Respuesta del microbioma intestinal de animales del sector avícola, porcino y piscícola al tratamiento con probióticos y extractos de aliáceas

Tesis Doctoral

Miguel Rabelo Ruiz

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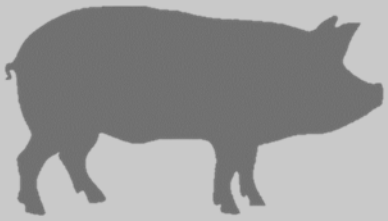

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ÍNDICE

LISTADO DE ABREVIATURAS

ADFI:	Average Daily Feed Intake / Consumo diario promedio de alimento
ADG:	Average Daily Gain / Ganancia diaria promedio
ADN:	Ácido desoxirribonucleico
AMR:	Antimicrobial Resistance / Resistencia a los antimicrobianos
ANOVA:	Analysis of Variance / Análisis de la varianza
AGP/APC:	Antibiotic Growth Promoters / Antibióticos Promotores del Crecimiento
ARNr:	Ácido ribonucleico ribosómico
BAL:	Bacterias del Ácido Láctico
DPH:	Days post hatching / Días tras la eclosión
FB:	Final Biomass / Biomasa final
FE:	Feed Efficiency / Eficiencia alimenticia
FCR:	Feed Conversion Ratio / Tasa de conversión del alimento
FMBW:	Final Mean Body Weight / Peso corporal medio final
FTU:	Unidades de fitasa
GLMM:	Generalized Linear Mixed Models / Modelos Lineales Generalizados Mixtos
GRAS:	Generally Recognized As Safe / Generalmente reconocido como seguro
IB:	Initial Biomass / Biomasa inicial
IMBW:	Initial Mean Body Weight / Peso corporal medio inicial
OTU:	Operative Taxonomic Units / Unidades Taxonómicas Operativas
PCoA:	Principal Coordinates Analysis / Análisis de Coordenadas Principales
PCR:	Reacción en cadena de la polimerasa
PTS:	Propil propano tiosulfinato
PTSO:	Propil propano tiosulfonato
RAS:	Recirculating Aquaculture System / Sistema de acuicultura recircularizada
SCFA:	Short-chain fatty acids / Ácidos grasos de cadena corta
SGR:	Specific Growth Rate / Tasa de crecimiento específico
TBG:	Total Biomass Gain / Ganancia total de biomasa
UE:	Unión Europea
WG:	Weight Gain / Ganancia de peso

RESUMEN

Los sectores avícola, porcino y piscícola son de gran importancia para la productividad de nuestro país, siendo España un referente a nivel Europeo. El aumento exponencial de la población de los últimos años, el cual se prevé que siga creciendo, hace necesaria la búsqueda de estrategias que permitan aumentar la producción de alimentos de origen animal. Durante años, se han conseguido mantener o aumentar los niveles productivos gracias en parte a la mejora genética de las razas destinadas a la producción, a la mejora en las dietas, y también por la utilización de antibióticos promotores del crecimiento (APCs). Sin embargo, el uso excesivo de estos compuestos ha conllevado a un incremento de la resistencia a los antimicrobianos (AMR, *Antimicrobial Resistance*), lo que originó su prohibición en alimentación animal por parte de la Unión Europea y de otros países. Además, en los últimos años se ha cuestionado la sostenibilidad de algunos de los compuestos utilizados de forma tradicional para la elaboración de dietas de animales. Todo esto ha hecho necesaria la búsqueda de nuevos compuestos capaces de: (1) aumentar la calidad nutricional y la sostenibilidad de las dietas; (2) incrementar la producción y el crecimiento de los animales; y (3) eliminar o reducir en lo posible la administración de antibióticos como promotores del crecimiento y como terapéuticos. En este contexto se han propuesto algunos compuestos como posibles alternativas, entre los que destacan enzimas, prebióticos, probióticos y extractos de plantas o fitobióticos.

Los probióticos son microorganismos vivos que, al ser administrados en cantidades adecuadas en la dieta, confieren un beneficio para la salud del organismo hospedador. Los beneficios ocasionados por los probióticos se producen a través de diversos mecanismos, como la inducción de inmunomodulación, la mejora de la función intestinal o la modulación las comunidades microbianas del intestino. Entre las bacterias empleadas como probióticos en la dieta de animales destacan bacterias pertenecientes a los géneros *Lactobacillus, Bifidobacterium, Bacillus* o *Enterococcus*. Dentro de este último género, la cepa *Enterococcus faecalis* UGRA10 presenta propiedades tecnológicas, funcionales y potencialmente probióticas, y ha mostrado resultados positivos para la salud, el crecimiento y la composición de la microbiota intestinal de pollos, cerdos y peces en estudios previos. En el **Capítulo I** de esta Tesis se ha estudiado la suplementación de la dieta de gallinas ponedoras con esta cepa potencialmente probiótica, mostrando una mejora en la producción de huevos. Las gallinas alimentadas con este probiótico mantuvieron la puesta de huevos a largo plazo (40 a 76 días), mientras

que las gallinas control sufrieron una disminución de la puesta durante este período. Además, la suplementación con esta cepa produjo un incremento de la diversidad microbiana en el íleon y en el ciego, el cual podría estar mediando en la mejora observada en la producción de huevos.

Por su parte, los extractos de plantas o fitobióticos, han sido ampliamente utilizados como suplementos en la dieta de numerosas especies animales por su capacidad para modular las comunidades microbianas del intestino y producir mejoras en su sistema inmunitario, y por su actividad antimicrobiana, antioxidante y antiestrés. Entre los fitobióticos empleados en alimentación animal destacan las especies del género *Allium*, las cuales presentan numerosos compuestos organosulfurados, como la alicina, el PTS (propil propano tiosulfinato) o el PTSO (propil propano tiosulfonato). Los resultados de estudios previos han mostrado sus posibles beneficios sobre la productividad, el crecimiento, la salud o la inmunidad de animales de granja como pollos de engorde o cerdos. En el **Capítulo II** de esta Tesis se han estudiado los efectos de un extracto de *Allium* rico en PTSO sobre la composición de la microbiota intestinal y la productividad de gallinas ponedoras. La adición de este extracto produjo un incremento en la puesta, así como un aumento del peso y la talla de los huevos tras sólo 30 días de experimento. Este aumento en la productividad estuvo acompañado de cambios en la composición de la microbiota intestinal que podrían resultar positivos para los animales, caracterizados principalmente por un aumento de especies potencialmente beneficiosas como *Lactococcus* en el íleon y *Lactobacillus* en el ciego, sugiriendo la posibilidad de utilizar este extracto como aditivo para la dieta de gallinas ponedoras.

En el **Capítulo III** se ha evaluado el efecto de la adición de un extracto de *Allium* sobre el crecimiento y las comunidades microbianas del intestino de lechones destetados a los 28 días de edad. Los resultados se han comparado con los obtenidos utilizando una dieta control y una dieta suplementada con un tratamiento antibiótico (colistina y óxido de zinc). Los lechones alimentados con el extracto de *Allium* mostraron valores de peso corporal, ganancia de peso diaria y tasa de conversión alimenticia similares a los obtenidos por los lechones del tratamiento antibiótico y mayores a los obtenidos con la dieta control. Además, los lechones del tratamiento experimental mostraron cambios en la estructura general de las comunidades microbianas de las regiones posteriores del intestino (ciego y colon), así como una disminución de la diversidad bacteriana del intestino en estas regiones intestinales. Además, esta dieta produjo un aumento de algunos

géneros relacionados con potenciales efectos beneficiosos en los animales, como son los géneros *Lactobacillus, Blautia* o *Megasphaera.* Los resultados obtenidos indican la posibilidad de utilizar el extracto de *Allium* con el fin de reducir el uso de colistina, uno de los principales antibióticos utilizados de forma masiva como promotor del crecimiento y como terapéutico para tratar la diarrea post-destete en lechones.

Por otra parte, los estudios relacionados con la utilización de fitobióticos en la dieta de animales acuáticos han aumentado en los últimos años, mostrando en la mayoría de los casos resultados beneficiosos para los animales. Sin embargo, la utilización de PTSO derivado de plantas del género *Allium* en la dieta de animales acuáticos no se ha explorado hasta la fecha. En los **Capítulos IV y V** se ha utilizado este compuesto en la dieta de juveniles de doradas y lubinas, estudiando las consecuencias de su adición sobre parámetros relacionados con el crecimiento y el microbioma intestinal. En ambos estudios se han obtenido resultados similares en relación al crecimiento, manteniendo los peces alimentados con PTSO valores de peso similares a los obtenidos con la dieta control a lo largo del experimento. Sin embargo, las lubinas suplementadas con PTSO sí que mostraron un aumento significativo del peso al final del experimento (Día 89) respecto a los peces control. En cuanto a la composición y la diversidad bacteriana, el PTSO tuvo efectos diferentes en ambas especies de peces, observándose un aumento de la diversidad microbiana del intestino en doradas, y una disminución en las lubinas suplementadas con PTSO. Además, en el intestino de lubinas se observaron cambios en las bacterias mayoritarias y minoritarias de las comunidades microbianas respecto a las lubinas control. No obstante, en ambas especies se observó una disminución en la abundancia del género *Vibrio* en la parte anterior y posterior del intestino. Este género incluye algunas especies de bacterias potencialmente patógenas de estos animales, por lo que su disminución podría resultar beneficiosa para la salud de los peces. Los resultados obtenidos, aunque muestran un comportamiento distinto en ambas especies de peces, parecen indicar la posibilidad de utilizar PTSO como potencial aditivo para la dieta de estas especies piscícolas.

Los últimos capítulos de esta Tesis se centran en el uso de microalgas como alternativa para tratar de reducir el uso de productos derivados del pescado en la dieta de peces. La harina de pescado se ha utilizado de forma masiva durante años como uno de los principales ingredientes de los piensos de animales acuáticos. Sin embargo, en la actualidad se está investigando el uso de compuestos que sean más sostenibles y que

permitan reducir el uso de harinas de pescado. En este contexto, se ha propuesto la utilización de microalgas como ingrediente y por lo tanto como alternativa para la dieta de peces, ya que son fáciles de producir y han demostrado ser eficaces en estudios previos. En el **Capítulo VI** se ha evaluado la inclusión de 5% y 10% de biomasa cruda e hidrolizada de *Arthrospira platensis* en la dieta de larvas de dorada. El uso de hidrolizados facilita la digestión de los alimentos, especialmente en etapas tempranas del desarrollo, dada la falta de la actividad enzimática necesaria para digerir algunos componentes de la dieta. Nuestros resultados no mostraron alteraciones en el crecimiento de las larvas suplementadas con biomasa de microalgas crudas e hidrolizadas, apareciendo valores de peso y longitud corporal similares a los de las larvas control. Además, las dietas que presentaban un 5% de biomasa de *A. platensis* tanto cruda como hidrolizada mostraron una composición de las comunidades microbianas similar a las de las larvas control, no afectando a la composición de su microbiota intestinal. La dieta que incluía un 10% de *A. platensis* hidrolizada produjo cambios solamente en las especies minoritarias, mientras que 10% de *A. platensis* cruda produjo alteraciones de las especies mayoritarias y una reducción de la diversidad microbiana. Estos resultados sugieren que la inclusión de 5% de *A. platensis* cruda y 5% y 10% de *A. platensis* hidrolizada podría ser útil para reducir los niveles de harina de pescado en el pienso, manteniendo el crecimiento y sin alterar el comportamiento y la composición de la microbiota intestinal de larvas de dorada.

Por último, en el **Capítulo VII** se ha utilizado una dieta complementada con biomasa cruda de microalgas y diferentes concentraciones de fitasa. La fitasa es una enzima que degrada el ácido fítico, liberando fósforo y otros nutrientes que pueden ser utilizados por los animales. Tanto la fitasa como otras enzimas se han utilizado como aditivos en dietas en animales terrestres y acuáticos, produciendo un aumento de la digestibilidad de los nutrientes y un incremento en el crecimiento de los animales. En nuestro estudio se ha utilizado una formulación que incluye 2,5% de biomasa cruda de microalgas (*Arhtrospira platensis* y *Nannochloropsis gaditana*), junto con diferentes concentraciones de fitasa (500, 1.000, 2.000 y 10.000 FTU/kg) en la dieta de juveniles de lubina. La inclusión de biomasa de microalgas junto niveles altos de fitasa (2.000 y 10.000 FTU/kg) produjo un aumento en el peso de los peces, siendo los peces alimentados con la dieta de 10.000 FTU/kg los que mostraron un mayor crecimiento. Estos resultados indican que el incremento del peso es proporcional a la concentración de fitasa, produciéndose mayor crecimiento a medida que aumenta la concentración de la enzima.

Con respecto a las comunidades microbianas intestinales, las dietas con 500, 1.000 y 2.000 FTU/kg produjeron cambios en la diversidad o en la composición microbiana del intestino, mientras que la dieta que contenía un 2,5% de biomasa de microalgas y 10.000 FTU/kg de fitasa no alteró la composición de la microbiota intestinal. Esta última dieta parece ser la más beneficiosa para los juveniles de lubina, produciendo un aumento significativo del crecimiento sin alterar la estructura de las comunidades microbianas intestinales.

En resumen, los resultados de esta Tesis muestran la importancia de suplementar la dieta de animales con diferentes aditivos, como potenciales probióticos, fitobióticos y microalgas solas o junto con preparados enzimáticos, permitiendo mejorar la calidad nutricional de las dietas y mantener o aumentar el crecimiento y los parámetros productivos de animales del sector avícola, porcino y piscícola, además de inducir en algunos casos cambios potencialmente beneficiosos en la composición de la microbiota intestinal. Serán necesarias más investigaciones en el futuro que permitan conocer la acción de estos compuestos sobre patógenos específicos de los distintos animales empleados, así como otros parámetros relacionados con la salud y el bienestar o la respuesta del sistema inmunitario de los animales, lo que completaría los resultados obtenidos en esta Tesis.

INTRODUCCIÓN

1. Importancia de los sectores avícola, porcino y piscícola en España

La ganadería en España representa uno de los sectores productivos más importante del país, siendo una referencia a nivel europeo. Este sector se encuentra representado por la cría de diferentes especies de animales, siendo la actividad porcina la de mayor peso en cuanto a producción. El sector porcino supuso en torno al 43% del valor de la producción total ganadera en el año 2020 (Figura I.1), con una producción estimada entorno a 5 millones de toneladas de carne. En este mismo año, el censo de ganado porcino se situó alrededor de los 32,7 millones de animales, representando el 22,4% del total de animales de la Unión Europea (en adelante UE), y convirtiendo a nuestro país en el principal productor de la UE, y superando a otros grandes países productores como Alemania o Francia (Ministerio de Agricultura Pesca y Alimentación, 2020c).

En cuanto al sector avícola, representa un 5,7% del valor de la producción final ganadera el sector avícola de puesta, y un 12,2% el sector avícola de carne (Figura I.1). La producción de huevos ha aumentado los últimos años, produciéndose en España en torno a 1.250 millones de docenas de huevos en el año 2020 (Ministerio de Agricultura Pesca y Alimentación, 2020b). Con respecto a la UE, España ocupa el tercer lugar en producción de huevos, correspondiente al 13% de la producción de la UE y siendo superada sólo por Alemania y Francia. En lo que se refiere al número de gallinas ponedoras, los últimos datos obtenidos en el año 2020 mostraron un censo superior a 47 millones de animales, lo que supone un aumento respecto a años anteriores (Ministerio de Agricultura Pesca y Alimentación, 2020b). En cuanto a la producción de carne de ave en España, en el año 2020 se produjeron 1,7 millones de toneladas, lo que supuso un 12,7% de la producción de la UE, convirtiendo a España en el segundo mayor productor tras Polonia (Ministerio de Agricultura Pesca y Alimentación, 2020a).

Por su parte, la acuicultura también representa una actividad con gran impacto económico en nuestro país, siendo el principal productor de acuicultura de los estados miembro de la UE. Los datos más recientes, sitúan la producción acuícola de España en torno a las 300.000 toneladas en el año 2020, colocándose a la cabeza de la EU. Sin embargo, considerando el valor de la producción, España ocupa la segunda posición con una producción superior a 560 millones de euros (APROMAR, 2021). Centrándonos solamente en la producción de peces (sin tener en cuenta la producción de moluscos), España se sitúa en segundo lugar de la UE, con una producción de en torno a 80.000 toneladas, con un valor aproximado de unos 450 millones de euros (Figura I.2). Las

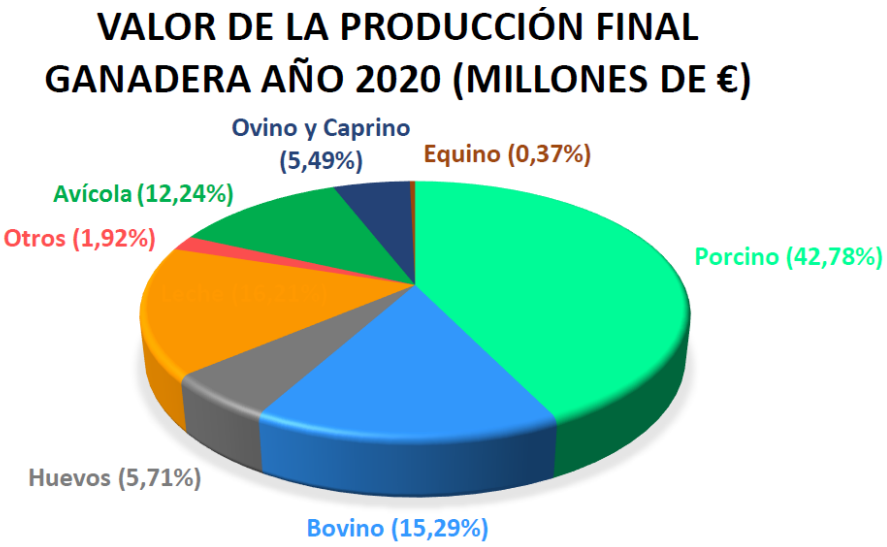

Figura I.1. Valor de la producción final ganadera en España en el año 2020. La mayor parte de la producción ganadera pertenece al ganado porcino, seguido del bovino, avícola y del sector avícola de puesta de huevos (Ministerio de Agricultura Pesca y Alimentación, 2020b).

principales especies de peces producidas en la UE incluyen la trucha arcoíris (*Onchorhynchus mykiss*), la dorada (*Sparus aurata*) y la lubina (*Dicentrarchus labrax*). Además, el informe *Fish to 2030* (The World Bank, 2013) considera que la producción de pescado seguirá creciendo de forma exponencial en los próximos años, pudiendo alcanzar niveles de producción cercanos a los 190 millones de toneladas anuales en el año 2030, siendo su componente principal el proveniente de la acuicultura.

Por otra parte, el aumento exponencial de la población mundial de los últimos años, la cual se estima que pueda alcanzar valores cercanos a los 9.700 millones de personas en el año 2050 y casi 11.000 millones en el año 2100 (United Nations, Department of Economic and Social Affairs, Population Division, 2019), ha llevado a la necesidad por parte de la industria agroalimentaria de buscar nuevas metodologías que sean capaces de incrementar los niveles productivos y al mismo tiempo aumentar el bienestar animal (Wu et al., 2020). Entre estos métodos, la suplementación de las dietas con aditivos de tipo alimentario ha resultado ser una herramienta muy eficaz, pues permite influir favorablemente el rendimiento y el bienestar animal, particularmente mediante modificaciones de la microbiota intestinal. El establecimiento de una microbiota intestinal adecuada constituye una barrera eficaz frente a patógenos (Ubeda et al., 2017), además de suponer un estímulo para el desarrollo del sistema inmunitario y aportar

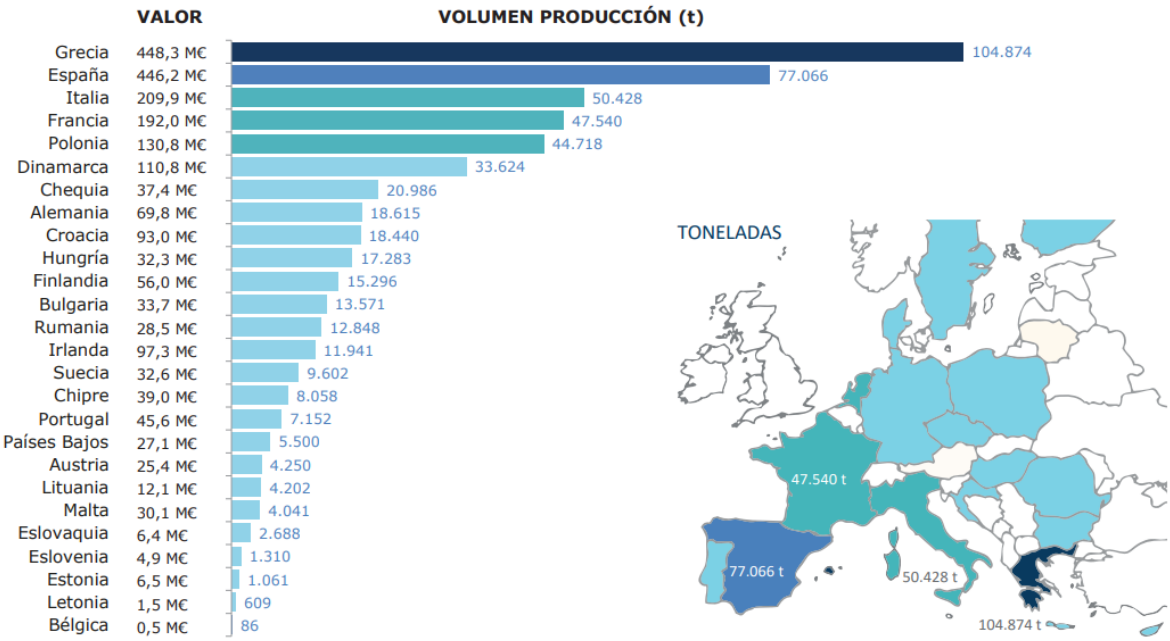

Figura I.2. Distribución de la producción de acuicultura de peces en los estados miembro de la UE por su cantidad (toneladas) y valor (millones de euros) en el año 2020. España se sitúa en segundo lugar en valor y volumen de producción, sólo por detrás de Grecia (tomado de APROMAR (2021)).

metabolitos necesarios para el bienestar intestinal, tanto en humanos como en animales (Duarte & Kim, 2021; Rescigno, 2014). De ahí la importancia de conocer la composición de microbiota intestinal de los animales, así como los principales factores que puedan alterarla.

2. Microbiota del tracto digestivo

La microbiota intestinal hace referencia a la comunidad de microorganismos que habitan un determinado ambiente del tracto gastrointestinal, incluyendo bacterias, hongos, protozoos, arqueas y levaduras (Turner, 2018). El tracto gastrointestinal es una de las regiones corporales que alberga una microbiota más abundante y compleja, llegando a niveles cercanos a 10^{14} bacterias totales en todo el tracto gastrointestinal (Kim & Isaacson, 2015; Thursby & Juge, 2017). Los microorganismos residentes en el tracto gastrointestinal otorgan diversos beneficios al hospedador, desempeñando papeles clave en funciones nutricionales, fisiológicas, inmunológicas y protectoras (Frick & Autenrieth, 2013; Gensollen et al., 2016), además de desempeñar un papel importante en la absorción y utilización de nutrientes (Frick & Autenrieth, 2013; Morgan & Huttenhower, 2012). Por otra parte, la microbiota intestinal también se encuentra comunicada con otros órganos y sistemas del cuerpo a través de los ejes intestino-cerebro, intestino-pulmones,

intestino-piel o intestino-hígado (Martínez et al., 2021). Esta comunicación se produce principalmente a través de metabolitos secundarios, siendo de especial importancia los ácidos grasos de cadena corta (*short-chain fatty acids* - SCFAs) producidos por el metabolismo de las bacterias intestinales (O'Riordan et al., 2022). Por tanto, la presencia de una microbiota intestinal beneficiosa puede afectar positivamente a la salud y las enfermedades relacionadas con diversos órganos y sistemas de humanos y animales (Morais et al., 2020).

Numerosos estudios en humanos y animales han demostrado que la composición de la microbiota intestinal está influenciada por numerosos factores, entre los que se incluyen el genotipo o especie del hospedador, la edad, el ambiente, la temperatura, la dieta o el uso de antibióticos, entre otros (Bian et al., 2016; Donaldson et al., 2015; Leeming et al., 2019; Wen & Duffy, 2017; Yukgehnaish et al., 2020). Cambios en estos factores pueden conllevar a alteraciones en su composición, lo que puede ocasionar distintas enfermedades. Sin embargo, algunos estudios han sugerido que los factores dietéticos son probablemente los determinantes más importantes que dan forma a la microbiota intestinal, por lo que pequeños modificaciones en la dieta pueden conllevar a diferencias en la composición (Ringø et al., 2016; Scott et al., 2013; Zmora et al., 2018). Por ello, resulta enormemente importante conocer su estructura y cómo se desarrolla en cada uno de los animales objeto de estudio de estudio de esta Tesis Doctoral, para así poder conocer cómo pueden afectar las dietas experimentales a su composición final y cómo afectan a su salud.

2.1. Microbiota intestinal del ganado porcino

La microbiota intestinal de los cerdos, al igual que la del resto de animales es estéril en el momento del nacimiento (Guevarra et al., 2019; Isaacson & Kim, 2012). Los primeros microorganismos que colonizan el tracto digestivo provienen del medio, principalmente del tracto vaginal, la piel y las heces de la madre (Isaacson & Kim, 2012). Durante las primeras horas de vida el tracto es colonizado rápidamente por microorganismos aerobios o facultativos, produciéndose una sucesión progresiva hacia anaerobios en los adultos (De Rodas et al., 2018). La participación de los primeros colonizadores crea un ambiente favorable para los anaerobios. Durante la fase de lactancia, la microbiota intestinal se mantiene bastante estable, siendo la leche materna el factor externo que más influye en las comunidades microbianas (Bian et al., 2016).

El destete es una de las etapas más críticas en el ciclo de vida de los cerdos, ya que genera estrés y cambios fisiológicos, ambientales y sociales en los lechones (Campbell et al., 2013; Gresse et al., 2017; Guevarra et al., 2018). Además, en la industria ganadera actual, el destete se suele realizar entre las 3-4 semanas de vida, mientras que de forma natural ocurre en torno a la semana 17, lo que conlleva un mayor grado de estrés para los lechones (Gresse et al., 2017). Por otra parte, el cambio de leche a comida sólida, con fuentes de energía más complejas, puede favorecer la aparición de modificaciones en la microbiota intestinal encaminados hacia el establecimiento de una comunidad microbiana característica de adultos (Bian et al., 2016). Todos estos cambios pueden dar lugar a una alteración de la microbiota, proceso que se conoce como disbiosis y que puede llevar a la aparición de infecciones así como a la aparición de diarrea post-destete en los lechones, lo cual conlleva importantes pérdidas económicas para los ganaderos, siendo una de las principales causas de muerte de los lechones (Guevarra et al., 2018; Rhouma et al., 2017). Se han llevado a cabo algunos estudios mediante técnicas de secuenciación masiva para determinar la composición de la microbiota intestinal de lechones, mostrando una predominancia de los filos *Firmicutes*, *Bacteroidetes* y *Proteobacteria*, que pueden llegar a representar casi el 100% de la comunidad microbiana del intestino. A nivel de género, el tracto gastrointestinal se encuentra colonizado principalmente por especies de *Lactobacillus, Clostridium, Bacteroides* o *Prevotella,* entre otros (Bian et al., 2016; Guevarra et al., 2018; Rodas et al., 2018).

