (*Ctenopharyngodon idella*) monocytes/macrophages, which lead to a promote innate defense against *Aeromonas hydrophila*.

Regulation of I-kappaB Kinase/NF-kappaB Signaling

NF-κB is an important factor for the maintenance of the immune homeostasis, by modulating the transcription of a diverse group of genes involved in many biological processes such as development, immunity, apoptosis, and cell differentiation in different cell types such as B and T cells, monocytes, chemokines, cytokines, among others (Dorrington and Fraser, 2019). In our study, we observed a modulation of the biological process "Regulation of I-kappaB kinase/NF-kappaB signaling (GO:0043122)". This modulation is in line with a recent study from our group, in which we demonstrated that a phytogenic feed additive from the olive fruit (*Olea europaea*), with biochemical and functional properties similar to MPLE, was also able to modulate biological process such as "Regulation of I-kappaB kinase/NF-kappaB signaling" (Salomón et al., 2021b).

Among the DEGs participating in this GO, the hub gene notch receptor 1 (*notch1*) is of special relevance. NOTCH proteins are transmembrane receptors of critical importance for several biological functions (Shin et al., 2014). In particular, *notch1* upregulates the expression of the cytokine IFN-γ in T cells through activation of NF-κB (Shin et al., 2014), which highlights the connection between NF-κB and IFN-γ biological processes. Moreover, we found another hub gene which is involved in the regulation of the NF-κB pathway; in particular, CCAAT enhancer-binding protein beta (*cebpb*) was upregulated in the head kidney of Atlantic salmon smolts fed the MPLE-supplemented diet. This gene has been well documented in direct association with NF-κB, whose function has been associated to the activation of pro-inflammatory mediators, such as chemokines, being also involved in migration, maturation, and activation of immune cells (Rebl et al., 2014). Last but not least, it has been demonstrated that the hub gene named *cd40* and its protein product (TNF receptor superfamily member 5), which is upregulated in our study, has been shown to be capable of stimulating the NF-κB pathway, as well as playing an essential role for the cooperation of T and B cells in responses to protein antigens (Gong et al., 2009; Hayden and Ghosh, 2014).

Leukocyte Activation Involved in Immune and B Cells Signaling Response

A successful immune response involves the tight control of a wide repertoire of processes such the leukocyte-mediated cells, among others (Rieger and Barreda, 2011). Leukocyte activation is mediated by several signaling pathways that interact to produce changes in binding protein affinity on the surface of neutrophils that mobilize the cytoskeleton for chemotaxis and phagocytosis, ultimately triggering a respiratory burst and degranulation (Rieger and Barreda, 2011). In our study, the "Leukocyte activation involved in immune response (GO:0002366)" was enriched. In this context, eight of the ten hub genes related to leukocyte activation (*fn1*, *notch1*, *grb2*, *rac2*, *rdx*, *ezr*, *smad3*, *abl1*) were upregulated. Similarly, a modulation of immune-related GOs such as "Leukocyte activation involved in immune response" were enriched in Atlantic salmon smolts fed a feed additive AQUOLIVE® as we mentioned above from a phytogenic derived from olive (*O. europaea*) fruit with similar biochemical and functional properties to the MPLE phytogenic tested in the current study (Salomón et al., 2021b). For instance, *notch1* a gene we have previously discussed highlighting its connection between NF-κB and IFN-γ as biological processes that have a key role in the differentiation of granulocytes, macrophages, and dendritic cells (Schroeder et al., 2003; Monsalve et al., 2009). This is relevant since, granulocytes, macrophages, dendritic cells, and B cells are recognized as a critical component of the innate and adaptive immunity against pathogens (Secombes and Wang, 2012). These results are in agreement with our transcriptomic data, since biological processes like "Neutrophil mediated immunity (GO:0002446)" and "Lymphocyte activation involved in immune response (GO:0002285)" were also enriched. In addition, we also found the upregulation of *rac2* among hub genes. In teleosts, RAC2 (Rac family small GTPase 2) is a member of the Rho family that plays an important role in the host defense-mediated neutrophil response, which is a critical first step in innate immunity (Deng et al., 2011; Tell et al., 2012). Moreover, loss of RAC2 activity has been shown to cause severe bacterial infections and deficits in neutrophil function in humans and mice (Tell et al., 2012).

Furthermore, some of these genes involved in the GO "Leukocyte activation involved in immune response" were also associated with the innate and adaptive immunity modulated by the MPLE-supplemented. For instance, fish fed with the MPLE diet showed an increase of the expression in the growth factor receptor bound protein 2 (*grb2*), which is an essential signal integrator that can interact with multiple genes to regulate signal transduction; thus, playing an important role in regulating B cells activation (Neumann et al., 2009). This is relevant, as B cell activation is essential the development of an effective antigen-specific humoral immune response (Scapigliati et al., 2018). Moreover, two hub genes (*rdx*, *ezr*), which are part of the family of proteins called ezrin-radixin-moesin (ERM), were also upregulated in our study. Several studies have demonstrated that ERM proteins are involved in the regulation of B cell function under healthy and disease conditions (Pore and Gupta, 2015). Therefore, the upregulation of these two genes, *rdx* and *ezr*, might confirm the immunomodulatory properties of the MPLE in Atlantic salmon. Therefore, the regulation of genes involved in leukocyte activation and B cells might suggest an increased systemic specific immune capacity promoted by the tested phytogenic feed additive. Thus, our transcriptional analysis seemed to indicate that the tested phytogenic activated leukocytes, which would promote host's disease resistance as the *in vivo* challenge with a pathogenic bacteria confirmed.

CONCLUSION

Phytogenics obtained from sage and lemon verbena included at 0.1% in diets promoted somatic growth and improved FCR in Atlantic salmon smolts. In addition, data from the microarray analysis of the head kidney samples indicated this feed additive enhanced the host's systemic immune response through the transcriptional regulation of innate immunity processes like leukocytes' activation. We showed that other pathways related to immunity were also enhanced by the tested functional feed additive, such as interferon-gamma-mediated signaling pathway, antigen processing and presentation of peptide antigen *via* MHC class II, autophagy, and regulation of i-kappaB kinase/NF-kappaB, promoting disease resistance when challenged with *A. salmonicida*. Altogether, this study indicated that the tested feed additive promotes systemic immunity and protects Atlantic salmon smolts against bacterial infections like *A. salmonicida*. According to these findings, we suggest that the combination of current vaccination practices coupled with the administration of MPLE may be a good strategy against furunculosis in salmonids. Furthermore, this phytogenic may be also of interest for other marine species like European sea bass (*Dicentrarchus labrax*) also suffering from emerging *A. salmonicida* infections in the Mediterranean basin.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Experimental procedures were conducted following the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and authorized by the Ethical Committee of IRTA (FUE-2020-01314717).

AUTHOR CONTRIBUTIONS

EG: conceptualization, resources, project administration, and funding acquisition. MF, EV-V, and FER-L: methodology. RS, FER-L, JF, and EV-V: formal analysis. RS: writing—original draft. MF, EV-V, RS, FER-L, EG, and LT: writing—review and editing. RS, FER-L, and EV-V: visualization. EG and EV-V: supervision. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work has been financially supported by the project "AQUOLIVE by NATAC," funded by European Union's Horizon 2020 Research and Innovation Program (Grant Agreement No. 830202) and partially by the Agencia Española de Investigación (grant AGL2016-76069-C2-2-R to Universitat Autònoma de Barcelona). FER-L and EV-V thank the support of Fondecyt Regular (No. 1211841) and Fondecyt iniciación grants (No. 11221308), respectively (ANID; Government of Chile). RS was supported by a Ph.D. grant from the government of Paraguay (BECAL). JF has been subsidized by the Industrial Ph.D. program of *Generalitat de Catalunya* and TECNOVIT-FARMFAES S.L. (No. 2017 DI 017). Collaboration between Ibero-American researchers has been done under the framework of the network LARVAplus "Strategies for the development and improvement of fish larvae production in Ibero-America" (117RT0521) funded by the Ibero-American Program of Science and Technology for Development (CYTED, Spain).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.828497/full#supplementary-material

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Conflict of Interest: JQ and JP-R are current NATAC BIOTECH S.L. employers.

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CAPÍTULO IV

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Frontiers in Immunology 12:737601

frontiers in Immunology

ORIGINAL RESEARCH
published: 12 November 2021
doi: 10.3389/fimmu.2021.737601

A Bioactive Extract Rich in Triterpenic Acid and Polyphenols from *Olea europaea* Promotes Systemic Immunity and Protects Atlantic Salmon Smolts Against Furunculosis

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In the present study, the modulation of the transcriptional immune response (microarray analysis) in the head kidney (HK) of the anadromous fish Atlantic salmon (*Salmo salar*) fed a diet supplemented with an olive fruit extract (AQUOLIVE®) was evaluated. At the end of the trial (133 days), in order to investigate the immunomodulatory properties of the phytogenic tested against a bacterial infection, an *in vivo* challenge with *Aeromonas salmonicida* was performed. A total number of 1,027 differentially expressed genes (DEGs) (805 up- and 222 downregulated) were found when comparing the transcriptomic profiling of the HK from fish fed the control and AQUOLIVE® diets. The HK transcripteractome revealed an expression profile that mainly favored biological processes related to immunity. Particularly, the signaling of i-kappa B kinase/NF-kappa and the activation of leukocytes, such as granulocytes and neutrophils degranulation, were suggested to be the primary actors of the innate immune response promoted by the tested functional feed additive in the HK. Moreover, the bacterial challenge with *A. salmonicida* that lasted 12 days showed that the cumulative survival was higher in fish fed the AQUOLIVE® diet (96.9 ± 6.4%) than the control group (60.7 ± 13.5%). These results indicate that the dietary supplementation of AQUOLIVE® at the level of 0.15% enhanced the systemic immune response and reduced the *A. salmonicida* cumulative mortality in Atlantic salmon smolts.

Keywords: feed additive, *Olea europaea*, aquaculture, *Aeromonas salmonicida*, challenge, systemic immunity, *Salmo salar*, immune homeostasis

OPEN ACCESS

Edited by:
Chris Hauton, University of Southampton, United Kingdom

Reviewed by:
Neeraj Sood, National Bureau of Fish Genetic Resources Indian Council of Agricultural Research (ICAR), India
Samad Rahimnejad, University of South Bohemia in České Budějovice, Czechia

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Specialty section:
This article was submitted to Comparative Immunology, a section of the journal Frontiers in Immunology

Received: *07 July 2021*
Accepted: *18 October 2021*
Published: *12 November 2021*

Citation:
Salomón R, Furones MD, Reyes-López FE, Tort L, Firmino JP, Esteban MA, Espinosa Ruiz C, Quintela JC, Pinilla-Rosas JM, Vallejos-Vidal E and Gisbert E (2021) A Bioactive Extract Rich in Triterpenic Acid and Polyphenols from Olea europaea Promotes Systemic Immunity and Protects Atlantic Salmon Smolts Against Furunculosis. Front. Immunol. 12:737601. doi: 10.3389/fimmu.2021.737601

1 INTRODUCTION

The worldwide production of farmed Atlantic salmon (*Salmo salar*) has progressively increased from 294 t in its inception in 1970 up to 2,615,962.4 t in 2019, with Norway and Chile being the main producers with 1,364,042 t (52.1%) and 701,731 t (26.9%), respectively (1). This flourishing industry has grown focusing their efforts on profitability, competitiveness, and sustainable development; however, disease is the biggest risk to the industry, since it undermines financing and market development. In particular, infectious diseases represent a major problem in worldwide salmon farming, despite the successful development and application of vaccines against a wide range of pathogens and the implementation of management practices for fighting against parasites (2). In this sense, intensified production systems and climate change will favor the occurrence of disease outbreaks due to the farming of more stressed and immuno-compromised animals in farms, and the evolution and spread of more virulent pathogens. This qualifies aquatic animal diseases as one of the major limiting factors for aquaculture development (3, 4).

Although in recent years, there has been a drastic reduction in antibiotic use in some countries due to vaccination and improved husbandry practices, the use of antimicrobials is still a common practice in order to avoid and mitigate potential production and economic losses derived from outbreaks of pathogenic organisms (5, 6). In this sense, the academy and the industry have merged efforts in order to develop, test, and validate sustainable and environmentally friendly alternative treatments in order to prevent disease outbreaks and to reduce the use of chemotherapeutic drugs. Among the repertoire of tested strategies (7, 8), functional feeds are considered as one of the most affordable and sustainable preventive solutions (9). Feeds that provide physiological benefits beyond the animal's basic nutritional requirements are named as functional feeds, and their use has progressively gained attention within the aquaculture. Feed additives may be divided into different categories considering the purpose of their use (nutritional, sensorial, and functional additives), which also affects their chemical nature and mode of action (10, 11). In this sense, functional feed additives with immunomodulatory properties and capacity of relieving stress and promoting disease resistance in farmed animals are of interest as sustainable health management strategies. The most widely evaluated functional feed additives, as immunostimulants, are probiotics, prebiotics, symbiotics, acidifiers, nucleotides, and phytogenics (10, 12). Among them, phytogenics are reputed for their growth-promoting effects, as well as their antimicrobial, antioxidant, anti-inflammatory, immunostimulant, and anti-stress properties (10), representing a promising effective and sustainable prophylactic tool to be implemented in health management in front of bacterial and parasitic infections (13, 14).

Fruits and leaves of the olive oil tree (*Olea europaea* L.) contain significant amounts of hydrophilic and lipophilic bioactives including flavones, phenolic acids, phenolic alcohols, secoiridoids, and hydroxycinnamic acid derivates (15). As a result of their anti-inflammatory, antioxidant, and antimicrobial actions, olive-derived phytogenics have shown beneficial health effects in human (16–18) and livestock (19–21) health. However, limited information is available on their effects on aquaculture fish species (22). In pigs (20) and fish (22), an olive-oil bioactive extract, containing a mixture of triterpenic acid and polyphenols, had anti-inflammatory and immunomodulatory properties in the intestine, while it also enhanced the integrity of the epithelium. In addition, a recent study showed that these compounds were able to reduce systemic inflammation in cattle (21). Regardless of these results, little is known about the immunomodulatory effects of this olive-oil bioactive extract on the systemic immune response and its potential use as a functional feed additive in aquafeeds for promoting disease resistance in fish.

The objective of the present study was to evaluate the effects of a diet supplemented with an olive-oil bioactive extract rich in polyphenols and triterpenic acid (AQUOLIVE®; NATAC Biotech SL, Spain) on the systemic immune response and disease resistance in Atlantic salmon smolts. For this purpose, Atlantic salmon parrs were smoltified with a diet supplemented with AQUOLIVE®. The levels of several humoral immune parameters were measured and the transcriptomic profiling of the head kidney (HK) analyzed by means of a microarray, whereas the potential protection of the tested feed additive was validated by means of an *in vivo* challenge with a pathogenic bacteria (*Aeromonas salmonicida*). This bacterium was chosen because it is the causative agent of furunculosis, which has been recognized as a threat for the salmon industry, reaching mortality rates up to 50%, even though it may be controlled by the administration of antibiotics and oil-based vaccines (2). However, assessing alternative more sustainable and affordable strategies based on the administration of functional feeds is advisable.

2 MATERIAL AND METHODS

2.1 Diets

To evaluate the immunomodulatory properties of the phytogenic obtained from olive fruit, two isoproteic (40% crude protein), isolipid (22% crude fat), and isoenergetic (21.6 MJ/kg gross energy) diets were formulated in order to fulfill the nutritional requirements of juvenile Atlantic salmon (23). Diets named as control and AQUOLIVE® were formulated to contain 17.5% fishmeal LT70, 2.5% fish protein concentrate, 55% plant-protein sources (soy protein concentrate, wheat and corn gluten faba beans, and wheat meal), and 10% fish oil and only differed in their content of the tested phytogenic (0.15%). The AQUOLIVE® was obtained by NATAC Biotech SL (proximate composition: 69.23% carbohydrates, 8.19% crude lipids, 0.41% crude proteins, 9.11% salts, and 3.06% water) which contained 10% olive bioactive compounds (8.0% triterpenic acid and 2% polyphenols).

Diets were manufactured by Sparos Lda. The main ingredients were ground (below 250 µm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed

according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 2 and 3 mm) by means of a low-shear extruder (P55; Italplast S.R.L., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientific, Oldham, UK) for 4 h at 45°C. Samples of each diet were taken for proximate composition analysis (24) and additive quantification (information provided by the manufacturer). Feeds were stored at 4°C during the experimental period (146 days) in order to prevent their oxidation. The list of ingredients and the proximate composition of experimental diets are shown in **Table 1**.

2.2 Fish and Experimental Design

A total of 1,500 unvaccinated Atlantic salmon parrs were obtained from a commercial fish farm (SARL SALMO, Gonneville-le-Thiel, France) and transported by road to IRTA-Sant Carles de la Ràpita research facilities (Sant Carles de la Ràpita, Spain). Once at IRTA facilities, fish were acclimated in two 2,000-l tanks connected to an open-flow system (water temperature: 12°C ± 1.5°C) for 2 weeks under a natural photoperiod. During the acclimation period, fish were fed commercial feed (T2-2 Royal Optime, Skretting; proximate composition: 44% crude protein; 21% crude fat; 6.9% crude ash; 2.9% crude fiber) to apparent satiation.

Before the start of the nutritional trial, parrs (n = 696) were gently anesthetized (50 mg/l tricaine methanesulfonate, MS-222, Sigma-Aldrich, Madrid, Spain) and individually measured in body weight (BW) and standard length (SL) to the nearest 0.1 g and 1 mm, respectively. Fish measuring 55.0 ± 0.1 g and 16.2 ± 0.2 mm in BW and SL, respectively, were distributed homogeneously among the 12 experimental tanks (n = 58 fish per tank; 6 replicate tanks per experimental diet). During the trial that lasted 133 days, fish were fed at the daily rate of 3.0% based on the stocked biomass by means of automatic feeders (ARVO-TEC T Drum 2000; ARVO-TEC, Finland). Feed ration was evenly distributed in six meals per day from 07:00 to 17:00 h and regularly adjusted by means of intermediate samplings along the trial according to the stocked biomass in order to guarantee apparent satiation.

TABLE 1 | List of ingredients and proximal composition of experimental diets: control and a basal diet supplemented with AQUOLIVE®.

Ingredients, %	Control diet	AQUOLIVE® diet
Fishmeal LT70	17.5	17.5
Soy protein concentrate	20.0	20.0
Fish protein concentrate	2.5	2.5
Wheat gluten	9.0	9.0
Corn gluten	5.0	5.0
Faba beans	5.0	5.0
Wheat meal	16.23	16.08
Fish oil	12.0	12.0
Vitamin and mineral premix	1.0	1.0
Soy lecithin	0.5	0.5
Vitamin C35%	0.07	0.07
Monocalcium phosphate	3.0	3.0
Rapeseed oil	7.0	7.0
Betaine HCl	1.0	1.0
DL-Methionine	0.2	0.2
AQUOLIVE®	–	0.15
Proximate composition		
Crude protein, %	40.03	40.02
Crude fat, %	22.15	22.15
Fiber, %	1.75	1.74
Starch, %	13.02	12.93
Ash, %	8.74	8.89
Gross Energy, MJ/kg	21.60	21.58

The experiment consisted of two different periods with regard to the smoltification process of Atlantic salmon juveniles. During the parr phase (47 days; December 19–February 4), water temperature and pH (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 12.2 ± 1.0°C, 7.4 ± 0.3, and 9.4 ± 0.8 mg/l (mean ± SD), respectively (**Supplementary Figure 1**). The water flow rate in experimental tanks was maintained at approximately 9.0 l/min (open-flow system), which guaranteed two full tanks' water renewal per hour. The photoperiod was 8 h light: 16 h darkness.

Smoltification started on February 5 and lasted 10 days. During this period, water salinity was increased progressively at *ca.* 3 ppt per day until reaching 35 ppt according to SARL SALMO recommendations. The water temperature, pH, and oxygen levels during this period were 12 ± 0.1°C, 7.4 ± 0.3, and 9.6 ± 0.2 mg/l (**Supplementary Figure 1**). The photoperiod during the smoltification period was 24 h light, 0 h darkness. Once fish were transferred to seawater on February 14, the water quality and temperature were maintained by means of a water recirculation system (IRTAmar®; Spain) that maintained adequate water quality through UV, biological, and mechanical filtration. The water quality parameters during the rest of the trial were 12.1 ± 0.2°C, 7.4 ± 0.3, and 9.5 ± 0.2 mg/l. Ammonia and nitrite were ≤0.07 and 0.14 mg/l, respectively. Ammonia and nitrites were measured twice per week by means of a portable spectrophotometer (Lovibond MD600, Tintometer GmbH, Germany) using the VARIO Ammonia Salicylate F10 mL (Tintometer GmbH, Germany) and NitriVer® 3 Nitrite Reagent (Permachem® Reagent, Hach Lange, GmbH) assays. The photoperiod during the smolt stage was 24 h light: 0 h darkness. The illumination system for the smolt phase consisted of a led illumination system (Celer, Spain) with a light temperature of 4,000 K and light intensity of 1,540 lumens. At the end of the trial, all fish were netted, anaesthetized with MS-222 as previously described, and individually weighted.

2.3 Humoral Immune Parameters

After fish were measured, blood (*ca.* 3 ml) was taken from anaesthetized fish (n = 3 fish per tank) by caudal puncture using heparinized vacutainers with 21 G needles (BD Vacutainer® containing lithium heparin 68 IU) and immediately centrifuged (3,000 × *g* for 15 min at 4°C) to separate plasma.

2.3.1 Peroxidase Activity

The peroxidase activity in plasma samples was measured according to Quade and Roth (25). Samples without plasma were used as blanks. Plates were read at λ = 450 nm in a plate reader (SPECTROstar Nano, BMG LABTECH, Ortenberg,

Germany). The peroxidase activity present in each sample was expressed as units/mL.

2.3.2 Protease Activity

The protease activity of plasma was quantified using the azocasein hydrolysis assay (26). Aliquots of 10 µl of plasma were incubated overnight at RT and in agitation with 100 µl of ammonium bicarbonate buffer and 125 µl of 2% azocasein (Sigma-Aldrich) in sterile Eppendorfs. The reaction was stopped by adding 250 µl of 10% trichloroacetic acid (TCA). The mixtures were centrifuged (6,000 × *g* 5 min), 100 µl of the supernatants transferred to a flat-bottomed 96-well plate, and 100 µl of 1 N NaOH added. Optical density was read at λ = 450 nm using a plate reader. Plasma was replaced by trypsin (5 mg/ml, Sigma-Aldrich) for the positive controls (100% of protease activity) or by ammonium bicarbonate buffer for the negative controls (0% of protease activity). The activity for each sample was expressed as % protease activity in relation to the controls.

2.3.3 Antiprotease Activity

The antiprotease activity of plasma was determined by the ability of plasma to inhibit trypsin activity (27). Briefly, 10 µl of plasma samples were incubated (10 min, 22°C) with the same volume of standard trypsin solution (5 mg/ml). After adding 100 µl of 100 mM ammonium bicarbonate buffer and 125 µl of buffer containing 2% azocasein (Sigma-Aldrich), samples were incubated (2 h, 30°C) and, following the addition of 250 µl of 10% TCA, a new incubation (30 min, 30°C) was done. The mixture was then centrifuged (1,500 × *g* 10 min) being the supernatants transferred to a 96-well plate in triplicate containing 100 µl well^{-1} of 1 N NaOH, and the optical density read at λ = 450 nm using a plate reader. For positive control, buffer replaced plasma and trypsin, and for negative control, buffer replaced the plasma. Activity for each sample was expressed as % antiprotease activity in relation to the controls.

2.3.4 Lysozyme Activity

Plasma lysozyme activity was measured by using a turbidimetric method (28) with some modifications. Samples of 20 µl of plasma diluted 1:10 with 0.04 M NaH_2PO_4–Na_2HPO_4 buffer, pH 6.2, were placed in a flat-bottomed 96-well plate. To each well, 200 µl of freeze-dried *Micrococcus lysodeikticus* in the above buffer (0.3 mg/ml, Sigma-Aldrich) was added as lysozyme substrate. The reduction in absorbance at 450 nm was measured over 15 min at 3-min intervals at RT in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min. The units of lysozyme present in plasma were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma-Aldrich). The lysozyme activity for each sample was expressed as µg/mL of hen egg white lysozyme eq. activity.