Tras el proceso de destete, los lechones adquieren una microbiota similar a la de los animales adultos, llegando a una comunidad clímax bastante estable y característica de cada individuo (Kim et al., 2011). Los grupos mayoritarios presentes en la microbiota intestinal de cerdos adultos son los mismos que en lechones (*Firmicutes*, *Bacteroidetes* y *Proteobacteria*), produciéndose tras el destete cambios en la abundancia relativa de algunos grupos mayoritarios, como una disminución de *Lactobacillus* y un aumento de *Proteobacteriaceae*, *Clostridium* y *Prevotella* (Crespo-Piazuelo et al., 2018; Gresse et al., 2017; Holman et al., 2017; Looft et al., 2014). Este último género aparece principalmente en regiones intestinales posteriores (ciego y colon), en las cuales se produce un aumento del filo *Bacteroidetes* y una reducción de *Firmicutes* y *Proteobacteria* (Crespo-Piazuelo et al., 2018; Looft et al., 2014) (Figura I.3). Sin embargo, esta microbiota puede sufrir variaciones ya que se encuentra influenciada por factores internos, como el genotipo del

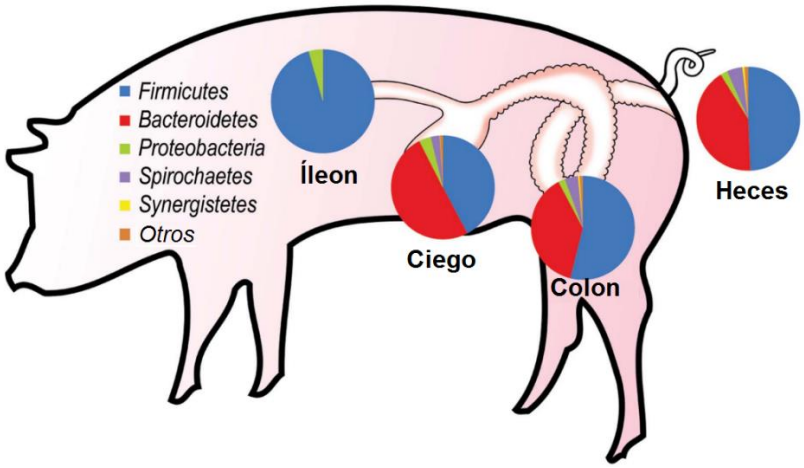

Figura I.3. Abundancia relativa de los filos presentes en la microbiota intestinal de diferentes regiones del tracto gastrointestinal de cerdos. El íleon está colonizado principalmente por *Firmicutes*, mientras que en el ciego y el colon se produce un aumento de *Bacteroidetes* (modificada de Looft et al., 2014).

hospedador, la edad o el sexo, y externos como el ambiente, el sistema de cría, la dieta o administración de antibióticos (Bian et al., 2016; Guevarra et al., 2019; Pajarillo et al., 2014). Otro de los factores que afecta a la composición de la microbiota intestinal es la región del tracto gastrointestinal, produciéndose un gradiente creciente de microorganismos autóctonos a medida que se avanza en el tracto gastrointestinal (Crespo-Piazuelo et al., 2018; Holman et al., 2017).

2.2. Microbiota intestinal del ganado avícola

A diferencia de los mamíferos, el embrión de las aves se desarrolla en un huevo, sin contacto directo con el cuerpo materno (Ding et al., 2017). El tracto gastrointestinal de aves entra en contacto con microorganismos exógenos en el momento del nacimiento, encontrándose microbiota en el tracto gastrointestinal desde el primer día de vida (Ballou et al., 2016). Conforme el hospedador va creciendo, su microbiota se va haciendo más diversa, hasta llegar a una microbiota estable y muy compleja en el adulto (Pan & Yu, 2013; Pourabedin & Zhao, 2015; Shang et al., 2018). Diversos estudios han mostrado el papel fundamental de la microbiota intestinal en la salud, la absorción de nutrientes, el desarrollo del sistema inmunitarito, la resistencia a enfermedades y la productividad de las aves (Clavijo & Flórez, 2018; Pan & Yu, 2013; Qi et al., 2019; Shang et al., 2018). La microbiota de las aves, al igual que la del resto de animales, se encuentra influenciada por múltiples factores como el género (Lumpkins et al., 2008), la edad (Ballou et al., 2016),

el genotipo del hospedador (Pan & Yu, 2013; Zhao et al., 2013), la dieta (Oakley et al., 2014), las condiciones ambientales (Shang et al., 2018) o el material de suelo (Cressman et al., 2010; Nordentoft et al., 2011).

A nivel general, el intestino de pollos y gallinas se encuentra principalmente colonizado por bacterias de los filos *Firmicutes* y *Bacteroidetes*, que representan entre ambos filos en torno al 90% del total de la población microbiana, seguido de bacterias pertenecientes a los filos *Proteobacteria, Actinobacteria* y *Deferribacteres* (Oakley et al., 2014; Pourabedin & Zhao, 2015; Qi et al., 2019; Shi et al., 2019). El género más abundante en la microbiota intestinal es *Lactobacillus*, seguido de *Bacteroides, Enterococcus* y diversos géneros pertenecientes a la clase *Clostridia* (Ding *et al*., 2017; Rychlik, 2020). Sin embargo, la microbiota no es constante a lo largo del intestino, con diferencias a lo largo de las distintas regiones. El tracto gastrointestinal de pollos y gallinas está formado por el buche, proventrículo, molleja, duodeno, yeyuno, íleon, ciego, colon y cloaca, teniendo cada región una función metabólica diferente, lo cual conlleva modificaciones en la comunidad microbiana de cada región (Clavijo & Flórez, 2018; Shang et al., 2018; Yeoman et al., 2012). El buche se encuentra principalmente colonizado por especies de la familia *Lactobacillaceae*; el proventrículo y la molleja también se encuentran colonizados por *Lactobacillaceae* y por algunas especies del filo *Proteobacteria*; la microbiota del duodeno, yeyuno e íleon es bastante similar, incluyendo especies de las familias *Lactobacillaceae* y *Clostridiaceae*, así como *Enterococcus* y *Escherichia coli*/*Shigella* (Figura I.4); el ciego por su parte es la región con mayor densidad y diversidad bacteriana, estando colonizado mayoritariamente por bacterias de las familias *Ruminococcaceae, Lachnospiraceae* y *Clostridiaceae* (Figura I.4); por último la microbiota de colon, cloaca y fecal son bastante parecidas entre sí y muy similares a la microbiota del ciego, ya que el tiempo de retención en esta última parte es bastante corto y el contenido que sale del ciego es muy similar a las heces (Clavijo & Flórez, 2018; Pourabedin & Zhao, 2015; Rychlik, 2020).

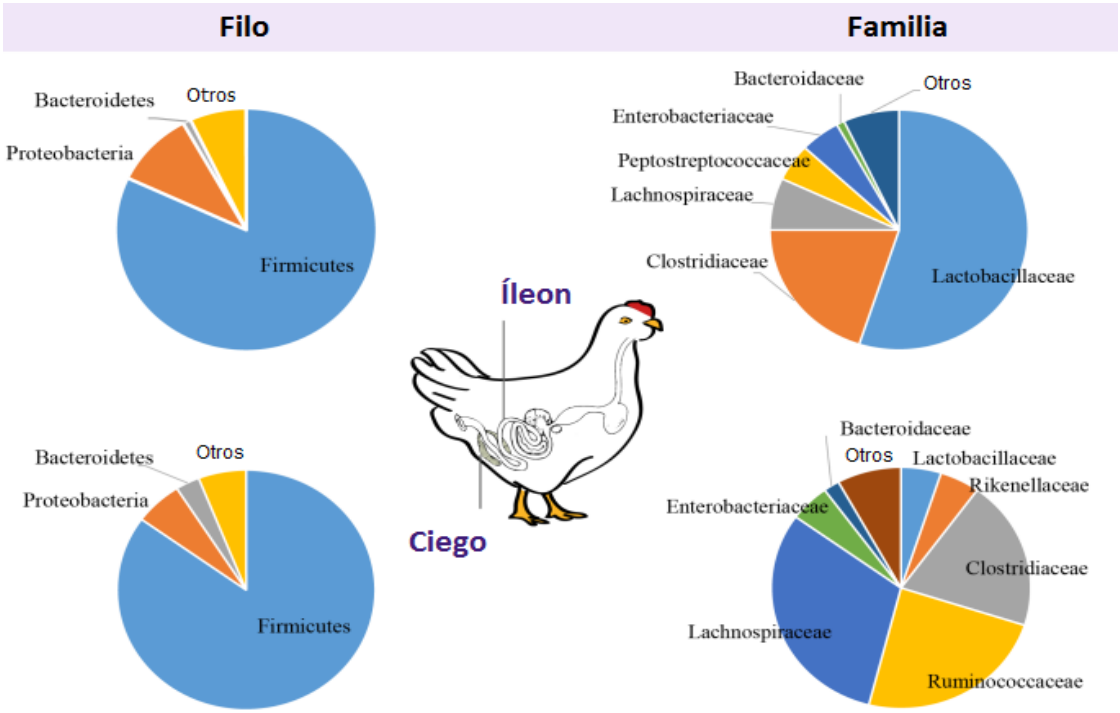

Figura I.4. Descripción de la abundancia relativa de los filos y familias bacterianas presentes en las comunidades microbianas del íleon y el ciego de aves de corral (imagen modificada de Pourabedin & Zhao, 2015).

2.3. Microbiota intestinal de peces

La colonización intestinal de las larvas de peces se produce a través de los huevos, el agua y los primeros alimentos. Esta colonización inicial es específica de especie, con diferencias controladas por la variación y composición de las proteínas de membrana de la superficie de los huevos (Larsen, 2014). Hasta el momento de la eclosión de los huevos las larvas permanecen estériles, y en el momento de la eclosión entran en contacto con las bacterias de la cubierta de los huevos. Posteriormente empiezan a beber agua, lo que ayuda que la microbiota intestinal se diversifique (Egerton et al., 2018). A medida que los individuos van creciendo, también aumenta su diversidad microbiana. Diversos estudios sobre microbiota intestinal de peces han mostrado gran diversidad a nivel tanto intra- como interespecífico. Los factores que pueden afectar a esta diversidad incluyen la dieta, estación, hábitat, sistema de crianza, edad, sexo, filogenia y genética del hospedador (Egerton et al., 2018; Piazzon et al., 2019). Por otra parte, también se ha comprobado que la densidad y el tipo de microbiota intestinal puede cambiar dentro de un mismo individuo, apareciendo diferencias en la microbiota presente en distintas partes del intestino, asociadas a diferencias fisiológicas de los animales (Clements et al., 2014;

Jones et al., 2018). El intestino de los peces se puede dividir en tres secciones principales: el tracto anterior (*foregut*), donde se lleva a cabo la absorción de nutrientes; el tracto medio (*midgut*), donde se realiza el transporte de macromoléculas, así como reacciones de inmunoestimulación; y el tracto posterior (*hindgut*), encargado de la captura de antígenos y de la estimulación del sistema inmunitario (Egerton et al., 2018; Estruch et al., 2015).

Estudios realizados mediante secuenciación masiva han señalado los filos *Proteobacteria*, *Firmicutes* y *Bacteroidetes* como los que presentan una abundancia relativa mayor en las comunidades microbianas del intestino de peces criados en sistemas de acuicultura, llegando a suponer entre los tres filos cerca del 90% de las bacterias, y siendo el filo de *Proteobacteria* el que presenta una mayor prevalencia (Ghanbari et al., 2015; Nikouli et al., 2018). Algunos trabajos mostraron también altos niveles de *Actinobacterias* (Estruch et al., 2015; Piazzon et al., 2019). A nivel de género, los más comunes son *Vibrio, Pseudomonas, Photobacterium, Acinetobacter, Acinetobacter, Aeromonas, Clostridium* y *Moraxella*, así como algunos géneros de bacterias del ácido láctico (*Lactobacillus, Streptococcus* o *Carnobacterium*) (Egerton et al., 2018; Estruch et al., 2015; Kormas et al., 2014). Con respecto a la microbiota intestinal de doradas (*Sparus aurata*) y lubinas (*Dicentrarchus labrax*), especies objeto de estudio de esta Tesis, ambas presentan una composición bacteriana similar. Los estudios realizados utilizando doradas y lubinas provenientes de acuicultura también han mostrado una predominancia de los filos *Proteobacteria, Firmicutes* y *Actinobacteria*, y niveles más bajos de *Bacteroidetes* (Estruch et al., 2015; Kormas et al., 2014; Nikouli et al., 2018). Con respecto a los géneros bacterianos, los que tienen mayor representación en el tracto gastrointestinal de ambas especies son *Photobacterium, Vibrio, Lactobacillus, Pseudomonas, Corynebacterium, Propionibacterium* y *Clostridium* (Estruch et al., 2015; Kormas et al., 2014).

3. Uso de antibióticos en la industria animal

Los antibióticos se han administrado en la industria animal desde su descubrimiento y durante más de 60 años para el control, tratamiento y la prevención de enfermedades infecciosas (Allen et al., 2013; Thacker, 2013). Se ha estimado que en torno al 50% de los antibióticos utilizados en América del Norte y Europa estaban destinados a animales de consumo (Capita & Alonso-Calleja, 2013). Estos, además, se han utilizado en alimentación animal desde los años 40 como agentes promotores del crecimiento

(APC, Antibióticos Promotores del Crecimiento) (Capita & Alonso-Calleja, 2013; Seal et al., 2013). Durante este periodo, las empresas ganaderas han incorporado dosis subterapéuticas de antibióticos como promotores del crecimiento (APCs) a la dieta de los animales, permitiendo mantener así altos niveles productivos del sector. Los posibles mecanismos de acción mediante los cuales los APCs aumentan el rendimiento de los animales incluyen la reducción de la carga bacteriana total, la eliminación de patógenos, el adelgazamiento de la mucosa intestinal y la modulación de su sistema inmunitario (Dibner & Richards, 2005; Niewold, 2007). Sin embargo, la mayoría de los estudios sugieren que el principal efecto de los APC parece estar relacionado con modificaciones en el equilibrio de las comunidades que conforman la microbiota intestinal, provocando cambios en los procesos digestivos y metabólicos de los animales, que se traducen en una mayor absorción de nutrientes y aumento significativo de peso (Kim et al., 2012; Lin et al., 2013; Unno et al., 2015). El efecto de estos compuestos sobre la composición de la microbiota intestinal podría explicar una disminución en la competencia por los nutrientes o la reducción de metabolitos microbianos que reducen el crecimiento (Capita & Alonso-Calleja, 2013; Looft et al., 2012). Además, con su uso no solo se lograba disminuir el riesgo potencial de desarrollar enfermedades infecciosas por patógenos como *Campylobacter*, *Salmonella*, *Listeria* o *Clostridium perfringens*, sino también incrementar la producción de carne. Sin embargo, el uso abusivo de los APC en la industria ganadera conlleva diferentes riesgos asociados, como la presencia de residuos de antibióticos en la comida de origen animal o la liberación de antibióticos al ambiente a través de las heces, y, sobre todo, la aparición y diseminación de genes o bacterias resistentes a la mayoría de agentes antimicrobianos utilizados extensivamente en ganadería (Capita & Alonso-Calleja, 2013; Ferri et al., 2017).

Se han encontrado evidencias de que los genes de resistencia a los antibióticos pueden transferirse a la microbiota humana, lo que hace que, en la actualidad, la resistencia a los antimicrobianos (AMR, *Antimicrobial Resistance*) se haya convertido en una amenaza global para la salud pública, siendo un problema de primer orden tanto para la salud de los animales como la humana (World Health Organization, 2021). La preocupación por la aparición de resistencia a los antibióticos ha ido creciendo y extendiéndose a nivel nacional e internacional, llegando a definirse por algunos organismos como una pandemia global o como uno de los mayores desafíos sanitarios del siglo XXI (World Health Organization, 2021). Dondequiera que se usen

antimicrobianos se pueden encontrar reservorios de resistencia, como en la microbiota humana y animal, sus entornos asociados, el agua, el suelo, el medio ambiente y muchos otros nichos ecológicos (Ferri et al., 2017; McEwen & Collignon, 2018; Woolhouse & Ward, 2013) (Figura I.5). Sin embargo, es ampliamente reconocido que su consumo es el principal factor de riesgo para la aparición de resistencia. Con respecto a los animales, hay numerosos casos de uso inapropiado de antibióticos, entre los que se encuentra su uso a dosis subterapéuticas para promover el crecimiento animal (Capita & Alonso-Calleja, 2013).

Estos problemas asociados al uso masivo de antimicrobianos hicieron que en los últimos 30 años saltara la alarma, concluyendo finalmente su prohibición en el caso de antibióticos promotores del crecimiento a partir de 2006 por parte de la UE (EC Regulation 1831, 2003). Estas restricciones se han ido extendiéndose a los países miembros de la UE y posteriormente por otros países como Estados Unidos, México o Corea del Sur, donde se ha prohibido o se ha limitado su uso (Maron et al., 2013; U.S. FDA, 2017; Unno et al., 2015).

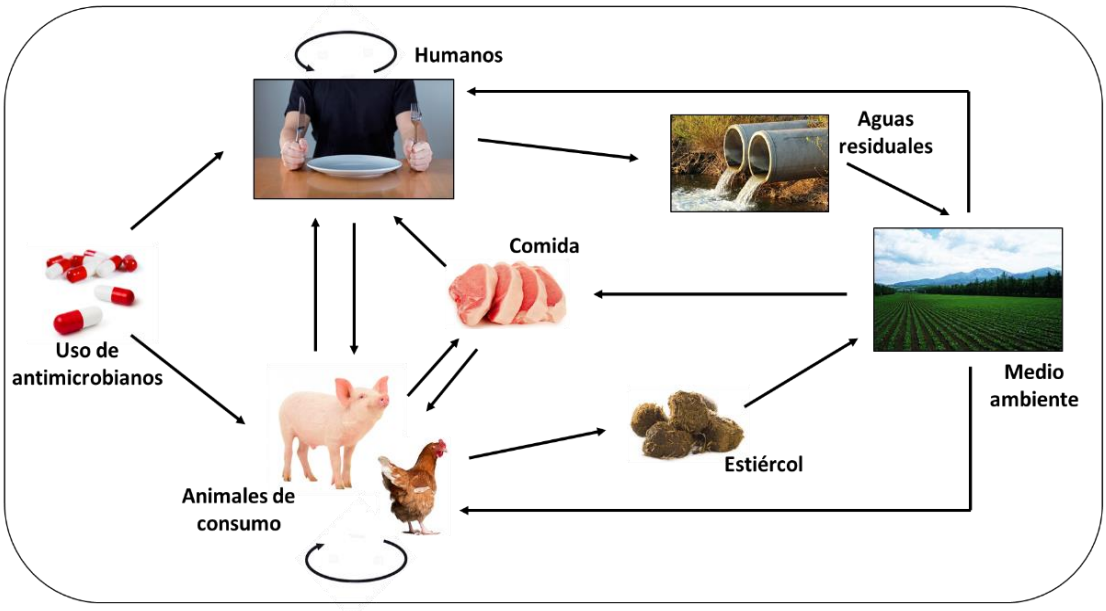

Figura I.5. Diferentes vías de propagación de bacterias resistentes a los antimicrobianos, pudiendo llegar de forma directa e indirecta a los humanos y a los animales de consumo.

4. Alternativas al uso de antibióticos

La prohibición de los antibióticos como promotores del crecimiento ha supuesto un aumento en la muerte de animales debido a infecciones por microorganismos patógenos, lo que puede llevar a un incremento del uso de antibióticos con fines terapéuticos, con los riesgos que esto conlleva (Capita & Alonso-Calleja, 2013). Además, esta prohibición ha llevado consigo un aumento en los costes de producción de los animales y por tanto un incremento en el precio final de los productos (Teillant & Laxminarayan, 2015). Todo esto, sumado al aumento de la población, hace necesaria la búsqueda nuevas estrategias para aumentar la producción de proteína animal reduciendo o eliminando el uso de antibióticos. En este contexto, diversos países han comenzado a desarrollar nuevas políticas y programas que permitan erradicar o reducir en lo posible el uso indiscriminado de estos compuestos en la industria agroalimentaria (Founou et al., 2016). Se ha mostrado un interés creciente por el desarrollo de nuevas productos y complementos biológicos capaces de: (1) reemplazar total o parcialmente el uso de antibióticos como promotores del crecimiento, (2) restringir su uso terapéutico cuando sea posible, y (3) tratar de evitar la aparición de resistencia en los microorganismos asociados a la industria ganadera (Cheng et al., 2014). Numerosos compuestos y aditivos de tipo alimentario se han propuesto como alternativas viables a los APCs, destacando los péptidos antimicrobianos, ácidos orgánicos, enzimas, probióticos, prebióticos o fitobióticos, entre otros (Cheng et al., 2014; Seal et al., 2013) (Figura I.6).

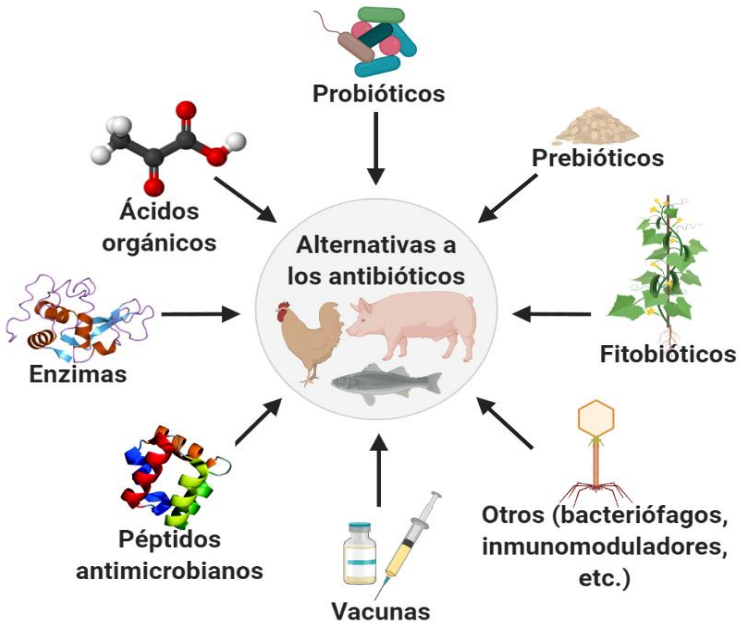

Figura I.6. Posibles compuestos y aditivos utilizados como alternativas a los antibióticos como terapéuticos y como promotores del crecimiento en alimentación de animales de consumo humano.

4.1. Enzimas

Las enzimas son proteínas biológicamente activas que facilitan la hidrólisis química de nutrientes en compuestos más pequeños que posibilitan su digestión y absorción. Algunas enzimas pueden facilitar la digestión de compuestos vegetales presentes en los alimentos, como celulosa o pectina, las cuales no pueden ser degradadas de forma natural. Esta capacidad para degradar compuestos y facilitar la absorción se ha relacionado con una mejora del rendimiento de los animales, caracterizada por un aumento del peso corporal, de la tasa de conversión del alimento o de la productividad (Thacker, 2013). Sin embargo, el mecanismo por cual las enzimas pueden actuar como promotores del crecimiento no se conoce con detalle, pero puede incluir cambios en la composición de la microbiota, prevención del daño causado por partes de las plantas en el interior del intestino o la descomposición de moléculas en compuestos potencialmente prebióticos (Huyghebaert et al., 2011). La mayor parte de las enzimas que se utilizan en la industria animal, entre las que destacan fitasas, carbohidrasas y proteasas, suelen estar producidas por bacterias, hongos o levaduras (Gadde et al., 2017; Thacker, 2013). La adición de estas enzimas en la dieta ha mostrado efectos beneficiosos sobre parámetros relacionados con la salud y el crecimiento de animales del sector avícola, porcino y piscícola, como una mayor digestibilidad de nutrientes, un aumento de la tasa de crecimiento o un incremento en la actividad enzimática intestinal (Castillo & Gatlin, 2015; Lei et al., 2017; Walk et al., 2018; Zuo et al., 2015).

La fitasa es una enzima que lleva a cabo la hidrolisis del ácido fítico, un compuesto abundante en el material vegetal, especialmente en semillas y fibras, e indigerible por parte de los animales. El ácido fítico sirve principalmente como almacenamiento de fósforo, pero también se puede asociar con cationes cargados positivamente, como calcio, magnesio, zinc, cobre, hierro o potasio, reduciendo su biodisponibilidad para los animales (Kumar et al., 2012). La presencia de ácido fítico también afectar de forma directa al estado fisiológico de los animales, produciendo efectos negativos en su crecimiento, la utilización de nutrientes y la absorción de minerales (Kumar et al., 2012). Además, se ha demostrado que dietas ricas en ácido fítico disminuyen la expresión de genes relacionados con el apetito y la absorción de glucosa en lechones y peces (Liu et al., 2014; Woyengo et al., 2012). Por tanto, la acción de la fitasa permite liberar el fósforo y otros minerales asociados al ácido fítico, aumentando con ello su disponibilidad para la absorción por parte de los animales (Cao et al., 2007; Romano & Kumar, 2018) (Figura I.7). Esta enzima

se encuentra de forma natural en los componentes vegetales y es producida por algunas bacterias intestinales, sin embargo, en algunos casos resulta insuficiente, pudiendo ocasionar deficiencias de fósforo y otros minerales que pueden conllevar a efectos adversos en los animales, como una disminución de la mineralización de los huesos (Romano & Kumar, 2018). Esto hace necesaria la suplementación de algunas dietas con fitasa exógena, mostrando en diversas investigaciones un aumento del crecimiento y una mejora de la salud de animales del sector porcino, avícola y piscícola. En pollos de engorde, la inclusión de fitasa ha conseguido mejorar la salud gastrointestinal, produciendo un incremento de SCFAS y un aumento de las poblaciones de *Lactobacillus* y *Enterococcus* en el íleon, los cuales pueden actuar como promotores del crecimiento (Ptak et al., 2015). En peces, la suplementación de la dieta con fitasa también ha mostrado resultados positivos. La adición de esta enzima en la dieta del pez dorado (*Carassius auratus*) produjo un aumento de peso y de la utilización de fosforo, así como una mejora en la tasa de conversión del alimento (Nie et al., 2017). En tilapia del Nilo, la acción de la fitasa también conllevó a un aumento de parámetros relacionados con el peso, produjo un incremento en los niveles de proteína y lípidos, y mejoró la respuesta inmune (Norag et al., 2018). Sin embargo, el estudio de los efectos de la fitasa sobre la composición y la diversidad de la microbiota intestinal mediante técnicas de secuenciación masiva, y su relación con parámetros de crecimiento de peces es prácticamente desconocido.

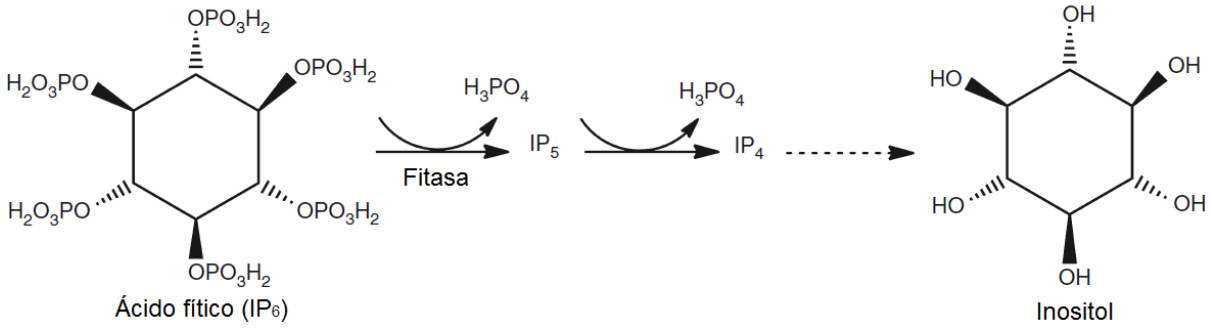

Figura I.7. Hidrólisis del ácido fítico por parte de la fitasa, liberando ácido fosfórico y generando inositol como producto final de la reacción (imagen modificada de Herrera Bravo de Laguna et al., 2015).

4.2. Probióticos

Los probióticos se definen como microorganismos vivos que, cuando se administran en cantidades adecuadas, confieren un beneficio para la salud del organismo hospedador (Hill et al., 2014). Las características más importantes que deben de presentar los microorganismos utilizados como probióticos son resistir las condiciones estomacales e intestinales, fijarse al epitelio intestinal, no ser patógenos y generar efectos beneficiosos para la salud (FAO, 2016). Los efectos beneficiosos que se atribuyen al consumo de probióticos suelen estar asociados a diversos mecanismos, los cuales incluyen la inducción de inmunomodulación, el aumento de la digestibilidad de nutrientes, la producción de sustancias antimicrobianas, la supresión de patógenos, la modulación de la microbiota y la mejora de la función de barrera del epitelio intestinal (FAO, 2016; Markowiak & Ślizewska, 2018). La actividad probiótica está asociada a género, especie o cepa, pudiendo emplearse una mezcla de diferentes organismos. Son numerosas las especies del dominio *Bacteria* y también algunas levaduras las que tradicionalmente se han utilizado como probióticos en alimentación animal para mejorar la salud y los parámetros productivos de los animales. La mayoría de estas especies están relacionadas con las bacterias del ácido láctico (BAL), como algunas especies de géneros de *Lactobacillaceae* (*Levilactobacillus, Limosilactobacillus, Lentilactobacillus, Lactobacillus, Lacticaseibacillus, Lactiplantibacillus,* entre otros), así como especies de *Enterococcus, Streptococcus, Lactococcus, Leuconostoc, Pediococcus, Bacillus, Bifidobacterium* o *Propionibacterium,* y finalmente entre los eucariotas, las especies de *Saccharomyces o Aspergillus* (Cheng et al., 2014; Gadde et al., 2017). De todos estos microorganismos, algunos de los más ampliamente utilizados como probióticos en alimentación animal incluyen a especies de los géneros *Bacillus* y *Enterococcus* y de la familia *Lactobacillaceae*.

Dentro de la familia *Lactobacillaceae*, numerosos géneros se han utilizado como probióticos en aves de corral, cerdos y peces, siendo de gran importancia las bacterias anteriormente englobadas dentro del género *Lactobacillus*. Diferentes estudios han mostrado un aumento del peso y una mejora de la productividad en pollos de engorde, lechones y peces tras la suplementación de una dieta enriquecida con diferentes especies lactobacilos (Cho et al., 2011; Gadde et al., 2017; Mookiah et al., 2014; Veizaj-Delia et al., 2010). La adición de bacterias pertenecientes a este grupo también ha conllevado otros efectos en los animales, como una mejora en la salud intestinal y cambios en la

composición de su microbiota, caracterizados por un aumento de bacterias beneficiosas o la inhibición de potenciales patógenos como determinadas cepas de *E. coli* (Gadde et al., 2017; Pieper et al., 2009). En un estudio de Wang et al. (2019) en el cual se ha suplementado la dieta de lechones destetados con una mezcla de *Lactobacillus fermentum* y *Pediococcus acidilactici*, se ha asociado con una alteración de la estructura de las comunidades microbianas del ciego y el colon, debida principalmente al aumento de las poblaciones de lactobacilos en la microbiota intestinal. En pollos, una dieta probiótica que incluía *Lactobacillus acidophilus* produjo igualmente un aumento de los lactobacilos y una reducción de *E. coli* en el ciego de pollos de engorde (Zhang & Kim, 2014).

Con respecto al género *Bacillus*, distintas especies pertenecientes a este género se han empleado como potenciales probióticos en alimentación animal, siendo *Bacillus subtilis* la especie comúnmente utilizada. La adición de esta especie ha conseguido no solo mejorar la salud en cerdos, aves y diversas especies acuícolas, sino también mejoras en su crecimiento y su sistema inmunitario (Ahmed et al., 2014; Luise et al., 2019; Martínez Cruz et al., 2012; Park & Kim, 2014). La suplementación de la dieta con este probiótico también ha mostrado cambios en la composición de la microbiota intestinal. En un estudio realizado por Khan & Chousalkar (2021), la inclusión de varias cepas de *Bacillus* no produjo alteraciones en el comportamiento general de las comunidades microbianas del intestino de gallinas, pero sí que produjo un aumento de las poblaciones de los filos *Bacteroidetes* y *Proteobacteria*, y una reducción de algunos patógenos como por ejemplo *Salmonella*. Esta reducción de patógenos también se ha visto en un estudio de Abou-Kassem et al. (2021) realizado con codornices, donde también disminuyeron las poblaciones de *E. coli* y otros coliformes en las aves suplementadas con *Bacillus toyonensis*.

Por su parte, algunas bacterias pertenecientes al género *Enterococcus* también se han empleado como potenciales probióticos en alimentación animal, siendo *Enterococcus faecium* la especie más utilizada. La adición de esta especie en la dieta de lechones ha mostrado un aumento del crecimiento y mejoras en su sistema inmunitario, así como un incremento de bacterias potencialmente beneficiosas (lactobacilos) y una disminución de coliformes en la microbiota intestinal (Mallo et al., 2010; Sukegawa et al., 2014). La adición de la cepa probiótica *E. faecium* NCIMB 10415 en pollos de engorde también produjo un aumento del peso corporal y de la tasa de conversión del alimento, así como un incremento en los niveles de bacterias del ácido láctico en el íleon y las heces (Samli

et al., 2007). Otra especie potencialmente probiótica de este género es *Enterococcus faecalis*, la cual se ha utilizado en estudios con pollos, cerdos y también en acuicultura, mostrando resultados positivos en el crecimiento y la salud animal, así como cambios en la composición bacteriana del intestino, reduciendo los niveles de patógenos y favoreciendo a las bacterias beneficiosas (Baños et al., 2019; Li et al., 2017; Rodriguez-Estrada et al., 2013; Song et al., 2016). En gallinas, la suplementación de la dieta con *E. faecalis* produjo un aumento de *Bacteroidetes* y una reducción de *Firmicutes* (Song et al., 2019), lo cual se ha relacionado con un aumento del peso de las gallinas durante la producción de huevos (Videnska et al., 2014). La cepa UGRA10 de *E. faecalis* presenta propiedades tecnológicas, funcionales y potencialmente probióticas, como son la resistencia a altas concentraciones de bilis o la capacidad para estimular la respuesta inmunitaria en animales (Baños, 2016; Cebrián et al., 2012). Además, *E. faecalis* UGRA10 se ha utilizado como suplemento para la dieta de trucha arcoíris, consiguiendo reducir los niveles del patógeno *Lactococcus garviae* (Baños et al., 2019). Sin embargo, hasta la fecha no se han realizado estudios que investiguen los efectos potencialmente probióticos de esta cepa sobre la producción de huevos y la microbiota intestinal de gallinas ponedoras, por lo que su uso se aborda por primera vez en el Capítulo I de esta Tesis.

4.3. Extractos de plantas o fitobióticos

Los extractos de plantas, también conocidos como fitobióticos, son compuestos bioactivos naturales derivados de plantas y que incorporados en la alimentación animal pueden mejorar la productividad (Gheisar & Kim, 2018). Son numerosos los productos derivados de las plantas que han mostrado propiedades beneficiosas y que se han categorizado como potenciales fitobióticos en alimentación animal, particularmente por sus capacidades para modular la microbiota intestinal, reforzar la respuesta inmunitaria y también en algunos casos por sus actividades como antimicrobianos, antioxidantes y antiestrés (Hashemi & Davoodi, 2011; Vondruskova et al., 2010). Estas propiedades beneficiosas suelen ser derivadas de los componentes activos de las plantas, siendo la mayoría de ellos metabolitos secundarios como terpenoides, compuestos fenólicos, glucósidos y alcaloides (Huyghebaert et al., 2011). Aunque los mecanismos de acción de estas sustancias no están totalmente aclarados, se han propuesto varios mecanismos relacionados con su actividad biológica y sus efectos sobre la salud y el crecimiento de

los animales. Entre estos, se incluyen la ruptura de la membrana celular de patógenos (antimicrobianos), la modificación de la superficie celular, la activación del sistema inmunitario (inmunomodulación) o la promoción de bacterias beneficiosas en el tracto intestinal (Diaz-Sanchez et al., 2015).

En los últimos años, numerosos compuestos potencialmente fitobióticos procedentes de diferentes especies de plantas, entre las que se incluyen el tomillo, orégano, romero, ajo, cebolla, jengibre, comino, cilantro o canela, entre otras, se han utilizado por su potencial para mejorar la calidad nutricional de algunas dietas y tratar de reducir el uso de antibióticos en alimentación animal, tanto en la industria avícola, porcina y acuicultura. Algunos de estos compuestos han mostrado un aumento del crecimiento y la productividad, y/o una mejora de la tasa de conversión del alimento en aves, cerdos y peces (Gadde et al., 2017; Liu et al., 2018; Reverter et al., 2014; Windisch et al., 2008). En este sentido, cerdos alimentados con extracto de ajo, tomillo, clavo y orégano han mostrado un incremento del peso corporal y una mejora en su rendimiento de carne (Liu et al., 2008; Oetting et al., 2006; Tatara et al., 2008). La adición de orégano, estevia o comino también han mostrado resultados similares en pollos de engorde, con aumento del peso y mejora de la tasa de conversión del alimento (Jahan et al., 2016; Peng et al., 2016; Sadeghi et al., 2016). Con respecto a los peces, meros, lenguados y tilapias del Nilo alimentados con extractos de plantas como ajo, jengibre o albahaca, han mostrado una mayor ganancia de peso que los peces alimentados con dietas tradicionales (revisado en Reverter et al., 2014). Por otra parte, el uso de estos compuestos además de suponer una mejora en los parámetros productivos de los animales, también han mostrado capacidad de inducir otros cambios, como alteraciones morfológicas, mejoras en el sistema inmunitario de los animales o cambios beneficiosos en la composición de la microbiota intestinal (aumento de cepas beneficiosas y disminución de patógenos) (Clavijo & Flórez, 2018; Lee & Gao, 2012; Ren et al., 2019). Por ejemplo, la utilización de ingredientes activos derivados de plantas (carvacrol, cinamaldehido y eugenol) produjo un incremento en las poblaciones de algunas especies con características probióticas del género *Lactobacillus* en el buche de gallinas ponedoras (Ren et al., 2019). Con respecto a la reducción de patógenos, algunos compuestos derivados de orégano o canela han mostrado una disminución de los niveles de *E. coli* o *Eimeria*, y por tanto la reducción de la mortalidad de aves y cerdos (Gheisar & Kim, 2018). La adición de harina de hoja de cilantro vietnamita y de betel ha también ha mostrado una disminución de bacterias

patógenas en el intestino de pollos de engorde, como *E. coli*, *Salmonella* y *Staphylococcus aureus* (Basit et al., 2020).

Dentro de los fitobióticos, algunos de los más importantes son los extractos de plantas del género *Allium*, principalmente los procedentes de ajo (*Allium sativum*) y cebolla (*Allium cepa*). Son muchos los trabajos que han demostrado que algunos compuestos derivados del ajo tienen actividad biológica relacionada con la salud y el crecimiento, principalmente en alimentación animal (Guillamón et al., 2021; Valenzuela-Gutiérrez et al., 2021). Estudios realizados por Yan et al. (2012) en cerdos mostraron un aumento del crecimiento y de la digestibilidad de nutrientes de los animales suplementados con polvo de ajo. Otro estudio realizado con cerdas lactantes demostró un aumento del peso y un mayor desarrollo de los órganos del tracto gastrointestinal en las cerdas suplementadas con extractos de ajo (Tatara et al., 2005). Resultados similares se han obtenido en pollos, con una mejora de los parámetros productivos y de la actividad metabólica, y una reducción de *E. coli* en el microbioma intestinal (Aji et al., 2011; Ren et al., 2019). También se han obtenido efectos beneficiosos utilizando extractos de *Allium* en acuicultura; así el trabajo de Büyükdeveci et al. (2018) con trucha arcoíris suplementada con extracto de ajo mostró un aumento de la tasa de crecimiento y una mayor abundancia relativa de *Clostridium* y *Exiguobacterium* en la microbiota del tracto gastrointestinal, los cuales pueden aportar ácidos grasos esenciales, vitaminas y lípidos a los peces, mejorando así la salud y el crecimiento.

Los beneficios para la salud de los animales inducidos por el suplemento alimenticio con productos derivados del ajo, parecen estar relacionados con compuestos bioactivos organosulfurados como ajoeno, alicina, isoalicina, propil propano tiosulfinato (PTS) o propil propano tiosulfonato (PTSO) (Guillamón et al., 2021; Valenzuela-Gutiérrez et al., 2021). La alicina ha sido uno de los compuestos más estudiados, mostrando la suplementación de la dieta de cerdos con este compuesto un aumento de la diversidad microbiana y un incremento en los niveles de *Prevotella*, el cual está relacionado con la fermentación y la hidrólisis de proteínas y carbohidratos (Liu et al., 2018). La alicina también ha mostrado un aumento del crecimiento de diferentes especies de peces, además de mostrar capacidad antimicrobiana frente a numerosas bacterias patógenas de peces como *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Streptococcus agalactiae* o *Staphylococcus aureus*, entre otras (Lee & Gao, 2012). Algunos estudios han evaluado los efectos de otros dos compuestos organosulfurados

derivados del género *Allium*, como el propil propano tiosulfinato (PTS), y especialmente el propil propano tiosulfonato (PTSO) (Guillamón et al., 2021). Estas moléculas se generan cuando se producen daños en los tejidos de plantas del género *Allium* y la enzima alinasa degrada los compuestos iniciales presentes en las células, produciendo estos compuestos organosulfurados (Lira et al., 2020) (Figura I.8). La adición de PTSO a la dieta de pollos de engorde produjo una reducción de los niveles de potenciales patógenos como *Salmonella*, *Campylobacter* y especies de *Clostridium* (Peinado et al., 2013; Peinado et al., 2012). Otro estudio con pollos realizado por Ruiz et al. (2015) mostró el efecto del PTSO en la composición de la microbiota asociada a la mucosa del íleon, produciendo un aumento de las bifidobacterias. Por su parte, la suplementación de la dieta de gallinas ponedoras con PTSO produjo un aumento del peso de los huevos y mostró un incremento en las poblaciones de *Lactobacillus* y *Bifidobacterium*, y una reducción de enterobacterias en las heces de los animales en un estudio realizado utilizando técnicas dependientes de cultivo (Abad et al., 2021). El PTSO también se ha utilizado en la industria porcina, obteniendo resultados positivos en el tracto gastrointestinal de cerdas, caracterizados por una reducción de patógenos como *E. coli y S. typhimurium* (Ruiz et al., 2010), y provocando una reducción de procesos diarreicos e inflamación causada por *Escherichia coli* en lechones (Liu et al., 2013). Además, en un estudio reciente de Lira et al. (2020) confirmó la seguridad del PTSO a nivel toxicológico y a las dosis empleadas. Todos estos resultados hacen que estos compuestos organosulfurados puedan ser

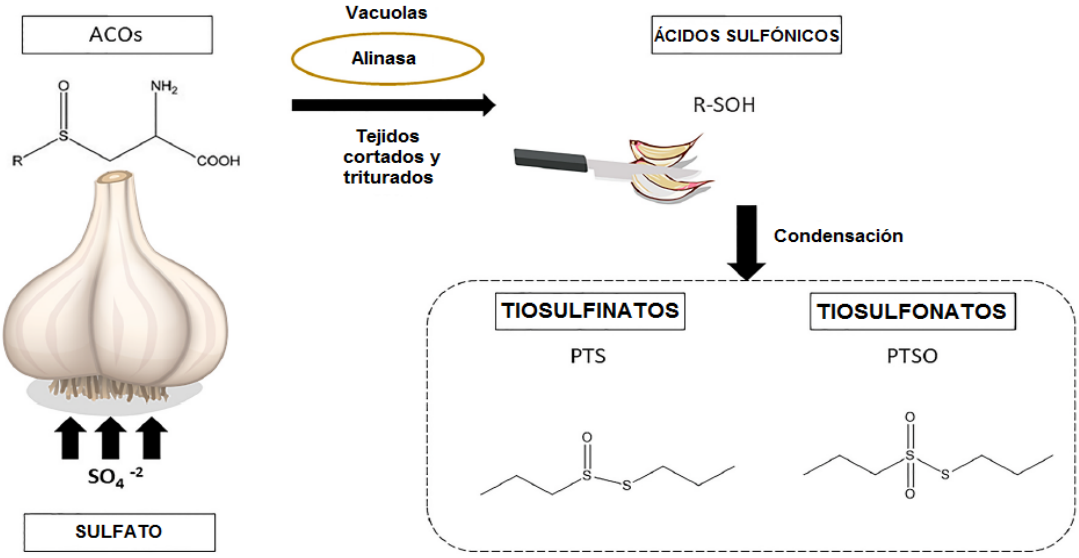

Figura I.8. Vías bioquímicas y estructuras químicas de los compuestos organosulfurados. S-alqu(en)il-cisteína sulfóxido (ACOs); Propil-propano-tiosulfonato (PTSO); Propil-propano-tiosulfinato (PTS) (imagen modificada de Lira et al., 2020).

alternativas en la preparación de dietas seguras y que permitan reducir o eliminar el uso de antibióticos con fines terapéuticos y promotores del crecimiento, pudiendo además mejorar el rendimiento y la salud de los animales. Sin embargo, pocos trabajos han utilizado técnicas de secuenciación masiva para estudiar en profundidad los efectos de los extractos derivados de *Allium* sobre la microbiota intestinal y su relación con el crecimiento y la productividad de animales de los sectores avícola, porcino y piscícola. Por tanto, el objetivo de los Capítulos II a V de esta Tesis se centrará en investigar el uso de estos compuestos en gallinas ponedoras, lechones, doradas y lubinas.

5. Uso de harina y aceite de pescado, problemas asociados y posibles alternativas

En los últimos años se ha planteado otro problema asociado a la alimentación de especies piscícolas, como es el uso abusivo de harina y aceite de pescado para la elaboración de dietas (Gasco et al., 2018). El incremento constante de la producción en la acuicultura en los últimos años y el uso excesivo de estos compuestos derivados del pescado, ha redundado paralelamente en un incremento de los precios. Además, la formulación de dietas para peces elaboradas a base de harina y aceite de pescado actualmente no se considera sostenible, al menos a largo plazo (Gasco et al., 2018; Oliva-Teles et al., 2015). Por otra parte, puede que no sea éticamente correcto recolectar peces para elaborar piensos de especies acuáticas, los cuales podrían utilizarse directamente como alimento para humanos (Olsen & Hasan, 2012). Por tanto, ha surgido la necesidad de reducir su uso, buscando para ello alternativas que puedan satisfacer los requerimientos nutricionales de los peces y mejorar en lo posible la salud y su crecimiento.

Los piensos suplementados con extractos de plantas han sido considerados como alternativa a los compuestos derivados del pescado por su gran disponibilidad y bajos precios. Una de sus principales aplicaciones es como sustitutos de las harinas y aceites de pescado en ciertas dietas, sin embargo, sigue siendo un desafío su empleo en aquellas especies carnívoras donde se ha asociado a la aparición de efectos adversos, como la disminución en el crecimiento y la salud de los peces (Gasco et al., 2018). Otra de las propuestas desarrollada en los últimos años ha sido el uso de microalgas como alternativa a los compuestos derivados de pescado en las dietas de acuicultura (Shah et al., 2018). El término microalga utilizado en ficología aplicada generalmente incluye a las algas microscópicas y las bacterias fotosintéticas (como las cianobacterias) (García et al., 2017). De aquí en adelante se utilizará el término microalga para referirse a estos grupos.

Introducción

Las microalgas tienen gran potencial para su uso en acuicultura, dado que presentan una gran fuente de proteínas, lípidos, vitaminas, minerales, pigmentos, entre otros (Roy & Pal, 2014). Estos aditivos han mostrado mejoras en el crecimiento de especies acuáticas debido al aumento de triglicéridos y proteínas en músculo, también un aumento en los niveles de ácidos grasos omega-3, una mayor resistencia a las enfermedades y una mayor actividad fisiológica (Ansari et al., 2021). Además, las microalgas presentan otras ventajas adicionales, como su capacidad de crecer en numerosos tipos de hábitats, e incluso muchas de las especies tienen mayor capacidad de producción que las plantas, por lo cual se pueden multiplicar con muy pocos requerimientos nutricionales (Hemaiswarya et al., 2011). Sin embargo, las microalgas también presentan ciertos inconvenientes, como su elevado coste de producción, la posibilidad de contaminación de los cultivos y la presencia de paredes celulares poco digeribles en ciertas especies (Shah et al., 2018). Esta dificultad para digerir las microalgas puede provocar inflamación intestinal, incremento de la permeabilidad intestinal y alteraciones en los procesos de digestión y absorción de nutrientes (Cerezo-Ortega et al., 2021). Todo esto puede provocar problemas de salud en los animales, por lo que algunas investigaciones han propuesto el uso de hidrolizados de microalgas, los cuales han mostrado un aumento en la absorción de nutrientes, una mayor actividad enzimática del intestino, y han conseguido mantener o aumentar el crecimiento de los peces (Galafat et al., 2020). Así, en los últimos años ha aumentado exponencialmente el número de estudios sobre el uso de microalgas crudas e hidrolizadas en dietas para animales acuáticos, indicando su gran potencial como aditivos para piensos de acuicultura (Chen et al., 2021; Roy & Pal, 2014; Shah et al., 2018). Algunas de las microalgas más utilizadas en la elaboración de dietas para acuicultura incluyen especies de los géneros *Chlorella*, *Scenedesmus*, *Nanofrustulum*, *Tetraselmis*, *Arthrospira* o *Nannochloropsis* (Chen et al., 2021; Shah et al., 2018).

El género *Arthrospira* (*Cyanobacteria*) es conocido por su alto contenido en proteínas, ácidos grasos poliinsaturados como el ácido gamma-linolénico, vitaminas A y B_{12}, minerales y pigmentos con actividad antioxidante como los carotenoides (Galafat et al., 2020). Dentro de este género, la especie *A. platensis* (también llamada *Spirulina platensis* (Guiry & Guiry, 2021)) es una de las más ampliamente utilizadas para la elaboración de piensos para acuicultura. *A. platensis* es una cianobacteria verde azulada de agua dulce, con un contenido en proteínas cercano al 70%, con alto contenido en vitaminas, minerales y pigmentos, y un perfil de aminoácidos y ácidos grasos equilibrado

(Niccolai et al., 2019; Ronda et al., 2012). En los últimos años, las investigaciones se han centrado en el uso de esta especie como posible sustituto a la harina de pescado o como aditivo funcional para piensos de acuicultura (Rosas et al., 2019). La inclusión de *A. platensis* en la dieta se ha evaluado en diferentes especies de peces, no apareciendo resultados negativos en su crecimiento, ni afectando a la salud o la utilización de nutrientes de los animales (revisado en Rosas et al., 2019). En un estudio reciente de Akter et al. (2021) con *Ompok pabda,* especie perteneciente a los silúridos, se observó que la inclusión de *A. platensis* lleva asociada un aumento del peso corporal y de la tasa de crecimiento, así como un incremento en el contenido aminácidos. En otros estudios no aparecieron diferencias significativas crecimiento y en la utilización de nutrientes de juveniles de dorada suplementados con *Arthrospira*, pero sí que se observaron mejoras en la actividad de las enzimas intestinales (tripsina, quimotripsina y leucina aminopeptidasa) y un aumento de la longitud y la capacidad de absorción de las microvellosidades intestinales (Galafat et al., 2020). No obstante, sólo un par de trabajos recientes han estudiado los efectos de la inclusión de *A. platensis* sobre la composición y la diversidad de la microbiota intestinal. El trabajo realizado por Rosenau et al. (2021) ha mostrado un aumento de la diversidad microbiana del pez gato africano (*Clarias gariepinus*) suplementado con *A. platensis*, sin afectar a la estructura general de las comunidades microbianas. En otro estudio, la utilización *A. platensis* en mero gigante de Sabah produjo una mejora en el crecimiento de los peces y modificaciones en el perfil de las bacterias dominantes del intestino, caracterizadas por un aumento de especies del género *Vibrio* (Man et al., 2020).

Por su parte, *Nannochloropsis gaditana* es una microalga que se caracteriza por su alto contenido en ácido grasos poliinsaturados, como ácido eicosapentanoico (EPA), ácido araquidónico (ARA) y ácido docosahexanoico (DHA), de especial importancia en la nutrición de especies marinas en etapas tempranas del desarrollo (Ayala et al., 2020). Además, presenta un alto contenido proteico de alta calidad, de vitaminas y pigmentos (como clorofila a y carotenoides) (Cerezo-Ortega et al., 2021; Wang et al., 2014). Estas características hacen a *N. gaditana* una de las microalgas más utilizadas en nutrición de especies de acuicultura, especialmente durante las etapas larvarias (Jorge et al., 2019). Los efectos de *N. gaditana* sobre el crecimiento y la salud han sido estudiados en algunas investigaciones recientes utilizando diferentes especies de peces como modelo de estudio, siendo la dorada la especie más ampliamente estudiada. Un estudio reciente llevado a

cabo por Ayala et al. (2020) utilizando 2,5 y 5% de *N. gaditana* en la dieta de juveniles de dorada mostró un aumento del tamaño y el peso corporal. Por otra parte, la inclusión de extractos lipídicos de *N. gaditana* tampoco mostró diferencias en el crecimiento de juveniles de dorada, pero sí aparecieron mejoras en la composición y la pigmentación del músculo (Sales et al., 2021). También se ha observado que *N. gaditana* no provoca alteraciones de la actividad enzimática intestinal de doradas (Jorge et al., 2019). En lubinas, sí que se ha observado una mejora en la respuesta enzimática antioxidante del intestino y del hígado de los peces alimentados con *Nannochloropsis* sp. (Castro et al., 2020). Con respecto a la acción de esta microalga sobre la microbiota intestinal, el trabajo de Cerezo-Ortega et al. (2021) no ha mostrado cambios en la composición y en la funcionalidad de la microbiota intestinal de doradas tras la adición de 5% de biomasa hidrolizada de *N. gaditana*, sugiriendo la posibilidad de emplearla como alternativa a la harina de pescado.