2.3.5 Bactericidal Activity

Two pathogenic bacteria for fish (*Vibrio anguillarum* and *Vibrio harveyi*) were used in the bactericidal assays. All bacterial strains were grown from 1 ml of stock culture that had been previously frozen at −80°C. The two bacteria were cultured for 48 h at 25°C in Tryptic Soy Agar (TSA, Difco Laboratories) and then inoculated in Tryptic Soy Broth (TSB, Difco Laboratories), both supplemented with NaCl to a final concentration of 1% (w/v). Bacteria in the TSB medium were then cultured at the same temperature, with continuous shaking (100 rpm) for 24 h. Exponentially growing bacteria were resuspended in sterile PBS and adjusted to 10^8 colony forming units (CFU) per mL.

Bactericidal activity was determined following the method of Stevens et al. (29) using the MTT assay, which is based on the reduction of the yellow soluble tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, Sigma-Aldrich) into a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase (30). Samples of 20 µl of plasma were added in a flat-bottomed 96-well plate. PBS was added to some wells instead of the samples and served as a positive control. Aliquots of 20 µl of the bacteria previously cultured were added, and the plates were incubated for 5 h at 25°C. After that, 25 µl of MTT (1 mg/ml) was added to each well and the plates were newly incubated for 10 min at 25°C to allow the formation of formazan. Plates were then centrifuged (2,000 g, 10 min), with the precipitates dissolved in 200 µl of DMSO and transferred to a new flat-bottom 96-well plate. The absorbance of the dissolved formazan was measured at 570 nm in a plate reader. Bactericidal activity was expressed as the percentage of no viable bacteria, calculated as the difference between absorbance of bacteria surviving compared to the absorbance of bacteria from positive controls (100%).

2.4 Bacterial Challenge

In order to investigate the immunomodulatory properties of the phytogenic compounds against bacterial infection, an experimental bacterial challenge with the strain IRTA-17-44 of *A. salmonicida* subsp. *salmonicida* (courtesy of HIPRA culture collection, coded: AS8074) was performed at the end of the nutritional trial. Bacterial suspensions of the selected strain were prepared from a stock stored in glycerol at -80°C. The inoculum was grown in TSA at 23.0 ± 1.0°C for 48 h. The bacterial inoculum was prepared to an OD of λ = 550 nm of 1.2, corresponding to a density of 10^8 CFU/ml previously established by serial dilutions and plate counting. The bacterial suspension was 10-fold serially diluted in sterile PBS, to prepare the desired inoculum, which was confirmed by CFU's plate counting. Prior to the challenge trial, an *A. salmonicida* (IRTA-17-44) lethal dose of 50% (LD_{50}) was determined for the experimental conditions to be assayed. For this purpose, 30 control Atlantic salmon were injected intraperitoneally (IP) with 0.2 ml of three concentrations of *A. salmonicida* inoculum, 10^6, 10^7, and 10^8 CFU/mL (10 fish injected with each inoculum concentration). Ten additional fish were injected with PBS as methodological control. The concentration of 10^7 CFU/mL was established as the nearest LD_{50} (data not shown).

For the challenge trial, 32 Atlantic salmon smolts (BW = 194.0 ± 29.1 g) per each dietary treatment were randomly distributed (https://www.randomizer.org) into quadruplicate tanks (four tanks per dietary treatment), with eight fish per tank (stocking density = 14–16 kg m^{-3}). During the acclimation period (5 days), fish were fed *ad libitum* with the same

experimental diets used in the nutritional assay. After acclimation, fish were anaesthetized and IP injected with 0.2 ml of 10^7 CFU/ml of *A. salmonicida* (IRTA-17-44).

Both the establishment of the *A. salmonicida* LD_{50} and the challenge trial were performed at IRTA's biosafety challenge room, in 32 cylindrical tanks (100 l) connected to a RAS unit (IRTAmar®) equipped with real-time control of oxygen and temperature, mechanical filtration, biofiltration, and ultraviolet disinfection of the water. The outflow water was chlorinated, followed by ozone treatment before being discharged. The water quality conditions in terms of temperature and salinity were 13.1 ± 1.1°C and 32.3 ± 0.4 ppt, respectively.

Fish mortality occurring after 12 h post-injection (hpi) was considered to be induced by the pathogen infection rather than handling stress, since no casualties were found in the control group injected with PBS. During the duration of the challenge (12 days), smolts were supervised every 2 h, six times per day, including weekends. In order to avoid unnecessary suffering, when the animals became moribund (i.e., loss of equilibrium, swollen abdomens, hemorrhaging in the anal area, and erratic swimming), they were euthanized with an overdose of MS-222. At the end of the experiment, all the remaining fish were sacrificed following the same procedure.

Confirmation of cause of death was determined by the recovery of the bacteria from all moribund animals, followed by specific PCR using *A. salmonicida* specific primers (31). For this purpose, animals were aseptically opened and a tissue sample of HK was taken and plated on TSA, incubated at 23°C for 72 h. Bacterial colonies were collected from the agar using sterile toothpicks and placed into 200 µl of DNA extraction lysis buffer containing proteinase K, and extractions performed following the manufacturer's protocol (DNeasy Blood and Tissue Kit, Qiagen, Spain). Extracted DNA was evaluated by spectrophotometry to determine the purity and concentration prior to PCR analysis. Amplification was performed in 25-µl reactions containing Taq polymerase buffer (1×), 0.5 U of Taq polymerase, $MgCl_2$ (2 mM), dNTPs (900 µM), and 1 µM of each primer specific for *A. salmonicida* [forward primer: 5′-CGGTTTTGGCGCAGTGACG-3′ and reverse primer: 5′-AGGCGCTCGGGTTGGCTATCT-3′; Beaz-Hidalgo et al. (31)]. The conditions for amplification were as follows: initial denaturation of template DNA at 95°C for 10 min, followed by 30 cycles of 1 min at 92°C, 1 min at 55°C, and 1 min at 72°C with a final extension step of 5 min at 72°C. Reactions lacking DNA, and containing genomic DNA of *A. salmonicida*, were used as negative and positive controls, respectively. PCR products were separated on a 1.2% (w/v) agarose gel and visualized using ethidium bromide staining. The presence of bands with a size of 422 bp was considered as a positive result.

2.5 Transcriptional Analysis

2.5.1 RNA Isolation and Quality Control

At the end of the nutritional assay, the total RNA from the HK of individual fish (n = 18 fish per dietary treatment) was extracted using TRI reagent (Sigma-Aldrich, Sant Louis, MO, USA), according to the manufacturer's instructions. Total RNA concentration and purity were quantified using a NanoDrop-2000® spectrophotometer (Thermo Scientific, USA) and stored at -80°C for further analysis. Samples were diluted to 133.33 ng/µl concentration and checked for integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Spain). All the samples used in this study were selected by the criteria of a RNA Integrity Number (RIN) value >8.5. Three pooled samples for each diet were used for microarray hybridization. Each pool consists in n = 1 fish from each replicate tank per treatment (n = 18 fish per diet, total N = 36 fish) (**Figure 1**). The information regarding individual variability was lost with this choice.

2.5.2 Microarray Design and Analysis

Transcriptional analysis was carried out using the custom-commercial *Salmo salar* oligonucleotide microarray platform (AMADID 084881; Gene Expression Omnibus (GEO) access number: GPL28080, Agilent Technologies; USA). Data presented in this manuscript are available in the GEO accession number GSE179142.

The transcriptomic analysis of HK samples from Atlantic salmon smolts was conducted as described by Reyes-López et al. (32). One-color microarray was carried out according to the manufacturer's protocols. In brief, 200 ng of total RNA from each pooled samples was reverse transcribed with Agilent One Color RNA Spike-In Kit (Agilent Technologies, USA). Then, total RNA was used as template for Cyanine-3 (Cy3)-labeled cRNA synthesis and amplification with the Quick Amp Labeling Kit (Agilent Technologies). cRNA samples were purified using the RNeasy Micro Kit (Qiagen). Dye incorporation and cRNA yield were checked with the NanoDrop ND-2000® spectrophotometer. Then, 1.5 mg of Cy3-labeled cRNA with specific activity >6.0 pmol Cy3 mg-1 cRNA was fragmented at 60°C for 30 min, and then the samples were mixed with hybridization buffer and hybridized to the array (ID 084881, Agilent Technologies) at 65°C for 17 h using the Gene Expression Hybridization Kit (Agilent Technologies). The microarray washes were conducted as recommended by the manufacturer using Gene Expression Wash Buffers (Agilent Technologies) and stabilization and drying solutions (Agilent Technologies). Microarray slides were scanned with an Agilent Technologies Scanner (model G2505B); spot intensities and other quality control features were extracted with Agilent's Feature Extraction software version 10.4.0.0 (Agilent Technologies). Quality reports were checked for each array. The identification of differential expressed genes was done, as described elsewhere (33). In brief, the bioinformatic package STARS (Nofima, Norway) was used for data processing and mining (34). After filtration of low-quality spots, Lowess normalization of log2-expression ratios (ER) was performed. The differentially expressed genes (DEGs) were selected by difference between the control and the experimental diet following an unpaired *t*-test. Expression values with a p-value < 0.05 were considered statistically significant.

2.5.3 Functional Network Analyses: Interactomes

The complete map of interactions that can occur in a living organism (interactome) was obtained from the DEGs obtained in the microarray-based transcriptomic analysis (transcripteractome). The analysis was performed as described elsewhere (32). In brief, the Search Tool for the Retrieval of Interacting Genes (STRING) public repository version 10.0

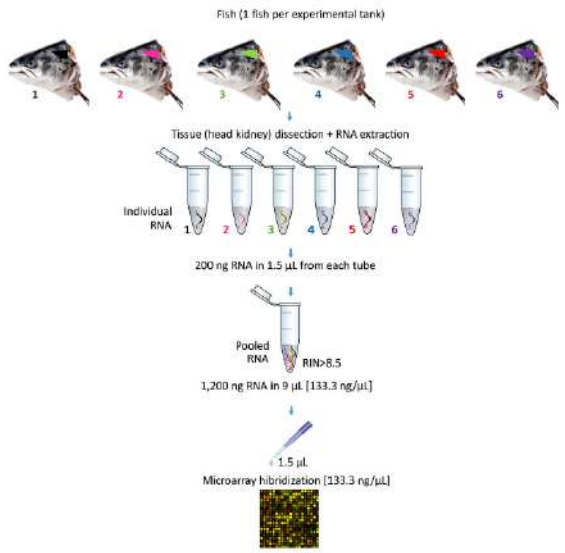


FIGURE 1 | Representation of pooling procedure for microarray hybridization of RNA samples. The diagram describes the pooling of one of the three pools used for each experimental diet. In particular, six individual samples of RNA were obtained from the head kidney of six Atlantic salmon (*S. salar*). Each of sampled fish came from one of the six experimental tank replicates per diet. The pooled RNA was prepared using 200 ng of each individual RNA in 1.5 µl (final volume of the pooled RNA = 9 µl; RNA concentration of the pooled RNA = 133.33 ng/µl). Prior to microarray hybridization, samples of pooled RNA were checked for quality and integrity. Then, pooled RNA were hybridized (1.5 µl; final RNA concentration = 133.33 ng/µl).

(https://string-db.org) was used (35). The protein–protein interaction (PPI) network for the differentially expressed genes was conducted with a high-confidence interaction score (value = 0.4). The mechanisms of response in which DEGs are involved were obtained from a comparative analysis based on *Homo sapiens* as a reference organism in order to extract the maximum information currently available. Thus, an *H. sapiens* acronym was assigned based on *S. salar* transcript annotation using UniProt (36) and GeneCards (37) databases. For those genes with no annotation match in salmon, an orthologue *H. sapiens* Entrez Gene was assigned based on sequence homology. To do it, we selected the best tBlastX (NCBI) hit for the DEG query sequence for *S. salar* and the human transcriptome database. We only consider those matches with at least E value $\leq 1e^{-10}$. The UniProt and GeneCards databases were used to confirm match of the gene acronym tag between both species. Gene ontology (GO) pathway enrichment analysis for biological processes (GO_BiologicalProcess-EBI-UniProt-GOA-ACAP-ARAP_10.11.2020_00h00) was obtained using the ClueGO v2.5.7 (38) app through the Cytoscape 3.8.2 (39) platform. The statistical analysis used was Enrichment/Depletion (two-sided hypergeometric test) with a p-value cutoff = 0.05 and corrected by Benjamini–Hochberg; a GO Fusion was performed to avoid redundant terms with a kappa score threshold = 0.4 in order to propose more stringent GO terms associated with the mechanism of response for the experimental diet incorporating the tested phytogenic. In addition, grouping of the GO terms was conducted when the sharing-group percentage was above 50, a p-value of < 0.05 was considered as significant. The statistically significant GOs obtained from the enrichment analysis were assigned to each one of the nodes represented in the functional network. The nodes classified in different clusters according to their functionality were represented with ClueGO v2.5.7.

2.6 Ethics Statement

All animal experimental procedures complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and were authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain) for the use of laboratory animals (FUE-2020-01314717).

2.7 Statistics

Growth performance was compared between groups with a t-test ($p < 0.05$). For the challenge trial, the mortality was registered in both experimental diets and data were represented using Kaplan–Meier mortality curves (40). The percent survival was calculated using the Mantel–Cox log-rank test. To construct the hierarchical heatmap, the Heatmapper server was used (41). Results related to the immune parameters were expressed as means ± standard error of mean (SEM). The normality of the variables was confirmed by the Shapiro–Wilk test while the homogeneity of variance was confirmed by the Levene test. Data were statistically analyzed by Student's t-test ($p < 0.05$) to determine significant differences between experimental groups. All the data were analyzed by the computer application SPSS for Windows® (version 15.0, SPSS Inc., Chicago, USA). All the determinations were performed in triplicates.

3 RESULTS

3.1 Growth Performance

After the 133-day of nutritional trial, no significant differences were observed in growth (252.3 ± 9.2 g *vs.* 240.2 ± 19.3 g) and Fulton's conditions factor (K = 1.2 ± 0.2 *vs.* 1.3 ± 0.1) between smolts fed the control diet and diet containing 0.15% AQUOLIVE® ($p > 0.05$), respectively.

3.2 Non-Specific Humoral Immune Parameters

At the end of the feeding trial, there were no significant differences in the humoral immunity (peroxidase, lysozyme, antiprotease, protease, and bactericidal activity) among Atlantic salmon smolts fed both diets (**Figure 2**; $p > 0.05$).

3.3 Head Kidney Transcriptomic and Microarrays

In order to determine the modulatory effect of the dietary supplementation with phytochemicals obtained from olive fruit

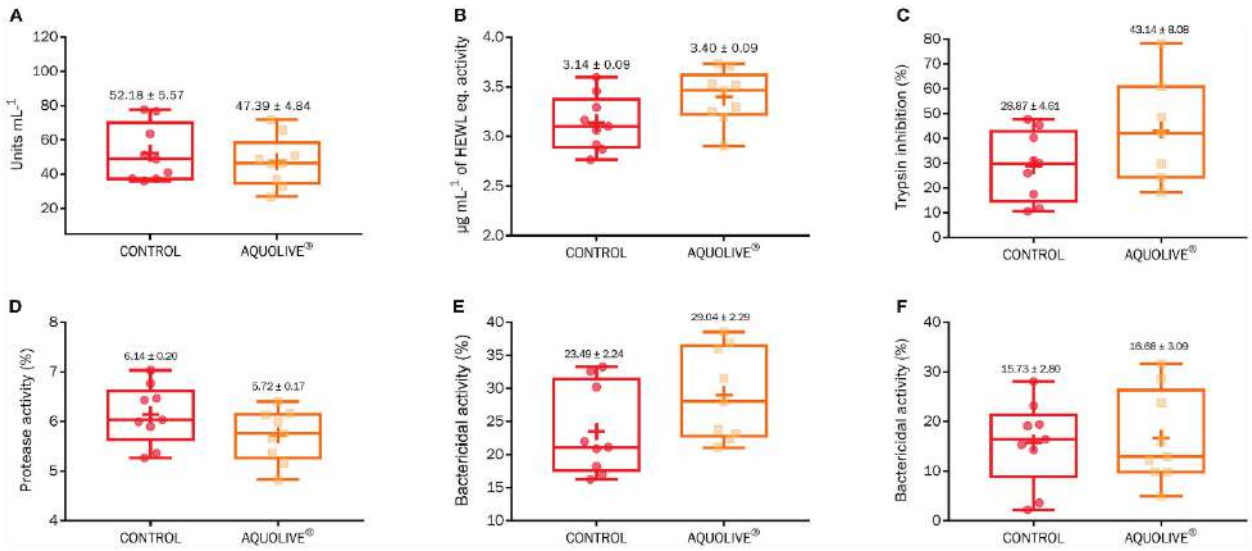


FIGURE 2 | Box-plot of humoral immunity (**A**, peroxidase; **B**, lysozyme; **C**, antiprotease; **D**, protease; **E**, bactericidal activity against *Vibrio anguillarum*; **F**, bactericidal activity against *Vibrio harveyi*) parameters measured in Atlantic salmon (*S. salar*) plasma fed the control diet and diet containing 0.15% AQUOLIVE®. Box-plot central lines indicate the median, and the plus symbol indicates the mean of data.

upon the Atlantic salmon HK transcriptome, a microarray-based transcriptomic analysis was conducted (**Figure 3**). A total number of 1,027 DEGs were found when comparing the transcriptomic profiling of the HK from Atlantic salmon fed the control and AQUOLIVE® diets ($p < 0.05$; **Supplementary Table 1**). In the case of upregulated genes, most of the transcripts (525) were identified in the 0.8 < log2 absolute fold change (|log2 FC|) < 1.4 interval. Then, 238 transcripts were identified in the 1.4 < |log2 FC| < 2.5 interval, 41 transcripts in the 2.5 < |log2 FC| < 5.0, and only one single gene in the |log2 FC| > 5.0. For the downregulated genes, 185 transcripts were identified in the 0.8 < |log2 FC| < 1.4 interval. Thirty-six other transcripts were grouped in the 1.4 < |log2 FC| < 2.5 interval, meanwhile only 1 DEG was included in the 2.5 < |log2 FC| < 5.0 expression interval. The detailed analysis of the gene absolute log2 fold change (|log2 FC|) revealed that genes were mostly upregulated in fish fed the AQUOLIVE® diet (78.4% of DEGs), while its gene modulation was moderate in terms of FC intensity (**Figure 3A**). Results from the three-principal component of the PCA analysis revealed a segregation pattern among dietary treatments pools. Differential gene expression patterns between the control and AQUOLIVE® groups are shown in **Figure 3B**. In addition, when representing DEGs intensity values from the pooled samples, a common segregation among profiles was observed in the hierarchical clustering heatmap for the HK transcriptomic response between AQUOLIVE® and control diet ($p < 0.05$; **Figure 3C**).

3.4 Enrichment Analyses

An enrichment analysis was carried out in order to determine those biological processes represented for the differentially expressed genes in HK response (**Figure 4**). For the enriched biological processes in HK of the Atlantic salmon (**Figure 4A**) fed with AQUOLIVE®, 10 representative groups were identified in the transcripteractome: "regulation of extent of cell growth" (4.76%; GO:0061387), "cellular response to ionizing radiation" (4.76%; GO:0071479), "signal transduction by p53 class mediator" (4.76%; GO:0072331), "positive regulation of cysteine-type endopeptidase activity" (4.76%; GO:2001056), "intracellular signal transduction" (4.76%; GO:0035556), "receptor metabolic process" (4.76%; GO:0043112), "regulation of i-kappaB kinase/NF-kappaB signaling" (9.52%; GO:0043122), "regulation of protein-containing complex disassembly" (9.52%; GO:0043244), "cellular macromolecule metabolic process" (9.52%; GO:0044260), and "leukocyte degranulation" (42.86%; GO:0043299) (**Figure 4B**).

According to the enrichment results, three main representative clusters of genes related to immunity were identified in the transcripteractome among the totality of biological processes obtained from the enrichment analysis: (1) "i-kappaB kinase/NF-kappaB signaling" (**Figure 5**), "leukocyte degranulation" (**Figure 6**), and "signal transduction by p53 class mediator" (**Figure 7**). **Table 2** summarizes the most relevant DEGs in terms of FC in fish fed the AQUOLIVE® diet in relation to the abovementioned biological processes.

As mentioned above, three main clusters regarding the dietary regulation of biological processes related to HK immunity were identified. For the cluster of "regulation of i-kappaB kinase/NF-kappaB signaling", two nodes were observed including "I-kappaB kinase/NF-kappaB signaling" (GO:0007249; 19 upregulated genes; 2 downregulated genes) and "regulation of I-kappaB kinase/NF-kappaB signaling" (GO:0043122;

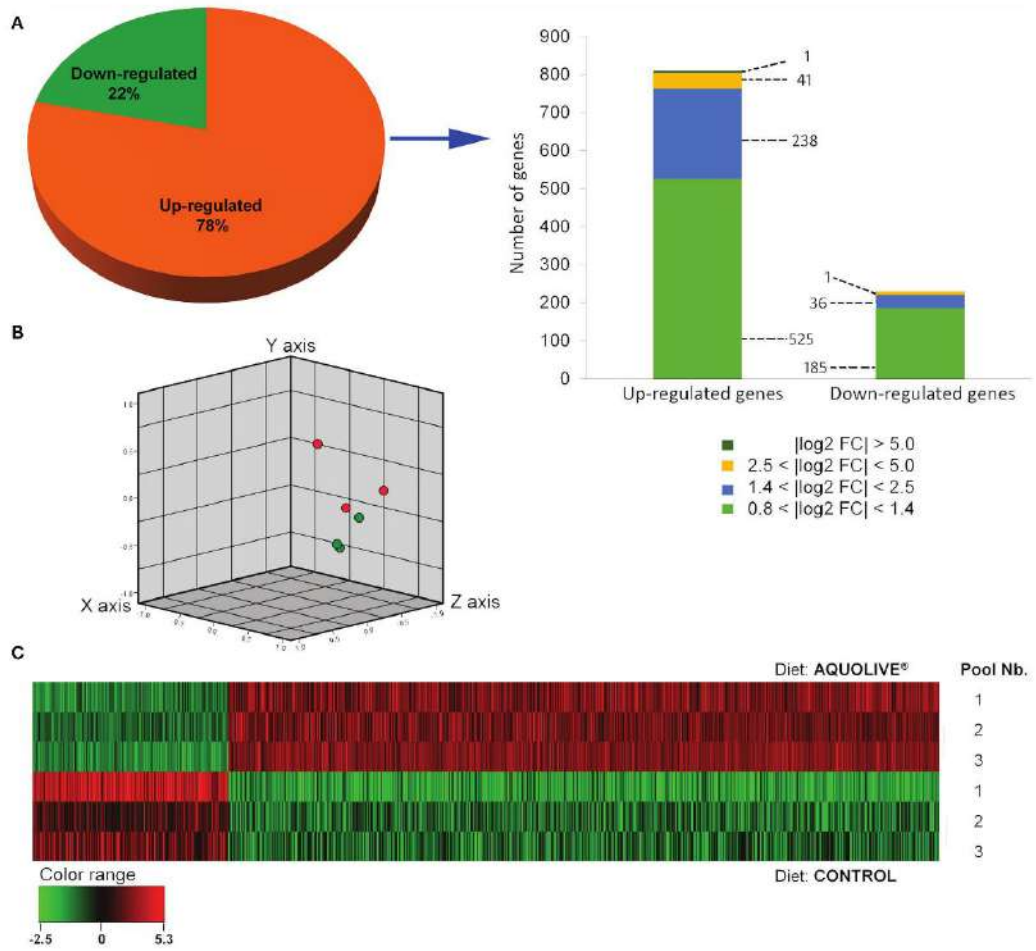


FIGURE 3 | Differential expression analysis of the Atlantic salmon (*S. salar*) head kidney transcriptomic response to AQUOLIVE® diet. **(A)** Distribution (pie chart) of the differential expressed genes (DEGs) obtained from the microarray-based transcriptomic analysis response fed a diet supplemented with a phytochemicals from olive fruit. Absolute log2 fold-change (|log2 FC|) indicates the magnitude interval of response. **(B)** Principal component analysis (PCA) of the DEGs for the Atlantic salmon head kidney in response to the control (red node) and phytogenic-supplemented diet (green node). **(C)** Hierarchical clustering of the Atlantic salmon head kidney transcriptomic response for the control and AQUOLIVE® diet, based in similitude patterns of the DEGs detected from three sample pools per dietary group. Data of the six microarrays are depicted. The normalized intensity values (log2) obtained for each microarray analyzed for control (replicates 1, 2, and 3) and AQUOLIVE® group (replicate 1, 2, and 3) are shown.