Teniendo en cuenta lo expuesto en este apartado, los efectos potenciales de *A. platensis* y *N. gaditana* sobre la composición y la diversidad de la microbiota intestinal de doradas y lubinas en etapas tempranas del desarrollo siguen siendo prácticamente desconocidos. Por tanto, en esta Tesis se va a estudiar la inclusión de *A. platensis* cruda e hidrolizada en larvas de dorada (Capítulo VI), y la adición de biomasa cruda de *A. platensis* y *N. gaditana* junto con diferentes niveles de fitasa en la dieta de juveniles de lubina (Capítulo VII).

6. Referencias

Abad, P., Arroyo-Manzanares, N., Ariza, J. J., Baños, A., & García-Campaña, A. M. (2021). Effect of Allium Extract Supplementation on Egg Quality, Productivity, and Intestinal Microbiota of Laying Hens. *Animals*, *11*(1), 41. https://doi.org/10.3390/ani11010041

Abou-Kassem, D. E., Elsadek, M. F., Abdel-Moneim, A. E., Mahgoub, S. A., Elaraby, G. M., Taha, A. E., … Ashour, E. A. (2021). Growth, carcass characteristics, meat quality, and microbial aspects of growing quail fed diets enriched with two different types of probiotics (Bacillus toyonensis and Bifidobacterium bifidum). *Poultry Science*, *100*(1), 84–93. https://doi.org/10.1016/J.PSJ.2020.04.019

Ahmed, S. T., Islam, M. M., Mun, H. S., Sim, H. J., Kim, Y. J., & Yang, C. J. (2014). Effects of Bacillus amyloliquefaciens as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. *Poultry Science*, *93*(8), 1963–1971. https://doi.org/10.3382/ps.2013-03718

Aji, S. B., Ignatius, K., Ado, A. A. Y., Nuhu, J. B., Abdulkarim, A., Aliyu, U., … Numan, P. T. (2011). Effects of Feeding Onion (Allium cepa) and Garlic (Allium sativum)

on Some Performance Characteristics of Broiler Chickens. *Research Journal of Poultry Sciences*, *4*(2), 22–27. https://doi.org/10.3923/rjpscience.2011.22.27

Akter, T., Hossain, A., Islam, M. R., Hossain, M. A., Das, M., Rahman, M. M., … Abdel-Tawwab, M. (2021). Effects of spirulina (Arthrospira platensis) as a fishmeal replacer in practical diets on growth performance, proximate composition, and amino acids profile of pabda catfish (Ompok pabda). *Journal of Applied Aquaculture*. https://doi.org/10.1080/10454438.2021.1936740

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., & Casey, T. A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in Microbiology*, *21*(3), 114–119. https://doi.org/10.1016/J.TIM.2012.11.001

Ansari, F. A., Guldhe, A., Gupta, S. K., Rawat, I., & Bux, F. (2021). Improving the feasibility of aquaculture feed by using microalgae. *Environmental Science and Pollution Research International*, *28*(32), 43234–43257. https://doi.org/10.1007/S11356-021-14989-X

APROMAR. (2021). La Acuicultura en España. *Asociación Empresarial de Acuicultura de España (APROMAR)*.

Ayala, M. D., Galián, C., Fernández, V., Chaves-Pozo, E., de la Serrana, D. G., Sáez, M. I., … Arizcun, M. (2020). Influence of Low Dietary Inclusion of the Microalga Nannochloropsis gaditana (Lubián 1982) on Performance, Fish Morphology, and Muscle Growth in Juvenile Gilthead Seabream (Sparus aurata). *Animals*, *10*(12), 1–18. https://doi.org/10.3390/ANI10122270

Ballou, A. L., Ali, R. A., Mendoza, M. A., Ellis, J. C., Hassan, H. M., Croom, W. J., & Koci, M. D. (2016). Development of the chick microbiome: How early exposure influences future microbial diversity. *Frontiers in Veterinary Science*, *3*(JAN). https://doi.org/10.3389/fvets.2016.00002

Baños, A. (2016). Aplicación de la tecnología de las barreras en el desarrollo de as-48 como bioconservante alimentario. Estudio de probiosis de una cepa productora de as-48. Tesis Doctoral, Universidad de Granada.

Baños, A., Ariza, J. J., Nuñez, C., Gil-Martínez, L., García-López, J. D., Martínez-Bueno, M., & Valdivia, E. (2019). Effects of Enterococcus faecalis UGRA10 and the enterocin AS-48 against the fish pathogen Lactococcus garvieae. Studies in vitro and in vivo. *Food Microbiology*, *77*, 69–77. https://doi.org/10.1016/j.fm.2018.08.002

Basit, M. A., Kadir, A. A., Loh, T. C., Aziz, S. A., Salleh, A., Zakaria, Z. A., & Idris, S. B. (2020). Comparative Efficacy of Selected Phytobiotics with Halquinol and Tetracycline on Gut Morphology, Ileal Digestibility, Cecal Microbiota Composition and Growth Performance in Broiler Chickens. *Animals*, *10*(11), 2150. https://doi.org/10.3390/ani10112150

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E. G., Mackie, R., … Zhu, W. (2016). Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environmental Microbiology*, *18*(5), 1566–1577. https://doi.org/10.1111/1462-2920.13272

Büyükdeveci, M. E., Balcázar, J. L., Demirkale, İ., & Dikel, S. (2018). Effects of garlic-supplemented diet on growth performance and intestinal microbiota of rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *486*, 170–174. https://doi.org/10.1016/J.AQUACULTURE.2017.12.022

Campbell, J. M., Crenshaw, J. D., & Polo, J. (2013). The biological stress of early weaned

piglets. *Journal of Animal Science and Biotechnology*. BioMed Central. https://doi.org/10.1186/2049-1891-4-19

Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., … Li, D. (2007). Application of microbial phytase in fish feed. *Enzyme and Microbial Technology*, *40*(4), 497–507. https://doi.org/10.1016/J.ENZMICTEC.2007.01.007

Capita, R., & Alonso-Calleja, C. (2013). Antibiotic-Resistant Bacteria: A Challenge for the Food Industry. *Critical Reviews in Food Science and Nutrition*, *53*(1), 11–48. https://doi.org/10.1080/10408398.2010.519837

Castillo, S., & Gatlin, D. M. (2015). Dietary supplementation of exogenous carbohydrase enzymes in fish nutrition: A review. *Aquaculture*, *435*, 286–292. https://doi.org/10.1016/J.AQUACULTURE.2014.10.011

Castro, C., Coutinho, F., Iglesias, P., Oliva-Teles, A., & Couto, A. (2020). Chlorella sp. and Nannochloropsis sp. Inclusion in Plant-Based Diets Modulate the Intestine and Liver Antioxidant Mechanisms of European Sea Bass Juveniles. *Frontiers in Veterinary Science*, *7*, 1094. https://doi.org/10.3389/FVETS.2020.607575/BIBTEX

Cebrián, R., Baños, A., Valdivia, E., Pérez-Pulido, R., Martínez-Bueno, M., & Maqueda, M. (2012). Characterization of functional, safety, and probiotic properties of Enterococcus faecalis UGRA10, a new AS-48-producer strain. *Food Microbiology*, *30*(1), 59–67. https://doi.org/10.1016/J.FM.2011.12.002

Cerezo-Ortega, I. M., Zeo-Sánchez, D. E. Di, García-Márquez, J., Ruiz-Jarabo, I., Sáez-Casado, M. I., Balebona, M. C., … Tapia-Paniagua, S. T. (2021). Microbiota composition and intestinal integrity remain unaltered after the inclusion of hydrolysed Nannochloropsis gaditana in Sparus aurata diet. *Scientific Reports*, *11*(1), 1–16. https://doi.org/10.1038/s41598-021-98087-5

Chen, F., Leng, Y., Lu, Q., & Zhou, W. (2021). The application of microalgae biomass and bio-products as aquafeed for aquaculture. *Algal Research*, *60*, 102541. https://doi.org/10.1016/J.ALGAL.2021.102541

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., & Yuan, Z. (2014). Antibiotic alternatives: The substitution of antibiotics in animal husbandry? *Frontiers in Microbiology*, *5*, 217. https://doi.org/10.3389/fmicb.2014.00217

Cho, J. H., Zhao, P. Y., & Kim, I. H. (2011). Probiotics as a dietary additive for pigs: A review. *Journal of Animal and Veterinary Advances*. https://doi.org/10.3923/javaa.2011.2127.2134

Clavijo, V., & Flórez, M. J. V. (2018). The gastrointestinal microbiome and its association with the control of pathogens in broiler chicken production: A review. *Poultry Science*, *97*(3), 1006–1021. https://doi.org/10.3382/ps/pex359

Clements, K. D., Angert, E. R., Montgomery, W. L., & Choat, J. H. (2014). Intestinal microbiota in fishes: What's known and what's not. *Molecular Ecology*. Blackwell Publishing Ltd. https://doi.org/10.1111/mec.12699

Crespo-Piazuelo, D., Estellé, J., Revilla, M., Criado-Mesas, L., Ramayo-Caldas, Y., Óvilo, C., … Folch, J. M. (2018). Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. *Scientific Reports*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-30932-6

Cressman, M. D., Yu, Z., Nelson, M. C., Moeller, S. J., Lilburn, M. S., & Zerby, H. N. (2010). Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. *Applied and Environmental Microbiology*, *76*(19),

6572–6582. https://doi.org/10.1128/AEM.00180-10

Cruz, P. M., Ibáñez, A. L., Hermosillo, O. A. M., & Saad, H. C. R. (2012). Use of Probiotics in Aquaculture. *International Scholarly Research Network*, 13. https://doi.org/10.5402/2012/916845

Diaz-Sanchez, S., D'souza, D., Biswas, D., & Hanning, I. (2015). Botanical alternatives to antibiotics for use in organic poultry production. *Poultry Science*, *94*(6), 1419–1430. https://doi.org/10.3382/ps/pev014

Dibner, J. J., & Richards, J. D. (2005). Antibiotic Growth Promoters in Agriculture: History and Mode of Action. *Poultry Science*, *84*(4), 634–643.

Ding, J., Dai, R., Yang, L., He, C., Xu, K., Liu, S., … Meng, H. (2017). Inheritance and establishment of gut microbiota in chickens. *Frontiers in Microbiology*, *8*(OCT), 1–11. https://doi.org/10.3389/fmicb.2017.01967

Donaldson, G. P., Lee, S. M., & Mazmanian, S. K. (2015). Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology*, *14*(1), 20–32. https://doi.org/10.1038/nrmicro3552

Duarte, M. E., & Kim, S. W. (2021). Intestinal microbiota and its interaction to intestinal health in nursery pigs. *Animal Nutrition*. https://doi.org/10.1016/J.ANINU.2021.05.001

EC Regulation 1831. (2003). EC Regulation 1831/2003. *Https://Eur-Lex.Europa.Eu/Legal-Content/ES/TXT/HTML/?Uri=CELEX:32003R1831&from=EN*.

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The gut microbiota of marine fish. *Frontiers in Microbiology*, *9*(MAY), 1–17. https://doi.org/10.3389/fmicb.2018.00873

Estruch, G., Collado, M. C., Peñaranda, D. S., Vidal, A. T., Cerdá, M. J., Martínez, G. P., … Moreau, C. S. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (Sparus aurata) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. *PLoS ONE*, *10*(8), 1–22. https://doi.org/10.1371/journal.pone.0136389

FAO. (2016). Probiotics in animal nutrition - Production, impact and regulation by Yadav S. Bajagai, Athol V. Klieve, Peter J. Dart and Wayne L. Bryden. Editor Harinder P.S. Makkar. *FAO Animal Production and Health*.

FDA, U. S. (2016). FDA Reminds Retail Establishments of Upcoming Changes to the Use of Antibiotics in Food Animals. *U.S. Food and Drugs Administration*.

Ferri, M., Ranucci, E., Romagnoli, P., & Giaccone, V. (2017). Antimicrobial resistance: A global emerging threat to public health systems. *Critical Reviews in Food Science and Nutrition*, *57*(13), 2857–2876. https://doi.org/10.1080/10408398.2015.1077192

Founou, L. L., Founou, R. C., & Essack, S. Y. (2016). Antibiotic resistance in the food chain: A developing country-perspective. *Frontiers in Microbiology*, *7*, 1881. https://doi.org/10.3389/FMICB.2016.01881/BIBTEX

Frick, J. S., & Autenrieth, I. B. (2013). The gut microflora and its variety of roles in health and disease. *Current Topics in Microbiology and Immunology*, *358*, 273–289. https://doi.org/10.1007/82_2012_217

Gadde, U., Kim, W. H., Oh, S. T., & Lillehoj, H. S. (2017). Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. *Animal Health Research Reviews*, *18*(01), 26–45.

https://doi.org/10.1017/S1466252316000207

Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Jérez-Cepa, I., Mancera, J. M., & Alarcón, F. J. (2020). Evaluation of Arthrospira sp. enzyme hydrolysate as dietary additive in gilthead seabream (Sparus aurata) juveniles. *Journal of Applied Phycology*, *32*(5), 3089–3100. https://doi.org/10.1007/s10811-020-02141-0

García, J. L., de Vicente, M., & Galán, B. (2017). Microalgae, old sustainable food and fashion nutraceuticals. *Microbial Biotechnology*, *10*(5), 1017. https://doi.org/10.1111/1751-7915.12800

Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., & Caruso, G. (2018). *Feeds for the Aquaculture Sector - Current Situation and Alternative Sources* (1st ed.). Springer International Publishing. https://doi.org/10.1007/978-3-319-77941-6

Gensollen, T., Iyer, S. S., Kasper, D. L., & Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune. *Science*, *352*(6285), 539–544. https://doi.org/10.1126/science.aad9378

Ghanbari, M., Kneifel, W., & Domig, K. J. (2015). A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture*. Elsevier. https://doi.org/10.1016/j.aquaculture.2015.06.033

Gheisar, M. M., & Kim, I. H. (2018). Phytobiotics in poultry and swine nutrition–a review. *Italian Journal of Animal Science*, *17*(1), 92–99. https://doi.org/10.1080/1828051X.2017.1350120

Gresse, R., Chaucheyras-Durand, F., Fleury, M. A., de Wiele, T. Van, Forano, E., & Blanquet-Diot, S. (2017). Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health. *Trends in Microbiology*, *25*(10), 851–873. https://doi.org/10.1016/j.tim.2017.05.004

Guevarra, R. B., Hong, S. H., Cho, J. H., Kim, B. R., Shin, J., Lee, J. H., … Kim, H. B. (2018). The dynamics of the piglet gut microbiome during the weaning transition in association with health and nutrition. *Journal of Animal Science and Biotechnology*, *9*(1). https://doi.org/10.1186/S40104-018-0269-6

Guevarra, R. B., Lee, J. H., Lee, S. H., Seok, M. J., Kim, D. W., Kang, B. N., … Kim, H. B. (2019). Piglet gut microbial shifts early in life: Causes and effects. *Journal of Animal Science and Biotechnology*, *10*(1), 1–10. https://doi.org/10.1186/s40104-018-0308-3

Guillamón, E., Andreo-Martínez, P., Mut-Salud, N., Fonollá, J., & Baños, A. (2021). Beneficial Effects of Organosulfur Compounds from Allium cepa on Gut Health: A Systematic Review. *Foods*, *10*(8), 1680. https://doi.org/10.3390/FOODS10081680

Guiry, M. D., & Guiry, G. M. (2021). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. Retrieved from https://ww.algaebase.org

Hashemi, S. R., & Davoodi, H. (2011). Herbal plants and their derivatives as growth and health promoters in animal nutrition. *Veterinary Research Communications*. https://doi.org/10.1007/s11259-010-9458-2

Hemaiswarya, S., Raja, R., Kumar, R. R., Ganesan, V., & Anbazhagan, C. (2011). Microalgae: A sustainable feed source for aquaculture. *World Journal of Microbiology and Biotechnology*, *27*(8), 1737–1746. https://doi.org/10.1007/S11274-010-0632-Z/FIGURES/2

Herrera Bravo de Laguna, I., Toledo Marante, F. J., & Mioso, R. (2015). Enzymes and

bioproducts produced by the ascomycete fungus Paecilomyces variotii. *Journal of Applied Microbiology*, *119*(6), 1455–1466. https://doi.org/10.1111/JAM.12934

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., … Sanders, M. E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/nrgastro.2014.66

Holman, D. B., Brunelle, B. W., Trachsel, J., & Allen, H. K. (2017). Meta-analysis To Define a Core Microbiota in the Swine Gut. *MSystems*, *2*(3). https://doi.org/10.1128/MSYSTEMS.00004-17

Huyghebaert, G., Ducatelle, R., & Immerseel, F. Van. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *Veterinary Journal*, *187*(2), 182–188. https://doi.org/10.1016/j.tvjl.2010.03.003

Isaacson, R., & Kim, H. B. (2012). The intestinal microbiome of the pig. *Animal Health Research Reviews*, *13*(1), 100–109. https://doi.org/10.1017/S1466252312000084

Jahan, M. S., Khairunnesa, M., Afrin, S., & Ali, M. S. (2016). Dietary black cumin (Nizella sativa) seed meal on growth and meat yield performance of broilers. *SAARC Journal of Agriculture*, *13*(2), 151–160. https://doi.org/10.3329/sja.v13i2.26576

Jones, J., DiBattista, J. D., Stat, M., Bunce, M., Boyce, M. C., Fairclough, D. V, … Huggett, M. J. (2018). The microbiome of the gastrointestinal tract of a range-shifting marine herbivorous fish. *Frontiers in Microbiology*, *9*(AUG), 2000. https://doi.org/10.3389/fmicb.2018.02000

Jorge, S. S., Enes, P., Serra, C. R., Castro, C., Iglesias, P., Teles, A. O., & Couto, A. (2019). Short-term supplementation of gilthead seabream (Sparus aurata) diets with Nannochloropsis gaditana modulates intestinal microbiota without affecting intestinal morphology and function. *Aquaculture Nutrition*, *25*(6), 1388–1398. https://doi.org/10.1111/ANU.12959

Khan, S., & Chousalkar, K. K. (2021). Functional enrichment of gut microbiome by early supplementation of Bacillus based probiotic in cage free hens: a field study. *Animal Microbiome*, *3*(1), 1–18. https://doi.org/10.1186/S42523-021-00112-5

Kim, H. B., Borewicz, K., White, B. A., Singer, R. S., Sreevatsan, S., Tu, Z. J., & Isaacson, R. E. (2011). Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary Microbiology*, *153*(1–2), 24–133. https://doi.org/10.1016/j.vetmic.2011.05.021

Kim, H. B., Borewicz, K., White, B. A., Singer, R. S., Sreevatsan, S., Tu, Z. J., & Isaacson, R. E. (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(38), 15485–15490. https://doi.org/10.1073/pnas.1205147109

Kim, H. B., & Isaacson, R. E. (2015). The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Veterinary Microbiology*, *177*(3–4), 242–251. https://doi.org/10.1016/J.VETMIC.2015.03.014

Kormas, K. A., Meziti, A., Mente, E., & Frentzos, A. (2014). Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (Sparus aurata). *MicrobiologyOpen*, *3*(5), 718–728. https://doi.org/10.1002/mbo3.202

Kumar, V., Sinha, A. K., Makkar, H. P., Boeck, G. De, & Becker, K. (2012). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, *96*(3), 335–364. https://doi.org/10.1111/J.1439-0396.2011.01169.X

Larsen, A. M. (2014). *Studies on the Microbiota of Fishes and the Factors Influencing Their Composition*. Auburn University.

Lee, J.-Y., & Gao, Y. (2012). Review of the Application of Garlic, Allium sativum, in Aquaculture. *Journal of the World Aquaculture Society*, *43*(4), 447–458. https://doi.org/10.1111/j.1749-7345.2012.00581.x

Leeming, E. R., Johnson, A. J., Spector, T. D., & Roy, C. I. Le. (2019). Effect of Diet on the Gut Microbiota: Rethinking Intervention Duration. *Nutrients*, *11*(12), 2862. https://doi.org/10.3390/NU11122862

Lei, X. J., Cheong, J. Y., Park, J. H., & Kim, I. H. (2017). Supplementation of protease, alone and in combination with fructooligosaccharide to low protein diet for finishing pigs. *Animal Science Journal*, *88*(12), 1987–1993. https://doi.org/10.1111/asj.12849

Li, P., Niu, Q., Wei, Q., Zhang, Y., Ma, X., Kim, S. W., … Huang, R. (2017). Microbial shifts in the porcine distal gut in response to diets supplemented with Enterococcus Faecalis as alternatives to antibiotics. *Nature Publishing Group*. https://doi.org/10.1038/srep41395

Lin, J., Hunkapiller, A. A., Layton, A. C., Chang, Y.-J., & Robbins, K. R. (2013). Response of Intestinal Microbiota to Antibiotic Growth Promoters in Chickens. *Foodborne Pathogens and Disease*, *10*(4), 331–337. https://doi.org/10.1089/fpd.2012.1348

Lira, A. C., Prieto, A. I., Baños, A., Guillamón, E., Moyano, R., Jos, A., & Cameán, A. M. (2020). Safety assessment of propyl-propane-thiosulfonate (PTSO): 90-days oral subchronic toxicity study in rats. *Food and Chemical Toxicology*, *144*(111612). https://doi.org/10.1016/j.fct.2020.111612

Liu, G. M., Wei, Y., Wang, Z. S., Wu, D., Zhou, A. G., & Liu, G. L. (2008). Effects of herbal extract supplementation on growthperformance and insulin-like growth factor (IGF)-Isystem in finishing pigs. *Journal of Animal and Feed Sciences*, *17*(4), 538–547. https://doi.org/10.22358/jafs/66681/2008

Liu, L., Liang, X. F., Li, J., Yuan, X., Zhou, Y., & He, Y. (2014). Feed intake, feed utilization and feeding-related gene expression response to dietary phytic acid for juvenile grass carp (Ctenopharyngodon idellus). *Aquaculture*, *424–425*, 201–206. https://doi.org/10.1016/J.AQUACULTURE.2013.12.044

Liu, S., He, L., Jiang, Q., Duraipandiyan, V., Al-Dhabi, N. A., Liu, G., … Yin, Y. (2018). Effect of dietary α-ketoglutarate and allicin supplementation on the composition and diversity of the cecal microbial community in growing pigs. *Journal of the Science of Food and Agriculture*, *98*(15), 5816–5821. https://doi.org/10.1002/JSFA.9131

Liu, Y, Song, M., Che, T. M., Almeida, J. A. S., Lee, J. J., Bravo, D., … Pettigrew, J. E. (2013). Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. *Journal of Animal Science*, *91*(11), 5294–5306. https://doi.org/10.2527/jas.2012-6194

Liu, Yanhong, Espinosa, C. D., Abelilla, J. J., Casas, G. A., Lagos, L. V., Lee, S. A., … Stein, H. H. (2018). Non-antibiotic feed additives in diets for pigs: A review. *Animal Nutrition*, *4*(2), 113–125. https://doi.org/10.1016/j.aninu.2018.01.007

Looft, T., Allen, H. K., Cantarel, B. L., Levine, U. Y., Bayles, D. O., Alt, D. P., …

Stanton, T. B. (2014). Bacteria, phages and pigs: The effects of in-feed antibiotics on the microbiome at different gut locations. *ISME Journal*, *8*(8), 1566–1576. https://doi.org/10.1038/ismej.2014.12

Looft, T., Johnson, T. A., Allen, H. K., Bayles, D. O., Alt, D. P., Stedtfeld, R. D., … Stanton, T. B. (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(5), 1691–1696. https://doi.org/10.1073/PNAS.1120238109

Luise, D., Bertocchi, M., Motta, V., Salvarani, C., Bosi, P., Luppi, A., … Trevisi, P. (2019). Bacillus sp. probiotic supplementation diminish the Escherichia coli F4ac infection in susceptible weaned pigs by influencing the intestinal immune response, intestinal microbiota and blood metabolomics. *Journal of Animal Science and Biotechnology*, *10*(74). https://doi.org/10.1186/s40104-019-0380-3

Lumpkins, B. S., Batal, A. B., & Lee, M. (2008). The effect of gender on the bacterial community in the gastrointestinal tract of broilers. *Poultry Science*, *87*(5), 964–967. https://doi.org/10.3382/ps.2007-00287

Mallo, J. J., Rioperez, J., & Honrubia, P. (2010). The addition of Enterococcus faecium to diet improves piglet's intestinal microbiota and performance. *Livestock Science*, *133*(1–3), 176–178. https://doi.org/10.1016/j.livsci.2010.06.057

Man, Y. B., Zhang, F., Ma, K. L., Mo, W. Y., Kwan, H. S., Chow, K. L., … Wong, M. H. (2020). Growth and intestinal microbiota of Sabah giant grouper reared on food waste-based pellets supplemented with spirulina as a growth promoter and alternative protein source. *Aquaculture Reports*, *18*, 100553. https://doi.org/10.1016/J.AQREP.2020.100553

Markowiak, P., & Ślizewska, K. (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathogens*, *10*(1), 1–20. https://doi.org/10.1186/S13099-018-0250-0

Maron, D. F., Smith, T. J. S., & Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: An international regulatory and economic survey. *Globalization and Health*, *9*(1). https://doi.org/10.1186/1744-8603-9-48

Martínez, J. E., Vargas, A., Pérez-Sánchez, T., Encío, I. J., Cabello-Olmo, M., & Barajas, M. (2021). Human Microbiota Network: Unveiling Potential Crosstalk between the Different Microbiota Ecosystems and Their Role in Health and Disease. *Nutrients*, *13*(9), 2905. https://doi.org/10.3390/NU13092905

McEwen, S. A., & Collignon, P. J. (2018). Antimicrobial Resistance: A One Health Colloquium. *Microbiology Spectrum*, *6*(2), 1–26. https://doi.org/10.1128/microbiolspec.ARBA-0009-2017

Ministerio de Agricultura Pesca y Alimentación. (2020a). EL SECTOR DE LA AVICULTURA DE CARNE EN CIFRAS: Principales Indicadores Económicos. *Subdirección General de Producciones Ganaderas Cinegéticas, Dirección General de Producciones y Mercados Agrarios*. https://www.mapa.gob.es/es/ganaderia/estadisticas/indicadoreseconomicossectoravicoladecarne2020_parapublicar_tcm30-419674.pdf

Ministerio de Agricultura Pesca y Alimentación. (2020b). EL SECTOR DE LA AVICULTURA DE PUESTA EN CIFRAS: Principales Indicadores Económicos. *Subdirección General de Producciones Ganaderas Cinegéticas, Dirección General de Producciones y Mercados Agrarios*. https://www.mapa.gob.es/es/ganaderia/estadisticas/indicadorespuesta20

20_parapublicar_tcm30-381335.pdf

Ministerio de Agricultura Pesca y Alimentación. (2020c). EL SECTOR DE LA CARNE DE CERDO EN CIFRAS: Principales Indicadores Económicos. *Subdirección General de Producciones Ganaderas y Cinegéticas, Dirección General de Producciones y Mercados Agrarios*. https://www.mapa.gob.es/es/ganaderia/estadisticas/indicadoreseconomicossectorporcino2020_tcm30-379728.pdf

Mookiah, S., Sieo, C. C., Ramasamy, K., Abdullah, N., & Ho, Y. W. (2014). Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. *Journal of the Science of Food and Agriculture*, *94*(2), 341–348. https://doi.org/10.1002/jsfa.6365