17 upregulated genes; 2 downregulated genes). In the "leukocyte degranulation" cluster, the other nine nodes were identified including "myeloid leukocyte activation" (GO:0002274; 29 upregulated genes; 13 downregulated genes), "leukocyte activation involved in immune response" (GO:0002366; 34 upregulated genes; 14 downregulated genes), "myeloid cell activation involved in immune response" (GO:0002275; 27 upregulated genes; 12 downregulated genes), "exocytosis" (GO:0006887; 38 upregulated genes; 18 downregulated genes), "granulocyte activation" (GO:0036230; 26 upregulated genes; 11 downregulated genes), "leukocyte degranulation" (GO:0043299; 26 upregulated genes; 12 downregulated genes) "neutrophil degranulation" (GO:0043312; 25 upregulated genes; 11 downregulated genes), and "vesicle-mediated transport" (GO:0016192; 79 upregulated genes; 35 downregulated genes). Lastly, one single-node cluster was identified including "signal transduction by p53 class mediator" (GO:0072331; 20 upregulated genes; 3 downregulated genes).

3.5 *In Vivo* Bacterial Challenge Test

During the *in vivo* bacterial challenge test with *A. salmonicida* (intraperitoneal injection: 1×10^7 CFU/ml), mortality in smolts was observed between 4 and 9 days post-injection (**Figure 8**). The Kaplan–Meier survival curves showed significant differences in terms of Atlantic salmon smolt survival depending on the dietary condition considered (**Figure 8A**; $p < 0.05$). In particular, smolts fed the AQUOLIVE® diet showed higher survival rates (96.9 ± 6.4%, mean ± standard deviation) in comparison to their

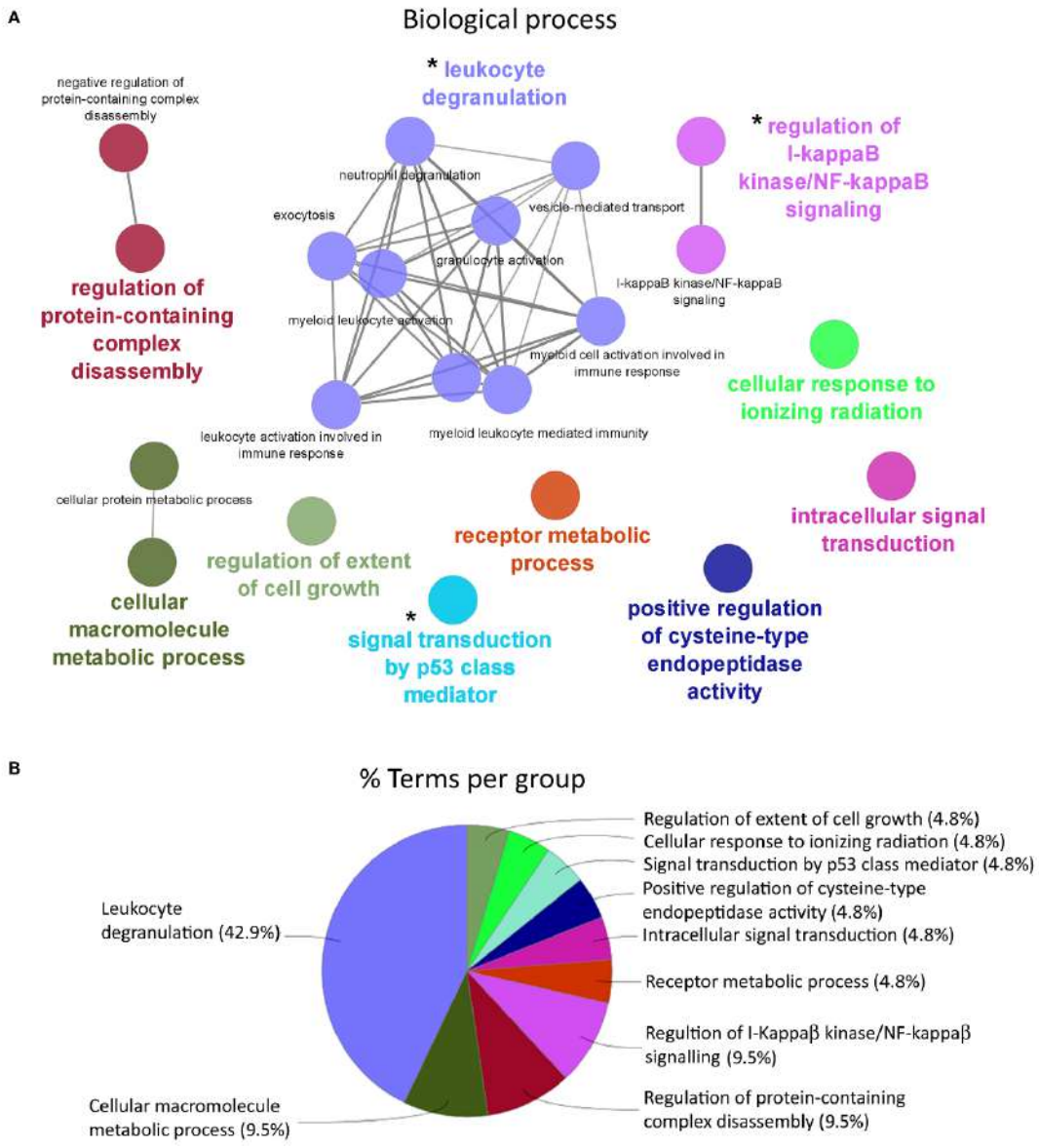


FIGURE 4 | Functional enrichment network analysis for biological processes based on the total number of differential expressed genes (DEGs) in the head kidney of Atlantic salmon (*S. salar*) fed with AQUOLIVE® diet. **(A)** Different biological processes represented in head kidney transcripteractome in response to the phytochemicals obtained from the olive fruit. Each color indicates a cluster of closely related biological processes. The colored biological term denominates the leading group term. The lines into the cluster indicated the close relationship between biological processes. The asterisks (*) indicate the three main clusters related to immune response pathways regulated by the feed additive. **(B)** Overview chart with functional groups including specific terms for DEGs of the Atlantic salmon head kidney transcriptomic response to AQUOLIVE®-supplemented diet, distribution of the biological processes according to their percentage of representation upon the total enriched terms is shown.

congeners fed the control diet (60.7 ± 13.5%). To confirm the cause of death, species-specific PCR was performed from bacterial colonies recovered from HK smears of all moribund fish during the bacterial challenge assay. Confluent pure bacterial growth was obtained from all animals, from which *A. salmonicida* was confirmed in all cases by means of PCR as shown in **Figure 8B**.

4 DISCUSSION

The market for sustainable products and feed additives is increasingly growing. The number of studies focused on the use of a wide variety of phytogenics as sustainable tools to be implemented in aquaculture production has dramatically increased in the last years. This has been mainly due to

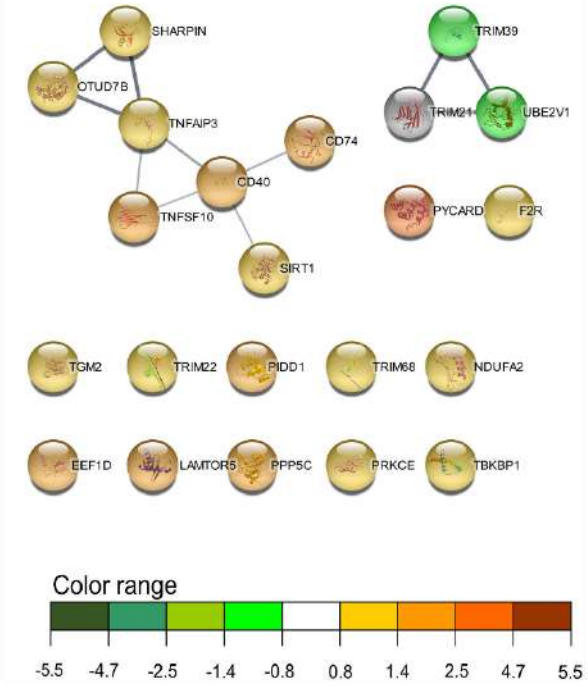


FIGURE 5 | I-kappaB kinase/NF-kappaB signaling transcripteractome of the differentially expressed genes (DEGs) related to the immune pathway in the head kidney of juvenile Atlantic salmon (*S. salar*) fed the AQUOLIVE® diet (see also **Supplementary Table 2**). Color range indicates the modulation in terms of log2 fold change (log2 FC) intensity of each node.

phytogenics' growth-promoting, antimicrobial, immunostimulant, antioxidant, and anti-inflammatory properties (13, 42). In this study, we have evaluated a new phytogenic feed additive rich in triterpenic compounds and polyphenols derived from olive fruit (AQUOLIVE®) on the systemic immune response and disease resistance in Atlantic salmon smolts. In this context, a total number of 1,027 DEGs (805 up- and 222 downregulated) were found when comparing the transcriptomic profiling of the HK from fish fed the control and AQUOLIVE® diets. Moreover, the bacterial challenge lasted 12 days at the end of the assay, showing that the cumulative survival was higher in fish fed the AQUOLIVE® diet (96.9 ± 6.4%) than in fish from the control group (60.7 ± 13.5%).

Previous studies on the inclusion of bioactive compounds derived from the olive industry have been conducted. Particularly, it has been shown that a diet with olive oil bioactive extract rich in triterpenic compounds enhanced the innate immune function and integrity in the intestine of gilthead seabream (*Sparus aurata*) (22). Additionally, a phytogenic with similar bioactive compounds than AQUOLIVE® showed a tightly controlled systemic immune response in an *ex vivo* assay using splenocytes stimulated by lipopolysaccharide (LPS) (43). Regarding the dietary supplementation of olive leaf extracts, Navruz et al. (44) reported that common carp (*Cyprinus carpio*) showed an improved immune response and survival rates against *Edwardsiella tarda*. Similarly, in rainbow trout (*Oncorhynchus mykiss*) feed phytogenic compounds derived from olive leaf extract (OLE) showed an enhancement of the expression of immune-related genes, such as pro-inflammatory cytokines like *tnfα*, *il1-β*, and *il-8*, as well as disease resistance against *Yersinia ruckeri* (45). The abovementioned results are in agreement with the results obtained in our study when Atlantic salmon smolts fed the AQUOLIVE® diet showed higher disease resistance in front of the pathogenic bacteria *A. salmonicida* than their congeners fed the control diet.

4.1 Transcription Factors

In order to investigate the immunomodulatory properties of the phytogenic tested, the modulation of the transcriptional immune response in the HK of the anadromous fish Atlantic salmon fed AQUOLIVE® diet was evaluated by means of a microarray analysis. This is of special relevance, since in order to achieve a proper immune response, a wide repertoire of biological processes at cellular and molecular levels, including transcription factors, are usually involved, as described in the following. The dietary supplementation of AQUOLIVE® in the HK of Atlantic salmon shows modulation of different biological processes related to transcription factors such as "signal transduction by p53 class mediator" and "i-kappa B kinase/NF-kappa B signaling", among others. Different studies have evidenced that there is a transcriptional cross talk between nuclear factor κB (NF-κB) and p53 (46, 47). In particular, NF-κB may be considered as a transcriptional regulator of p53 and *vice versa*. In fact, NF-κB was found to be able to recognize κB sites on the p53 promoter and thereby activate its expression (47). p53 is part of the innate and adaptive immune system, as well as detect DNA damage, repair, and recombination, besides playing an important role in infectious diseases, killing, and limiting viral and bacterial replication (48). In line with this, it has been shown in different fish species that p53 is an important mediator of innate antiviral and antibacterial immunity (49–51). On the other hand, the NF-κB pathway is well known as a central mediator in the regulation of several cytokines, chemokines, antimicrobial peptides, and interferon-stimulated genes, playing a critical role in regulating the survival, activation, and differentiation of innate and adaptive immune cells (52, 53). Moreover, it has been demonstrated that upon bacterial infection, the cytoplasmic NF-κB is rapidly activated and translocated into the nucleus to stimulate the expression of antimicrobial peptides fighting against pathogenic organisms (54). The gene coding for the P53-induced death domain protein (*pidd1*) was upregulated in the HK of Atlantic salmon smolts fed the AQUOLIVE®-supplemented diet in comparison to their congeners fed the control diet. This gene is reputed for playing an essential role in NF-κB and caspase-2 activation. It has been shown in the literature that PIDD1 expression causes spontaneous activation of caspase-2 and sensitization to apoptosis by genotoxic stimuli (55, 56). In this sense, *casp-2* expression is involved in the regulation of p53 in response to

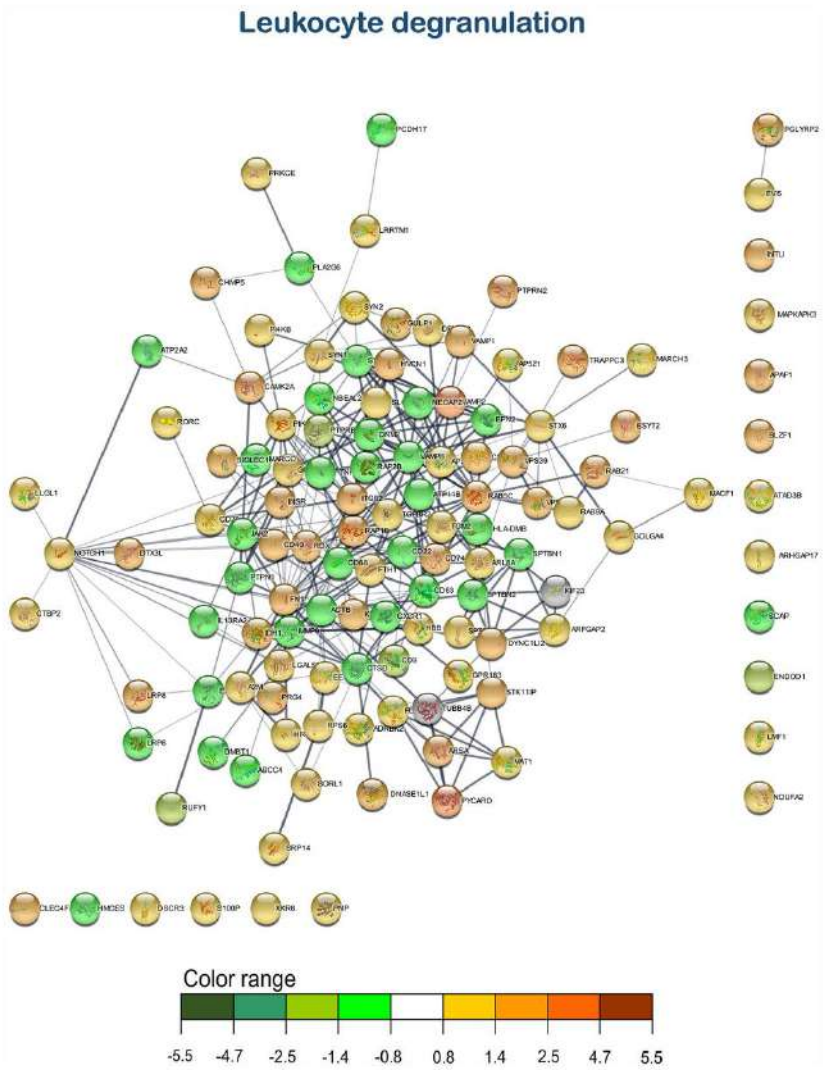


FIGURE 6 | Leukocyte degranulation transcripteractome of the differentially expressed genes (DEGs) related to the immune pathway in the head kidney of juvenile Atlantic salmon (*S. salar*) fed the AQUOLIVE® diet (see also **Supplementary Table 3**). Color range indicates the modulation in terms of log2 fold change (log2 FC) intensity of each node.

cellular stress and DNA damage to prevent the proliferation and accumulation of damaged or aberrant cells (56). *Casp-2* was significantly downregulated in the HK of fish fed the AQUOLIVE®-supplemented diet, thus leading us to a possible homoeostatic scenario. Another gene involved in the abovementioned biological processes that deserves attention is the PYD and CARD Domain Containing (*pycard*), which was upregulated in our samples from the HK of fish fed the AQUOLIVE® diet. PYCARD is a dual regulator in NF-κB activation pathways and plays a distinct role in innate defense systems through the inflammasome (57, 58). This is relevant, since it has been shown that inflammasome activation plays a critical role in activating innate immunity (59). The inflammasome consists of caspase-1 and caspase-5 enzymes, Pycard/Asc, and NAPL1, a pyrin domain-carrying protein, which shares a structural homology with NODs (nucleotide-binding oligomerization domain-like receptors). In the presence of certain stimuli (e.g., a specific pathogen cell-surface proteins), the caspase-1 scaffold within the inflammasome is activated, which induces the inflammatory response (59, 60). Therefore, it might provide us an answer to the increased disease resistance of Atlantic salmon smolts fed with the tested phytogenic and *in vivo* challenged with *A. salmonicida*, obtaining a higher survival when compared to fish fed the control diet (60).

4.2 Cell Response

The HK is one of the most important organs in fish due to its role in endocrine and hematopoietic functions, and it is a major

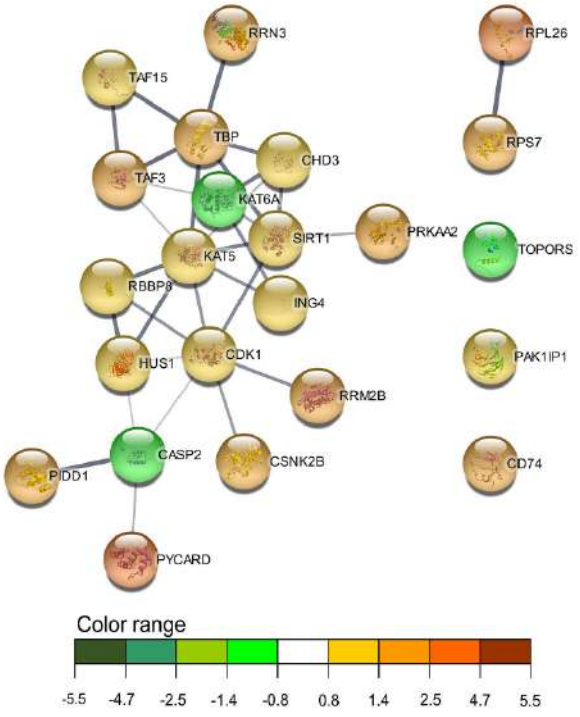
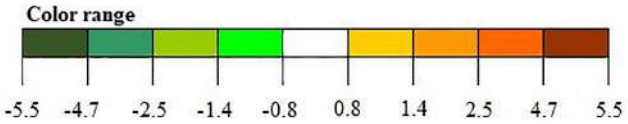


FIGURE 7 | Signal transduction by p53 class mediator transcripteractome of the differentially expressed genes (DEGs) related to the immune pathway in the head kidney of juvenile Atlantic salmon (*S. salar*) fed the AQUOLIVE® diet (see also **Supplementary Table 4**). Color range indicated the modulation in terms of log2 fold change (log2 FC) intensity of each node.

TABLE 2 | List of the most relevant DEGs related to the three main representative biological processes identified by the transcripteractome in fish fed the AQUOLIVE® diet.

Gene description	Gene acronym	FC (log2)	*p*-value
Ribosomal protein L26	*rpl26*	3.43	0.02429
Vesicle-associated membrane protein 2	*vamp2*	3.40	0.01714
PYD and CARD domain containing	*pycard*	2.72	0.00004
RAB21, member RAS oncogene family	*rab21*	2.35	0.00369
RAB5B, member RAS oncogene family, b	*rab5b*	2.20	0.04218
RAS related protein 1b	*rap1b*	2.17	0.03414
CD40 molecule	*cd40*	2.02	0.00121
TNF superfamily member 10	*tnfsf10*	1.85	0.03433
P53-induced death domain protein 1	*pidd1*	1.66	0.01492
CD74 molecule	*cd74*	1.63	0.00496
RAB9A, member RAS oncogene family, b	*rab9a*	1.10	0.00110
CD28 molecule	*cd28*	1.08	0.03644
TNF alpha-induced protein 3	*tnfaip3*	0.88	0.03090
Alpha-2-macroglobulin	*a2m*	0.80	0.04309
CD68 molecule	*cd68*	-0.86	0.00752
CD9 molecule	*cd9*	-0.89	0.02258
CD63 molecule	*cd63*	-0.95	0.01869
CD22 molecule	*cd22*	-1.02	0.01087
Caspase 2	*casp-2*	-1.31	0.03100
Protein tyrosine phosphatase receptor type b	*ptprb*	-1.81	0.00296
Endonuclease domain-containing 1	*endod1*	-1.86	0.03239



secondary lymphoid organ in the body (61). Our findings showed that the tested feed additive regulates several biological processes in the HK related to the host's immunity. In particular, these biological processes were related to innate immune effector key cell functions of vertebrate innate immunity (62), such as "leukocyte activation", "granulocyte activation", "neutrophil degranulation", "exocytosis", and "vesicle-mediated transport", among others. In addition, granulocytes are the main phagocytic cells in the HK and are also involved in the innate immunity as antigen-presenting cells (63). Moreover, neutrophils are one of the three types of granulocytes identified in fish (64, 65), whereas neutrophilic granulocytes are the most abundant in salmonids (66). As their main function is arriving first at the site of the infection and having a central role in host tissue protection by killing pathogenic microorganisms and stimulating lymphocytes and other immune cells, neutrophils are an essential part of the innate immune system (67). In addition, under normal conditions, neutrophils are rarely found in tissues since they are recruited from blood and hematopoietic organs. However, fish neutrophils are not so abundantly present in the bloodstream contrarily to mammals, since they are stored in hematopoietic reservoirs, which may be interpreted as a disadvantage for rapid migration and effective resolution of infection and inflammation events (68). In fact, the dietary supplementation of olive extract or similar bioactive compounds has been reported to enhance hematological and other immune parameters in different animal species, such as reducing inflammation and oxidative stress and enhancing the intestinal immune function, among others (20–22, 69, 70). As previously mentioned, "exocytosis" and "vesicle-mediated transport" were also modulated by AQUOLIVE®; this is especially relevant since exocytosis is recognized by its important role in the immune response participating in neutrophil function (71). For instance, genes like vesicle-associated membrane protein 2 (*vamp2*) showed an upregulation when compared to the control diet. VAMP2 is known to participate in different cell types, including neutrophils, monocytes, and eosinophils, regulating exocytosis, since it is predominantly in the membrane of secretory vesicles (72, 73). Thus, the membrane densities of VAMP2 correspond to the exocytic potential of the different storage vesicles, strongly suggesting a functional role of this protein in neutrophil degranulation (71). This is of special relevance, since it has been shown that individuals with decreased or missing neutrophil degranulation had higher incidence of bacterial and fungal infections (74). Therefore, an increase in neutrophil degranulation could lead to enhanced disease resistance and reduced mortality rates in individual fish, as occurred in our

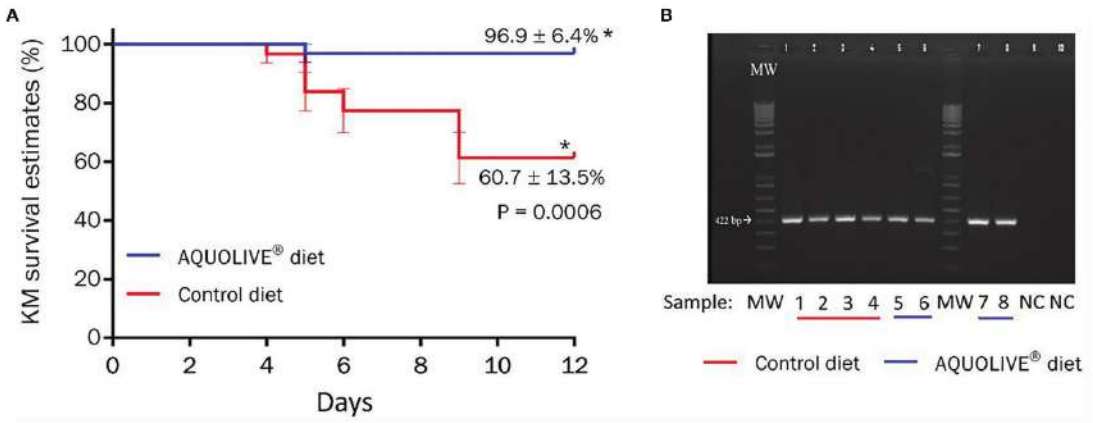


FIGURE 8 | Results of the bacterial challenge conducted in Atlantic salmon smolts intraperitoneally injected with 1×10^7 CFU/ml of *A. salmonicida*. **(A)** Kaplan–Meier (KM) survival curves (%) for Atlantic salmon smolts intraperitoneally injected with *A. salmonicida* (1×10^7 CFU/ml) during the 12-day challenge trial period. Data correspond to the mean ± standard error (four replicates tanks per experimental diet; n = 8 fish per tank). The asterik (*) indicates statistically significant differences among dietary treatments (t-test; $p < 0.05$). **(B)** Specific PCR of bacterial colonies recovered from smears of head kidney from moribund Atlantic salmon smolts during the bacterial challenge test with *A. salmonicida*. MW = molecular weight standard; lanes 1–6 are samples recovered from moribund fish (1–3: control diet; 4–6: AQUOLIVE® diet); lanes 7–8 are positive control genomic DNA from *A. salmonicida*; lanes 9–10 are negative control lacking template DNA.

bacterial challenge (75). Additionally, transcriptional regulation of vesicle-mediated transport by dietary administration of AQUOLIVE® resulted in the positive regulation of several genes encoding the RAB family of GTPases (*rab21*, *rab5b*, *rap1b*, *rab9a*), recognized for participation in the regulation of exocytosis as leading regulators of membrane trafficking and directing inflammation and immune cellular responses (76, 77). In this sense, phenolic compounds from olive tree leaves have been described to regulate vesicle and exocytic processes (78). Therefore, we hypothesize that the machinery implied in the activation of biological processes observed by dietary AQUOLIVE® may be inherent to the activation of processes of secretory protein translocation by vesicles.