Morais, L. H., Schreiber, H. L., & Mazmanian, S. K. (2020). The gut microbiota–brain axis in behaviour and brain disorders. *Nature Reviews Microbiology*, *19*(4), 241–255. https://doi.org/10.1038/s41579-020-00460-0

Morgan, X. C., & Huttenhower, C. (2012). Chapter 12: Human Microbiome Analysis. *PLoS Computational Biology*, *8*(12). https://doi.org/10.1371/journal.pcbi.1002808

Niccolai, A., Zittelli, G. C., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Research*, *42*(April). https://doi.org/10.1016/j.algal.2019.101617

Nie, X., Chen, S., Zhang, X., Dai, B., & Qian, L. (2017). Effects of neutral phytase on growth performance and phosphorus utilization in crucian carp (Carassius auratus). *Journal of Zhejiang University: Science B*, *18*(10), 886–896. https://doi.org/10.1631/jzus.B1600280

Niewold, T. A. (2007). The Nonantibiotic Anti-Inflammatory Effect of Antimicrobial Growth Promoters, the Real Mode of Action? A Hypothesis. *Poultry Science*, *86*(4), 605–609. https://doi.org/10.1093/ps/86.4.605

Nikouli, E., Meziti, A., Antonopoulou, E., Mente, E., & Kormas, K. (2018). Gut Bacterial Communities in Geographically Distant Populations of Farmed Sea Bream (Sparus aurata) and Sea Bass (Dicentrarchus labrax). *Microorganisms*, *6*(3), 92. https://doi.org/10.3390/microorganisms6030092

Norag, M. A. A., El-Shenawy, A. M., Fadl, S. E., Abdo, W. S., Gad, D. M., Rashed, M. A., & Prince, A. M. (2018). Effect of phytase enzyme on growth performance, serum biochemical alteration, immune response and gene expression in Nile tilapia. *Fish & Shellfish Immunology*, *80*, 97–108. https://doi.org/10.1016/J.FSI.2018.05.051

Nordentoft, S., Mølbak, L., Bjerrum, L., Vylder, J. De, Immerseel, F. Van, & Pedersen, K. (2011). The influence of the cage system and colonisation of Salmonella Enteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 pyrosequencing. *BMC Microbiology*, *11*, 187. https://doi.org/10.1186/1471-2180-11-187

O'Riordan, K. J., Collins, M. K., Moloney, G. M., Knox, E. G., Aburto, M. R., Fülling, C., … Cryan, J. F. (2022). Short chain fatty acids: Microbial metabolites for gut-brain axis signalling. *Molecular and Cellular Endocrinology*, *546*, 111572. https://doi.org/10.1016/J.MCE.2022.111572

Oakley, B. B., Lillehoj, H. S., Kogut, M. H., Kim, W. K., Maurer, J. J., Pedroso, A., … Cox, N. A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiology Letters*, *360*(2), 100–112. https://doi.org/10.1111/1574-6968.12608

Oetting, L. L., Utiyama, C. E., Giani, P. A., Ruiz, U. D. S., & Miyada, V. S. (2006). Effects of herbal extracts and antimicrobials on apparent digestibility, performance, organs morphometry and intestinal histology of weanling pigs. *Revista Brasileira de Zootecnia*, *35*(4), 1389–1397. https://doi.org/10.1590/S1516-35982006000500019

Oliva-Teles, A., Enes, P., & Peres, H. (2015). *Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish*. *Feed and Feeding Practices in Aquaculture*. Elsevier Ltd. https://doi.org/10.1016/b978-0-08-100506-4.00008-8

Olsen, R. L., & Hasan, M. R. (2012). A limited supply of fishmeal: Impact on future increases in global aquaculture production. *Trends in Food Science & Technology*, *27*(2), 120–128. https://doi.org/10.1016/J.TIFS.2012.06.003

Pajarillo, E. A. B., Chae, J. P., Balolong, M. P., Kim, H. B., Seo, K. S., & Kang, D. K. (2014). Pyrosequencing-based analysis of fecal microbial communities in three purebred pig lines. *Journal of Microbiology*, *52*(8). https://doi.org/10.1007/s12275-014-4270-2

Pan, D., & Yu, Z. (2013). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), 108–119. https://doi.org/10.4161/gmic.26945

Park, J. H., & Kim, I. H. (2014). Supplemental effect of probiotic Bacillus subtilis B2A on productivity, organ weight, intestinal Salmonella microflora, and breast meat quality of growing broiler chicks. *Poultry Science*, *93*(8), 2054–2059. https://doi.org/10.3382/ps.2013-03818

Peinado, M. J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L. A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Animal Feed Science and Technology*, *181*(1–4), 87–92. https://doi.org/10.1016/j.anifeedsci.2013.03.001

Peinado, M. J., Ruiz, R., Echavarri, A., & Rubio, L. A. (2012). Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. *Poultry Science*, *91*(9), 2148–2157. https://doi.org/10.3382/ps.2012-02280

Peng, Q. Y., Li, J. D., Li, Z., Duan, Z. Y., & Wu, Y. P. (2016). Effects of dietary supplementation with oregano essential oil on growth performance, carcass traits and jejunal morphology in broiler chickens. *Animal Feed Science and Technology*, *214*, 148–153. https://doi.org/10.1016/j.anifeedsci.2016.02.010

Piazzon, M. C., Naya-Català, F., Simó-Mirabet, P., Picard-Sánchez, A., Roig, F. J., Calduch-Giner, J. A., … Pérez-Sánchez, J. (2019). Sex, age, and bacteria: How the intestinal microbiota is modulated in a protandrous hermaphrodite fish. *Frontiers in Microbiology*, *10*(OCT), 1–15. https://doi.org/10.3389/fmicb.2019.02512

Pieper, R., Janczyk, P., Urubschurov, V., Korn, U., Pieper, B., & Souffrant, W. B. (2009). Effect of a single oral administration of Lactobacillus plantarum DSMZ 8862/8866 before and at the time point of weaning on intestinal microbial communities in piglets. *International Journal of Food Microbiology*, *130*(3), 227–232. https://doi.org/10.1016/j.ijfoodmicro.2009.01.026

Pourabedin, M., & Zhao, X. (2015). Prebiotics and gut microbiota in chickens. *FEMS Microbiology Letters*, *362*(15). https://doi.org/10.1093/femsle/fnv122

Ptak, A., Bedford, M. R., Świątkiewicz, S., Zyła, K., & Józefiak, D. (2015). Phytase Modulates Ileal Microbiota and Enhances Growth Performance of the Broiler Chickens. *PLOS ONE*, *10*(3), e0119770. https://doi.org/10.1371/JOURNAL.PONE.0119770

Qi, Z., Shi, S., Tu, J., & Li, S. (2019). Comparative metagenomic sequencing analysis of cecum microbiotal diversity and function in broilers and layers. *3 Biotech*, *9*(8), 1–10. https://doi.org/10.1007/s13205-019-1834-1

Ren, H., Vahjen, W., Dadi, T., Saliu, E. M., Boroojeni, F. G., & Zentek, J. (2019). Synergistic effects of probiotics and phytobiotics on the intestinal microbiota in young broiler chicken. *Microorganisms*, *7*(12). https://doi.org/10.3390/microorganisms7120684

Rescigno, M. (2014). Intestinal microbiota and its effects on the immune system. *Cellular Microbiology*, *16*(7), 1004–1013. https://doi.org/10.1111/CMI.12301

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*, *433*, 50–61. https://doi.org/10.1016/j.aquaculture.2014.05.048

Rhouma, M., Fairbrother, J. M., Beaudry, F., & Letellier, A. (2017). Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. *Acta Veterinaria Scandinavica*, *59*(1), 1–19. https://doi.org/10.1186/S13028-017-0299-7

Ringø, E., Zhou, Z., Vecino, J. L. G., Wadsworth, S., Romero, J., Krogdahl, … Merrifield, D. L. (2016). Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition*, *22*(2), 219–282. https://doi.org/10.1111/ANU.12346

Rodas, B. De, Youmans, B. P., Danzeisen, J. L., Tran, H., & Johnson, T. J. (2018). Microbiome profiling of commercial pigs from farrow to finish. *Journal of Animal Science*, *96*(5), 1778–1794. https://doi.org/10.1093/jas/sky109

Rodriguez-Estrada, U., Satoh, S., Haga, Y., Fushimi, H., & Sweetman, J. (2013). Effects of inactivated enterococcus faecalis and mannan oligosaccharide and their combination on growth, immunity, and disease protection in rainbow trout. *North American Journal of Aquaculture*, *75*(3), 416–428. https://doi.org/10.1080/15222055.2013.799620

Romano, N., & Kumar, V. (2018). Phytase in animal feed. *Enzymes in Human and Animal Nutrition: Principles and Perspectives*, 73–88. https://doi.org/10.1016/B978-0-12-805419-2.00004-6

Ronda, S. R., Bokka, C. S., Ketineni, C., Rijal, B., & Allu, P. R. (2012). Aeration effect on Spirulina platensis growth and γ-linolenic acid production. *Brazilian Journal of Microbiology*, *43*(1), 12–20. https://doi.org/10.1590/S1517-83822012000100002

Rosas, V. T., Poersch, L. H., Romano, L. A., & Tesser, M. B. (2019). Feasibility of the use of Spirulina in aquaculture diets. *Reviews in Aquaculture*, *11*(4), 1367–1378. https://doi.org/10.1111/RAQ.12297

Rosenau, S., Oertel, E., Mott, A. C., & Tetens, J. (2021). The Effect of a Total Fishmeal Replacement by Arthrospira platensis on the Microbiome of African Catfish (Clarias gariepinus). *Life*, *11*(6), 558. https://doi.org/10.3390/LIFE11060558

Roy, S. Sen, & Pal, R. (2014). Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics. *Proceedings of the Zoological Society*, *68*(1). https://doi.org/10.1007/s12595-013-0089-9

Ruiz, R, Peinado, M. J., Aranda-Olmedo, I., Abecia, L., Suárez-Pereira, E., Melle, C. O., … Rubio, L. A. (2015). Effects of feed additives on ileal mucosa-associated microbiota composition of broiler chickens. *Journal of Animal Science*, *93*(7), 3410–

3420. https://doi.org/10.2527/jas.2015-8905

Ruiz, Raquel, García, M. P., Lara, A., & Rubio, L. A. (2010). Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. *Veterinary Microbiology*, *144*(1–2), 110–117. https://doi.org/10.1016/j.vetmic.2009.12.025

Rychlik, I. (2020). Composition and function of chicken gut microbiota. *Animals*, *10*(1). https://doi.org/10.3390/ani10010103

Sadeghi, G., Karimi, A., Shafeie, F., Vaziry, A., & Farhadi, D. (2016). The Effects of purslane (Portulaca oleracea L.) powder on growth performance, carcass characteristics, antioxidant status, and blood metabolites in broiler chickens. *Livestock Science*, *184*, 35–40. https://doi.org/10.1016/j.livsci.2015.12.003

Sales, R., Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Cerón-García, M. C., … Alarcón, F. J. (2021). Effects of dietary use of two lipid extracts from the microalga Nannochloropsis gaditana (Lubián, 1982) alone and in combination on growth and muscle composition in juvenile gilthead seabream, Sparus aurata. *Algal Research*, *53*, 102162. https://doi.org/10.1016/J.ALGAL.2020.102162

Samli, H. E., Senkoylu, N., Koc, F., Kanter, M., & Agma, A. (2007). Effects of Enterococcus faecium and dried whey on broiler performance, gut histomorphology and intestinal microbiota. *Archives of Animal Nutrition*, *61*(1), 42–49. https://doi.org/10.1080/17450390601106655

Scott, K. P., Gratz, S. W., Sheridan, P. O., Flint, H. J., & Duncan, S. H. (2013). The influence of diet on the gut microbiota. *Pharmacological Research*. https://doi.org/10.1016/j.phrs.2012.10.020

Seal, B. S., Lillehoj, H. S., Donovan, D. M., & Gay, C. G. (2013). Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. *Animal Health Research Reviews*, *14*(1), 78–87. https://doi.org/10.1017/S1466252313000030

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Chowdhury, M. A. K., Parsaeimehr, A., … Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology*, *30*(1), 197–213. https://doi.org/10.1007/s10811-017-1234-z

Shang, Y., Kumar, S., Oakley, B., & Kim, W. K. (2018). Chicken gut microbiota: Importance and detection technology. *Frontiers in Veterinary Science*, *5*(OCT). https://doi.org/10.3389/fvets.2018.00254

Shi, S., Qi, Z., Gu, B., Cheng, B., Tu, J., Song, X., … Li, S. (2019). Analysis of high-throughput sequencing for cecal microbiota diversity and function in hens under different rearing systems. *3 Biotech*, *9*(12), 1–11. https://doi.org/10.1007/s13205-019-1970-7

Song, D., Wang, Y. W., Hou, Y. J., Dong, Z. L., Wang, W. W., & Li, A. K. (2016). The effects of dietary supplementation of microencapsulated Enterococcus faecalis and the extract of Camellia oleifera seed on growth performance, immune functions, and serum biochemical parameters in broiler chickens. *Journal of Animal Science*, *94*(8), 3271–3277. https://doi.org/10.2527/jas.2016-0286

Song, D., Wang, Y. W., Lu, Z. X., Wang, W. W., Miao, H. J., Zhou, H., … Li, A. K. (2019). Effects of dietary supplementation of microencapsulated Enterococcus fecalis and the extract of Camellia oleifera seed on laying performance, egg quality, serum biochemical parameters, and cecal microflora diversity in laying hens. *Poultry Science*, *98*(7), 2880–2887. https://doi.org/10.3382/PS/PEZ033

Sukegawa, S., Ihara, Y., Yuge, K., Rao, S., Oka, K., Arakawa, F., … Morimatsu, F. (2014). Effects of oral administration of heat-killed Enterococcus faecium strain NHRD IHARA in post-weaning piglets. *Animal Science Journal = Nihon Chikusan Gakkaihō*, *85*(4), 454–460. https://doi.org/10.1111/asj.12163

Tatara, M. R., Sliwa, E., Dudek, K., Gawron, A., Piersiak, T., Dobrowolski, P., … Studziński, T. (2008). Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared in artificial sow. *Annals of Agricultural and Environmental Medicine*, *15*(1), 63–69.

Tatara, M. R., Śliwa, E., Dudek, K., Mosiewicz, J., & Studziński, T. (2005). Effect of aged garlic extract and allicin administration to sows during pregnant and lactation on body weight gain and gastrointestinal tract development of piglets. Part I. *Bull Vet Inst Pulawy*.

Teillant, A., & Laxminarayan, R. (2015). Economics of Antibiotic Use in U.S. Swine and Poultry Production. *The Magazine of Food, Farm, and Resource Issues*.

Thacker, P. A. (2013). Alternatives to antibiotics as growth promoters for use in swine production: A review. *Journal of Animal Science and Biotechnology*, *4*(1), 1–12. https://doi.org/10.1186/2049-1891-4-35

The World Bank. (2013). *Fish to 2030: Prospects for Fisheries and Aquaculture*. *International Bank for Reconstruction and Development. WORLD BANK REPORT NUMBER 83177-GLB*. Retrieved from https://www.fao.org/3/i3640e/i3640e.pdf

Thursby, E., & Juge, N. (2017). Introduction to the human gut microbiota. *Biochemical Journal*, *474*(11), 1823–1836. https://doi.org/10.1042/BCJ20160510

Turner, P. V. (2018). The role of the gut microbiota on animal model reproducibility. *Animal Models and Experimental Medicine*, *1*(2), 109–115. https://doi.org/10.1002/ame2.12022

Ubeda, C., Djukovic, A., & Isaac, S. (2017). Roles of the intestinal microbiota in pathogen protection. *Clinical & Translational Immunology*, *6*(2), e128. https://doi.org/10.1038/CTI.2017.2

United Nations, Department of Economic and Social Affairs, Population Division. (2019). *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423)*.

Unno, T., Kim, J., Guevarra, R. B., & Nguyen, S. G. (2015). Effects of antibiotic growth promoter and characterization of ecological succession in swine gut microbiota. *Journal of Microbiology and Biotechnology*, *25*(4), 431–438. https://doi.org/10.4014/jmb.1408.08063

Valenzuela-Gutiérrez, R., Lago-Lestón, A., Vargas-Albores, F., Cicala, F., & Martínez-Porchas, M. (2021). Exploring the garlic (Allium sativum) properties for fish aquaculture. *Fish Physiology and Biochemistry*, *47*(4), 1179–1198. https://doi.org/10.1007/S10695-021-00952-7

Veizaj-Delia, E., Piu, T., Lekaj, P., & Tafaj, M. (2010). Using combined probiotic to improve growth performance of weaned piglets on extensive farm conditions. *Livestock Science*, *134*(1–3), 249–251. https://doi.org/10.1016/j.livsci.2010.06.155

Videnska, P., Sedlar, K., Lukac, M., Faldynova, M., Gerzova, L., Cejkova, D., … Rychlik, I. (2014). Succession and Replacement of Bacterial Populations in the Caecum of Egg Laying Hens over Their Whole Life. *PLOS ONE*, *9*(12), e115142. https://doi.org/10.1371/JOURNAL.PONE.0115142

Vondruskova, H., Slamova, R., Trckova, M., Zraly, Z., & Pavlik, I. (2010). Alternatives

to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: A review. *Veterinarni Medicina*, *55*(5), 199–224. https://doi.org/10.17221/2998-VETMED

Walk, C. L., Pirgozliev, V., Juntunen, K., Paloheimo, M., & Ledoux, D. R. (2018). Evaluation of novel protease enzymes on growth performance and apparent ileal digestibility of amino acids in poultry: enzyme screening. *Poultry Science*, *97*(6), 2123–2138. https://doi.org/10.3382/ps/pey080

Wang, D., Ning, K., Li, J., Hu, J., Han, D., Wang, H., … Xu, J. (2014). Nannochloropsis Genomes Reveal Evolution of Microalgal Oleaginous Traits. *PLOS Genetics*, *10*(1), e1004094. https://doi.org/10.1371/JOURNAL.PGEN.1004094

Wang, S., Yao, B., Gao, H., Zang, J., Tao, S., Zhang, S., … Wang, J. (2019). Combined supplementation of Lactobacillus fermentum and Pediococcus acidilactici promoted growth performance, alleviated inflammation, and modulated intestinal microbiota in weaned pigs. *BMC Veterinary Research*, *15*(1), 239. https://doi.org/10.1186/s12917-019-1991-9

Wen, L., & Duffy, A. (2017). Factors Influencing the Gut Microbiota, Inflammation, and Type 2 Diabetes. *The Journal of Nutrition*, *147*(7), 1468S-1475S. https://doi.org/10.3945/JN.116.240754

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, *86*(14), E140–E148. https://doi.org/10.2527/jas.2007-0459

Woolhouse, M. E. J., & Ward, M. J. (2013). Sources of antimicrobial resistance. *Science*, *341*(6153), 1460–1461. https://doi.org/10.1126/science.1243444

World Health Organization. (2021). Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report. *Geneva: World Health Organization*.

Woyengo, T. A., Weihrauch, D., & Nyachoti, C. M. (2012). Effect of dietary phytic acid on performance and nutrient uptake in the small intestine of piglets. *Journal of Animal Science*, *90*(2), 543–549. https://doi.org/10.2527/JAS.2011-4001

Wu, G., Bazer, F. W., & Lamb, G. C. (2020). *Introduction: significance, challenges and strategies of animal production*. (F. W. Bazer, G. C. Lamb, & G. Wu, Eds.), *Animal Agriculture*. Academic Press. https://doi.org/10.1016/B978-0-12-817052-6.00001-X

Yan, L., Meng, Q. W., & Kim, I. H. (2012). Effects of fermented garlic powder supplementation on growth performance, nutrient digestibility, blood characteristics and meat quality in growing-finishing pigs. *Animal Science Journal*, *83*(5), 411–417. https://doi.org/10.1111/j.1740-0929.2011.00973.x

Yeoman, C. J., Chia, N., Jeraldo, P., Sipos, M., Goldenfeld, N. D., & White, B. A. (2012). The microbiome of the chicken gastrointestinal tract. *Animal Health Research Reviews / Conference of Research Workers in Animal Diseases*. https://doi.org/10.1017/S1466252312000138

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B. A., & Arockiaraj, J. (2020). Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. *Reviews in Aquaculture*, *12*(3), 1903–1927. https://doi.org/10.1111/raq.12416

Zhang, Z. F., & Kim, I. H. (2014). Effects of multistrain probiotics on growth performance, apparent ileal nutrient digestibility, blood characteristics, cecal

microbial shedding, and excreta odor contents in broilers. *Poultry Science*, *93*(2), 364–370. https://doi.org/10.3382/PS.2013-03314

Zhao, L., Wang, G., Siegel, P., He, C., Wang, H., Zhao, W., … Meng, H. (2013). Quantitative Genetic Background of the Host Influences Gut Microbiomes in Chickens. https://doi.org/10.1038/srep01163

Zmora, N., Suez, J., & Elinav, E. (2018). You are what you eat: diet, health and the gut microbiota. *Nature Reviews Gastroenterology & Hepatology*, *16*(1), 35–56. https://doi.org/10.1038/s41575-018-0061-2

Zuo, J., Ling, B., Long, L., Li, T., Lahaye, L., Yang, C., & Feng, D. (2015). Effect of dietary supplementation with protease on growth performance, nutrient digestibility, intestinal morphology, digestive enzymes and gene expression of weaned piglets. *Animal Nutrition*, *1*(4), 276–282. https://doi.org/10.1016/J.ANINU.2015.10.003

OBJETIVOS

A lo largo de la Introducción se ha puesto de manifiesto el interés en la búsqueda de compuestos que puedan mejorar los parámetros relacionados con el crecimiento y la productividad de los animales, o que produzcan cambios beneficiosos en la composición de la microbiota intestinal y que permitan en paralelo reducir la utilización de compuestos poco sostenibles y/o de antibióticos como agentes promotores del crecimiento y terapéuticos. Por ello, el objetivo general de esta Tesis ha sido evaluar la influencia de varios tipos de compuestos sobre la producción y el crecimiento, así como sobre la composición y la diversidad de las comunidades microbianas del intestino de animales del sector avícola, porcino y piscícola. Los compuestos utilizados en esta Tesis incluyen la cepa potencialmente probiótica *Enterococcus faecalis* UGRA10, fitobióticos derivados de plantas del género *Allium,* biomasa de *Arthrospira platensis* cruda e hidrolizada, y biomasa de microalgas junto con la enzima fitasa. Para alcanzar este objetivo general se plantearon los siguientes objetivos específicos:

1) Estudiar la inclusión de la cepa *Enterococcus faecalis* UGRA10 como potencial probiótico para la dieta de gallinas ponedoras. Para ello se van a explorar los efectos de su ingesta sobre el peso, la talla y el número de huevos puestos por gallinas ponedoras, y se van analizar los cambios producidos por esta cepa en la composición y la diversidad de la microbiota intestinal tras 76 días de experimento **(Capítulo I)**.

2) Comprobar los efectos de un extracto fitobiótico obtenido de plantas del género *Allium* sobre el tamaño y la producción de huevos, y sobre las comunidades bacterianas del íleon y el ciego de gallinas ponedoras **(Capítulo II)**.

3) Evaluar el uso de un extracto de *Allium* como alternativa a la colistina, uno de los principales antibióticos utilizados como promotores del crecimiento en lechones destetados, y estudiar su efecto sobre el crecimiento y la microbiota de diferentes regiones del tracto intestinal **(Capítulo III)**.

4) Determinar cómo afecta al peso corporal y a las comunidades microbianas del intestino de juveniles de dorada **(Capítulo IV)** y juveniles de lubina **(Capítulo V)** la suplementación de la dieta con el compuesto organosulfurado propil propano tiosulfonato (PTSO), obtenido de plantas del género *Allium.*

5) Analizar los efectos de la suplementación de la dieta de larvas de dorada con biomasa cruda e hidrolizada de *Arthrospira platensis* sobre el crecimiento de los

animales y la composición de las comunidades bacterianas intestinales. Con este objetivo se pretende determinar la posibilidad de emplear biomasa cruda e hidrolizada de microalgas como aditivo para la dieta de larvas de dorada, lo que permitiría reducir el uso de harinas de pescado **(Capítulo VI)**.

6) Analizar la relación entre el uso de dietas con microalgas (*Arthrospira platensis* y *Nannochloropsis gaditana*) y diferentes concentraciones de la enzima fitasa con el peso corporal y la composición de la microbiota intestinal de juveniles de lubinas. La incorporación de esta enzima podría actuar sobre los componentes de la dieta, aumentando su digestibilidad y, con ello, incrementar el crecimiento de los animales **(Capítulo VII)**.

MATERIAL Y MÉTODOS

En este apartado se describe de forma general la metodología empleada en los experimentos llevados a cabo en esta Tesis Doctoral. Al final de este apartado, en las Figuras M.2 a M.8, se resumen los esquemas de los diseños experimentales que se han realizado en cada uno de los capítulos de esta Tesis. Una descripción más detallada de cada diseño experimental se expone en cada uno de los capítulos específicos de la memoria. Un esquema general de los pasos seguidos durante los diferentes estudios de microbiota intestinal se muestra en la Figura M.9.

1. Medios de cultivo

Para el cultivo bacteriano se emplearon diversos medios de cultivo, tanto generales como selectivos, detallados en las Tablas M.1 a M.5. Los medios se esterilizaron en autoclave siguiendo las instrucciones de cada casa comercial, lo que generalmente correspondió con una temperatura de 121ºC durante un tiempo de 15 minutos. Para elaborar medios sólidos se adicionó agar a una concentración del 1,5 %.

Tabla M.1. Composición del medio de triptona y soja (TSB) utilizado como medio nutritivo de uso general. La composición se expresa en gramos por litro de agua.

Medio de triptona y soja (TSB, Scharlau)	
Peptona de caseína	17 g
Peptona de soja	3 g
Sodio cloruro	5 g
Fosfato dipotásico	2,5 g
D (+) Glucosa	2,5 g

Tabla M.2. Composición del medio Wilkins-Chalgren utilizado como medio selectivo para bacterias anaerobias totales. La composición se expresa en gramos por litro de agua.

Medio Wilkins-Chalgren (Scharlau)	
Triptona	10 g
Gelatina de peptona	10 g
Extracto de levadura	5 g
Glucosa	1 g
Cloruro sódico	5 g
L-Arginina	1 g
Piruvato sódico	1 g
Vitamina K1	0,0005 g
Hemina	0,005 g

Tabla M.3. Composición del medio Slanetz-Bartley, empleado para la detección de *Enterococcus* sp. La composición se expresa en gramos por litro de agua.

Medio Slanetz-Bartley (Scharlau)	
Triptosa	20 g
Glucosa	2 g
Fosfato dipotásico	4 g
Azida de sodio	0,4 g
Extracto de levadura	5 g
2,3,5-Trifeniltetrazolio cloruro (TTC)	0,1 g

Tabla M.4. Composición del medio de uso general de Infusión de Cerebro Corazón (BHI). La composición se expresa en gramos por litro de agua.

Medio de Infusión de Cerebro Corazón (BHI, Scharlau)	
Extracto de cerebro	12,5 g
Extracto de corazón	5 g
Peptona	10 g
Cloruro sódico	5 g
Fosfato disódico	2,5 g
Dextrosa	2 g

Tabla M.5. Composición del medio Mueller-Hinton, utilizado para los estudios de inhibición. La composición se expresa en gramos por litro de agua.