4.3 Innate and Adaptive Response

The expression of several genes (*cd9*, *cd22*, *cd28*, *cd63*, *cd68*, *cd74*) associated with innate and adaptive immunity was modulated by the AQUOLIVE®-supplemented diet as well. For example, the expression of the gene coding for the CD9 molecule was downregulated in the HK of fish fed the AQUOLIVE® diet. CD9 was found to be extensively present in Atlantic salmon IgM^+ B cells (79), also known to encode tetraspanins, which are key players in the recruitment of leukocytes into inflammation sites and regulation of several steps of the immune response (80). Castro et al. (81) reported that *cd9* transcription in IgM^+ B lymphocytes was modulated in the presence of bacteria and virus, in particular, *cd9* was downregulated in rainbow trout in response to a virus, thus revealing a role for this molecule in this antigen-specific lymphocyte response. Therefore, the downregulation of this gene in accordance with our results could suggest a migratory capacity of B cells in response to bacterial or viral infection. Furthermore, the downregulation of the CD63 molecule, another tetraspanin, was also modulated by the tested feed additives. Particularly, it was observed that *cd63* levels were downregulated when exposed *in vivo* in response to a virus, suggesting a possible increase of the antigen-presenting capacity of IgM^+ cells, as suggested by Castro et al. (81). In this way, the tetraspanin family has been shown to play an important role in influencing MHC II antigen presentation and $CD4^+$ T cell stimulation (82). Importantly, Petersen et al. (82) showed that a knockdown of CD63 in the B lymphoblastoid cell line may play a role in participating in the modulation of cell-surface-initiated signals, which can trigger exosomal secretion and lead to increased $CD4^+$ T cell recognition. Nevertheless, further studies need to be addressed properly to give us the proper meaning of the downregulation of *cd63* regarding the AQUOLIVE®-based feed additive in the HK of Atlantic salmon. Additionally, *cd68* was downregulated by the AQUOLIVE®-supplemented diet. This gene is a transmembrane protein with a suspected role in phagocytic activities of tissue macrophages, and it has also been found in granules of neutrophils, as well as in certain epithelial cells (83). Von Rhaden et al. (83) have shown that the upregulation of *cd68* in macrophages was involved in the inflammatory response. Under present experimental conditions, the downregulation of *cd68* may indicate a tight control of the inflammatory response. However, only a few studies were carried out on *cd68* in fish. Thus, the exact function in *cd68* with regard to its nutritional regulation by phytogenics is unclear and further studies are needed. On the other hand, *cd28* and *cd74* both were upregulated in the fish fed the AQUOLIVE®-supplemented diet. In particular, CD28 is probably the most important fish T cell co-stimulatory receptors, playing a key part in interactions between lymphocytes and antigen-presenting cells (84). Moreover, CD74 plays a specific role as an important component in the functional presentation of MHC class II-restricted antigens and as a cytokine receptor (85). Therefore, our results are in agreement with

another transcriptomic study in which virus-challenged Atlantic salmon had increased expression of both *cd28* and *cd74* genes in the experimental group compared to the control group, resulting in increased resistance to pancreas disease caused by salmonid alphavirus, which is a severe contagious disease in farmed Atlantic salmon (86). In this sense, we found evidence for the activation of specific immunity genes such as B and T lymphocyte activity or MHC class II antigen presentation, suggesting the stimulation of the innate and the adaptive immune response as well through the tested feed additives.

4.4 Inflammatory Response and Immune Signaling

Genes that are involved in response to tumor necrosis factor (TNF) family members (*cd40*, *tnfsf10*, *tnfaip3*) were also upregulated by the AQUOLIVE® diet. Particularly, the TNF family plays an especially important role in the immune system; many of these molecules are essential in the regulation of B cell biology and B cell-mediated immune responses (87). Interestingly, it has been demonstrated that the TNF receptor superfamily member 5 (*cd40*) is capable of stimulating the non-canonical NF-κB pathway, in addition to playing an essential role for T and B cell cooperation in response to protein antigens (88, 89). TRAIL, also known as TNF superfamily member 10 (*tnfsf10*), was positively modulated by the tested feed additive, and it has been reported to be involved in the immune response, specifically under parasite infections, and B cell differentiation and survival in front of bacterial and viral infections (87, 90). Biswas et al. (91) reported that the upregulation of the *tnfsf10* gene in Japanese pufferfish (*Takifugu rubripes*) indicated a probable role of this gene in inducing apoptosis in virus-infected cells. In addition, TRAIL was recognized as a critical mediator of the p53 response in the apoptotic pathway (92). Last but not least, the tumor necrosis factor alpha-induced protein 3 (*tnfaip3*) was also upregulated by the AQUOLIVE® diet. TNFAIP3 is a zinc finger domain-containing protein, which is recognized to be a negative regulator of NF-κB signaling (93), thereby negatively regulating the transcription of other pro-inflammatory cytokines and, consequently, controlling the inflammatory response. Therefore, the present results suggest a hypothesis that the tested feed additive promoted an immune homeostatic effect.

Our study also revealed that the ribosomal protein L26 (*rpl26*) was upregulated in the HK of fish fed the phytogenic-supplemented diet, which is involved in the abovementioned "signal transduction by p53 class mediator" biological process. This gene is located at the ribosomal subunit interface of the 60S subunit inside the cell (94). Interestingly, several studies have demonstrated the role of the *rpl26* gene as a phagocytosis-activating protein, thus being highly involved in the immune response, since phagocytosis is a major mechanism used to remove pathogens and cell debris (95–97). Furthermore, it has been possible to demonstrate that the *rpl26* gene has a strong ability to bind p53 mRNA and thereby to stimulate p53 translation, as previously indicated (98, 99). In fact, there is also evidence that the aforementioned function of RPL26 as a phagocytosis-activating protein into the cells may be facilitated by the alpha-2-macroglobulin (*α2M*) (100). Interestingly, *α2m* was also upregulated in fish fed dietary AQUOLIVE®. Moreover, this immune-related gene is known to be the most widely studied protease inhibitor that mainly functions to maintain body fluid homeostasis and is also involved in acute-phase reactions and defense against pathogens that secrete proteolytic enzymes. In this sense, *α2M* plays an important role in restricting the ability of bacteria to invade and grow during the infective process (101). This may be of particular relevance, since fish fed the AQUOLIVE® diet demonstrated higher survival (96.9 ± 6.4%) in comparison to fish fed the control diet (60.7 ± 13.5%). It has been found that some highly adapted pathogenic bacteria, like *A. salmonicida*, can evade the host defense mechanisms producing a highly toxic serine protease, which can resist some antiproteases (102, 103). However, *α2M* has the capacity to inhibit the serine protease of *A. salmonicida*, thus reducing susceptibility to furunculosis among salmonids (102–104). These transcriptomic results from the HK of smolts at the end of the nutritional trial are in agreement with different mortality rates observed between experimental groups when challenged with this pathogenic bacterium.

In addition to evaluating by microarray analysis the potential immunomodulatory effects of the tested plant extract used in this study, the authors wanted to extend these possible effects with other parameters (i.e., humoral immune markers). For this purpose, different humoral immune parameters were evaluated in plasma at the end of the nutritional assay. This evaluation of plasmatic immune parameters (peroxidase, protease, antiprotease, lysozyme, and bactericidal activity) revealed no significant immunostimulant effect of the tested feed additive. These results might be supported by the hypothesis that the use of additives does not always have the expected immunological response if fish are not exposed to a real threat (outbreaks of diseases or a bacterial challenge trial) (43, 105), and also to the fact that the unnecessary activation of immune response would affect the energy budget (106), which may potentially affect growth performance. Nevertheless, it should be noted that in the presence of a pathogen *stimulus*, this basal condition was affected and apparently enhanced when we observed at the DEG analysis of fish fed the AQUOLIVE®-supplemented diet.

5 CONCLUSIONS

In summary, analysis of the HK transcriptomic profiling response to a diet supplemented with 0.15% AQUOLIVE® revealed a gene expression profile that favors biological processes particularly related to immunity. This mechanism activates effector leukocytes such as granulocytes, which differentiate into neutrophils, suggesting an innate immune response promoted by the tested functional feed additive in the HK. The immune response promoted by AQUOLIVE® dietary is also supported by the active control of vesicular transport and

exocytosis. The overall results of our study highlighted the main biological processes induced by this dietary AQUOLIVE® which might be responsible for the better performance, as shown by lower mortality rates in fish fed this additive when they were challenged with *A. salmonicida*. Altogether, this study indicated that the tested feed additive, rich in triterpenic and polyphenolic compounds from *O. europaea*, promotes systemic immunity and protects Atlantic salmon smolts against *A. salmonicida*. Thus, the combination of current vaccination practices conducted by the industry coupled with the administration of AQUOLIVE® may represent a good strategy against furunculosis. In addition, this phytogenic may be also of interest for other marine species like European sea bass (*Dicentrarchus labrax*) suffering from furunculosis (107). Moreover, these results indicate that these phytogenics may be a promising tool to be implemented in sustainable and environmentally responsible aquaculture industry in the post-antibiotic era.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

All animal experimental procedures were complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and the guidelines of the Spanish laws (law 32/2007 and RD 1201/2015) and authorized by the Ethical Committee of the Institute for Research and Technology in Food and Agriculture (IRTA, Spain) for the use of laboratory animals (FUE-2020-01314717).

AUTHOR CONTRIBUTIONS

Conceptualization, EG. Methodology, MDF, EV-V, FER-L, ME, CE. Formal analysis, RS, FER-L, JF, EV-V. Resources, EG. Writing original draft, RS; writing review and editing, MDF, EV-V, RS, FER, EG, LT; visualization, RS, FER-L, EV-V; supervision, EG, EV-V; project administration, EG; funding acquisition, EG. All authors have read and agreed to the published version of the manuscript.

FUNDING

This work has been financially supported by the project "AQUOLIVE by NATAC", funded by the European Union's Horizon 2020 research and innovation program (Grant Agreement Nb. 830202). FER-L thanks the support of Fondecyt Regular grant (Nb. 1211841; ANID; Government of Chile). RS is supported by a PhD grant from the government of Paraguay (BECAL). JF has been subsidized by the Industrial PhD program of *Generalitat de Catalunya* and TECNOVIT-FARMFAES S.L. (Nb. 2017 DI 017). The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. Collaboration between Ibero-American researchers has been done under the framework of the network LARVAplus "Strategies for the development and improvement of fish larvae production in Ibero-America" (117RT0521) funded by the Ibero-American Program of Science and Technology for Development (CYTED, Spain).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.737601/full#supplementary-material

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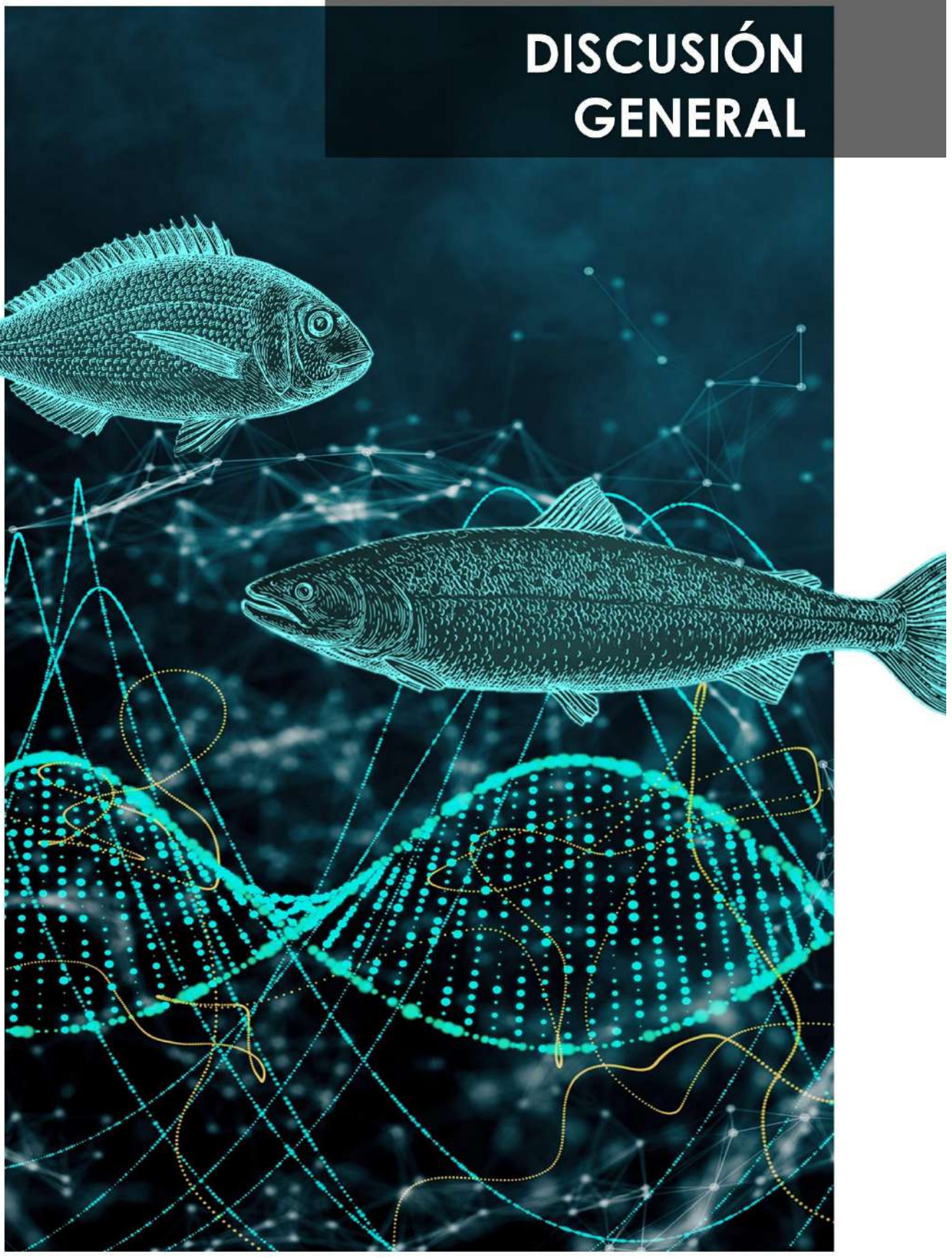
Conflict of Interest: JQ and JP are current NATAC BIOTECH S.L. employers.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DISCUSIÓN GENERAL



DISCUSIÓN

Los fitogénicos como estrategia sostenible para prevenir los brotes de enfermedades en acuicultura

En los últimos 20 años, los consumidores de los países de ingresos bajos y altos se han beneficiado de la disponibilidad y el acceso a los alimentos acuáticos durante todo el año gracias al continuo crecimiento de la acuicultura y su posicionamiento y relevancia en el abastecimiento de los mercados (Naylor et al., 2021). Para abastecer la creciente demanda de pescado y marisco derivada de un crecimiento demográfico constante y de los cambios en los patrones de consumo de proteína de origen acuático por parte de la población mundial, la acuicultura seguirá siendo la fuerza motriz del crecimiento de la producción mundial de pescado, prolongando una tendencia de décadas. Por lo tanto, se espera que la producción acuícola mundial aumente de 179 millones de toneladas en 2018 a 204 millones de toneladas en 2030 (un 15% más que en 2018) (FAO, 2020).

Sin embargo, la rápida expansión de la acuicultura aumenta su propia vulnerabilidad frente a muchos desafíos importantes que obstaculizan el desarrollo del sector. En algunos casos, socavando su capacidad para alcanzar los resultados sostenibles necesarios y afectando negativamente a la opinión de los consumidores y a la credibilidad del sector. De hecho, se prevé que la tasa media de crecimiento anual de la acuicultura se reduzca del 4,6% en 2007-2018 al 2,3% en 2019-2030. Se espera que una serie de factores contribuyan a esta desaceleración, incluyendo la adopción y aplicación más amplia de las regulaciones en favor del cuidado medioambiental, la disminución de la disponibilidad de agua y lugares de producción adecuados, la disminución de las ganancias de productividad, el aumento de los brotes de enfermedades de los animales acuáticos relacionados con las prácticas de producción intensiva, y las limitaciones asociadas al cambio climático (FAO, 2020).

La intensificación de los sistemas de producción y el cambio climático facilitan la aparición de brotes de enfermedades ya que pueden favorecer la presencia de animales estresados e inmunocomprometidos, y la evolución y propagación de patógenos cada vez más virulentos. Esto hace que las enfermedades de los animales acuáticos sean uno de los principales factores limitantes del desarrollo sostenible de la acuicultura (Reverter et al., 2020). A pesar de los esfuerzos desplegados en la mejora de la vigilancia y la gestión de las enfermedades en acuicultura, distintos estudios estiman que en las próximas décadas surgirán y se propagarán rápidamente patógenos, hasta ahora desconocidos, que causarán enfermedades nuevas, causando importantes pérdidas de producción aproximadamente cada tres a cinco años (FAO, 2020). Para prevenir y mitigar estas pérdidas económicas, en algunos de los principales países productores se sigue observando el uso profiláctico indiscriminado de antibióticos y productos químicos asociados a las prácticas acuícolas intensivas (Lulijwa et al., 2020; Schar et al., 2020). Sin embargo, el uso recurrente de tales terapéuticos tiene graves efectos secundarios en el sistema acuícola, no sólo por la inmunosupresión de los animales, sino también por la selección y aparición de cepas más virulentas y bacterias resistentes a los antibióticos (Reverter et al., 2020). Además, el uso indiscriminado de antibióticos supone un grave problema de salud pública (Adegoke et al., 2018). En la Unión Europea (Reglamento 1831/2003/CE), las amenazas de resistencia a los antibióticos y la prohibición de los antibióticos como promotores del crecimiento en los alimentos para animales de granja han promovido a la industria ganadera y acuícola la adopción de sistemas de producción libres de antibióticos (Vincent et al., 2019; Reverter et al., 2020), así como el desarrollo de tratamientos alternativos más sostenibles y respetuosos con el medio ambiente (Dawood et al., 2018). Con el fin de garantizar los beneficios sociales y medioambientales de los que la acuicultura se enorgullece, es obligatorio aplicar prácticas de producción acuícola sostenibles. En este contexto y con el objetivo de prevenir brotes de enfermedades y reducir el uso de fármacos quimioterapéuticos en la acuicultura, se han propuesto estrategias alternativas/complementarias, como la vacunación y la administración de

suplementos alimenticios funcionales (Miccoli et al., 2021; Reverter et al., 2021), entre otras (Barrett et al., 2020).

Sin embargo, aunque la vacunación haya sido demostrada como una excelente herramienta de prevención de enfermedades en acuicultura, todavía es una técnica utilizada en casos específicos para un patógeno en concreto, presentando una eficacia limitada contra las infecciones por múltiples agentes. Además, el tiempo y los costes asociados al desarrollo de vacunas pueden limitar su disponibilidad y aplicación frente a un amplio repertorio de organismos patógenos, lo que hace que en la actualidad no existan vacunas eficaces contra varias enfermedades de importancia económica, como las infecciones víricas y parasitarias (Miccoli et al., 2021). Por otro lado, dado que las enfermedades están íntimamente relacionadas con el estado fisiológico e inmunológico de los peces, el uso de piensos funcionales que aporten beneficios para la salud más allá de su valor nutricional ha recibido una importante atención en la última década como estrategias preventivas en cuanto a la gestión sanitaria de las instalaciones acuícolas (Dawood et al., 2018; Waagbø y Remø, 2020).

En este escenario, entre los diferentes aditivos funcionales de tipo zootécnico para piensos que pueden utilizarse en acuicultura, los fitogénicos se definen como combinaciones estandarizadas, específicas y basadas en la ciencia de compuestos bioactivos que se encuentran en plantas con eficacia comprobada e impacto sostenible en animales, personas y/o en medio ambiente. Los fitogénicos suelen ser extractos de plantas aromáticas y aceites esenciales caracterizados por su riqueza en compuestos biológicamente activos (Suphoronski et al., 2019; Christaki et al., 2020). En peces de cultivo, se están estudiado cada vez más un amplio espectro de aditivos fitogénicos debido principalmente a sus propiedades promotoras del crecimiento, antimicrobianas, inmunoestimulantes, antioxidantes, antiinflamatorias y sedantes (Franz et al., 2020). Por este amplio espectro de acción, los fitogénicos representan una prometedora herramienta profiláctica eficaz y sostenible para ser implementada en la gestión sanitaria en instalaciones acuícolas frente a infecciones ya sean de tipo bacteriano, vírico, parasitario y/o fúngico (Reverter et al., 2021).

En el contexto de esta tesis doctoral, se han estudiado dos aditivos zootécnicos de tipo fitogénico con propiedades inmunomoduladoras, compuestos que han sido analizados teniendo en cuenta los resultados de indicadores clave del rendimiento productivo (crecimiento y uso del alimento) y resultados asociados a la respuesta inmunitaria transcripcional de tres tejidos asociados a la inmunidad local (intestino) y a la inmunidad sistémica (bazo y riñón cefálico). Estos estudios se han realizado en dos especies de peces de relevancia acuícola, la primera de ellas y de ámbito local es la dorada (*Sparus aurata*), siendo una de las especies de peces marinos más importantes que se cultivan en el Mediterráneo, mientras que la segunda especie considerada en esta tesis has sido el salmón del Atlántico (*Salmo salar*), principal especie acuícola a nivel mundial, tanto por su valor económico como por su volumen de negocio internacional. Estas dos especies acuícolas fueron alimentadas con dos aditivos diferentes y que son objeto de la presente tesis doctoral, (1) una combinación de extractos de dos plantas medicinales como son la salvia y la hierbaluisa (MPLE, por sus siglas en inglés: *medicinal plant leaf extract*; nomenclatura utilizada en los Capítulos I, II y III [Salomón et al., 2020; 2021a; 2022]); y (2), un aditivo obtenido del extracto de la aceituna (AQUOLIVE®) (nomenclatura utilizada en el Capítulo IV; [Salomón et al., 2021b]). A continuación, se presenta la discusión general de los resultados obtenidos en esta tesis doctoral, tanto a nivel de variables relacionados con el rendimiento productivo de los animales, como a nivel de su respuesta inmunitaria.

Efectos de los fitogénicos y sus compuestos bioactivos obtenidos de extractos de la salvia, la hierbaluisa y la aceituna en el rendimiento productivo de los peces

En términos productivos y de retorno económico, el crecimiento (aumento de la biomasa) se ha considerado tradicionalmente como uno de los principales puntos finales e indicador clave de rendimiento a la hora de evaluar diferentes formulaciones de piensos. En base a la literatura consultada, los efectos sobre el

rendimiento productivo en diferentes especies de peces con respecto a fitogénicos derivados de la salvia, la hierbaluisa y la aceituna se encuentran resumidos en el ***Apéndice 1***. Es importante destacar que la mayoría de estos trabajos han sido publicados en los últimos años, lo que demuestra el creciente interés actual por la investigación de los fitogénicos y su aplicación en producción animal. En este contexto, los extractos de salvia, la hierbaluisa y la aceituna han demostrado actuar como promotores del crecimiento en varias especies de peces, como son la tilapia del Nilo (*Oreochromis niloticus*) (El Kholy et al., 2012; Mahmoud et al., 2014), la trucha arcoíris (*Oncorhynchus mykiss*) (Sönmez et al., 2015; Hoseinifar et al., 2020b), la carpa común (*Cyprinus carpio*) (Gholipourkanani et al., 2017; Sokooti et al., 2021), la dorada (Gisbert et al., 2017) y el esturión siberiano (*Acipenser baerii*) (Adel et al., 2021). En algunos de estos trabajos donde se observa un mayor crecimiento somático, también se traducen en diferencias significativas en ciertos indicadores claves de rendimiento como un menor factor de conversión alimenticia (FCA) o una mayor tasa de crecimiento (SGR, por sus siglas en inglés: *specific growth rate*) como ha sido en el caso de la tilapia del Nilo (El Kholy et al., 2012; Mahmoud, 2014), trucha arcoíris (Sönmez et al., 2015; Hoseinifar et al., 2020a), carpa común (Karimi Pashaki et al., 2018; Sokooti et al., 2021) y esturión siberiano (Adel et al., 2021).

Con respecto a los efectos en el rendimiento productivo en peces alimentados con dietas suplementadas con los fitogénicos derivados de las dos plantas medicinales (salvia y hierbaluisa) utilizadas en esta tesis, ejemplos en base a la literatura son expuestos y desarrollados a continuación. Por ejemplo, se ha visto que dietas suplementadas con 0,15% de un polvo de hojas de salvia durante 90 días en tilapias hibridas (*Oreochromis niloticus x Oreochromis aureus*), promovieron tanto el crecimiento somático, como la tasa específica de crecimiento, además de reducir significativamente el FCA (El-Kholy et al., 2012) (*Apéndice 1*). Asimismo, Mahmoud et al. (2014) han indicado que el crecimiento somático, el FCA y la tasa específica de crecimiento se vieron positivamente afectados en tilapias del Nilo alimentadas durante 84 días con una combinación de fitogénicos (aceite esencial en 0,05%) que contenía laurel (*Laurus nobilis*), hinojo (*Foeniculum vulgare*) y salvia (*Apéndice I*).

Igualmente, Sönmez et al. (2015) han demostrado que el aceite esencial de salvia con una inclusión de 500 mg kg^{-1} en la dieta tiene un efecto positivo en el incremento de peso y la tasa de crecimiento (*Apéndice I*). Por otro lado, y con respecto a la hierbaluisa, Gholipourkanani et al. (2017) han informado de un efecto positivo en cuanto al peso final en carpas comunes alimentadas con una dieta suplementada con un aceite esencial de hierbaluisa en 0,15 mL kg^{-1} durante 30 días. Además, en esturiones siberianos alimentados con una dieta suplementada con un extracto crudo de hierbaluisa en 5 mg kg^{-1} durante 56 días mostraron rendimientos positivos en cuanto al incremento de peso, el FCA y la tasa de crecimiento (Adel et al., 2021) (*Apéndice I*). Estos resultados están de acuerdo con nuestros trabajos, donde la inclusión en la dieta del aditivo obtenido de la combinación de extractos de dos plantas medicinales (salvia y hierbaluisa), MPLE, afectó positivamente el crecimiento de la dorada, así como la eficiencia alimentaria y la tasa específica de crecimiento (Capítulo I [Salomón et al., 2020]). Igualmente, cuando este aditivo fue incluido en la dieta basal utilizando el mismo porcentaje de inclusión (0,1%) en salmón del Atlántico también afectó de manera significativa el crecimiento somático de los animales y el FCA (Capítulo III [Salomón et al., 2022]). Es importante tener en cuenta que la dieta basal utilizada en gran parte de esta tesis doctoral ha sido formulada con un bajo porcentaje de harina de pescado (7% los estudios de dorada y 17,5% en los trabajos en salmón del Atlántico), apoyando de esta manera el cambio de paradigma de la industria hacia la formulación de dietas menos dependientes de materias primas derivadas de la pesca como la harina de pescado (Froehlich et al., 2018). Los resultados de la presente tesis doctoral, en donde las dos especies objeto de estudio han demostrado una mejora significativa en términos de crecimiento, en el caso de la dorada, que ha crecido un 8,5% más en cuanto a los peces alimentados con la dieta control (Salomón et al., 2020), y en el caso del salmón en el que se ha observado un 11,5% de aumento respecto a los salmones alimentados con la dieta control (Salomón et al., 2022). Estos resultados están en concordancia con resultados previos similares observados en vertebrados superiores (Pastorelli et al., 2012; Casamassima et al., 2013), así como también en otras especies de peces

dulceacuícolas alimentadas con dietas funcionales que contienen fitogénicos derivados de la salvia y/o hierbaluisa. Si bien en los trabajos anteriormente citados no se menciona cuál es su composición en compuestos potencialmente bioactivos, estos resultados promotores del crecimiento podrían atribuirse al contenido de dichos aditivos caracterizados en compuestos triterpenoides, como el ácido ursólico (Wang et al., 1999; Lu y Foo, 2000). En particular, se ha visto que el ácido ursólico tiene efectos sobre la capacidad promotora del crecimiento muscular mediante la hipertrofia de las fibras musculares, resultados que se han demostrado tanto en ratones (Kunkel et al., 2012) como en la trucha arcoíris (Fernández-Navarro et al., 2006). En el caso de la hierbaluisa, uno de sus compuestos más abundantes es el ácido verbascósido (Sánchez-Marzo et al., 2019); compuesto que tiene también efectos promotores sobre el crecimiento (Pastorelli et al., 2012; Casamassima et al., 2013). El mecanismo promotor del crecimiento del ácido verbascósido parece ser distinto al reportado por la salvia, siendo éste atribuible probablemente a la función protectora de la hierbaluisa a nivel intestinal, al promover la digestión y absorción de nutrientes y por consiguiente promover la disponibilidad de nutrientes para el organismo e indirectamente el crecimiento del animal (Adel et al., 2021). Así, por ejemplo, Pastorelli et al. (2012) pusieron de manifiesto en ganado porcino que una dieta enriquecida con ácido verbascósido (10 mg kg^{-1}) durante 56 días supuso una mejora en el crecimiento de los animales en comparación con la dieta control. Por otra parte, en corderos alimentados con dos dosis diferentes (2,5 mg y 5 mg por animal por día) con un suplemento a base de ácido verbascósido (extracto de hojas) durante 42 días, mostraron una diferencia significativa de ganancia de peso respecto a los corderos alimentados con la dieta control (Casamassima et al., 2013). Estos resultados están en línea con los obtenidos en la presente tesis, tanto en dorada como en salmón del Atlántico, si bien la aproximación analítica llevada a cabo no nos permite esclarecer los mecanismos de acción de dichos fitogénicos sobre los animales. No obstante, podemos hipotetizar que no deben ser distintos a los reportados en vertebrados superiores.

Por el contrario, otros estudios sobre la salvia y la hierbaluisa no han informado de efectos positivos sobre el crecimiento y la tasa específica de crecimiento en especies de peces como la tilapia del Nilo (Aydin et al., 2018), la trucha arcoíris (Hoseinifar et al., 2020a), y el esturión beluga (*Huso huso*) (Dadras et al., 2020), y/o la eficiencia alimentaria en la carpa común (Gholipourkanani et al., 2017). Pese a ello, no es fácil esclarecer por qué algunos fitogénicos pueden tener propiedades positivas sobre el crecimiento y eficiencia alimentaria de los animales, pues las razones pueden ser muy diversas. Por ejemplo, bajo condiciones experimentales óptimas y formulación de dietas con altos niveles de harina de pescado, los efectos positivos de determinados aditivos pueden verse potencialmente enmascarados al no darse las condiciones nutricionales necesarias para visualizar los efectos positivos esperados del uso de dichos compuestos. A su vez, la composición variable y no estandarizada de los distintos extractos de plantas, así como su origen y método de extracción, también pueden repercutir en su composición a nivel de compuestos funcionales, y por lo tanto influenciar de forma diferente en los organismos (Länger et al., 2018; Firmino et al., 2021c). Por estos factores, no siempre es fácil y/o posible extraer conclusiones derivadas de la comparación de distintos estudios, pues tal y como se ha mencionado, no siempre los autores de dichos trabajos se aproximan de forma similar, tanto conceptual como experimentalmente, a dar respuesta a una misma pregunta, pues el estudio de cómo afectan estos fitogénicos al rendimiento productivo de los animales no acostumbra a ser el objetivo final de dichos estudios, sino que éstos están enfocados a evaluar cómo dichos fitogénicos promueven la condición general del animal a partir de sus propiedades funcionales.

Por otro lado, con respecto a estudios basados en dietas funcionales con compuestos bioactivos derivados de la aceituna también se han visto resultados positivos en cuanto al rendimiento productivo. Dichos estudios y sus correspondientes resultados se encuentran resumidos en el ***Apéndice 1***. A título de ejemplo, doradas alimentadas con una dieta que contenía un extracto bioactivo de aceite de oliva (0,17%), rico en compuestos fenólicos como hidroxitirosol y tirosol durante 90 días, mostraron una mejora significativa en cuanto a su peso final

(Gisbert et al., 2017). Igualmente, Hoseinifar et al. (2020b) encontraron un incremento significativo en el peso final de truchas arcoíris alimentadas con una inclusión de 0,5 g kg^{-1} en la dieta de extractos de aceituna ricos en polifenoles y vitamina E durante 42 días. Asimismo, carpas alimentadas con 200 mg kg^{-1} de extractos de hoja de olivo, ricos en compuestos bioactivos, tales como la oleuropeína y sus derivados como el hidroxitirosol durante 75 días, mostraron una diferencia significativa de ganancia de peso respecto a los peces alimentados con la dieta control (Sokooti et al., 2021). Estos resultados no están en acuerdo con los obtenidos en los salmones del Atlántico alimentados con la dieta con una inclusión del 0,15% de AQUOLIVE®, donde no se registraron diferencias a nivel de peso final (Capítulo IV; Salomón et al., 2021b). Nuestros resultados están en línea con los publicados por Baba et al. (2018) donde truchas arcoíris alimentadas con un porcentaje de inclusión de 0,1% de compuestos fitogénicos derivados del extracto de hoja de olivo no registraron diferencias en cuanto al crecimiento. Asimismo, Karimi Pashaki et al. (2018) han indicado que carpas alimentadas con una dieta que incluía extracto de hoja de olivo (1 g kg^{-1}) durante 8 semanas no afectó al crecimiento de los animales. Igualmente, no se registraron diferencias significativas en cuanto a rendimiento productivo (peso final, FCA y tasa de crecimiento) en carpas alimentadas con 0,1% de extractos de hoja de olivo durante 8 semanas (Rajabiesterabadi et al., 2020a). Esta disparidad de resultados podría venir explicado por distintos factores, tales como: 1) el origen variable y poco estandarizado de los fitogénicos (es decir, materias primas obtenidas en los mercados locales), 2) la utilización de diferentes formas de suplementación (formato polvo, aceite esencial, extractos, etc.) y su dosis de inclusión, 3) los diferentes periodos de administración y formulaciones de las dietas, entre otros (Vallejos-Vidal et al., 2016). Asimismo, otra posible respuesta, no solo a nivel de diferencias bibliográficas, sino más bien tratando de explicar estas diferencias respecto al aditivo MPLE, el cual sí afectó de forma positiva el rendimiento productivo tanto en dorada como en salmón del Atlántico, pueden verse reflejadas con la ausencia de un desafío nutricional, como la inclusión del aditivo en una dieta baja en harina de pescado, que posiblemente podría revelar el

potencial promotor del crecimiento de los fitogénicos. Asimismo, con el efecto a nivel del músculo por parte de la salvia y de una mayor absorción y digestión de nutrientes en el intestino por parte de la hierbaluisa como hemos mencionado anteriormente, podrían darnos pistas claras de las diferencias entre aditivos en cuanto al rendimiento productivo. Igualmente, la ausencia de un proceso estandarizado para llevar a cabo estos estudios entre distintos grupos de investigación, tanto a nivel de la caracterización de los fitogénicos, composición de la dieta basal como en lo relativo a las condiciones experimentales del estudio, limita la interpretación y comparación entre ellos. Independientemente de estos factores, los fitogénicos probados en esta tesis no fueron elegidos por su potencial efecto positivo sobre el crecimiento somático, eficiencia alimentaria o tasa específica de crecimiento, sino por sus propiedades antioxidantes, antimicrobianas, antiinflamatorias e inmunoestimulantes, entre otras (Dawood et al., 2021), y por consiguiente, sobre su potencial efecto sobre la salud del animal y protector efecto sobre agentes patógenos, efectos que discutirán con mayor detalle en los siguientes apartados de la presente tesis doctoral.

Propiedades inmunomoduladoras del fitogénico obtenido del extracto de la salvia y hierbaluisa (MPLE)

Con respecto al MPLE, la tesis se centró en descifrar los posibles efectos inmunomoduladores de la salvia y la hierbaluisa en las especies objeto de estudio de la presente tesis, que son bien conocidas por sus propiedades, entre ellas, antiinflamatorias, antioxidantes, inmunoestimulantes y relajantes, entre otras. Los beneficios de estas plantas medicinales se encuentran bien caracterizados en vertebrados superiores (Funes et al., 2009; Caturla et al., 2011; Funes et al., 2010; Walch et al., 2011; Martins et al., 2015; Ben Khedher et al., 2018; Rasouli et al., 2020), pero el conocimiento de sus efectos en peces es más bien limitado (Abdellatief et al., 2018; Hoseinifar et al., 2020a; Adel et al., 2021). En este contexto, los resultados obtenidos de la respuesta inmune local a nivel del intestino (Capítulo II, [Salomón

et al., 2021a]), como también a nivel sistémico, a nivel del bazo (Capítulo I, [Salomón et al., 2020]) y del riñón cefálico (Capítulo III, [Salomón et al., 2022]), en las dos especies acuícolas modelo incluidas en esta tesis doctoral se discuten a continuación.

Las propiedades inmunomoduladoras del aditivo MPLE en cada uno de los tejidos diana de las dos diferentes especies utilizadas en la presente tesis fueron determinadas mediante: 1) un análisis transcriptómico basado en microarrays en el riñón cefálico y el intestino, con un posterior análisis asociado a un enriquecimiento funcional para identificar aquellos genes expresados diferencialmente que pueden tener una asociación con respuestas biológicas particulares; y 2) la aplicación de metodologías complementarias para apoyar y/o validar los estudios moleculares. En cuanto a las metodologías complementarias utilizadas, se realizó un ensayo *ex vivo* con esplenocitos de doradas alimentadas previamente con las dietas experimentales durante 92 días, los cuales se incubaron con un patrón molecular asociado a un patógeno (del inglés *pathogen associated molecular patterns*, PAMP) como es el lipopolisacárido (LPS), con el fin de evaluar la respuesta inmunológica de dichas células a varios procesos inmunitarios (respuesta inmunitaria humoral, citoquinas pro y antiinflamatorias, marcadores de superficie celular y enzimas antioxidantes) (Capítulo I, [Salomón et al., 2020]). Por otro lado, y en el caso del salmón del Atlántico, al final del ensayo nutricional se realizó un reto bacteriano de tipo *in vivo* con el fin de validar las posibles propiedades inmunomoduladoras del aditivo, este ensayo se basó en un reto bacteriano, donde los peces fueron infectados (inyección intraperitoneal) con el patógeno *Aeromonas salmonicida* responsable de la furunculosis (Capítulos III y IV [Salomón et al., 2021b; 2022]). Ambas metodologías complementarias fueron herramientas eficaces para poder validar los posibles efectos inmunomoduladores por parte de los fitogénicos probados en la presente tesis.

Los análisis transcriptómicos realizados en riñón cefálico e intestino mostraron que estos dos tejidos respondieron positivamente a la administración de ambos fitogénicos, con algunas variaciones en las respuestas transcripcionales entre los

diferentes tejidos. Uno de los compuestos bioactivos más abundantes en la salvia es el triterpeno conocido como ácido ursólico (Hussain et al., 2017), mientras que en la hierbaluisa es el ácido verbascósido (Sánchez-Marzo et al., 2019). Se ha demostrado que el ácido ursólico afecta a las funciones inmunitarias en diferentes modelos animales experimentales. Raphael y Kuttan (2003) informaron de que el ácido ursólico (dosis: 50 µmoles kg^{-1} de peso corporal/dosis/animal, tiempo de administración: 5 días, método de aplicación: intraperitoneal) aumentaba la respuesta inmunitaria a nivel celular en ratón, tal y como se puso de manifiesto con el incremento en el número de glóbulos blancos totales de células positivas α-esterasa y de células formadoras de placas en el bazo. Asimismo, en este mismo estudio en oveja se observó un aumento significativo de la producción de anticuerpos específicos tras la administración de un antígeno, confirmando su efecto positivo sobre la respuesta inmunitaria (Raphael y Kuttan, 2003). Estos resultados estarían de acuerdo con los resultados observados en la presente tesis (ensayo *ex vivo* con esplenocitos de dorada; Capítulo I), confirmando el efecto inmunoestimulante a nivel celular del ácido ursólico (Salomón et al., 2020). Además, Xu et al. (2015) estudiaron el efecto del ácido ursólico (dosis: 20 y 100 mg^{-1} kg^{-1} $día^{-1}$, método de aplicación: intraperitoneal) sobre las funciones inmunitarias en un modelo experimental de *miastenia gravis* (Xu et al., 2015), enfermedad autoinmune mediada por células B y dependiente de células T que se caracteriza por una debilidad muscular fatigable (Zuckerman et al., 2010). En el citado estudio, el ácido ursólico indujo la apoptosis en las células mononucleares de los ganglios linfáticos, aumentando también los niveles de IL-10 (citoquina antiinflamatoria) y disminuyendo los de IL-17A en las células de ganglios linfáticos, promoviendo así una respuesta inmunitaria de tipo celular. La dosis alta, 100 mg^{-1} kg^{-1} $día^{-1}$, aumentó el número de células T $CD4^+$ y redujo los niveles de anticuerpos (inmunoglobulina G) en el suero (Xu et al., 2015). Estos resultados no hacen más que apoyar la idea de que el MPLE es capaz de promover una respuesta inmune del tipo celular frente un potencial agente infeccioso (Salomón et al., 2020), tal y como se ha demostrado en doradas alimentadas con el aditivo MPLE. Los datos obtenidos de la

transcriptómica por PCR cuantitativa de los esplenocitos estimulados con un PAMP (LPS) al final del ensayo nutricional proporcionaron los conocimientos y respuestas básicas para lograr descifrar el papel inmunomodulador del aditivo probado, donde se ha visto un patrón de expresión de los genes implicados en la respuesta inmunitaria inespecífica (lisozima [*lys*] e inmunoglobulina M [*IgM*]), así como de las citoquinas proinflamatorias (factor de necrosis tumoral [*tnf-a*] e interleucina 1 beta [*il-1β*]) y antiinflamatorias (factor de crecimiento transformante beta 1 [*tgf-β1*] e interleucina 10 [*il-10*]), y del marcador de superficie de las células T (*cd4*). En particular, nuestro estudio reveló un aumento en los niveles de *il-10*, al igual que lo informado por Xu et al. (2015). IL-10 es una citoquina antiinflamatoria reconocida por su papel en suprimir las respuestas inmunitarias (Zou y Secombes, 2016). Además, antecedentes anteriores en peces teleósteos han propuesto un papel importante para esta citoquina en el control del proceso proinflamatorio y la resolución de procesos infecciosos (Reyes-Cerpa et al., 2014; Reyes-López et al., 2015). Esto es de suma importancia ya que, al evaluar la condición inmune, la evaluación de los genes asociados a la respuesta antiinflamatoria es importante porque regulan y reducen la expresión de citoquinas proinflamatorias (Reyes-Cerpa et al., 2013) cuando es necesario, para prevenir daños colaterales en los tejidos del huésped y evitar el desperdicio de recursos bioenergéticos (Moore et al., 2001). Además, tal y como se ha mencionado anteriormente, en un experimento con ratas, donde se estudió el efecto del ácido ursólico (dosis: 20 y 100 mg^{-1} kg^{-1} $día^{-1}$) sobre las funciones inmunitarias en un modelo experimental sobre una enfermedad autoinmune, se reportó un aumentó en el número de células T $CD4^+$ y una reducción de los niveles de anticuerpos (Ig G) en el suero con la dosis alta, 100 mg^{-1} kg^{-1} $día^{-1}$ (Xu et al., 2015). Esto es importante ya que el $CD4^+$ es una glicoproteína transmembrana perteneciente a la superfamilia de las inmunoglobulinas que se expresa en la superficie de las células T, siendo éstas últimas las principales protagonistas del sistema inmunitario adaptativo de cualquier vertebrado y esenciales para la inmunidad mediada por células (Laing et al., 2006; Castro et al., 2011). Además, estos leucocitos $CD4^+$ se denominan también como linfocitos T

"ayudantes" porque ayudan a regular/activar la respuesta de las células $CD8^+$ (linfocitos T "asesinos naturales") mediante la secreción de citoquinas (Laing et al., 2006; Castro et al., 2011). En particular, nuestro estudio *ex vivo* reveló una regulación al alza del *cd4*$^+$ en las células del bazo de los peces alimentados con el aditivo cuando fueran expuestas a un PAMP de tipo bacteriano, sugiriendo así el MPLE podría estar involucrado en la regulación de la actividad de los linfocitos, lo que sugiere la estimulación de la inmunidad sistémica a través de un estricto control de la respuesta inmunitaria íntimamente regulada por mecanismos tanto pro- y antiinflamatorios, además de la activación de poblaciones de células inmunitarias $CD4^+$, lo que permitiría al organismo combatir una posible infección bacteriana. Otro claro ejemplo de las propiedades inmunomoduladoras del ácido ursólico fue el informado por Jang et al. (2009), donde este compuesto (dosis: 0,05%, tiempo de administración: 4 semanas, método de aplicación: intraperitoneal) activó las células T aumentando significativamente la producción de IL-2 e IFN-γ en estas células.

Por otra parte, las especies reactivas de oxígeno (ROS, por sus siglas en inglés: *reactive oxygen species*), componen un importante mecanismo de defensa implicado en la activación de la respuesta inmune, incluyendo la activación de las células T (Chen et al., 2018). En condiciones fisiológicas normales, las ROS participan en varias funciones, como la defensa del huésped (es decir, la defensa contra microorganismos patógenos), la regulación del ciclo celular y muchas vías de señalización celular (Hoseinifar et al., 2021). En concentraciones bajas o moderadas, las ROS no causan daños, pero en concentraciones altas, inducen modificaciones negativas de los componentes celulares como los lípidos, las proteínas y el ADN (Hoseinifar et al., 2021). Para evitar los efectos negativos de la producción natural de ROS, los organismos vivos desarrollaron un sistema de defensa antioxidante enzimático que incluye diferentes enzimas como la superóxido dismutasa (SOD) y la catalasa (CAT) (Mishra et al., 2015). Estos elementos del sistema de defensa antioxidante son capaces de proporcionar el equilibrio entre la producción y la eliminación de ROS en condiciones fisiológicas normales (Hoseinifar et al., 2021). Sin embargo, la falta de equilibrio entre la formación de ROS y la capacidad de los

organismos para hacer frente a las ROS puede causar estrés oxidativo (Chen et al., 2018; Hoseinifar et al., 2021). Además, se ha visto que, durante la respuesta inicial a un patógeno invasor, la activación de la respuesta inmunitaria innata también está acompañada por la generación de ROS dentro de las células fagocíticas, como los macrófagos y los neutrófilos, siendo un acontecimiento crítico para el inicio de la fagocitosis y la posterior destrucción de estos microorganismos (Seifried et al., 2007). Asimismo, en ratas se ha descubierto que las ROS derivadas de los macrófagos tienen una función de señalización inmunitaria al influir en la selección, maduración y diferenciación de las células T (Bartosz, 2009). Debido a esto, varios mecanismos celulares de autoprotección deben estar estrechamente regulados durante una respuesta inmune para evitar daños colaterales sobre el organismo (Hoseinifar et al., 2021). De este modo, y como hemos mencionado anteriormente, la SOD y la CAT son dos enzimas implicadas en la defensa celular contra procesos oxidativos, catalizando la reducción de radicales superóxidos y H_2O_2 (Otto y Moon, 1996). Para minimizar los efectos dañinos del ROS, estas dos enzimas antioxidantes tienen funciones relacionadas y se consideran la primera línea de defensa contra la toxicidad del oxígeno debido a sus efectos inhibidores sobre la formación de radicales libres (Li et al., 2009; Pandey et al., 2003). Además, la literatura nos indica que, para evitar el desequilibrio en la producción de ROS, los organismos aeróbicos podrían reducir la formación de ésta mediante la ayuda de componentes antioxidantes, producidos por la célula (como la cadena de transporte de electrones mitocondrial y las oxidasas NAD(P)H) u obtenidos a través de la dieta (vitaminas C, E y otros nutrientes y/o aditivos con propiedades antioxidantes) (Wang et al. 2013; Franco y Martínez-Pinilla 2017). Por lo que, nos abre la posibilidad de hipotetizar de que estos resultados que van en línea con los encontrados en el Capítulo I de la presente tesis (Salomón et al., 2020), donde esplenocitos de doradas alimentadas con la dieta suplementada con el fitogénico MPLE y expuestos al LPS mostraron una regulación al alza significativa y controlada en cuanto a genes implicados con enzimas de estrés antioxidativo (*mn-sod*, *cat*), pudo haber sido a través del efecto del aditivo en la dieta relacionada a la presencia de compuestos

triterpénicos (Rufino-Palomares et al., 2011) y polifenólicos (Sönmez et al., 2015) derivados del aditivo MPLE, otorgando así al pez una protección contra las especies reactivas del oxígeno y estimulando las defensas antioxidantes del organismo (John et al., 2001).