Medio Mueller-Hinton (Scharlau)	
Peptona	17,5 g
Infusión de carne	2 g
Almidón	1,5 g

2. Técnicas de extracción de ADN genómico

Para aislar el ADN de las muestras objeto de estudio se emplearon diversos métodos de extracción en función de las particularidades de cada tipo de muestra. Para las muestras de lechones (Capítulo III) y juveniles de doradas (Capítulo IV) se empleó un kit comercial (FavorPrep Stool DNA Isolation Mini Kit, Favorgen Biotech®) siguiendo las instrucciones del fabricante. Este kit se basa en una lisis mecánica con perlas de vidrio y en la separación del ADN mediante columnas de sílice. En el caso de gallinas ponedoras (Capítulos I y II), juveniles de lubina (Capítulos V y VII) y larvas de dorada (Capítulo VI), dado el bajo rendimiento obtenido con el kit anterior, especialmente con muestras de peces, se realizaron mediante la técnica MSOP (*Modified Salting Out Procedure*, Martín-Platero *et al.*, 2007) modificada con un paso inicial de lisis mecánica mediante el disruptor de células FastPrep® FP120 (BIO101, Thermo Savant), obteniéndose mejores rendimientos de extracción de ADN. El procedimiento se resume a continuación:

- Unos 100 mg de muestra se resuspendieron en 900 µL de solución de lisis (Solución II de MSOP (Martín-Platero et al., 2007)), junto con unos 100 mg de bolas de zirconio y se realizó una lisis mecánica mediante 2 pulsos de 30 segundos en el disruptor de células.
- A continuación, se añadieron 10 µL de ARNasa (10 mg/mL) y se incubaron las muestras a 37ºC durante 20 minutos. Después se añadieron 20 µL de Proteinasa K (10 mg/ml), se incubaron las muestras a 55ºC durante 15 minutos y posteriormente a 80ºC durante 10 minutos.
- Seguidamente se centrifugaron las muestras durante 3 minutos a 14.000 rpm y se recuperaron unos 600 µL de sobrenadante, a los cuales se adicionaron 200 µL de Solución III de MSOP (Martín-Platero et al., 2007), se agitaron mediante vórtex durante 30 segundos y se incubaron en hielo durante 15 minutos.

- Posteriormente se centrifugaron durante 10 minutos a 4ºC y 14.000 rpm, y se recuperaron unos 600 µL de sobrenadante, al cual se le adicionó un volumen igual de isopropanol para conseguir la precipitación del ADN. Las muestras se agitaron por inversión suave unas 40-50 veces hasta que se observó un precipitado blanquecino correspondiente al ADN.
- A continuación, las muestras se centrifugaron 10 minutos a 14.000 rpm y se descartó el sobrenadante. El sedimento, el cual contenía el ADN, se lavó con 1 ml de etanol 80%. El lavado con etanol al 80 % se realizó dos veces.
- Por último, se secaron las muestras durante 10 minutos en una centrífuga de vacío, y se resuspendieron en 200 µL de agua miliQ, almacenándose a -20ºC hasta su uso.

Las extracciones de ADN se comprobaron mediante electroforesis sumergida horizontal en gel de agarosa al 0,7 %, en TAE 1x como tampón de electroforesis. Las muestras se cargaron en los pocillos de los geles utilizando solución de carga. La composición del TAE y de la solución de carga se detallan en las Tablas M.6 y M.7. Posteriormente el gel se sumergió en una solución de bromuro de etidio (1 µg/ml en agua destilada) (Sigma-Aldrich) durante 15 minutos. Tras lavar en agua durante 15 minutos para eliminar el exceso de bromuro de etidio, el gel se visualizó mediante el transiluminador Gel Doc XR (Bio-Rad), el cual expone al gel a luz ultravioleta de 302 nm, pudiendo visualizar el ADN.

Tabla M.6. Composición del tampón TAE 50x.

Tampón TAE	
Tris base	242,0 g
Ácido acético glacial	57,1 mL
EDTA-Na_2 0,5 M, pH 8	100 mL
Agua destilada	Completar hasta 1 L

Tabla M.7. Composición de la solución de carga 10x.

Solución de carga	
Glicerol	50 % (v/v)
TE (10 mM Tris-HCl pH 8; EDTA 1 mM pH 8)	49,75 % (v/v)
Agua destilada	0,25 %

Para conocer el tamaño aproximado de los ADNs se utilizó como patrón de tamaños el marcador lambda *Hind* III (Thermo Fisher Scientific™). La concentración de ADN de las muestras extraídas se llevó a cabo mediante el espectrofotómetro NanoDrop™ 2000 (Thermo Fisher Scientific™). La relación A_{260}/A_{280} sirvió para comprobar la pureza del ADN, considerándose valores cercanos a 1,8 como ADN puro.

3. Identificación de las comunidades bacterianas mediante secuenciación masiva

La construcción de las librerías para la plataforma Illumina MiSeq se llevó a cabo mediante una estrategia de PCR en dos pasos (Figura M.1) (Caporaso et al., 2011). Todas las amplificaciones se realizaron en un termociclador Mastercycler® (Eppendorf) o iCycler 170-8720 (Bio-Rad). La ADN polimerasa utilizada fue la polimerasa iProof HF Master Mix (Bio-Rad) y los oligonucleótidos utilizados fueron suministrados por StabVida y/o Metabion.

Para la realización de las librerías se llevó a cabo en un primer paso la amplificación mediante PCR de una región hipervariable del gen del ARNr 16S. En los experimentos con gallinas (Capítulos I y II), lechones (Capítulo III), y doradas (Capítulos IV y VI) se amplificó la región V4. En los estudios con lubinas (Capítulos V y VII) se amplificaron las regiones V6-V8 del gen del ARNr 16S dada la aparición de amplificaciones inespecíficas y dímeros al utilizar los oligonucleótidos de la región V4 con este tipo de muestras.

Los oligonucleótidos utilizados para la realización de esta primera PCR en la cual se amplifica la región variable del gen del ARNr 16S se detallan en la Tabla M.8.

Tabla M.8. Secuencia nucleotídica de los oligonucleótidos empleados para amplificar diferentes regiones variables del gen del ARNr 16S.

Cebador	Secuencia (5`-3`)	Región variable ARNr 16S
U515	GTGCCAGCMGCCGCGGTAA	V4 (directo)
U515_bar1	TGGTCGTGCCAGCMGCCGCGGTAA	V4 (directo)
U515_bar2	ACGAAGTGCCAGCMGCCGCGGTAA	V4 (directo)
E786	GGACTACHVGGGTWTCTAAT	V4 (reverso)
B969F	ACGCGHNRAACCTTACC	V6-V8 (directo)
B969F_bar1	TGGTCACGCGHNRAACCTTACC	V6-V8 (directo)
B969F_bar2	ACGAAACGCGHNRAACCTTACC	V6-V8 (directo)
B969F_bar3	GTACCACGCGHNRAACCTTACC	V6-V8 (directo)
BA1406R	ACGGGCRGTGWGTRCAA	V6-V8 (reverso)

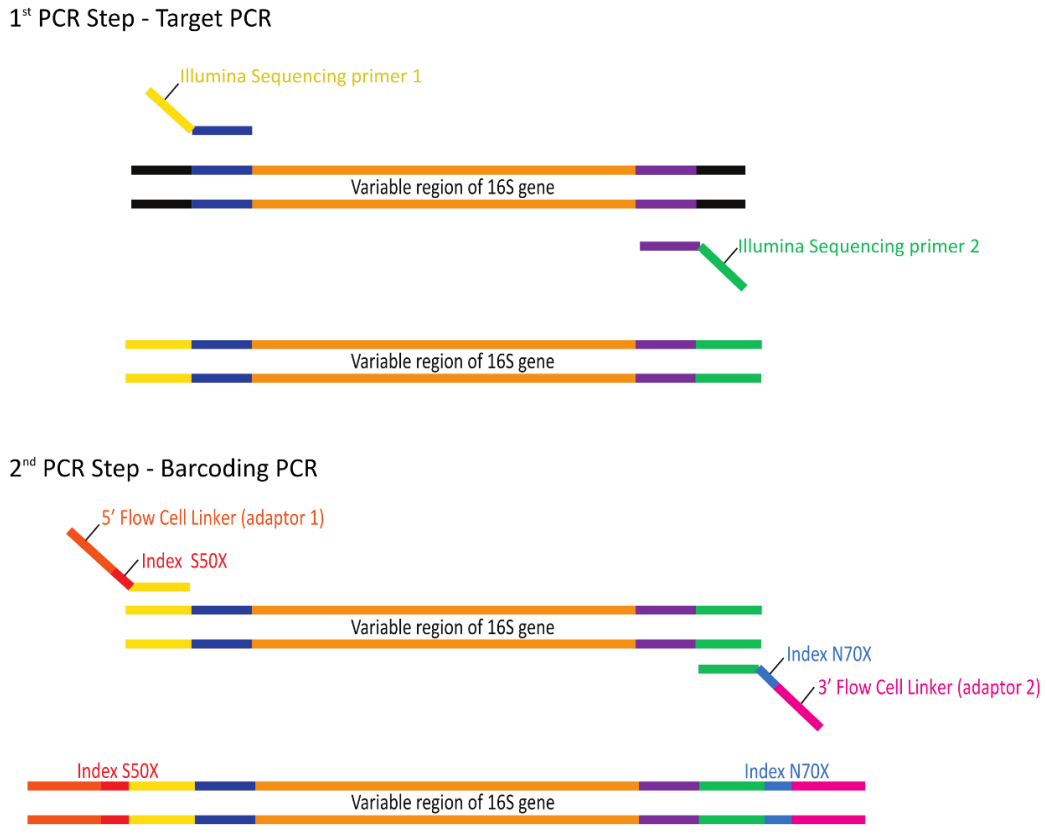

Figura M.1. Proceso de construcción de librerías de Illumina MiSeq mediante amplificación en 2 pasos. En el primer paso de PCR el gen diana de ARNr 16S se amplifica utilizando cebadores específicos de la región diana a amplificar, adicionados en el extremo 5`de la secuencia de cebadores de Illumina. En la segunda PCR, los cebadores utilizados solapan con la secuencia cebadora empleada en la primera PCR, y contienen los *barcodes* o códigos de barras específicos de cada muestra (S50X y N70X), además de los adaptadores de Illumina (modificado de Holm et al., 2019).

La composición general de la mezcla de amplificación y las condiciones de amplificación se detalla a continuación:

Compuesto	**Cantidad**	**Concentración final**
IProof HF Master Mix Bio-Rad (2x)	12,5 µL	1x
Cebador directo (3 µM)	2,5 µL	0,3 µM
Cebador reverso (3 µM)	2,5 µL	0,3 µM
H_2O	2,5 µL	-
ADN molde	5 µL	100-200 ng

Condiciones de la amplificación:

1) Desnaturalización inicial: 98ºC, 1 min.
2) Desnaturalización: 98ºC, 10 seg.
3) Hibridación: 52ºC, 20 seg.
4) Extensión: 72ºC, 15 seg.
5) El número de ciclos fue de 25 (incluyendo los pasos 2 a 4)
6) Terminación: 72ºC, 5 min.

Los productos de PCR se comprobaron mediante electroforesis sumergida horizontal en gel de agarosa al 1-1,5 %. El tampón de electroforesis utilizado fue TAE 1x. El proceso de visualización de geles de agarosa fue igual al empleado con los geles utilizados para comprobar las extracciones de ADN, sumergiendo el gel en bromuro de etidio durante 15 minutos, lavando posteriormente en agua abundante y visualizando en el transiluminador Gel Doc XR (Bio-Rad). En este caso, para conocer el tamaño aproximado de los amplicones se utilizaron como patrones los marcadores de pesos moleculares 1 kb o 100 bp (Thermo Fisher Scientific™).

Los productos de PCR se purificaron utilizando el kit de partículas magnéticas DNA Purification SPRI Magnetic Beads (Canvax®) siguiendo las instrucciones del fabricante. Este se basa en la técnica de inmovilización reversible en fase sólida (*Solid Phase Reversible Immobilization*, SPRI), mediante la cual el ADN se une de forma reversible a partículas magnéticas, permitiendo eliminar de la muestra oligonucleótidos, dímeros, dNTPs y otras impurezas. A continuación, se resume el procedimiento de purificación:

- Los productos de PCR se mezclaron con un volumen igual de solución de partículas magnéticas, se repipetearon hasta conseguir una mezcla homogénea y se dejaron 5 minutos a temperatura ambiente para aumentar la unión de las partículas magnéticas al ADN.
- A continuación, la placa con los productos de PCR se colocó encima de una placa magnética (Magnetic Stand-96, Thermo Fisher Scientific™), permitiendo al ADN unido a las partículas magnéticas pegarse a los imanes. Posteriormente, cuando las partículas magnéticas se habían unido a los imanes, se retiró el sobrenadante y se realizaron dos lavados con 200 µL de etanol 70%.
- Se dejó secar la placa de muestras durante 10 minutos para eliminar restos de etanol, se separó de la placa magnética y se añadieron 25 µL de agua y se repipetearon las muestras varias veces para eluir el ADN.
- Por último, se volvió a poner la placa con los productos de PCR sobre la placa magnética, permitiendo así separar las partículas magnéticas del producto de PCR, el cual se encuentra disuelto en el agua. Los productos de PCR purificados se transfirieron a una nueva placa o a tubos de microcentrífuga para su almacenamiento y uso posterior.

En un segundo paso se llevó a cabo una segunda amplificación utilizando los productos de PCR purificados como molde. En esta se introdujeron *barcodes* o códigos de barras específicos de cada muestra mediante el empleo de cebadores específicos, de forma que la combinación de estos códigos en los cebadores directo y reverso fue única para cada muestra. Estos cebadores solapan con los adaptadores de Illumina incluidos en la primera PCR (Figura M.1). La composición general de la mezcla de amplificación y las condiciones de amplificación se detalla a continuación:

Compuesto	**Cantidad**	**Concentración final**
IProof HF Master Mix Bio-Rad (2x)	12,5 µL	1x
Cebador N70(X) (3 µM)	3,3 µL	0,4 µM
Cebador S50(X) (3 µM)	3,3 µL	0,4 µM
H_2O	0,9 µL	-
ADN molde	5 µL	100-200 ng

Condiciones de la amplificación:

1) Desnaturalización inicial: 98ºC, 1 min.
2) Desnaturalización: 98ºC, 10 seg.
3) Hibridación: 55ºC, 20 seg.
4) Extensión: 72ºC, 15 seg.
5) El número de ciclos fue de 8 (incluyendo los pasos 2 a 4)
6) Terminación: 72ºC, 5 min.

Al igual que tras el primer paso de amplificación, tras esta segunda PCR se comprobó la amplificación mediante electroforesis sumergida horizontal en gel de agarosa al 1-1,5 % y se purificaron los productos de PCR utilizando el kit DNA Purification SPRI Magnetic Beads (Canvax®).

Finalmente, se determinó la concentración de cada una de las librerías utilizando Qubit® 3.0 Fluorometer (Thermo Fisher Scientific™). Las librerías se multiplexaron con todas las muestras, normalizando todas con los mismos nanomoles de ADN. Previamente a la secuenciación se comprobó la integridad de las librerías mediante la utilización de Bioanalyzer 2100 o D1000 ScreenTape (Agilent Technologies), los cuales permiten una separación de bandas de alta resolución y una cuantificación precisa del tamaño de los amplicones. Con la mezcla de las librerías se llevó a cabo la secuenciación masiva mediante la plataforma MiSeq de Illumina. Para las secuenciaciones se utilizaron los cartuchos Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), obteniendo lecturas pareadas de 2 x 300 pares de bases de longitud de lectura. La secuenciación de las muestras de los distintos experimentos se llevó a cabo en el Centro de Instrumentación Científica de la Universidad de Granada (CIC-UGR) o en el Instituto de Parasitología y Biomedicina "López-Neyra" de Granada.

4. Análisis de secuencias

A continuación, se muestra el flujo de trabajo común en el procesamiento y análisis de secuencias. Los análisis específicos de cada caso se detallan en los apartados de materiales y métodos correspondientes a cada uno de los capítulos de resultados de la presente Tesis.

Tras la secuenciación, las secuencias obtenidas se procesaron y analizaron utilizando el programa QIIME 2 (Quantitative Insights Into Microbial Ecology; Bolyen

et al., 2018; Caporaso et al., 2010), software abierto diseñado para el análisis de comunidades microbianas. En primer lugar, se procesaron las lecturas generadas por el secuenciador Illumina para su posterior análisis. Para ello se eliminaron los cebadores mediante *cutadapt trim-paired* (para lecturas solapadas) y *cutadapt trim-single* (para lecturas directas) (Martin, 2011), empleando los parámetros por defecto del método. Posteriormente se solaparon las lecturas directas (*forward*) y reversas (*reverse*) usando los parámetros por defecto de VSEARCH (Rognes et al., 2016). En aquellos casos en los que no se pudieron solapar ambas lecturas de manera satisfactoria se llevó a cabo el análisis utilizando exclusivamente las lecturas directas. A continuación, se realizó el filtrado de calidad utilizando el scritp *quality-filter q-score* (Bokulich et al., 2013). Las secuencias con más de 3 valores consecutivos de Phred inferiores a 20 se eliminaron de los análisis. Con las secuencias que pasaron el filtro de calidad se agruparon las lecturas en OTUs mediante Deblur, algoritmo destinado a eliminar errores de secuenciación (Amir et al., 2017) y el cual genera OTUs equivalentes a OTUs al 100% de identidad (también referidas en la literatura como *ASVs*; *Amplicon Sequence Variants*). Además, en este paso las secuencias se cortaron a la misma longitud de pares de bases. El tamaño elegido para cortar las secuencias en cada caso se seleccionó en función de la calidad de las lecturas, cortando en la posición en la cual la calidad media de las lecturas empieza a disminuir de forma significativa. A continuación, se llevó a cabo el alineamiento de secuencias y se creó el árbol filogenético utilizando *fragment insertion sepp*, un script implementado en QIIME 2 para incluir las secuencias en un árbol filogenético preconstruido (Janssen et al., 2018). A las secuencias representativas de cada OTU se les asigno la taxonomía empleando la base de datos de Greengenes (versión 13.08) agrupada al 99% de similitud como referencia (DeSantis et al., 2006). Finalmente, tras esta identificación taxonómica se eliminaron de la tabla de OTUs las secuencias correspondientes a cloroplastos, mitocondrias y ADN no bacteriano, puesto que los cebadores empleados eran específicos del Dominio *Bacteria*.

5. Estimas de diversidad

Para el análisis de diversidad de las muestras se calcularon los componentes alfa y beta de la diversidad microbiana de las comunidades objeto de estudio. Para controlar el esfuerzo de secuenciación, se llevó a cabo la rarefacción de las tablas de OTUs a una misma profundidad en cada experimento. Se seleccionó aquella profundidad que permitía

conservar el mayor número de muestras posible, manteniendo un número de secuencias razonable. Para estimar la diversidad alfa se calcularon los siguientes índices: Riqueza de OTUs; índice filogenético de Faith's Phylogenetic Diversity (PD) (Faith & Baker, 2006); índice de uniformidad de Pielou (Pielou, 1966); índice de diversidad de Shannon (Shannon, 1948); e índice de diversidad de Chao1 (Chao, 1984). Por otro lado, para estimar la diversidad beta se calcularon los siguientes índices: *Weighted UniFrac* y *Unweighted UniFrac*. Ambos índices basados en la métrica UniFrac (Lozupone & Knight, 2005), que incorpora información filogenética en las distancias composicionales. *Weighted UniFrac* pondera la distancia por la abundancia relativa, mientras que *Unweighted UniFrac* no, por lo que este último se centra en la presencia o ausencia de OTUs y, por tanto, las poblaciones minoritarias (*rare biosphere*) adquieren mayor peso (Lozupone *et al.*, 2007).

6. Análisis estadísticos

Para comprobar el efecto de los distintos tratamientos sobre los parámetros productivos animales en los diferentes experimentos se realizaron Modelos Lineales Generalizados Mixtos (GLMM), ejecutados en el programa Statistica 10.0 (StatSoft).

Las diferencias en la abundancia relativa de los distintos grupos bacterianos asociadas a los tratamientos experimentales se exploraron mediante el análisis LEfSe (*Linear Discriminant Analysis Effect Size*) (Segata et al., 2011), llevado a cabo en la plataforma de Galaxy (https://huttenhower.sph.harvard.edu/galaxy/).

Para estudiar el efecto de los distintos tratamientos sobre las matrices de distancia de beta diversidad se utilizó un test ANOVA con permutaciones (PERMANOVA), usando para ello el programa PRIMER-7.0.20 (Primer-e) implementado con el complemento (*plugin*) de PERMANOVA. Además, dichas matrices de beta diversidad se representaron gráficamente mediante Análisis de Coordenadas Principales (PCoA) utilizando el complemento EMPeror (Vázquez-Baeza et al., 2013, 2017) implementado en QIIME 2.

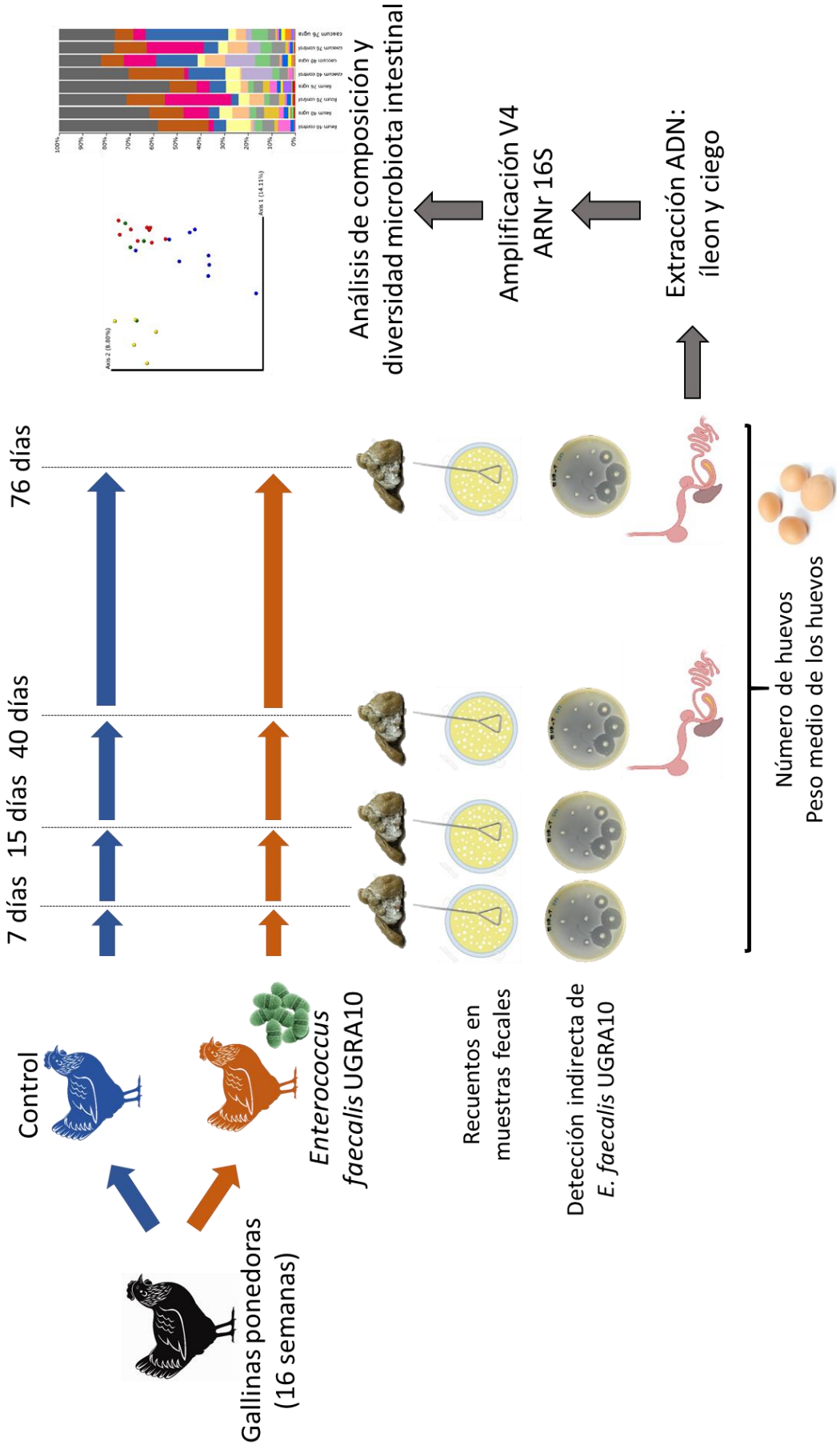

Figura M.2. Diseño experimental correspondiente al Capítulo I.

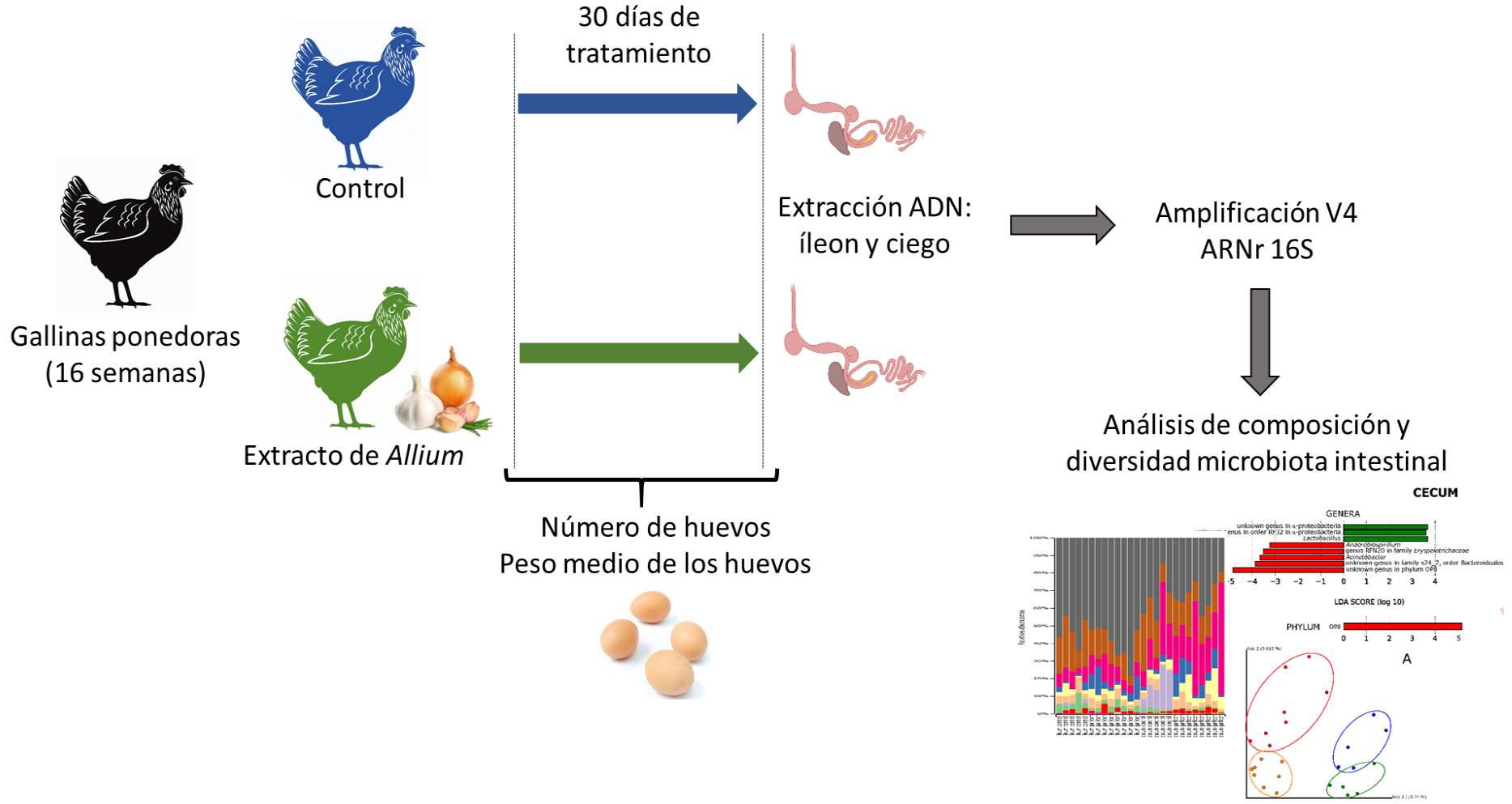

Figura M.3. Diseño experimental correspondiente al Capítulo II.