Varios son los trabajos en los que analizan parámetros inmunológicos en plasma para determinar los efectos de los aditivos alimentarios en la salud de los peces (Parrino et al., 2018; Fazio, 2019). A nivel plasmático, diferentes autores han indicado de las potenciales propiedades inmunomoduladoras de la salvia y la hierbaluisa. En un experimento con el esturión beluga, Dadras et al. (2020) han demostrado que, cuando juveniles de dicha especie fueron alimentados con una que contenía un extracto etanólico de salvia (120 ml kg^{-1}) durante 42 días, registraron un aumento en los niveles de lisozima y de la actividad de complemento (ACH50) en comparación con el grupo control, poniendo así en evidencia sus efectos beneficiosos sobre la respuesta inmunitaria humoral del animal. Esto es de importancia ya que, la actividad de la lisozima es un componente importante del sistema inmunitario innato de los peces con fluctuación en respuesta a factores de estrés e infecciones. En particular, la lisozima en peces se ha visto que funciona como una opsonina y activador del sistema de complemento y la fagocitosis siendo un mediador en la protección contra la invasión microbiana (Saurabh y Sahoo 2008). Por otro lado, la actividad de complemento desempeña un papel esencial a la hora de alertar al huésped de la presencia de posibles patógenos, así como en su eliminación. Además, la activación del sistema del complemento contribuye significativamente a la orquestación y desarrollo de una respuesta inmunitaria adquirida (Boshra et al., 2006). En relación con estos parámetros, Terzioğlu y Diler (2016) han indicado un incremento significativo de los niveles de lisozima en plasma en truchas arcoíris alimentadas con una dieta suplementada con 0,1% de inclusión de salvia en polvo. Por otro lado, Adel et al. (2021) han descrito que esturiones alimentados con extractos de hierbaluisa (20 mg kg^{-1}) durante 56 días mostraron índices hematológicos relacionados con la inmunidad, tales como el estallido respiratorio, lisozima, inmunoglobulinas (Ig) totales y la actividad ACH50,

incrementados de manera significativa, revelando que el uso del extracto de hierbaluisa podría mejorar la respuesta inmunitaria del esturión siberiano. Por su parte, Adeli et al. (2021) demostraron también un aumento significativo de lisozima e IgM en plasma en truchas arcoíris alimentadas con una dosis de inclusión de 7 g kg^{-1} de extracto de hierbaluisa en el alimento durante 42 días. La IgM en los peces teleósteos es una de las tres clases o tipos de Ig conocidos hasta la fecha (IgM, IgD e IgT) en comparación con las cinco clases de los mamíferos (Secombes y Ellis, 2012). En peces, la IgM puede tener un papel de opsonización en ciertas especies, ya sea de forma directa o a través de receptores para la IgM, o indirecta, a través de receptores para la actividad del complemento (Secombes y Ellis, 2012). La IgM es la inmunoglobulina más común en el plasma y mucosas como actor clave en la orquestación de las respuestas de memoria inmunitaria en los peces teleósteos (Parra et al., 2015). Sin embargo, dichos resultados están en desacuerdo con los obtenidos en nuestras doradas alimentadas con una dieta suplementada con un extracto de salvia y hierbaluisa, donde no se registraron diferencias significativas en los parámetros humorales no específicos, pero sí se observó una respuesta inmunitaria de tipo celular cuando las células del bazo fueron expuestas a LPS (Capítulo I; resultados del ensayo *ex vivo* [Salomón et al., 2020]). Por lo tanto, estos resultados podrían estar respaldados por la hipótesis de que el uso de aditivos no siempre tiene la respuesta inmunológica esperada si los peces no están expuestos a un agente estresor como pudiera ser un organismo patogénico (Álvarez-Rodríguez et al., 2018).

El efecto inmunomodulador de los fitogénicos derivados de la salvia y la hierbaluisa también se han demostrado mediante su evaluación *in vivo* al mejorar significativamente la supervivencia de los peces frente a desafíos patogénicos (Terzioğlu y Diler, 2016; Abdellatief et al., 2018; Metin et al., 2020; 2021; Adel et al., 2021). Por ejemplo, en tilapias del Nilo se llevó a cabo un reto bacteriano donde los peces fueron infectados vía inyección intraperitoneal con 0,2 mL de 10^7 unidad formadora de colonias (UFC) ml^{-1} del patógeno *Pseudomonas aeruginosa*, mostrando menores tasas de mortalidad (25% *vs*. 75%) en los peces alimentados con la dieta

que incluía 7,5 g kg^{-1} de salvia en polvo durante 28 días (Abdellatief et al., 2018). Así también, se ha visto que en esturiones alimentados durante 56 días con un extracto de hierbaluisa (20 mg kg^{-1}) y sometidos a un reto bacteriano, en este caso infectados vía inyección intraperitoneal con 0,1 mL de 10^6 UFC ml^{-1} del patógeno *Aeromonas hydrophila*, han demostrado diferencias significativas en cuanto a la tasa de supervivencia respecto de los peces alimentados con la dieta control (40% *vs*. 20%) (Adel et al., 2021). Los estudios referidos están en concordancia con los resultados presentados en esta tesis y que están descritos en el Capítulo III (Salomón et al., 2022), donde en particular, los salmones alimentados con la dieta suplementada con MPLE durante 133 días mostraron mayores tasas de supervivencia (90,6 ± 6,4%) en comparación con sus congéneres alimentados con la dieta control (60,7 ± 13,5%) cuando fueron infectados con *A. salmonicida* (0,2 mL de 10^7 UFC mL^{-1}). Los resultados obtenidos en la presente tesis son de especial relevancia ya que, se sugiere que la suplementación dietética del aditivo MPLE al 0,1% es capaz de modular la respuesta inmunitaria, mejorando la robustez frente enfermedades de tipo bacteriano y reduciendo así la mortalidad acumulada por una infección de *A. salmonicida*. Además, con respecto al compuesto bioactivo más abundante de la hierbaluisa, el ácido verbascósido, Avila et al. (1999) ya reportaron en la literatura aspectos sobre la bioactividad de la hierbaluisa, indicando que el ácido verbascósido aislado de la misma y de otras especies del género *Lippia* ejerce una actividad antimicrobiana (Kubica et al., 2020). Además, en el caso de especies relevantes para la acuicultura, algunos de los pocos estudios encontrados en peces han informado de una mejora en la inmunidad, a través de la expresión de genes relacionados con la respuesta innata humoral (*lys*) y citoquinas proinflamatorias (*tnf-a*, *il-1β,* e interleucina 8 [*il-8*]*)* mediante la suplementación con salvia o hierbaluisa en dietas destinadas a la tilapia del Nilo, truchas arcoíris y esturión siberiano (Abdellatief et al., 2018; Hoseinifar et al., 2020a; Adel et al., 2021). Estos resultados indican que el fitogénico utilizado en el presente estudio puede considerarse como una alternativa a los agentes antimicrobianos convencionales y usarse, así como herramienta profiláctica frente a enfermedades infecciosas de tipo bacteriano.

En cuanto al salmón del Atlántico alimentado con la dieta suplementada con 0,1% de MPLE y al estudio de la respuesta transcriptómica a nivel del **riñón cefálico**, uno de los principales órganos linfoides del pez (Tort, 2011), el análisis transcriptómico mostró un total de 1.178 genes expresados diferencialmente (DEGs, por sus siglas en inglés: *differential expressed genes*), de los cuales la mayoría de ellos muestran una regulación al alza (802 regulados al alza, y 376 a la baja) (Tabla suplementaria en el Capítulo IV; Salomón et al., 2022). A partir de los genes obtenidos de un análisis de expresión diferencial, se procedió a realizar una interpretación biológica de dichos genes basada en definiciones de ontología génica (GO, por sus siglas inglés: *gene ontology*) mediante un análisis de enriquecimiento. Dichas anotaciones GO revelaron un total de 185 procesos biológicos modulados de manera significativa en el riñón cefálico de los salmones del Atlántico alimentados con la dieta suplementada con MPLE (*Figura 1*). A partir de dicho análisis, se destaca la identificación de una serie de procesos biológicos relacionados con la inmunidad, como son por ejemplo el proceso biológico denominado "procesamiento de antígenos y presentación de antígeno peptídico vía CMH clase II (GO:0002495)" y el "interferón-gamma (IFN-γ) (GO:0060333)", entre otros. Estos resultados son de suma importancia, ya que el procesamiento y la presentación de antígenos son esenciales para desencadenar respuestas inmunitarias celulares y humorales, respuestas que están mediadas por linfocitos T y B (Vyas et al., 2008), siendo fundamentales para el inicio de la respuesta inmunitaria específica de antígeno (Yagamuchi y Dijkstra, 2019). Además, se ha demostrado que, en infecciones bacterianas, la señalización por parte del IFN-γ es capaz de influir en el procesamiento y presentación de antígenos a partir del complejo mayor de histocompatibilidad de clase II; contribuyendo así a la inmunidad a través de la mejora de las respuestas de células T específicas de patógenos, como son las $CD8^+$ y $CD4^+$ (Decker et al., 2005). Por lo tanto, los datos de nuestra tesis (Capítulo III [Salomón et al., 2022]), sugirieron que el aditivo MPLE podría estar involucrado en la regulación de la actividad de los linfocitos, lo que sugiere la estimulación de las respuestas inmunitarias tanto innatas como adaptativas. Estos resultados también

vendrían apoyados por los obtenidos y mencionados anteriormente en dorada con el mismo aditivo y la misma inclusión (0,1%), y que fueron obtenidos a partir de células del bazo estimuladas con LPS, tal y como indicaron los resultados de expresión del marcador *cd4* (Capítulo I [Salomón et al., 2020]).

Otro proceso biológico que fue modulado por el fitogénico probado fue el de la "autofagia (GO:0006914)", que también a su vez se encuentra modulado por el IFN-γ (Pereiro et al., 2019). La autofagia es una vía altamente conservada que juega un papel importante en la fisiología celular, las respuestas adaptativas al estrés y la respuesta inmune (Kuballa et al., 2012). De acuerdo con la literatura existente, diversos estudios han informado que la autofagia, así como el IFN-γ, cumplen un rol específico contra patógenos oportunistas como *Aeromonas* spp. en peces de cultivo (Pereiro et al., 2016; Yin et al., 2021); abriendo así la posibilidad de comprender y relacionar nuestros resultados relacionados con la mortalidad diferencial observada entre ambos grupos experimentales (Capítulo III [Salomón et al., 2022]). IFN-γ es probablemente una de las citoquinas más relevantes que orquestan la respuesta inmunitaria en los vertebrados (Pereiro et al., 2019). Este interferón es conocido por ser un potente activador de los macrófagos. Como hemos mencionado anteriormente, existe evidencia en peces que el IFN-γ puede inducir la autofagia. Los efectos de esta citoquina en el proceso de autofagia están mediados principalmente por dos vías independientes: la cascada de señalización Jak1-2/Stat1 y la vía PI3K/AKT/mTOR, aunque se ha descrito una tercera vía mediada por JAK1/2 y p38 MAPK (Pereiro et al., 2016; Yin et al., 2021). La evidencia acumulada sugiere que la autofagia es un mecanismo de defensa evolutivamente conservado para deshacerse de las bacterias a través del lisosoma en los huéspedes (Yin et al., 2021). Por ejemplo, Pereiro et al. (2016) informaron de una reducción de la mortalidad en el rodaballo (*Scophthalmus maximus*) cuando se les inyectó vía intraperitoneal con 50 µl de 5,5 x 10^5 UFC mL^{-1} del patógeno *A. salmonicida*. Estos resultados se atribuyeron al efecto del IFN-γ en las infecciones bacterianas, y a la participación de esta proteína en la respuesta inflamatoria (Pereiro et al., 2016). Por otro lado, Yin et al. (2021) pudieron demostrar el papel de la autofagia en los

macrófagos de la carpa herbívora (*Ctenopharyngodon idella*), lo que llevó a promover la defensa innata contra *A. hydrophila*. Siguiendo con los resultados presentados en el Capítulo III, también se ha visto una modulación del proceso biológico de "regulación de la señalización de I-kappaB quinasa/NF-kappaB (GO:0043122)". Estos resultados son de suma importancia ya que el NF-κB es un factor importante para el mantenimiento de la homeostasis inmune, al modular la transcripción de un grupo diverso de genes implicados en muchos procesos biológicos como el desarrollo, la inmunidad, la apoptosis y la diferenciación celular en diferentes tipos celulares como las células B y T, los monocitos, las quimiocinas y las citoquinas, entre otros (Dorrington y Fraser, 2019). Esta modulación va acorde a los resultados presentados en el Capítulo IV (Salomón et al., 2021b) de la presente tesis, en el que se ha demostrado que el fitógenico AQUOLIVE® con propiedades bioquímicas y funcionales similares al MPLE, también fue capaz de modular procesos biológicos asociados a la "regulación de la señalización de I-kappaB quinasa/NF-kappaB".

Por último, una respuesta inmunitaria satisfactoria implica el control de un amplio repertorio de procesos como el de las células mediadoras de los leucocitos, tales como granulocitos (neutrófilos, eosinófilos, basófilos), mastocitos, macrófagos, células dendríticas y células asesinas naturales (NK, por sus siglas en inglés *natural killer*), entre otros (Rieger y Barreda, 2011). La activación de los leucocitos está mediada por varias vías de señalización que interactúan para producir cambios en la afinidad de las proteínas de unión en la superficie de los neutrófilos que a su vez movilizan el citoesqueleto para la quimiotaxis y la fagocitosis, desencadenando finalmente un estallido respiratorio y la degranulación (Rieger y Barreda, 2011). En este contexto, en nuestro estudio se observó la modulación de procesos biológicos conocidos como la "activación leucocitaria implicada en la respuesta inmune (GO:0002366)", "inmunidad mediada por neutrófilos (GO:0002446)" y la "activación de linfocitos implicada en la respuesta inmunitaria (GO:0002285)". Esto es relevante ya que los neutrófilos, macrófagos, células dendríticas y células B son reconocidos como un componente crítico de la inmunidad innata y adaptativa contra los patógenos a través de procesos que contribuyen en la eliminación de

neutrófilos de la circulación. Esto es de suma importancia ya que, el mantenimiento de los neutrófilos circulantes y funcionales y su reclutamiento en los tejidos en respuesta a una lesión y/o infección microbiana son fundamentales para la defensa del huésped. Sin embargo, la eliminación homeostática de neutrófilos de la circulación está mediada por macrófagos *in situ*, aunque también en pequeña medida por las células dendríticas (Bratton y Henson, 2011). Ya que, si no se eliminan los neutrófilos moribundos, estos se desintegran y pueden contribuir aún más a la inflamación continua y la destrucción de tejidos (Bratton y Henson, 2011). Por lo tanto, la modulación de procesos biológicos implicados como los mencionados anteriormente, están relacionados con las funciones celulares clave de los efectores de la inmunidad innata de los vertebrados (Havixbeck et al., 2016), sugiriendo un aumento de la capacidad inmunológica sistémica específica promovida por el aditivo alimentario fitogénico probado y presentado en el Capítulo III de la presente tesis (Salomón et al., 2022).

Por el otro lado, y siguiendo la línea de los datos transcripcionales, los resultados obtenidos a nivel del **intestino** en doradas alimentadas con MPLE, han revelado un total de 506 DEGs (285 regulados al alza y 221 a la baja) al comparar el perfil transcriptómico del intestino de los peces alimentados con la dieta control (Capítulo II; Salomón et al., 2021a). El análisis de enriquecimiento realizado reveló un total de 161 procesos biológicos en el intestino de las doradas alimentadas con MPLE (*Figura 1*). Estos resultados mostraron que este aditivo fue capaz de promover la respuesta inmunitaria de tipo innato y adaptativo, especialmente a través de la modulación de los procesos implicados en la activación, diferenciación y selección de las células T. Además, como se ha mencionado anteriormente las células T son uno de los principales actores de la respuesta inmune adaptativa, siendo el intestino de los teleósteos un lugar importante de producción de células T (Scapigliati et al., 2018). En particular, las células T representan la principal población de leucocitos en el intestino de los teleósteos (Rombout et al., 2011) con una actividad citotóxica relevante (Fischer et al., 2006), además de desempeñar un papel importante en el reconocimiento de antígenos extraños y en la homeostasis intestinal (Salinas y Parra,

2015). Resultados en vertebrados superiores han mostrado que gallinas alimentadas con extracto de salvia (8,2 g kg^{-1}) durante 21 días y desafiadas vía oral con *Salmonella enteritidis* (0,2 mL de 10^8 ml^{-1} UFC) presentaron un mayor número de leucocitos y linfocitos a nivel intestinal, poniendo de manifiesto la actividad antimicrobiana de la salvia (Spišáková et al., 2013). Esto estaría de acuerdo con nuestros resultados descritos en el Capítulo II (Salomón et al., 2021a), donde en el intestino medio-anterior de doradas alimentadas con el MPLE, se ha observado una modulación de procesos biológicos asociados a la "activación de linfocitos (GO:0046649)" seguido por la "diferenciación de leucocitos (GO:0002521)", la "diferenciación de células T (GO:0030217)" y la "activación de las células T (GO:0042110)". Resultados que apoyan la idea de que el MPLE podría mejorar la condición y respuesta inmunitaria del intestino, y por lo tanto, la respuesta inmunitaria a nivel local.

Además, dicho fitogénico promovió los procesos relacionados con la integridad intestinal, a través de e una regulación diferencial de los genes implicados en uniones adherentes y uniones estrechas entre enterocitos. En este contexto es importante tener presente que el tracto gastrointestinal está recubierto por una gruesa capa de moco que forma la primera línea de defensa innata del huésped, y que ese moco a su vez está formado por glicoproteínas, las cuales son sintetizadas y secretadas por las células caliciformes, cuya función principal es la de lubricar el epitelio y protegerlo de los daños causados por sustancias nocivas (Cornick et al., 2015). En la presente tesis, el estudio de histoquímica del epitelio intestinal de los peces alimentados con la dieta suplementada con el fitogénico MPLE indicó que la administración del aditivo promovió un aumento del número de las células caliciformes intestinales, modificando así la composición de las glicoproteínas de las mucinas que componen el moco (aumento de la intensidad de tinción de las mucinas neutras, así como de las mucinas ricas en glicoconjugados carboxilados y débilmente sulfatados, en particular los ricos en residuos de ácido siálico), lo que sugiere un mecanismo de defensa del intestino a través de la modulación de las secreciones de mucinas promovidos por el aditivo. Las principales moléculas que se encuentran en el moco son las mucinas, que desempeñan un papel importante en

el mantenimiento de la barrera epitelial contra los patógenos (Johansson y Hansson, 2016). Las mucinas, tanto las secretadas como las unidas a la membrana, son glicoproteínas multifuncionales que contribuyen a la capa del moco protector. Algunas mucinas participan en vías de señalización que conducen a respuestas celulares coordinadas, como la proliferación, diferenciación y adhesión celular, la respuesta inmunitaria, la apoptosis, la adhesión/inhibición bacteriana y la secreción de productos celulares especializados (Pérez-Sánchez et al., 2013). Los resultados encontrados en la presente tesis están de acuerdo con la hipótesis de que los fitogénicos derivados de la salvia y la hierbaluisa podrían mejorar la condición y respuesta inmunitaria en el intestino (Capítulo II [Salomón et al., 2021a]), ya que la secreción continua de mucinas por parte de las células caliciformes promueva la microbiota comensal, conduce a la eliminación física de patógenos o toxinas adheridas (McGuckin et al., 2011). Otros estudios llevados a cabo tanto en peces, como en cerdos, ratas y gallinas han reportado un efecto protector en el intestino de los animales. Estos beneficios a nivel de la mucosa intestinal se han visto a través de la reducción de las lesiones histopatológicas inducidas por tóxicos (Hoseini et al., 2021) o desafíos bacterianos (Ngamkala et al., 2020), al estimular y replicar el crecimiento de las bacterias beneficiosas en el intestino, defendiéndolo del ataque microbiano, como también al estimular la proliferación y el crecimiento de enterocitos; aumentando la producción de enzimas digestivas y/o la capacidad antioxidante del intestino (Marcin et al., 2006; Čapkovičová et al., 2014; Mahmoud, 2014; Placha et al., 2015; Rasouli et al., 2020; Mashayekhi-sardoo et al., 2020; Jedidi et al., 2021; Adel et al., 2021). Además, en un modelo experimental de inflamación intestinal inducido por el sulfato de dextrano, que se asemeja a la enfermedad inflamatoria intestinal mediada por el sistema inmunitario en los seres humanos, la administración sistémica de verbascósido mejoró sustancialmente los patrones histológicos y los síntomas clínicos de la colitis, reduciendo así la secreción proinflamatoria de IFN-γ e inhibiendo la acción oxidativa relacionada con la NADPH-oxidasa (efecto antioxidante indirecto) en los macrófagos intestinales (Hausmann et al, 2007; Lenoir et al., 2011). Por lo tanto, la estimulación de la

inmunidad local a nivel intestinal por parte del aditivo MPLE se ve reflejado en el mantenimiento de la salud intestinal, con un aumento de la integridad del epitelio y las células calciformes, lo que sugiere que este fitogénico puede considerarse como un prometedor aditivo funcional sostenible para alimentos acuícolas que podrían mejorar la condición intestinal y la salud del tracto gastrointestinal (Capítulo II [Salomón et al., 2021a]).

Además de sus potenciales propiedades inmunomoduladoras ya mencionadas, algunos estudios han informado de resultados ventajosos sobre el estrés oxidativo asociado a la administración de fitogénicos como la salvia y la hierbaluisa en peces y otras especies animales (Pastore et al., 2012, Quirantes-Piné et al., 2013; Sönmez et al., 2015; Hoseinifar et al., 2020a; Adeli et al., 2021). Por ejemplo, en el caso de ratas, el verbascósido fue capaz de mejorar significativamente la actividad de las principales enzimas antioxidantes (catalasa, glutatión peroxidasa y glutatión reductasa), suprimiendo a la vez la mieloperoxidasa prooxidante relacionada con la inflamación en los linfocitos, eritrocitos y neutrófilos circulantes (Quirantes-Piné et al., 2013). En el caso de peces, y siguiendo la línea de posibles propiedades antioxidantes por parte de ambas plantas, Sönmez et al. (2015) han demostrado que el aceite esencial de la salvia con una inclusión de 500 mg kg^{-1} en la dieta durante 60 días tiene un efecto positivo plasmado en un aumento sobre la actividad de las enzimas antioxidantes en juveniles de truchas arcoíris. Por otro lado, truchas arcoíris alimentadas con un extracto en polvo de hierbaluisa (1%), han mostrado un aumento en la actividad de diversas enzimas antioxidantes (SOD, glutatión S-transferasa y glutatión peroxidasa) (Hoseinifar et al., 2020a). También por su parte, Adeli et al. (2021) han demostrado en truchas arcoíris alimentadas con extracto de hierbaluisa (7 g kg^{-1}) durante 42días un aumento significativo en términos de enzimas antioxidantes como es la SOD. La SOD es una metaloenzima que desempeña un papel importante en la protección de las células contra el daño oxidativo (Hoseinifar et al., 2021), atribuyendo una mejora en cuanto al efecto antioxidante por parte del extracto de hierbaluisa. Teniendo en cuenta dicha capacidad antioxidante descrita por los compuestos fenólicos en la salvia y la

hierbaluisa (Funes et al., 2009; Bulfon et al., 2014), los resultados descritos en el Capítulo I están en acuerdo con los mencionados anteriormente ya que los cambios en los niveles de expresión de *mn-sod* y *cat* en el cultivo primario celular de esplenocitos de dorada expuestos a LPS se produjeron como respuesta por parte de los peces alimentados con la dieta con la inclusión de 0,1% de MPLE (Salomón et al., 2020). Por lo tanto, estos datos sugieren que doradas alimentadas con 0,1% de MPLE tienen una mayor capacidad antioxidante que la dieta control, lo que puede estar relacionada a la presencia de compuestos triterpénicos (Rufino-Palomares et al., 2011) y polifenólicos (Sönmez et al., 2015). Por otra parte, resultados obtenidos en uno de nuestros estudios, doradas alimentadas con MPLE resultaron en una ligera pero significativa reducción de los niveles de peroxidación lipídica de las sustancias reactivas al ácido tiobarbitúrico (TBARs, por sus siglas en inglés*: thiobarbituric acid reactive substances*) en el filete de dorada, resultados que podrían venir asociados a las propiedades antioxidantes de dichos compuestos fenólicos (Funes et al., 2009; Bulfon et al., 2014). La prueba del TBARs se ha utilizado en anteriores estudios sobre la calidad de la carne del pescado al medir los niveles de peroxidación lipídica del filete (Menoyo et al. 2002; Mansour et al. 2006). En este sentido, la reducción de los niveles de TBARs fue acompañada de niveles significativos más bajos de CAT, GR, GST y GPx (Tabla 1; datos no publicados) en los filetes de las doradas alimentadas con la dieta suplementada con MPLE. Esto es de relevancia ya que por ejemplo la CAT y la GPx actúan juntas cuando los niveles de peroxidación lipídica son altos en la célula (estrés oxidativo), haciendo que estas dos enzimas tomen el peróxido de hidrógeno (H_2O_2), consecuentemente degradándolo y dividiéndolo en agua (H_2O) y oxígeno (O_2) (Hoseinifar et al., 2021).

Tabla 1. Niveles de peroxidación lipídica (TBARs) y actividad de las enzimas catalasa (CAT), glutatión reductasa (GR), glutatión S-transferasa (GST) y glutatión peroxidasa (GPX) en el filete de doradas alimentadas con las dietas experimentales. Distintas letras indican diferencias significativas entre grupos experimentales ($P < 0,05$). (Datos no publicados).

	DIETA CONTROL	DIETA MPLE
TBARs (mmol MDA/100 g)	1,120 ± 0,05 b	0,879 ± 0,11 a
CAT (nmol/ min/mg prot)	1,99 ± 0,21 b	1,58 ± 0,21 a
GR (nmol/ min/mg prot)	1,71 ± 0,08 b	1,01 ± 0,21 a
GST (nmol/ min/mg prot)	450,1 ± 11,2 b	341,8 ± 19,8 a
GPX (nmol/ min/mg prot)	3,84 ± 0,43 b	2,11 ± 0,22 a

Propiedades inmunomoduladoras del fitogénico AQUOLIVE®

Los compuestos bioactivos más abundantes de la aceituna se dividen en dos grupos, por un lado están los alcoholes fenólicos, conocidos como hidroxitirosol y tirosol (Ghanbari et al., 2012), y por el otro, los triterpenos pentacíclicos, los ácidos oleanólico y maslínico (Blanco-Cabra et al., 2019). Resultados recientes han demostrado que extractos de la aceituna presentan propiedades antiinflamatorias, antioxidantes y antimicrobianas, indicando así que los fitogénicos derivados del olivo tienen efectos beneficiosos para la salud humana (Caramia et al., 2012; Gorzynik-Debicka et al., 2018; George et al., 2019) y del ganado (Morrison et al., 2017; Liehr et al., 2017; Cangiano et al., 2019).

A nivel plasmático, las inmunoglobulinas, la actividad ACH50 y la lisozima son algunos de los elementos claves de los parámetros inmunitarios humorales no específicos que se miden con frecuencia para evaluar el estado de salud de los peces tratados con dietas suplementadas con fitogénicos (Parrino et al., 2018; Dawood et

al., 2018; Fazio, 2019; Dawood et al., 2021). En este contexto, se ha visto que en un experimento donde carpas comunes alimentadas con una inclusión de 1 g kg^{-1} de un extracto de aceituna rico en compuestos bioactivos como la oleuropeína durante 60 días e inducidas a un estrés químico al ser expuestas por 3 horas a 0,5 mg L^{-1} de amoníaco no ionizado (NH_3), han demostrado un aumento significativo en parámetros inmunes humorales (Ig totales, lisozima y la actividad ACH50), en comparación a los peces control, sugiriendo que estas mejoras en las respuestas inmunitarias humorales serían beneficiosas en caso de toxicidad por amonio (Rajabiesterabadi et al. (2020b). En otro ejemplo se observó un aumento significativo respecto a los peces alimentados con la dieta control en los niveles de lisozima y la actividad de estallido respiratorio en carpas alimentadas con 200 mg kg^{-1} de extractos de hoja de olivo, ricos en compuestos bioactivos tales como la oleuropeína y sus derivados, como el hidroxitirosol durante 75 días (Sokooti et al., 2021). Por otra parte, se llevó a cabo un experimento donde truchas arcoíris han sido alimentadas con tres inclusiones diferentes (0,5 2,5 y 5 g kg^{-1} en la dieta) de extractos de aceituna, especialmente relacionados con compuestos bioactivos como polifenoles y vitamina E durante 42 días para evaluar la respuesta humoral no específica (niveles de lisozima, actividad ACH50 e Ig totales). Los resultados mostraron que sólo hubo un aumento significativo de los niveles de lisozima en los peces alimentados con la inclusión de 5 g kg^{-1} de extracto de aceituna respecto a los peces alimentados con la dieta control (Hoseinifar et al., 2020a). Estos últimos resultados están en concordancia con los observados y descritos en el Capítulo IV de la presente tesis (Salomón et al., 2021b), donde se evaluaron diferentes parámetros inmunitarios humorales en el plasma al final del ensayo nutricional (peroxidasa, proteasa, antiproteasa, lisozima y actividad bactericida), y en el cual no se ha revelado ningún efecto inmunoestimulante significativo a nivel humoral del aditivo alimentario AQUOLIVE®. Estos resultados podrían apoyarse en la hipótesis ya mencionada anteriormente y vista en el Capítulo I de esta tesis (Salomón et al., 2020), donde algunos autores han demostrado que el uso de aditivos no siempre tiene la respuesta inmunológica esperada cuando no se enfrenta a un

estrés biótico o abiótico. En concordancia con lo anterior, incluso algunos autores han mostrado que la administración de aditivos naturales puede resultar contraproducente (situación de distrés) debido al coste bioenergético de las respuestas inmunes prolongadas y potenciadas (Álvarez-Rodríguez et al., 2018). En los resultados de esta tesis, no hubo inhibición de la respuesta humoral, por lo que no hay evidencia de una situación de distrés en los peces alimentados con este aditivo. Debido a esto, se puede presumir que la dieta suplementada con un 0,15% de AQUOLIVE® no tuvo ningún efecto negativo sobre la inmunidad humoral en ausencia de un reto bacteriano, lo que podría indicar que el efecto inmunomodulador de este aditivo se ve potenciado solamente en presencia de un estímulo externo (Salomón et al., 2021b).

Además de sus efectos ya mencionados a nivel plasmático, algunos estudios han informado de los resultados ventajosos de la administración en la dieta de extractos de la aceituna. Uno de los escasos ejemplos de esta evaluación en peces son los resultados proporcionados por Gisbert et al. (2017), donde doradas alimentadas con una dieta que contenía un extracto bioactivo de aceite de oliva (0,17%), rico en compuestos fenólicos como hidroxitirosol y tirosol durante 90 días, ha conseguido una mejora en la función inmunitaria innata intestinal, además de una mejora de la integridad del epitelio (Gisbert et al., 2017). Además, en un estudio realizado por Martínez et al. (2019) han reportado la capacidad del hidroxitirosol de inhibir la expresión de citoquinas proinflamatorias, como *tnf-a* e *il-1β*, reduciendo así la inflamación y el dolor en pacientes tratados con hidroxitirosol, atribuyendo este efecto a las propiedades antiinflamatorias del mismo. Asimismo, se ha visto que este compuesto administrado vía inyecciones subcutáneas diarias de 100 µg de hidroxitirosol durante 5 días en ratas, fue capaz de disminuir los niveles de proteína de las citoquinas proinflamatorias IL-6 y TNF-α (Ramírez-Expósito y Martínez-Martos, 2018). Siguiendo la línea de los posibles efectos inmunomoduladores de los principales componentes bioactivos de la aceituna encontrados en la literatura; en un estudio reciente, se ha visto que en muestras de riñón cefálico del siluro rayado (*Pangasianodon hypophthalmus*) estimulados con 10 µg mL^{-1} de extractos de *Psidium*

guajava, una especie de pequeño árbol, rico en ambos polifenoles como el oleanólico y el maslínico, fue capaz de aumentar significativamente parámetros inmunes indicadores de la respuesta innata como el estallido respiratorio, óxido nítrico sintasa y lisozima (Nhu et al. 2020), sugiriendo una mejora de la misma en los peces estimulados con ambos polifenoles. Estos resultados están en concordancia con los presentados en el Capítulo IV (Salomón et al., 2021b) donde también se sugiere una respuesta inmunitaria innata en muestras de riñón cefálico promovida por el aditivo evaluado. En algunos casos, estos efectos beneficiosos se han asociado a la modulación al alza de ciertos genes implicados en la inmunidad de los peces como son las citoquinas proinflamatorias como *tnf-a*, *il1-β* e *il-8*, con una mejora significativa frente a retos bacterianos con patógenos en cuanto al estado de la salud de los peces alimentados con extractos de hoja de oliva (Baba et al., 2018; Zemheri-Navruz et al., 2019). A título de ejemplo, Zemheri-Navruz et al. (2019) han informado que la carpa común alimentada con una inclusión del 0,1% de extractos de hoja de olivo ricos en compuestos bioactivos tales como la oleuropeína, hidroxitirosol y verbascositis durante 60 días, mostró una respuesta inmunitaria mejorada en cuanto a tasas de supervivencia cuando los peces fueron infectados vía intraperitoneal con 0,1 mL de 10^8 UFC mL^{-1} del patógeno *Edwardsiella tarda*. Del mismo modo, en la trucha arcoíris alimentada con el mismo porcentaje de inclusión (0,1%) de compuestos fitogénicos derivados del extracto de hoja de olivo se ha observado un aumento en la expresión de citoquinas proinflamatorias como *tnfa*, *il1-β* e *il-8* relacionados con la inmunidad innata, así como una reducción significativa en la mortalidad producida por el patógeno *Yersinia ruckeri* (0,1 mL de 10^8 UFC mL^{-1}) (Baba et al., 2018). Los resultados mencionados están de acuerdo con los obtenidos en nuestro estudio descrito en el Capítulo IV (Salomón et al., 2021b) de esta tesis, cuando salmones del Atlántico han sido infectados vía intraperitoneal con 0,2 mL de 10^7 UFC mL^{-1} de patógeno *A. salmonicida* en un reto bacteriano donde los peces alimentados con la dieta suplementada con AQUOLIVE® mostraron una supervivencia acumulada mayor (96,9 ± 6,4%) que en los peces del grupo control (60,7 ± 13,5%). Esto es de especial relevancia, ya que para lograr una respuesta

inmunitaria adecuada suelen intervenir un amplio repertorio de procesos biológicos a nivel celular y molecular, como se describe a continuación. De este modo, cuando los salmones han sido alimentados con la dieta AQUOLIVE® se ha puesto en evidencia un perfil de expresión que favorecía principalmente procesos biológicos relacionados con la inmunidad. En particular, la suplementación en la dieta con AQUOLIVE® fue capaz de modular en el riñón cefálico del salmón del Atlántico diferentes procesos biológicos relacionados con factores de transcripción como "transducción de señales por mediador de clase p53 (GO:0072331)", "señalización de la quinasa I-kappaB/NF-kappaB (GO:0007249)", entre otros. Diferentes estudios han evidenciado que existe una conexión transcripcional entre el factor nuclear κB (NF-κB) y p53 (Webster y Perkins, 1999; Carrà et al., 2020). En particular, NF-κB puede considerarse como un regulador transcripcional de p53 y viceversa. De hecho, se descubrió que NF-κB podía reconocer sitios κB en el promotor p53 y, por lo tanto, activar su expresión (Carrà et al., 2020). El p53 forma parte del sistema inmunológico innato y adaptativo, además de detectar daño, reparación y recombinación del ADN, además de desempeñar un papel importante en enfermedades infecciosas, matando y limitando la replicación viral y bacteriana (Levine, 2020). Por otro lado, a nivel de respuesta celular, nuestros resultados mostraron que el aditivo probado reguló varios procesos biológicos en el riñón cefálico relacionados con funciones celulares claves de la inmunidad innata de los vertebrados (Havixbeck et al., 2016), tales como la "activación de los leucocitos (GO:1901135)", seguido por la "activación de granulocitos GO:0036230", la "degranulación de neutrófilos (GO:0043312)", "exocitosis (GO:0006887)", "transporte mediado por vesículas (GO:0016192), entre otros. En ese contexto, los granulocitos son las principales células fagocíticas en el riñón cefálico participando en la inmunidad innata como células presentadoras de antígenos (Cuesta et al., 2006). Además, los neutrófilos son uno de los tres tipos de granulocitos identificados en los peces (Zapata et al., 1996). Dado que su función principal es llegar primero al lugar de la infección y desempeñar un papel central en la protección de los tejidos del hospedador matando a los microorganismos patógenos y estimulando los

linfocitos y otras células inmunitarias, los neutrófilos son una parte esencial del sistema inmunitario innato (Secombes y Wang, 2012). Como se mencionó anteriormente, la "exocitosis" y el "transporte mediado por vesículas" también fueron modulados por AQUOLIVE®; esto es especialmente relevante ya que la exocitosis es reconocida por su importante papel en la respuesta inmune participando en la función de los neutrófilos (Faurschou y Borregaard, 2003). La exocitosis, también conocida como degranulación en los neutrófilos, es la liberación de mediadores preformados desde los gránulos. Los neutrófilos cumplen su función destacada de liberar compuestos proinflamatorios, como citoquinas, quimioquinas y enzimas digestivas, las cuales se almacenan en compartimentos intracelulares a través de la exocitosis (Sheshachalam et al., 2014). Además, macrófagos utilizan la exocitosis mediada por células para la producción de radicales superóxidos y la posterior eliminación de patógenos mediante la actividad del estallido respiratorio (Logan et al., 2003). De las evidencias anteriores, esto es de suma importancia, ya que se ha demostrado que los individuos con una degranulación de neutrófilos disminuida o ausente tienen una mayor incidencia de infecciones bacterianas y fúngicas (Segal, 2005). Por lo tanto, un aumento de la degranulación de los neutrófilos podría conducir a una mayor resistencia a la enfermedad y a una reducción de las tasas de mortalidad en los peces (Palić et al., 2006), tal y como ocurrió en nuestro desafío bacteriano. Por lo tanto, resultados obtenidos en el Capítulo IV (Salomón et al., 2021b) de la presente tesis, nos permite destacar los principales procesos biológicos inducidos por el aditivo AQUOLIVE®, que podrían ser responsables del efecto inmunomodulador sobre el organismo, como lo demuestran las tasas de mortalidad más bajas en los peces alimentados con este fitogénico frente a una infección ocasionada por *A. salmonicida*.

Análisis de representación de la relación de los procesos biológicos en diferentes tejidos y en diferentes especies

Desde un punto de vista global, y con el fin de identificar procesos biológicos similares entre los diferentes aditivos utilizados a lo largo de la tesis y su potencial funcionalidad entre ellos, se ha realizado un diagrama de Venn para representar la relación de los procesos biológicos (GOs). Para ello, se han relacionado los diferentes tejidos (riñón cefálico e intestino) en las diferentes especies utilizadas en la presente tesis, como es el caso la dorada y el salmón del Atlántico, alimentados con los diferentes fitogénicos (MPLE y AQUOLIVE®) (*Figura 1*).

Tal como se observa en la figura, se obtuvieron 185 procesos biológicos únicos en los salmones del Atlántico alimentados con el 0,1% de inclusión del aditivo MPLE, de los cuales comparte un total de 22 procesos biológicos con el intestino de las doradas alimentadas con el mismo fitogénico y otros 14 procesos biológicos se modularon también en el riñón cefálico de salmones del Atlántico, pero alimentados con una inclusión de 0,15% del aditivo AQUOLIVE® (*Figura 1*). En el intestino de las doradas alimentadas con 0,1% de inclusión del MPLE se obtuvieron un total de 161 procesos biológicos únicos, de los cuales 22 procesos biológicos fueron compartidos con el riñón cefálico de los salmones del Atlántico alimentados con la misma inclusión y el mismo aditivo (MPLE), y 3 procesos biológicos fueron modulados también en el riñón cefálico de los salmones del Atlántico alimentados con 0,15% de AQUOLIVE® (*Figura 1*). En cuanto al análisis del riñón cefálico de los salmones del Atlántico alimentados con 0,15% de inclusión del aditivo AQUOLIVE®, se obtuvieron un total de 21 procesos biológicos únicos, 14 de ellos compartidos con el mismo tejido (riñón cefálico) y con la misma especie (salmón del Atlántico), pero alimentados con el aditivo MPLE, y 3 procesos biológicos compartidos con el intestino de las doradas alimentadas con MPLE (*Figura 1*). Sorprendentemente, sólo 3 procesos biológicos fueron compartidos entre los tres tejidos analizados (*Figura 1*), evidenciando divergencias dependientes del tejido y

sus respectivas respuestas inmunes, ya sean a nivel local (intestino) o sistémico (riñón cefálico) con respecto a la administración de los diferentes fitogénicos utilizados en la presente tesis. No obstante, los estudios incluidos en esta tesis han contribuido activamente a ampliar la información del transcriptoma de la dorada y del salmón del Atlántico, además de proporcionar un conocimiento más profundo de las respuestas específicas de los tejidos analizados por medio de estímulos dietéticos, tal y como se comenta a lo largo de la presente tesis.

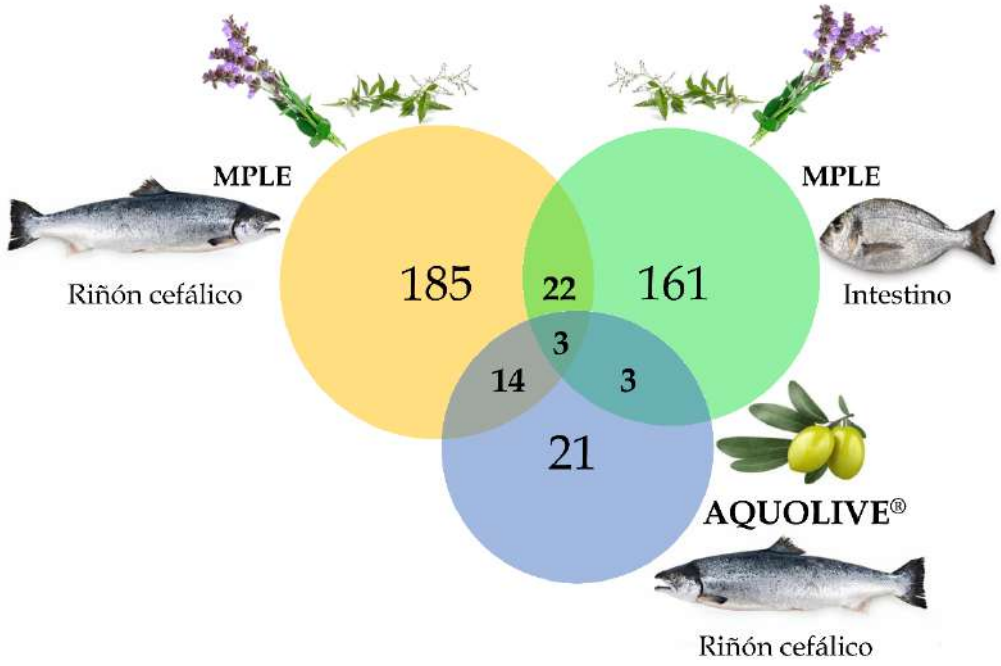


Figura 1. Diagrama de Venn para representar la relación de los procesos biológicos (GOs) en diferentes tejidos (riñón cefálico e intestino) y en diferentes especies, en este caso la dorada (*Sparus aurata*) y el salmón del Atlántico (*Salmo salar*) alimentados con diferentes fitogénicos (MPLE y AQUOLIVE®) con el fin de identificar potenciales funciones similares entre ambos aditivos.

En este contexto, la cantidad de GOs en el intestino (161 procesos biológicos únicos) no fue sustancialmente diferente de la observada en el riñón cefálico de los salmones alimentados con el mismo fitogénico (MPLE) (185 procesos biológicos únicos)

(*Figura 1*), a diferencia del riñón cefálico de los salmones del Atlántico alimentados con el aditivo AQUOLIVE®, donde sólo se obtuvieron 21 procesos biológicos únicos modulados (*Figura 1*). Esta diferencia en la susceptibilidad de los tejidos puede ser consecuencia de la combinación de varios factores, como la funcionalidad particular del riñón cefálico y el modo de acción de los compuestos bioactivos de los fitogénicos, en especial del aditivo MPLE, si bien también hay que tener en cuenta de que el intestino está constantemente expuesto a una gran variedad de antígenos extraños presentes en los alimentos y la microbiota intestinal (Satitsuksanoa et al., 2018). Los tejidos linfoides asociados al intestino desempeñan un papel importante en la limitación de las respuestas inflamatorias a las bacterias residentes y proteínas alimentarias (Chinthrajah et al., 2016). Para mantener la tolerancia los tejidos linfoides asociados al intestino deben de discriminar los antígenos propios de los ajenos, reconociendo a los patógenos que pueden causar inflamación de los tejidos o enfermedades intestinales. La interrupción de este proceso se produce cuando los tejidos linfoides asociados al intestino no cumplen sus funciones. Los posibles mecanismos de tolerancia pueden implicar el reconocimiento de antígenos alimentarios por parte de las células dendríticas, la inducción robusta de células T reguladoras, así como de células B reguladoras. La tolerancia a los alimentos la adquieren principalmente las células dendríticas, las células epiteliales del intestino y el microbioma intestinal. Un subconjunto de células dendríticas $CD103^+$ son capaces de inducir células reguladoras T que expresan citoquinas antiinflamatorias. Las células T anérgicas también contribuyen a la tolerancia, al reducir el número de células efectoras. Al igual que las células T reguladoras, las células B reguladoras suprimen las células T efectoras y contribuyen a la tolerancia inmunitaria a los alérgenos alimentarios. Por lo tanto, el sistema inmunitario de la mucosa intestinal ha desarrollado mecanismos no sólo para detectar y eliminar patógenos, sino esencialmente para evitar una respuesta inmunitaria perjudicial exacerbada a los antígenos alimentarios y microbiota (Satitsuksanoa et al., 2018), lo que puede explicar la respuesta moderada en comparación con el riñón cefálico. Además, dado que la metodología transcriptómica aplicada en nuestros estudios (es decir, el

transcripteractoma) no se utiliza habitualmente en la evaluación de los aditivos alimentarios, existe un vacío en la literatura disponible que limita la exploración y discusión de los resultados actuales con los de otros estudios. Por otro lado, en un trabajo reciente de nuestro grupo de investigación, Firmino et al. (2021c), propuso la posibilidad de que compuestos bioactivos de fitogénicos derivados de la familia Lamiaceae como son los polifenoles y los terpenos desplieguen una actividad inmunomoduladora a través de la activación de los canales iónicos de potencial receptor transitorio (TRP, por sus siglas en inglés: *transient receptor potential*). En los vertebrados superiores, los canales TRP se expresan ampliamente en varios tipos celulares que incluyen la mayoría de los componentes de tejidos linfoides como de mucosa. Mediante el mantenimiento de la homeostasis del calcio intracelular, se sabe que estos canales regulan varias funciones celulares, como la percepción de estímulos, la producción y secreción de moléculas inflamatorias, la migración e incluso la fagocitosis (Feske et al., 2015; Khalil et al., 2018; Clement et al., 2020). Esto es de especial interés ya que, en la presente tesis, ambos aditivos comparten ciertos compuestos bioactivos, como son los terpenos y polifenoles, y en particular, el aditivo MPLE que contiene extractos de salvia, el cual pertenece a la familia Lamiaceae. Además, existen pruebas de que tanto el ácido ursólico, compuesto bioactivo de la salvia, como el ácido oleanólico, compuesto bioactivo de la aceituna, desempeñan un papel en el sistema quimiosensorial a través de la activación de los canales iónicos TRP (Rodrigues et al., 2012; Soares et al., 2019). Por ejemplo, Rodrigues et al. (2012) confirmó los efectos antinociceptivos y antiinflamatorios de la salvia mediante la modulación de la activación de los TRP, en particular sobre los receptores TRPV1 y TRPA1, además de la inhibición de la liberación de mediadores inflamatorios en ratas. Por otro lado, en peces cebra (*Danio rerio*), Soares et al. (2019) han demostrado que el ácido oleanólico puede interactuar como inhibidor de la nocicepción orofacial mediada por el TRPV1. Además, en los peces, junto con los receptores tipo Toll (TLR), se ha demostrado que la activación de los canales TRP modulan los procesos inflamatorios mediante la activación de la vía de señalización TRP/$Ca2^{+}$/TAK1/NF-kB (Galindo-Villegas et al., 2016), lo que podría sugerir

también en nuestro caso que la activación celular mediada por los canales TRP puede ser la base de las propiedades inmunomoduladoras de los aditivos probados en la presente tesis. Sin embargo, esta hipótesis necesita más investigación para poder realmente esclarecer el modo exacto de acción promovidos por los fitogénicos probados en la presente tesis, el cual podría ser uno de los siguientes pasos a dar respecto a futuras perspectivas tanto académicas como industriales.