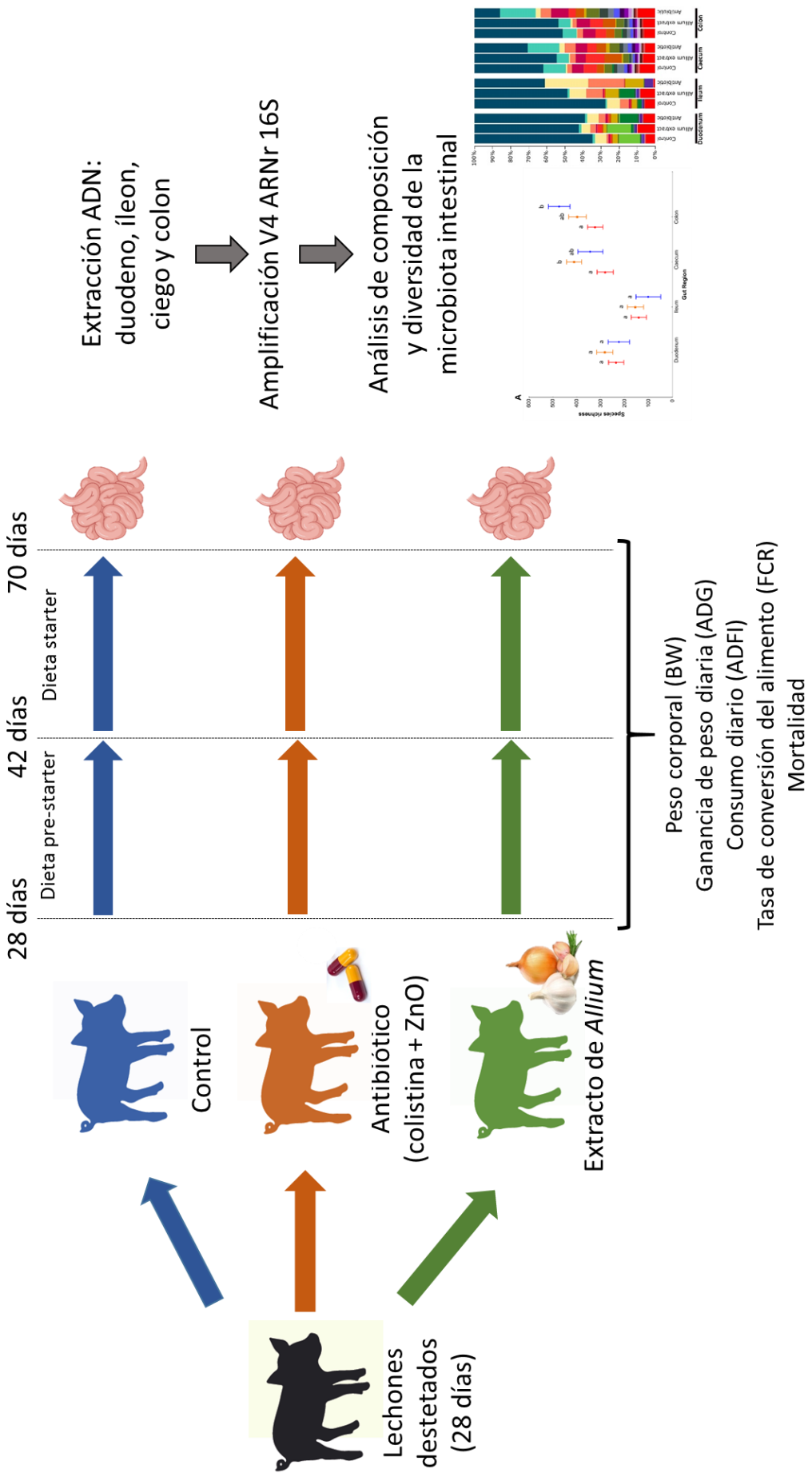

Figura M.4. Diseño experimental correspondiente al Capítulo III.

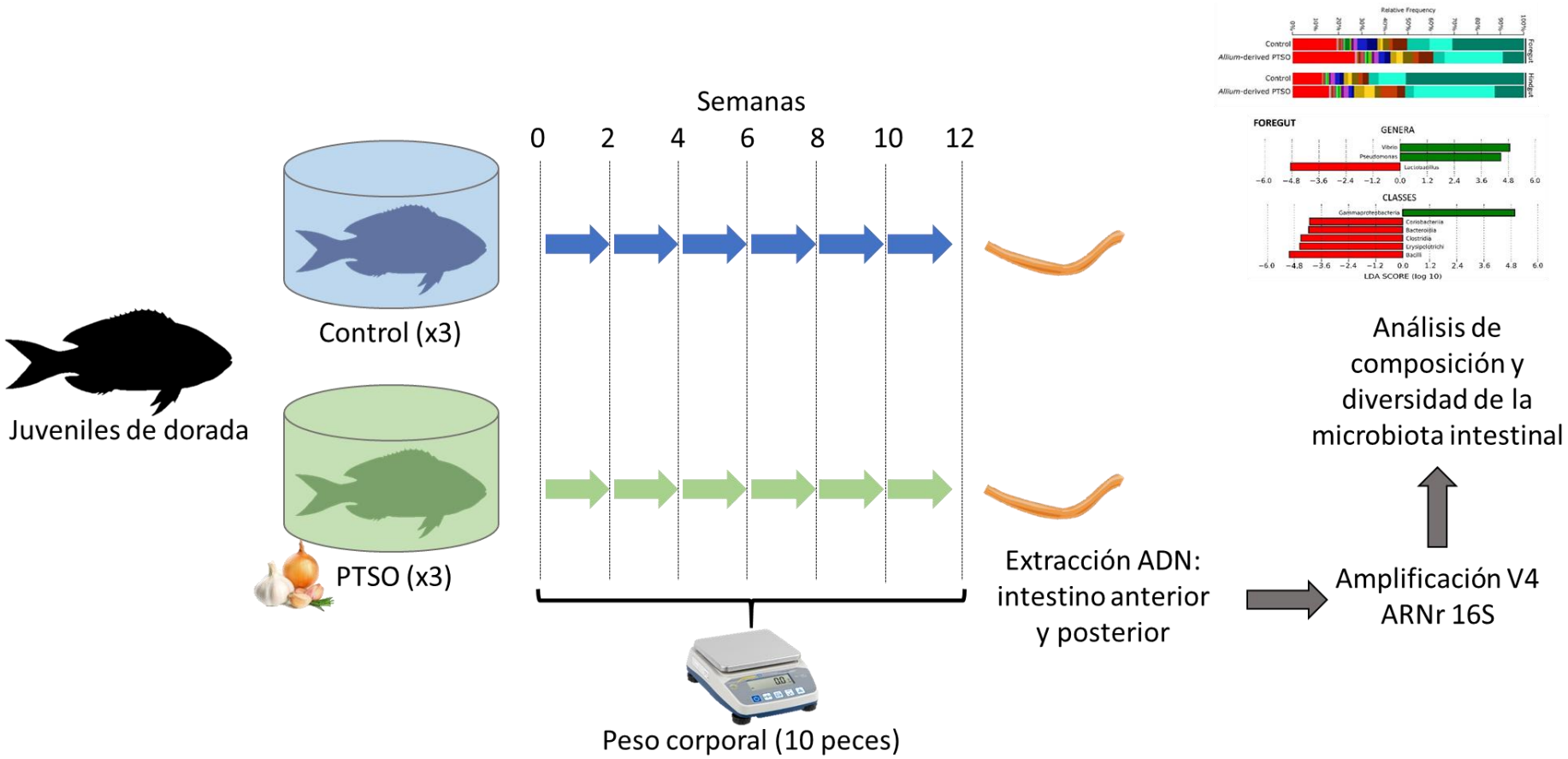

Figura M.5. Diseño experimental correspondiente al Capítulo IV. PTSO: Propil propano tiosulfonato.

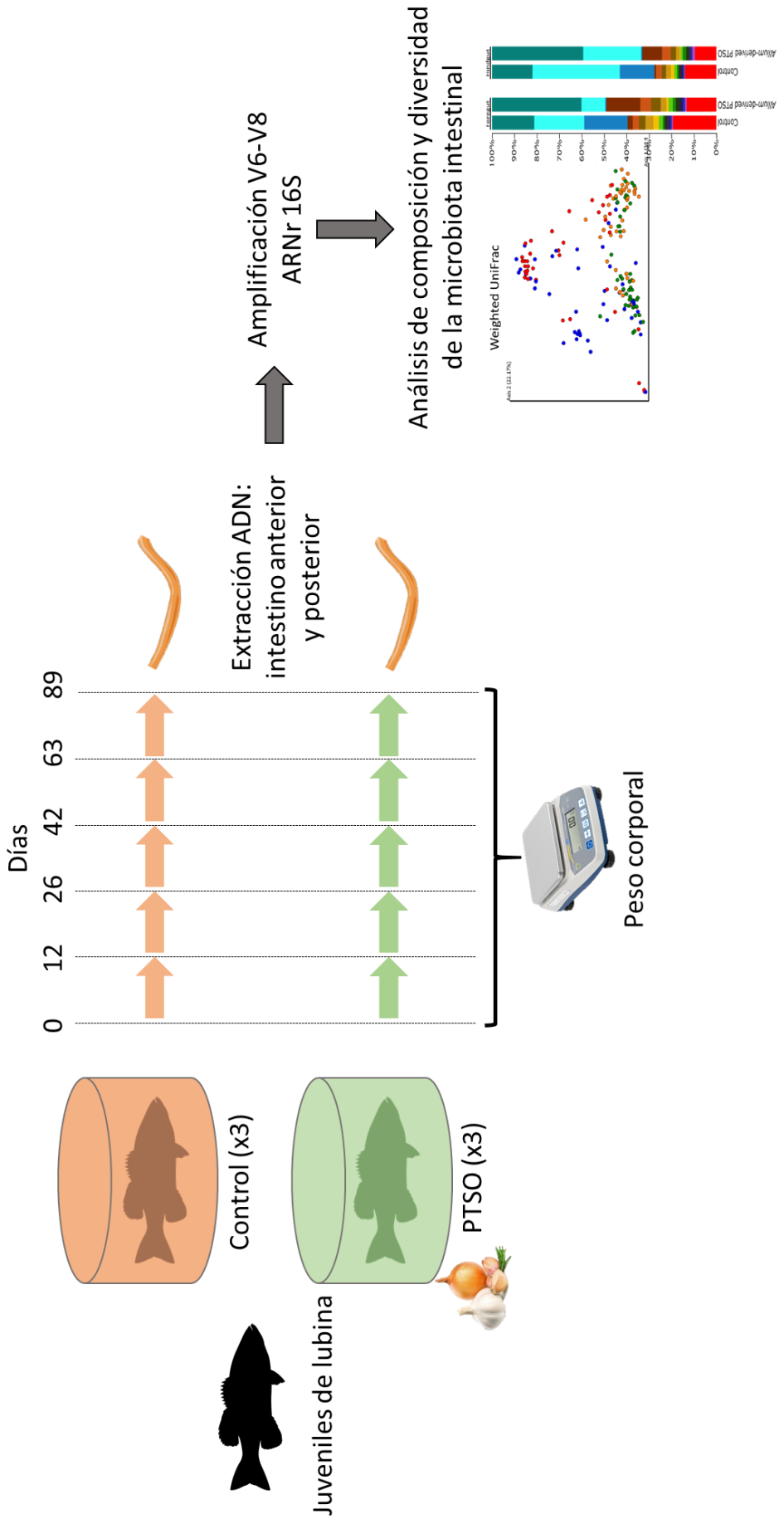

Figura M.6. Diseño experimental correspondiente al Capítulo V. PTSO: Propil propano tiosulfonato.

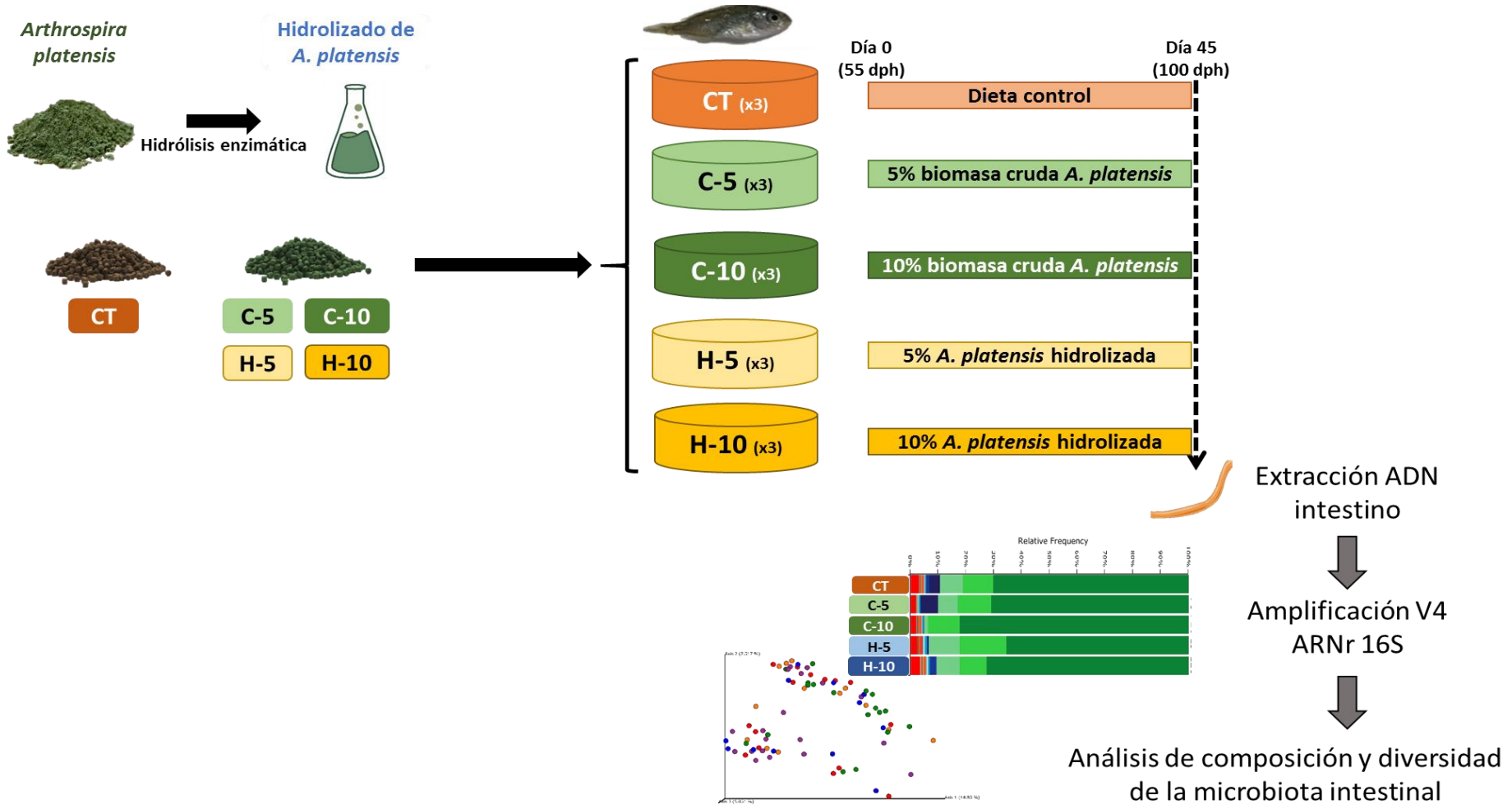

Figura M.7. Diseño experimental correspondiente al Capítulo VI. CT: Dieta control, C-5: Dieta suplementada con 5% de *A. platensis* cruda, C-10: Dieta suplementada con 10% de *A. platensis* cruda, H-5: Dieta suplementada con 5% de *A. platensis* hidrolizada, H-10: Dieta suplementada con 10% de *A. platensis* hidrolizada.

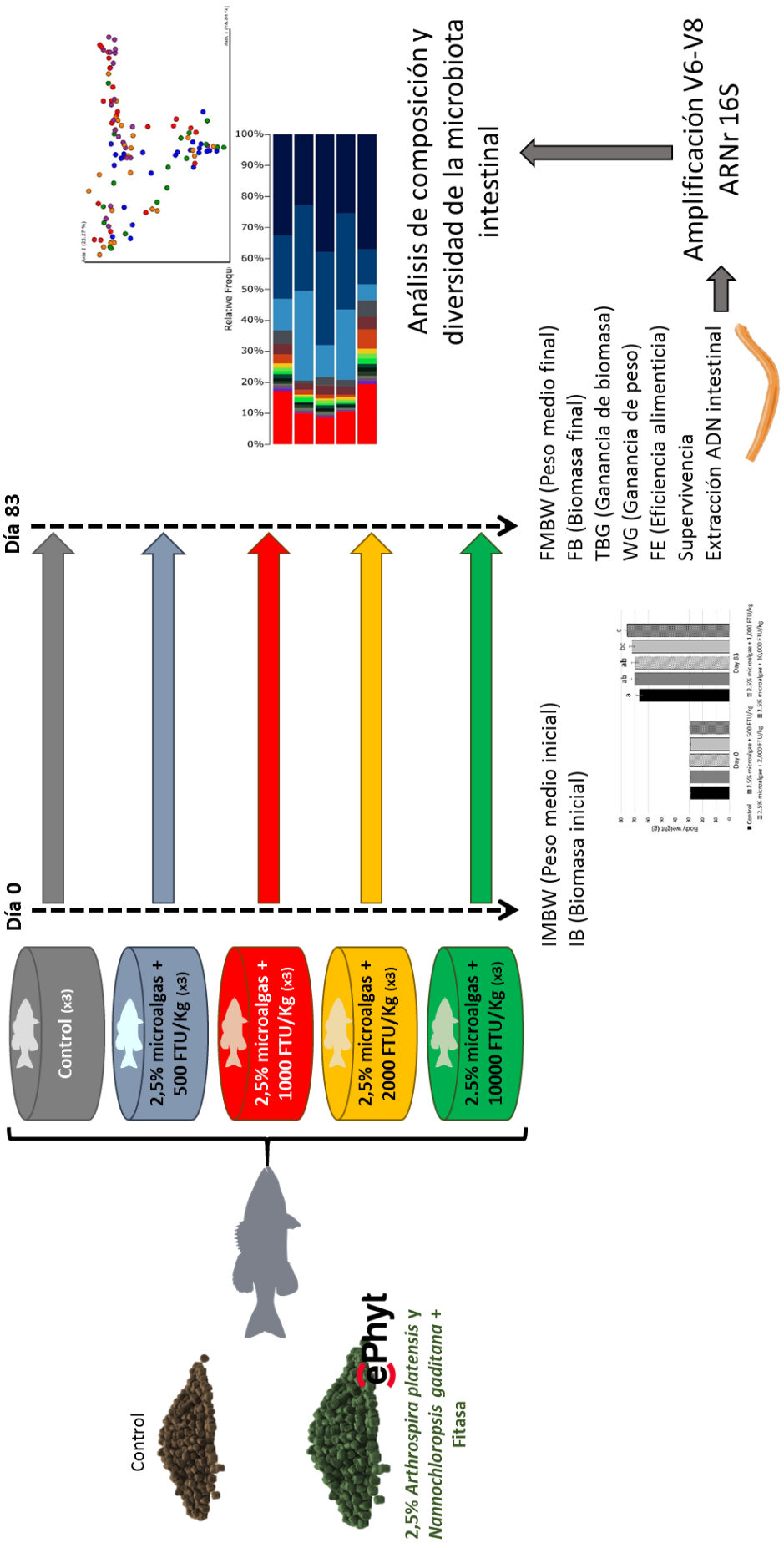

Figura M.8. Diseño experimental correspondiente al Capítulo VII. FTU: Unidades de fitasa.

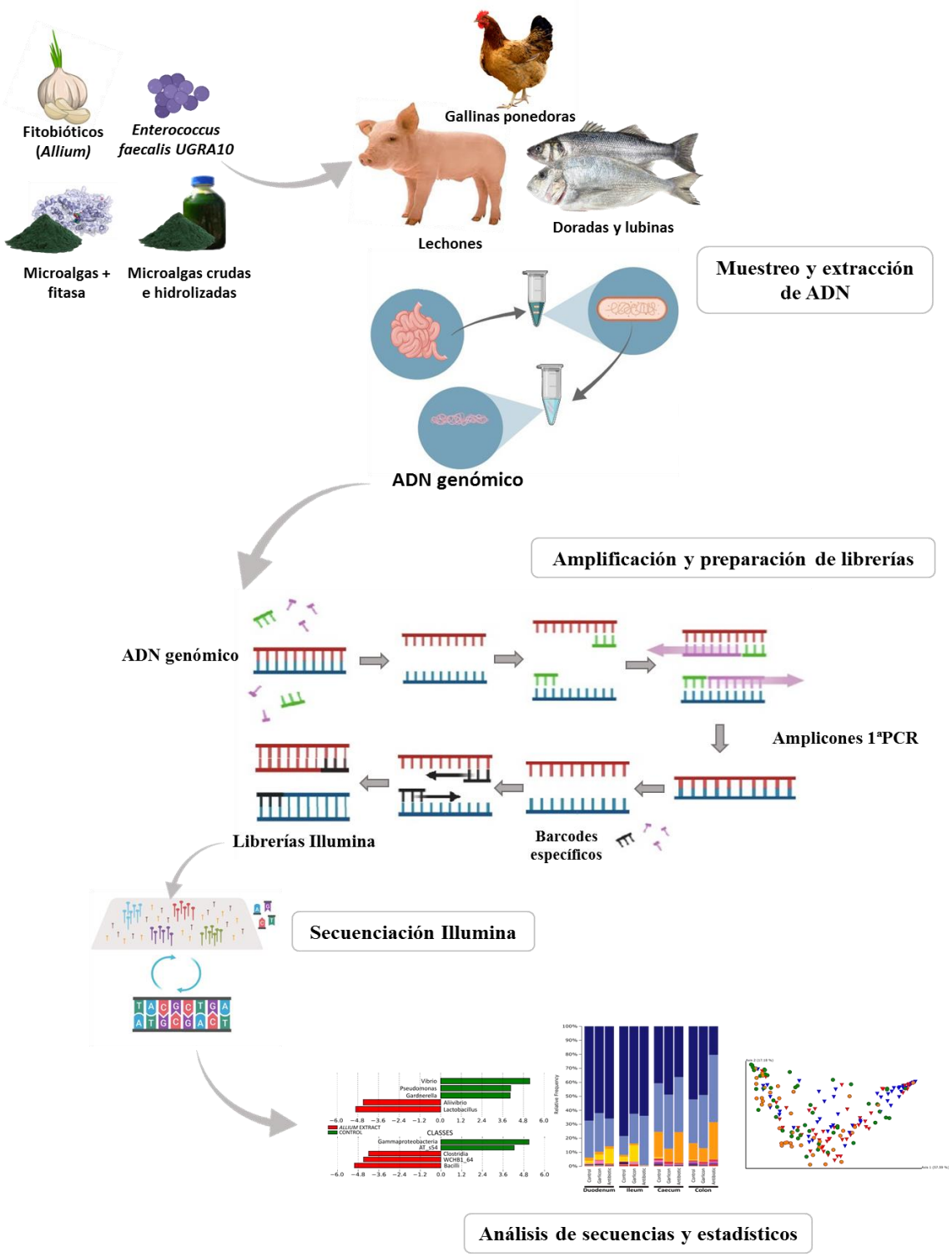

Figura M.9. Esquema general de los pasos seguidos durante los diferentes estudios de microbiota intestinal. En primer lugar, se extrajo el intestino de los distintos animales suplementados con las diferentes dietas experimentales, a partir del cual se llevó a cabo la extracción del ADN genómico de las bacterias presentes en la microbiota intestinal. El ADN se utilizó como molde para amplificar el gen del ARNr 16S, obteniéndose las librerías de Illumina tras dos pasos de amplificación por PCR. Las librerías se secuenciaron mediante la plataforma MiSeq de Illumina, obteniéndose millones de secuencias con las que se realizaron análisis de composición y diversidad microbiana, así como análisis estadísticos.

7. Referencias

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Xu, Z. Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(9), 1091. https://doi.org/10.1038/s41587-019-0252-6

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Chao, A. (1984). Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, *11*(4), 265–270.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Faith, D. P., & Baker, A. M. (2006). Phylogenetic Diversity (PD) and Biodiversity Conservation: Some Bioinformatics Challenges. *Evolutionary Bioinformatics*, *2*, 121–128. https://doi.org/10.1177/117693430600200007

Holm, J. B., Humphrys, M. S., Robinson, C. K., Settles, M. L., Ott, S., Fu, L., … Ravel, J. (2019). Ultrahigh-Throughput Multiplexing and Sequencing of >500-Base-Pair Amplicon Regions on the Illumina HiSeq 2500 Platform. *MSystems*, *4*(1). https://doi.org/10.1128/MSYSTEMS.00029-19

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MySistems*, *3*(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, *73*(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Analytical Biochemistry*, *366*(1), 102–104. https://doi.org/10.1016/J.AB.2007.03.010

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Pielou, E. C. (1966). Measurement of diversity in different types of biological collections. *Journal of Theoretical Biology*, *13*(C), 131–144. https://doi.org/10.1016/0022-5193(66)90013-0

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*(10). https://doi.org/10.7717/PEERJ.2584

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shannon, C. E. (1948). A Mathematical Theory of Communication. *The Bell System Technical Journal*, *27*, 379-423,623-656.