En consecuencia, como hemos señalado anteriormente, el riñón cefálico de los salmones del Atlántico alimentados con la dieta enriquecida con el aditivo MPLE comparte un total de 22 procesos biológicos con el intestino de las doradas alimentadas con el mismo fitogénico (*Figura 1*). De los 22 GOs compartidos, aquellos que están particularmente relacionados con la inmunidad, son los siguientes: GO:0016192, transporte mediado por vesículas; GO:0006887, exocitosis; GO:0045055, regulación de los procesos de exocitosis; GO:0002366, activación de los leucocitos que participan en la respuesta inmunitaria; y el GO:0045321, activación de leucocitos. Dichos procesos biológicos compartidos son de suma importancia en términos de posibles propiedades inmunológicas por parte del aditivo probado, ya que a través de la literatura existente encontramos que la exocitosis es reconocida por su importante papel en la respuesta inmune participando en la función de los leucocitos (Faurschou y Borregaard, 2003), siendo la exocitosis mediada por macrófagos para la producción de radicales superóxidos y la posterior eliminación de patógenos mediante la actividad del estallido respiratorio (Logan et al., 2003). Además, los datos del análisis tanto de las muestras de riñón cefálico como de intestino indicaron que este aditivo alimentario mejora la respuesta inmunitaria sistémica del huésped a través de la regulación transcripcional de procesos de inmunidad innata como la activación de los leucocitos, lo que sugiere un efecto inmunomodulador por parte del aditivo MPLE a nivel tanto sistémico (riñón cefálico) y local (intestino), tal y como fue desarrollado a lo largo de la presente tesis, en particular en los Capítulos II y III (Salomón et al., 2021a; Salomón et al., 2022). Estos resultados apoyan la hipótesis central sobre la que se articuló la presente tesis doctoral, que mediante el uso de fitogénicos sería posible modular la respuesta

inmunológica de los peces de crianza, tales como la dorada y el salmón del Atlántico.

Siguiendo con los restantes GOs compartidos entre el riñón cefálico de los salmones del Atlántico y el intestino de las doradas alimentadas con el mismo aditivo (MPLE), los siguientes procesos biológicos están relacionados principalmente con procesos metabólicos. Entre ellos, el GO:0043170, proceso metabólico de las macromoléculas; GO:0016043, organización de los componentes celulares; GO:0006725, proceso metabólico celular de los compuestos aromáticos; GO:0006139, proceso metabólico de compuestos que contienen nucleobases; GO:0006996, organización de los orgánulos; GO:0051246, regulación del proceso metabólico de las proteínas; GO:0044248, proceso catabólico celular; GO:1901575, proceso catabólico de sustancias orgánicas; GO:0030163, proceso catabólico de las proteínas, GO:0043933, organización del complejo proteico; GO:0009057, proceso catabólico de macromoléculas; GO:1901135, proceso metabólico derivado de carbohidratos; y el GO:0006511, proceso catabólico de proteínas dependiente de la ubiquitina. Considerando la estrecha relación entre la dieta y el estado del intestino y las consecuencias sobre el organismo y la salud en general, la evaluación de las interacciones entre los ingredientes de la dieta y el intestino es de especial relevancia debido a la amplia gama de funciones que se han asociado al tracto gastrointestinal (Calduch-Giner et al., 2016), ya que es el tejido principal que interactúa en primera persona con los compuestos bioactivos y los antígenos exógenos presentes en la dieta. En el riñón cefálico esta interacción con los compuestos bioactivos fitogénicos puede producirse a un nivel diferente debido al metabolismo de los compuestos y a su posible alteración a lo largo del organismo; por lo tanto, cabría esperar que la respuesta transcripcional fuera en consecuencia procesos biológicos relacionados con el metabolismo de proteínas. Sorprendentemente, la cantidad de GOs respecto al aditivo MPLE en el intestino no fue sustancialmente diferente de la observada en el riñón cefálico (de salmones alimentados con la misma inclusión y el mismo aditivo) a pesar de su distancia del sitio de absorción del fitogénico administrado a través de la dieta (*Figura 1*). Por lo tanto, esta regulación de los procesos biológicos

mencionados podría sugerir un intestino metabólicamente más activo, debido a una mayor síntesis de proteínas y con una mejor condición tisular, capaz de promover la respuesta inmunitaria de tipo innato y adaptativo, permitiendo al animal combatir eficientemente una agresión externa o tolerar mejor un proceso infeccioso, a través especialmente de la modulación de los procesos implicados en la activación, diferenciación y selección de las células T como se ha podido comprobar en el análisis del perfil transcriptómico en el Capítulo II (Salomón et al., 2021a) y III (Salomón et al., 2022) de la presente tesis doctoral.

Por otro lado, al comparar el mismo tejido (riñón cefálico) entre peces de la misma especie (salmón del Atlántico), pero alimentados con diferentes aditivos (MPLE *vs*. AQUOLIVE®) se observa que se comparten un total de 14 procesos biológicos (*Figura 1*). De los 14 GOs compartidos, 11 de ellos están particularmente relacionados con la inmunidad, como por ejemplo son, GO:0035556, transducción de señales intracelulares; GO:0016192, transporte mediado por vesículas; GO:0006887, exocitosis; GO:0002366, activación de los leucocitos que participan en la respuesta inmunitaria; GO:0002274, activación de leucocitos mieloides; GO:0002275, activación de células mieloides implicadas en la respuesta inmunitaria; GO:0002444, inmunidad mediada por leucocitos mieloides; GO:0043299, degranulación de leucocitos; GO:0007249, señalización de la quinasa I-kappaB/NF-kappaB; GO:0043122, regulación de la señalización de I-kappaB quinasa/NF-kappaB; GO:0072331, transducción de señales por el mediador de clase p53. Considerando esta comparación entre ambos tejidos de los salmones del Atlántico y el aditivo obtenido de extractos de la aceituna (AQUOLIVE®), la literatura existente nos indica que los compuestos fenólicos de la aceituna regulan los procesos vesiculares y de exocitosis (Giacometti et al., 2020). Al mismo tiempo, son compartidos procesos biológicos relacionados con factores de transcripción como la señalización de quinasa I-kappaB/NF-kappaB, la regulación de la señalización de I-kappaB quinasa/NF-kappaB y la transducción de señales por el mediador de clase p53. Siendo esto es de relevancia ya que diferentes estudios han evidenciado que p53 forma parte del sistema inmunológico innato y adaptativo

como hemos mencionado anteriormente (Levine, 2020). Además, y no menos importante, existe una conexión transcripcional entre el NF-κB y p53 (Webster y Perkins, 1999; Carrà et al., 2020), mediante el reconocimiento de sitios κB en el promotor p53 y, por lo tanto, activar su expresión (Carrà et al., 2020). Asimismo, con procesos biológicos relacionados con las funciones de las células efectoras de la inmunidad innata de los vertebrados (Havixbeck et al., 2016), como son la activación de los leucocitos que participan en la respuesta inmunitaria, la activación de leucocitos mieloides, la degranulación de leucocitos, entre otros. Por lo tanto, hipotetizando de que la maquinaria implicada y sus respectivos efectos en la activación de los procesos biológicos observados por ambos fitogénicos se comparten en ambas especies, dando por resultado la efectividad de ambos aditivos en cuanto a potenciales propiedades inmunomoduladoras como hemos visto en el Capítulo III (Salomón et al., 2022) y el Capítulo IV (Salomón et al., 2021b) de la presente tesis doctoral con respecto a ambos aditivos (MPLE y AQUOLIVE®) en el salmón del Atlántico.

Por último, los resultados en cuanto al riñón cefálico de los salmones alimentados con el aditivo AQUOLIVE® mostraron tan solo un total de 21 procesos biológicos únicos. Sorprendentemente, sólo 3 procesos biológicos son compartidos con los resultados de los intestinos de las doradas alimentadas con MPLE (*Figura 1*), siendo estos 3 procesos biológicos, los mismos 3 GOs compartidos entre los 3 tratamientos (*Figura 1*). Los 3 procesos biológicos compartidos son GO:0016192, transporte mediado por vesículas; GO:0002366, activación de los leucocitos que participan en la respuesta inmunitaria; y GO:0006887, exocitosis. A pesar del bajo número de procesos biológicos compartidos, dichos procesos están estrictamente involucrados con la inmunidad (Faurschou y Borregaard, 2003; Rieger y Barreda, 2011). La exocitosis el proceso por el que las células trasladan materiales del interior de la célula al líquido extracelular. Por ejemplo, cuando un macrófago engulle un patógeno, ciertas partes del patógeno ya no son necesarias y por consecuente el material es desechado a través de la exocitosis (Logan et al., 2003), lo que atribuiría

una vez más a las potenciales propiedades inmunomoduladoras de ambos fitogénicos probados a lo largo de la tesis presentada.

Futuras perspectivas de investigación académica e industrial

Los resultados de este trabajo de tesis apoyan la hipótesis planteada inicialmente que apunta a la capacidad de los fitogénicos sobre la modulación de la respuesta inmunológica del animal cuando son administrados en la dieta. En las dos especies de peces evaluadas, la dorada y el salmón del Atlántico, hemos registrado un amplio espectro inmunomodulador de los dos fitogénicos analizados. Los resultados obtenidos en esta tesis y el conocimiento añadido respecto al mecanismo de respuesta en el huésped brindan un nuevo impulso en el desarrollo y consolidación de nuevas herramientas nutricionales amigables con el medioambiente, como es el uso de fitogénicos, que representen actualmente una prometedora herramienta profiláctica eficaz y sostenible para ser implementada en la gestión sanitaria en instalaciones acuícolas frente a infecciones (Reverter et al., 2021). Aunado a esto, el uso de fitogénicos en la dieta puede ser utilizado en el manejo de brotes de enfermedades relacionadas con el aumento de los sistemas de producción intensivo a nivel mundial, mitigando el impacto negativo que trae consigo dichos eventos en una producción. En este contexto, otro aspecto para el desarrollo y su posterior aplicación de los aditivos probados en el sector de la acuicultura es la necesidad de evaluarlos en otras especies de peces relevantes para la acuicultura y bajo retos microbianos específicos para cada especie. Considerando los resultados positivos obtenidos en la presente tesis doctoral en condiciones controladas de laboratorio con ambas especies y aditivos, sería de vital importancia poder validar dichos resultados en entornos de cultivo reales para un posible futuro establecimiento del producto final en la cadena de valor de la acuicultura.

Otra línea de investigación fascinante que podría dar respuesta a las posibles preguntas derivadas de este estudio sobre la funcionalidad de los aditivos probados

es el estudio en profundidad de las interacciones compuestos-huésped-microbiota. Tales interacciones en otros tejidos caracterizados por cooperar con los microorganismos, como la piel y las branquias, son de igual importancia y, por lo tanto, la evaluación de esas interacciones que pueden ser moduladas por las estrategias dietéticas son bastante relevantes. En este contexto, la exploración de enfoques holísticos en los que se incluyan todos los órganos y sistemas diferentes, incluida la microbiota de distintas regiones del cuerpo, así como la combinación de herramientas *in vitro* o *ex vivo*, puede ser valiosa para proporcionar información sobre el complejo modo de acción de los compuestos bioactivos de los aditivos probados.

Además, aunque la composición de ambos aditivos ensayados, tanto nivel de inclusión y el periodo de administración se establecieron de acuerdo con la literatura existente y el know-how industrial, se requieren estudios adicionales que evalúen formulaciones optimizadas y protocolos de suplementación (es decir, periodo mínimo de administración para garantizar su efectividad, ajustes a las estaciones del año, formulación de la dieta basal, etc.) de acuerdo con diferentes escenarios de cultivo.

Considerando que la acuicultura suministrará la mayor parte de la proteína dietética acuática para el año 2050 (Stentiford et al., 2020), desempeñando un papel relevante en la seguridad y el suministro de alimentos y en la mitigación de la pobreza (Béné et al., 2016). El crecimiento sostenido de la acuicultura depende en gran medida de la intensificación de la producción (Little et al., 2018), de la formulación sostenible de los piensos (Ghamkhar y Hicks, 2020) y de la generación de condiciones de cultivo que favorezcan la salud y el bienestar de los peces (Stentiford et al., 2017). Por la tanto, el aporte de la presente tesis actual respecto a los resultados logrados y las posibilidades de desarrollo y mejora de ambos fitógénicos en la industria acuícola nos abre un abanico de líneas de investigación que hagan del sector de la acuicultura un sector cada vez más seguro para el consumidor y a su vez, sostenible y respetuoso con el medioambiente.

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CONCLUSIONS

CONCLUSIONS

1. The inclusion of a medicinal plant leaf extract (MPLE), rich in ursolic and verbascoside acid obtained from sage and lemon verbena at 0.1% in diets, promoted somatic growth and reduced feed conversion ratio (FCR) values in gilthead seabream (*Sparus aurata*) and Atlantic salmon (*Salmo salar*).

2. The administration of a feed additive at 0.1% MPLE in on-growing gilthead seabream enhanced their systemic immune response as indicated by changes in gene expression of a repertoire of markers in an *ex vivo* trial using splenocytes primary cell culture exposed to lipopolysaccharide (LPS).

3. The administration of a feed additive at 0.1% MPLE in on-growing gilthead seabream promoted local immunity in the intestine as indicated by the transcriptomic results through the modulation of those processes involved in T cell activation, differentiation and selection.

4. Data from the microarray analysis of the head kidney samples indicated that the MPLE enhanced systemic immunity in Atlantic salmon smolts through the transcriptional regulation of innate immunity processes like leukocytes' activation, and other pathways such as interferon-gamma-mediated signaling pathway, antigen processing and presentation of peptide antigen via MHC class II, autophagy, and regulation of i-kappaB kinase/NF- kappaB.

5. Results indicated that the tested feed additive, MPLE, protects Atlantic salmon smolts against furunculosis caused by *Aeromonas salmonicida*, reducing fish mortality when exposed to the bacterial pathogen.

6. The administration of AQUOLIVE® at 0.15%, a feed additive rich in triterpenic and polyphenolic compounds from *Olea europaea* did not promote somatic growth; however, significantly reduced FCR values in Atlantic salmon smolts.

7. Analysis of the head kidney transcriptomic profiling response to a diet supplemented with 0.15% of AQUOLIVE® revealed a gene expression profile that favored biological processes particularly related to systemic immunity in Atlantic salmon smolts.

8. Results indicated that the tested feed additive AQUOLIVE® protects Atlantic salmon smolts against *A. salmonicida*.

9. The combination of sage and lemon verbena extracts and the olive fruit extract can be used by the aquaculture industry as zootechnical additives with immunomodulatory properties, besides being an effective, safe, and environmentally eco-friendly tool to be used as a prophylactic strategy against infectious diseases and as an alternative to antimicrobial compounds.

CONCLUSIONES

1. La inclusión de un extracto de hojas de plantas medicinales (MPLE), rico en ácido ursólico y verbascósido obtenido de la salvia y la hierbaluisa al 0,1% en las dietas, promovió el crecimiento somático y redujo los valores del índice de conversión alimenticia (FCA) en la dorada (*Sparus aurata*) y el salmón del Atlántico (*Salmo salar*).

2. La administración del aditivo para piensos MPLE al 0,1% en doradas mejoró su respuesta inmunitaria sistémica, tal y como indicaron los cambios en la expresión génica de un repertorio de marcadores en un ensayo *ex vivo* en el que se utilizaron esplenocitos de un cultivo celular primario expuestos al lipopolisacárido (LPS).

3. La administración de un aditivo para piensos al 0,1% de MPLE en la dorada promovió la inmunidad local en el intestino, tal y como indican los resultados transcriptómicos, a través de la modulación de los procesos implicados en la activación, diferenciación y selección de las células T.

4. Los datos del análisis de microarrays de las muestras de riñón cefálico indicaron que el fitogénico MPLE mejoró la inmunidad sistémica de los salmones del Atlántico a través de la regulación transcripcional de los procesos relacionados a la inmunidad innata, como la activación de los leucocitos, y de otras vías como la vía de señalización mediada por el interferón-gamma, el procesamiento de antígenos y la presentación de antígenos peptídicos a través del MHC de clase II, la autofagia y la regulación de la quinasa i-kappaB/NF- kappaB.

5. Los resultados indicaron que el aditivo para piensos probado, MPLE, protege a los salmones del Atlántico contra la furunculosis enfermedad causada por *Aeromonas salmonicida*, reduciendo la mortalidad de los peces cuando son expuestos al patógeno bacteriano.

6. La administración de AQUOLIVE® al 0,15%, un aditivo para piensos rico en compuestos triterpénicos y polifenólicos de *Olea europaea*, no promovió el crecimiento somático; sin embargo, redujo significativamente los valores de FCA en los salmones del Atlántico.

7. El análisis de la respuesta del perfil transcriptómico del riñón cefálico a una dieta suplementada con 0,15% de AQUOLIVE® reveló un perfil de expresión génica que favorecía los procesos biológicos particularmente relacionados con la inmunidad sistémica en salmones del Atlántico.

8. Los resultados indicaron que el aditivo para piensos probado, AQUOLIVE®, protege a los salmones del Atlántico contra *A. salmonicida*.

9. La combinación de los extractos de salvia y hierbaluisa y el extracto de aceituna pueden ser utilizados por la industria acuícola como aditivos zootécnicos con propiedades inmunomoduladoras, además de ser una herramienta eficaz, segura y respetuosa con el medio ambiente para utilizada como estrategia profiláctica contra las enfermedades infecciosas y como alternativa a los compuestos antimicrobianos.

APÉNDICE

APÉNDICE 1

Apéndice 1. Resumen ampliado de la bibliografía utilizada en la presente tesis doctoral sobre los efectos nutricionales de los fitogénicos derivados de la salvia (*Salvia officinalis*), la hierbaluisa (*Lippia citriodora*) y la aceituna (*Olea europaea*) en el rendimiento productivo de diferentes especies de peces.

Origen del fitogénico	Forma de suplementación	Forma de administración	Dosis de inclusión	Nivel harina de pescado (%)	Periodo de administración	Principales compuestos bioactivos	Especie de pez	Rendimiento	Ámbito	Referencia
Salvia*										
	Polvo de hojas	Pellet /oral	0,15% 0,30% 0,60%	23%	90 días	N/I	Tilapia híbrida (*Oreochromis niloticus* x *Oreochromis aureus*)	↑ Peso final ↑ SGR ↑ Incremento de peso ↓ FCA ↑ Consumo de alimento	Dulceacuícola	El Kholy et al. (2012)
	Aceite esencial	Pellet /oral	500 mg kg^{-1} 1000 mg kg^{-1} 1500 mg kg^{-1}	N/I	60 días	N/I	Trucha arcoíris (*Oncorhynchus mykiss*)	↑ SGR ↑ Incremento de peso ↓ FCA	Dulceacuícola	Sönmez et al. (2015)

	Aceite esencial	Pellet /oral	0,25% 0,50% 1%	N/I	30 días	N/I	Tilpia del Nilo (*Oreochromis niloticus*)	↓ Peso final ↓ Incremento de peso ↓ SGR ↓ Consumo de alimento ↑ FCA	Dulceacuícola	Aydin et al. (2018)
	Extracto etanólico	Pellet /oral	30 mL kg-1 60 mL kg-1 120 mL kg^{-1}	N/I	42 días	N/I	Esturión beluga (*Huso huso*)	Sin efecto	Dulceacuícola	Dadras et al. (2020)
Hierbaluisa**										
	Aceite esencial	Pellet /oral	0,15 mL kg^{-1} 0,30 mL kg^{-1}	N/I	30 días	N/I	Carpa común (*Cyprinus carpio*)	↑ Peso final	Dulceacuícola	Gholipourkanani et al. (2017)
	Polvo de hojas	Pellet /oral	0,5% 1% 2%	N/I	42 días	N/I	Trucha arcoíris (*Oncorhynchus mykiss*)	Sin efecto	Dulceacuícola	Hoseinifar et al. (2020a)
	Extracto crudo	Pellet /oral	5 mg kg^{-1} 10 mg kg^{-1} 20 mg kg^{-1}	N/I	56 días	N/I	Esturión siberiano (*Acipenser baerii*)	↑ SGR ↑ Incremento de peso ↓ FCA	Dulceacuícola	Adel et al. (2021)

Aceituna* **										
			0,08%			9% de ácidos triterpénicos				
	Extracto de aceite	Pellet /oral	0,17%	39%	90 días	2% de polifenoles	Dorada (*Sparus aurata*)	↑ Peso final	Marina	Gisbert et al. (2017)
			0,42%			2% de alcoholes grasos				
			0,73%							
	Extracto de hoja	Pellet /oral	1 g kg^{-1} 5 g kg^{-1}	N/I	56 días	N/I	Carpa común (*Cyprinus carpio*)	Sin efecto	Dulceacuícola	Karimi Pashaki et al. (2018)
	Extracto de hoja	Pellet /oral	0,1% 0,25% 0,5% 1%	47%	60 días	N/I	Trucha arcoíris (*Oncorhynchus mykiss*)	Sin efecto	Dulceacuícola	Baba et al. (2018)
	Extracto de hoja	Pellet /oral	0,1% 0,5% 1%	16%	56 días	N/I	Carpa común (*Cyprinus carpio*)	Sin efecto	Dulceacuícola	Rajabiesterabadi et al. (2020a)
	Extracto de aceituna	Pellet /oral	0,5 g kg^{-1} 2,5 g kg^{-1} 5 g kg^{-1}	N/I	42 días	Vitaminas E (α- tocoferol, β-tocoferol, α-tocotrienol)	Trucha arcoíris (*Oncorhynchus mykiss*)	↑ Peso final ↑ SGR ↑ Incremento de peso ↓ FCA	Dulceacuícola	Hoseinifar et al. (2020b)

	Extracto de hoja	Pellet /oral	200 mg kg^{-1} 400 mg kg^{-1}	23%	75 días	N/I	Carpa común (*Cyprinus carpio*)	↑ Peso final ↑ SGR ↑ Incremento de peso ↓ FCA	Dulceacuícola	Sokooti et al. (2021)
	Extracto de aceituna	Pellet /oral	0,15%	17,5%	133 días	10% compuestos bioactivos de la oliva 8% compuestos triterpénicos 2% polifenoles	Salmón del Atlántico (*Salmo salar*)	↓ FCA	Marina	Capítulo IV (Salomón et al., 2021b)
Combinaciones fitogénicas										
Laurel (*Laurus nobilis*) Hinojo (*Foeniculum vulgare*) Salvia (*Salvia officinalis*)	Aceite esencial	Pellet /oral	0,05% 0,1%	18,0%	84 días	N/I	Tilpia del Nilo (*Oreochromis niloticus*)	↑ Peso final ↑ SGR ↑ Incremento de peso ↓ FCA ↑ Consumo de alimento	Dulceacuícola	Mahmoud et al. (2014)

Salvia (*Salvia officinalis*)						10% ácido ursólico		↑ Peso final		
Hierbaluisa (*Lippia citriodora*)	Extracto de hojas	Pellet /oral	0,1%	7%	92 días	3% otros compuestos triterpénicos 2% verbascósido <1% polifenoles	Dorada (*Sparus aurata*)	↓ FCA	Marina	Capítulo I (Salomón et al. 2020)
Salvia (*Salvia officinalis*)						10% ácido ursólico		↑ Peso final		
Hierbaluisa (*Lippia citriodora*)	Extracto de hojas	Pellet /oral	0,1%	17,5%	133 días	3% otros compuestos triterpénicos 2% verbascósido <1% polifenoles	Salmón del Atlántico (*Salmo salar*)	↓ FCA	Marina	Capítulo III (Salomón et al. 2022)

*Salvia (*Salvia officinalis*)

**Hierbaluisa (*Lippia citriodora*)

***Aceituna (*Olea europaea*)

N/I: no identificado o no evaluado