Vázquez-Baeza, Y, Gonzalez, A., Smarr, L., McDonald, D., Morton, J. T., Navas-Molina, J. A., & Knight, R. (2017). Bringing the Dynamic Microbiome to Life with Animations. *Cell Host & Microbe*, *21*(1), 7–10. https://doi.org/10.1016/J.CHOM.2016.12.009

Vázquez-Baeza, Yoshiki, Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

CAPÍTULOS

CAPÍTULO I

Egg Production in Poultry Farming Is Improved by Probiotic Bacteria

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ABSTRACT

Antimicrobial resistance (AMR) is one of the most serious threats for human health in the near future. Livestock has played an important role in the appearance of antibiotic-resistant bacteria, intestinal dysbiosis in farming animals, or the spread of AMR among pathogenic bacteria of human concern. The development of alternatives like probiotics is focused on maintaining or improving production levels while diminishing these negative effects of antibiotics. To this end, we supplied the potential probiotic *Enterococcus faecalis* UGRA10 in the diet of laying hens at a final concentration of 10^8 Colony Forming Units per gram (CFU/g) of fodder. Its effects have been analyzed by: (i) investigating the response of the ileum and caecum microbiome; and (ii) analyzing the outcome on eggs production. During the second half of the experimental period (40 to 76 days), hens fed *E. faecalis* UGRA10 maintained egg production, while control animals dropped egg production. Supplementation diet with *E. faecalis* UGRA10 significantly increased ileum and caecum bacterial diversity (higher bacterial operational taxonomic unit richness and Faith's diversity index) of laying hens, with animals fed the same diet showing a higher similarity in microbial composition. These results point out to the beneficial effects of *E. faecalis* UGRA10 in egg production. Future experiments are necessary to unveil the underlying mechanisms that mediate the positive response of animals to this treatment.

Keywords: bacterial community, egg production, *Enterococcus faecalis* UGRA10, high-throughput sequencing, laying hens, probiotics

INTRODUCTION

The emergence of antimicrobial resistance (AMR) is a worldwide issue in public health (Ferri et al., 2017; World Health Organization, 2018). This situation has been reached due to the abusive prescription of antibiotics, their inappropriate use by patients, and the abuse of these substances in livestock (Hecker et al., 2003; Levy & Marshall, 2004; Capita & Alonso-Calleja, 2013). The indiscriminate use of antibiotics in livestock is due to two major reasons. The first and most obvious one is disease control associated with intensive farming and animal overcrowding (McEwen & Fedorka-Cray, 2002; Gilchrist et al., 2007; Marshall & Levy, 2011). The second reason is the discovery of the effects of antibiotics as growth promoters (AGPs, reviewed in McEwen & Fedorka-Cray, 2002; Dibner & Richards, 2005). Although the mechanisms of action are still unclear, antibiotic imbalance alters the bacterial communities of the intestine, causes alterations in the digestive tract and in the metabolic processes, and increases nutrient absorption (Dibner & Richards, 2005; Miles et al., 2006; Niewold, 2007). In addition, antibiotics affect the immune system, via changes in bacterial community or directly altering the immune response (Bode et al., 2014; Yang et al., 2017). Therefore, one of the bacterial communities most affected by the use of antibiotics is the intestinal microbiome (McEwen & Fedorka-Cray, 2002; Wegener, 2003). These bacteria play a fundamental role in gut homeostasis, balance, and resilience (Clemente et al., 2012; Huttenhower et al., 2012). However, the appearance of undesirable collateral effects, especially affecting the distribution and selection of AMR genes in commensal bacteria, makes these bacteria risky to human health (Wegener, 2003) and makes it necessary to search alternatives to the use of antibiotics in livestock (Ferket, 2004).

Antibiotics have played a major role in maximizing poultry production (Dibner & Richards, 2005; McEwen & Fedorka-Cray, 2002). Thus, they have been used as growth promoters in broilers (Gadde et al., 2018; Wealleans et al., 2018), or egg production enhancers in laying hens (Park et al., 2016). This increase in productivity has been associated with the beneficial role of antibiotics in infection control in poultry farms (Gustafson & Bowen, 1997; McEwen & Fedorka-Cray, 2002; Singer & Hofacre, 2006). However, undesirable and collateral effects have appeared, especially affecting changes in the distribution and selection of AMR genes in commensal bacteria (Teuber, 2001; Diarra et al., 2007; Diarrassouba et al., 2007; Gyles, 2008). Some evidences point out the appearance of AMR in relation to the use of antibiotics in poultry. The relationship

between the use of fluoroquinolones and AMR in *Campylobacter* sp. has been evidenced (Alfredson & Korolik, 2007; Nelson et al., 2007). Multi-resistant strains of *Escherichia coli* have been associated with avian farms and have been found in chicken-derived products (Diarra et al., 2007; Diarrassouba et al., 2007; Thibodeau et al., 2008; Gyles, 2008; Osman et al., 2018). Several studies pointed out poultry and derivate-food as a reservoir and potential resource for *Salmonella* sp. resistant strains (Carramiñana et al., 2004; De Oliveira et al., 2005; Singh et al., 2010; Velasquez et al., 2018). Under this scenario of AMR, governments and institutions started to ban the use of antibiotics in livestock (European Commission, 2003, 2005; U.S. FDA, 2017). However, the prohibition of antibiotics in livestock in general, and in the poultry sector in particular, has caused an increase in incidence of infectious diseases by *Campylobacter jejuni* or *Clostridium perfringens* (reviewed in Van Immerseel et al., 2010; Alfredson & Korolik, 2007). Therefore, there is considerable concern and a certain need to replace antibiotics in disease control and as growth promoters (Joerger, 2003; Griggs & Jacob, 2005).

Different agents have been suggested to substitute antibiotics in poultry farming, such as prebiotics and probiotics (Patterson & Burkholder, 2003; Gaggìa et al., 2010). Most probiotics used in aviculture are bacteria that already exist in the digestive tract of animals and have properties of interest as signal modulators of intestinal cells, bacterial community stabilizers or competitors against undesirable bacterial species (de Vrese & Schrezenmeir, 2008; Kabir, 2009). Although *Enterococcus* species are not "generally recognized as safe" (GRAS, reviewed in Ogier & Serror, 2008), different *Enterococcus* species have been tested as probiotics (Fuller, 1989; Franz et al., 2011). The use of enterococci as probiotics remains controversial: while the probiotic benefits of some strains have been well-established, the increase in enterococcal diseases associated with human health and resistance to multiple antibiotics has raised concerns about their use (Franz et al., 2003). Despite this controversy, some strains are commercialized as probiotics, such as *Enterococcus faecium* SF68 (Cylactin, F. Hoffmann-Roche, S.A., Switzerland) and *Enterococcus faecalis* (Symbioflor, SymbioPharm, Germany) (Habermann et al., 2001; Fenimore et al., 2017; Torres-Henderson et al., 2017). *E. faecalis* has been pointed out as a potential substitute for antibiotics (Gaggìa et al., 2010). *E. faecalis* is a common bacterium in vertebrate gut, Gram+, facultative anaerobe, with a high degree of tolerance to pH (3–10) and salinity

[6.5% NaCl (w/v)] (Krieg & Holt, 1984; Schleifer & Kilpper-Bälz, 1984) which would allow gastrointestinal transit to the large intestine. Moreover, they are good producers of antagonistic substances, especially bacteriocins, also called enterocins, which allow a great interaction with the rest of the community (Foulquié-Moreno et al., 2006; Martín-Platero et al., 2006; Trmcic et al., 2011). In this sense, the strain *E. faecalis* UGRA10 isolated from an Andalusian goat cheese is a producer of the enterocin AS-48 and has very interesting technological properties: it is resistant to high concentrations of bile [up to 40% (w/v)]; shows a high antagonistic spectrum including Gram- and Gram+ bacteria (Cebrián et al., 2012) and seems to stimulate immune response in animals (Baños, 2016). Interestingly, this strain is harmless to mice and fish, and protects against some pathogenic strains such as *C. perfringens* in mice and *Lactococcus garvieae* in rainbow trout and zebrafish (Baños, 2016). These properties make *E. faecalis* UGRA10 an excellent candidate for probiotic and a model to test its implantation, its effects on the community and, especially, on health and production of animals.

We investigated the possible influence of *E. faecalis* UGRA10 on egg production and gut microbiome of laying hens when administered in the diet, combining the classical culture techniques with the latest high throughput sequencing.

MATERIAL AND METHODS

Laying hens and Farm Facilities

The experiment was performed at Granja Avícola Gil, SL, a laying hen farm (Alhendín, Granada, Spain). Laying hens were kept at 20 ± 2°C and 78 ± 3% relative humidity (average ± standard deviation), under a photoperiod of 16 h per day. The farm fulfilled the national regulations and the European directive for the protection of animal welfare in research (Directive 2010/63/EU, European Commission, 2010).

Experimental Design and Sampling Collection

Three production lines housed 180 experimental laying hens in groups of 6 hens per cage, with food and water *ad libitum*. All laying hens belonged to the same Hy Line brown variety and were placed in cages at the age of 16 weeks. Cage distribution between treatment groups was randomly assigned in three production lines. Hens were kept and fed during 2 weeks for acclimation.

Control hens (90 hens, 15 cages) received a basal fodder diet (45% fish flour, 35% soya, 8% granulated corn, 7% bran corn, and 5% sunflower bread) while experimental hens (90 hens, 15 cages) received the same diet but supplemented with the bacterium *E. faecalis* UGRA10 (see below).

Hens were first kept at the farm for 15 days for acclimation. Experiment started on April 8th, and egg production (number of eggs) of each treatment group was recorded every working day until the day 76. At this experimental farm, laying hens are slaughtered after 76 days. One day per week, eggs from each treatment were weighted and classified into size categories (S: <53 g; M: 53–63 g; L: 63–73 g; XL; >73 g) according to EU regulation (European Commission, 2008). Fecal samples were collected on days 7, 15, 40, and 76 of the experiment. On day 40, 13 hens of each group, they were euthanized by an intrathoracic injection of 2 mL/hen of the euthanasic T-61 (Intervet, Salamanca, Spain). On day 76, 10 hens from each group were euthanized following similar procedure. Immediately after slaughtering, hens were dissected and ileum and caecum were collected with sterile material, kept in sterile plastic bags, and transported directly to the lab where samples were processed. No animal died during the experimental period due to illness or malnutrition.

E. faecalis UGRA10 Production

Enterococcus faecalis UGRA10 was isolated by the Microbial Antagonism research group from the University of Granada (Cebrián et al., 2012). The bacteria were cultured in bioreactors in the DMC Research facilities located in Alhendín (Granada, Spain), as by-product of AS-48 bacteriocin production. Briefly, the strain was cultured from -80°C stock in Tryptic Soy Agar (Scharlau, S.L., Spain) and isolated colonies were inoculated into 2 L Brain Heart Infusion broth (BHI, Scharlau, S.L., Spain) and incubated at 28°C for 24 h. This primary inoculum was added (5%) to a broth based on Lactoalbumin Esprion 300 (E-300, DMW International, Holland) as described in Ananou et al. (2008) in a 20 L biofermenter (Biobech 20 Applikon Biotechnology, Delft, Netherlands), and then incubated at 28°C for 24 h. Afterward, 16 L of this culture were added of a 300 L of Lactoalbumin Esprion 300 in a CHEMAP fermenter (Compact Unit, Process Engineering Company, India) and incubated at 29°C for 24 h. All inocula and broths were adjusted at pH 6.5. Cells were collected by means of aM-500 ultra-filtration

equipment (BionetIngenieria, Spain). Afterward, cells were suspended in saline buffer and re-centrifuged twice. Final clean pellets were kept at -20°C.

Cells were encapsulated into β-cyclodextrin microstructures and kept at 4°C for up to 15 days. β-cyclodextrin is an excellent transport agent due to the lack of significant effects on hosts (Del Valle, 2004). In order to study cell viability included inside β-cyclodextrin, we cultured serial decimal dilutions of three samples of these microstructures (1 g of sample diluted in 10 mL of phosphate buffer) at different time intervals (0, 7, 15, and 30 days of storage) in TSA and incubated at 37°C for 48 h. Cells viability was reduced to 15–30 days of storage (Kruskal–Wallis, $H_{2,11} = 9.36$, $P = 0.025$). Following a conservative strategy, β-cyclodextrin complex was produced every 15 days in order to ensure cell viability. UGRA10-β-cyclodextrin microstructures were mixed with the food of experimental laying hens at a final concentration of 10^8 Colony Forming Units per gram (CFU/g) of fodder. Probiotic was administered daily throughout the experiment.

Enumeration of Microorganisms in Fecal Samples

Once in the lab, fecal samples were weighted, transferred to lab blender bags, and diluted 10-fold in phosphate saline buffer added with 0.5 g/L cysteine hydrochloride (for ensuring viability of anaerobic bacteria). The samples were homogenized using a stomacher lab blender (IUL Instruments, Spain) for 2 min. For each fecal treatment and sampling point (7, 15, 40, and 76 days), three sets of samples were collected. Each set consisted of a mixture of seven fecal samples from different cages with the same treatment.

Decimal serial dilutions of each set were performed and cultured in triplicate on Wilkins-Chalgren agar for total anaerobic bacteria (Scharlau, S.L., Spain) and Slanetz-Bartley agar for *Enterococcus* sp. (Scharlau, S.L., Spain). Plates were incubated at 37°C for 48 h in 2.5 L anaerobic chambers (Oxoid) and 2.5 L AnaeroGen Compact system (Thermo Scientific). Bacterial counts were expressed as CFU/g per gram of fecal sample.

***E. faecalis* UGRA10 Indirect Detection in Fecal Samples**

We tested the inhibition capacity of isolates from fecal samples against two indicator strains, *E. faecalis* S-47 and *E. faecalis* UGRA10. We expected the *Enterococcus* population in feces of the treated group to be dominated by *E.*

faecalis UGRA10, so most of the isolates will produce inhibition halos against S-47 (Cebrián et al., 2012). However, these halos will disappear in the presence of the original strain due to the fact that *E. faecalis* UGRA10 is immune to its own bacteriocin (Cebrián et al., 2012).

Indicator strains were cultured from the stock of the Lactic Acid Bacteria Laboratory in the University of Granada. After isolation in Brain Heart Infusion agar (BHA), colonies were cultured overnight in 6 mL BHI. The antagonistic activity was tested following Tagg & Mcgiven (1971). Stainless steel cylinders for antibiotics (diameter: 8 mm, height: 10 mm; Scharlab, S.L.) were placed on a layer of 10 mL Müller-Hilton agar (Scharlau, S.L.) buffered with phosphate buffer saline (pH 7.2, $M = 0.2$). Afterward, a 6 mL of BHA (Scharlau, S.L.) tempered around 55°C, were inoculated with one of the indicator strains (around 10^8 CFU per mL), shaken in a vortex and extended over the Müller-Hilton agar. Once the BHA solidified, the cylinders were taken out, leaving a circular hole in the BHA layer.

From Slanetz-Bartley agar plates, we selected 320 colonies randomly from plates for each treatment and sampling (2 treatments × 4 sampling times × 40 colonies). Each colony was incubated overnight in tubes containing 6 mL of BHI tubes, 1 mL was centrifuged and 100 µL of supernatant were added to the holes. Plates were incubated during 18–24 h at 37°C. Inhibition activity was measured as presence or absence of inhibition halo.

High-Throughput Sequencing

Bacterial total DNA from ileum and caecum samples was extracted by following the Modified Salting-Out Procedure by Martín-Platero et al. (2007). Amplicon PCR was performed from bacterial total DNA on the V4 region of the 16S rRNA gene by using the primer pair 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) – 786r (5′-GGACTACHVGGGTWTCTAAT-3′) with Golay barcodes on the forward primer. High-throughput sequencing was performed on Illumina Miseq platform in the Scientific Instrumental Center at the University of Granada (Spain). Ileum and caecum of 5 control and 10 treated hens were used in subsequent analyses, for both sampling times (on days 40 and 76). Six samples failed to amplify (see distribution in Supplementary Table S1). Sequences are available in the Sequence Read Archive (SRA) in the GenBank – NCBI webpage under Accession Nos. SAMN09603288 to SAMN9603361.

Subsequent analyses were performed with QIIME2 v2018.02 (Quantitive Insights In Microbial Ecology, Caporaso et al., 2010). Primer trimming, pair joining, and quality filtering were performed by using default parameters. Afterward, we used Deblur, a sub-operational-taxonomic-unit (sOTU) approach, in order to remove sequencing errors (Amir et al., 2017). We used the fragment insertion script implemented in QIIME2, a script that performs the sequence alignment and *de novo* phylogenetic tree (Janssen et al., 2018). Taxonomy assignment was based on Greengenes 13_08 with a similarity of 99% (DeSantis et al., 2006). Finally, chloroplasts and mitochondria were removed from the sOTU table, but Cyanobacteria were retained in subsequent analyses (Ley et al., 2005).

Statistical Analysis

We used general linear models (GLMs) to explore the effect of the treatment, sampling date and their interaction in different dependent variables: bacterial cultures, number of eggs (Supplementary Table S2), and different indexes of alpha diversity. We also explored the effects of egg size as factor (S, M, L, or XL as describe above). Whittaker (1972) defined diversity in three different levels: alpha diversity is the diversity found in a sample; beta diversity is the compositional difference between samples; and gamma diversity is the diversity at the regional scale. We calculated three different alpha diversity indexes from the sOTU table: bacterial operational taxonomic unit (OTU) richness (or number of observed OTUs), Faith's phylogenetic diversity index (Faith & Baker, 2006) and chao1 (Chao, 1984). Residuals of the dependent variables after analyses followed normal distribution (Kolmogorov–Smirnov normality test; $P > 0.20$) and were homoscedastic (Levene's test for homogeneity of variances, all $P > 0.19$). These results validate the use of parametric statistical tests. These analyses were performed in Statistica 10.0.

Beta diversity distance matrixes were calculated using Unifrac distance (Lozupone & Knight, 2005) based on a rarefied sOTU table at 1800 sequences depth per sample. Both weighted and unweighted Unifrac distance matrixes were used in subsequent analyses as we do not have *a priori* predictions in the effects of the independent variables (treatment, sampling date, and gut portion) on the bacterial community. Weighted Unifrac gives more importance to the most abundant bacteria as it takes into account sequence abundance per sOTU, while unweighted Unifrac gives similar weight to all bacterial sOTU present in the samples, i.e., it gives more importance

to the minority bacteria as it takes into account the presence or absence of a sOTU. Procrustes ANOVA was used to test these effects on both Unifrac distance matrixes (Collyer et al., 2015), using the geomorph (Adams & Otárola-Castillo, 2013) and vegan (Oksanen et al., 2016) packages. Principal Coordinate Analyses were performed and visualizations of the three first PCoA axes were plotted using Emperor 2018.2.0 (Vázquez-Baeza et al., 2013).

RESULTS

Alpha Diversity of Bacterial Community in Ileum and Caecum

Ileum microbiome of control hens on both 40 and 76 days were dominated at the class level by *Clostridia*, *Bacteroidia*, *Erysipelotrichi*, *Mollicutes*, *Deltaproteobacteria*, and *Bacilli* (Figure 1 and Supplementary Figure S1). These dominant classes were present in *E. faecalis* UGRA10 treated hens on day 40, although proportions of each class changed, being the class Clostridia the only dominant one (Figure 1 and Supplementary Figure S1). The ileum community in the control hens was very diverse at the genus level, dominated by *Desulfovibrio*, *Bacteroides*, an unknown genus of the order *Bacteroidales*, an unknown genus of the family *Ruminococcaceae* and an unknown genus of the family *Mogibacteriaceae* (Supplementary Figure S2). The bacterial community of samples from hens treated with *E. faecalis* UGRA10 was very similar, although other dominant genus as *Phascolarctobacterium* or *Megamonas* appeared (Supplementary Figure S2).

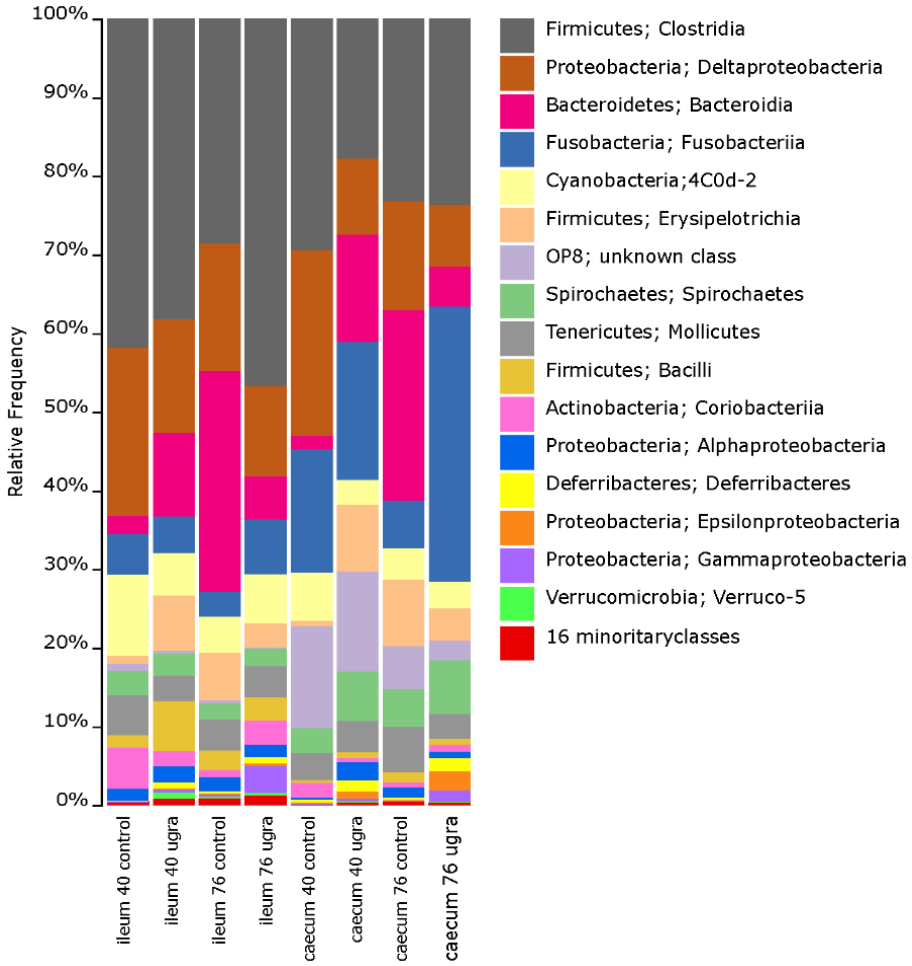

Figure 1. Bar plots of average relative bacterial abundance in gut regions of laying hens at the class level, grouped by sampling time and treatment. Classes in the legend are sorted by sequence relative abundance, from most abundant to least abundant. UGRA refers to hen fed *Enterococcus faecalis* UGRA10 at 10^8 CFU per gram of fodder per day; while 40 and 76 refers to the number of days after the experiment started.

Operational taxonomic unit richness in the ileum differed significantly between treatments throughout the experimental period (Table 1). While *E. faecalis* UGRA10 group kept similar levels of bacterial OTU richness, the control one experienced an increase in OTU richness until reaching similar levels to those of the treatment group at day 76 (Figure 2 and Supplementary Table S3). Similar pattern was marginally significant in the case of Faith's diversity index (see interaction term in Faith's diversity index in ileum, Table 1).

Table 1. General linear Models exploring the effects of treatment (control and *E. faecalis* UGRA10 administration) and sampling time (days 40 and 76) in the different alpha diversity indexes of the bacterial community of ileum and caecum of laying hens.

	Explanatory variables	**d.f.**	***F***	***P***
Ileum				
Species richness	Treatment	1,23	12.83	**0.002**
	Time	1,23	1.79	0.194
	Treatment x Time	1,23	5.18	**0.032**
Pielou evenness	Treatment	1,23	0.27	0.608
	Time	1,23	5.32	**0.030**
	Treatment x Time	1,23	1.52	0.230
Faith's diversity index	Treatment	1,23	10.70	**0.003**
	Time	1,23	1.40	0.249
	Treatment x Time	1,23	4.08	0.055
Shannon's diversity index	Treatment	1,23	1.24	0.277
	Time	1,23	1.60	0.219
	Treatment x Time	1,23	0.01	0.987
Caecum				
Species richness	Treatment	1,23	0.23	0.636
	Time	1,23	0.41	0.528
	Treatment x Time	1,23	0.00	0.966
Pielou evenness	Treatment	1,23	0.23	0.638
	Time	1,23	0.84	0.370
	Treatment x Time	1,23	0.41	0.529
Faith's diversity index	Treatment	1,23	0.72	0.405
	Time	1,23	1.21	0.283
	Treatment x Time	1,23	0.35	0.561
Shannon's diversity index	Treatment	1,23	0.26	0.618
	Time	1,23	0.29	0.597
	Treatment x Time	1,23	0.21	0.650

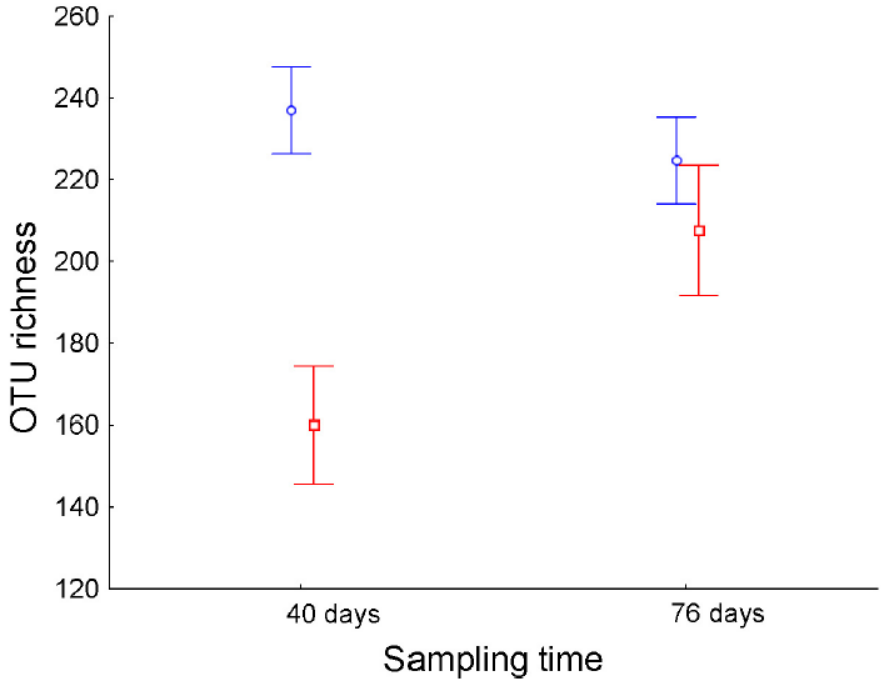

Figure 2. Average ± standard error of the mean of the bacterial OTU richness (number of different OTUs) of ileum of laying hens at different sampling times (in days) from control (n = 5, red) and hens supplemented with *E. faecalis* UGRA10 (n = 10, blue).

The bacterial community of caecum was more diverse than that of ileum (Figure 1 and Supplementary Figure S1). Major classes on days 40 and 76 in both experimental groups included *Clostridia*, *Deltaproteobacteria*, *Bacteroidia* and the unknown phylum *OP8* (Figure 1 and Supplementary Figure S1). The genera abundance between treatments and sampling date followed similar patterns. Bacterial community was dominated by *Phascolarctobacterium*, *Fusobacterium*, *Desulfovibrio*, an unknown genera belonging to the class *Cyanobacteria* and *Megamonas* (Supplementary Figures S2A,B). Alpha diversity was very similar between treatment groups, showing no significant changes throughout the experimental period (Table 1).

Effects of Treatment and Sampling Date on Beta Diversity

In the ileum region, changes of bacterial communities throughout the experiment period varied from one treatment to another (see Figure 3 and the interaction term in Table 2). Interestingly, control communities on day 76 overlapped with bacterial communities of *E. faecalis* UGRA10 treatment.

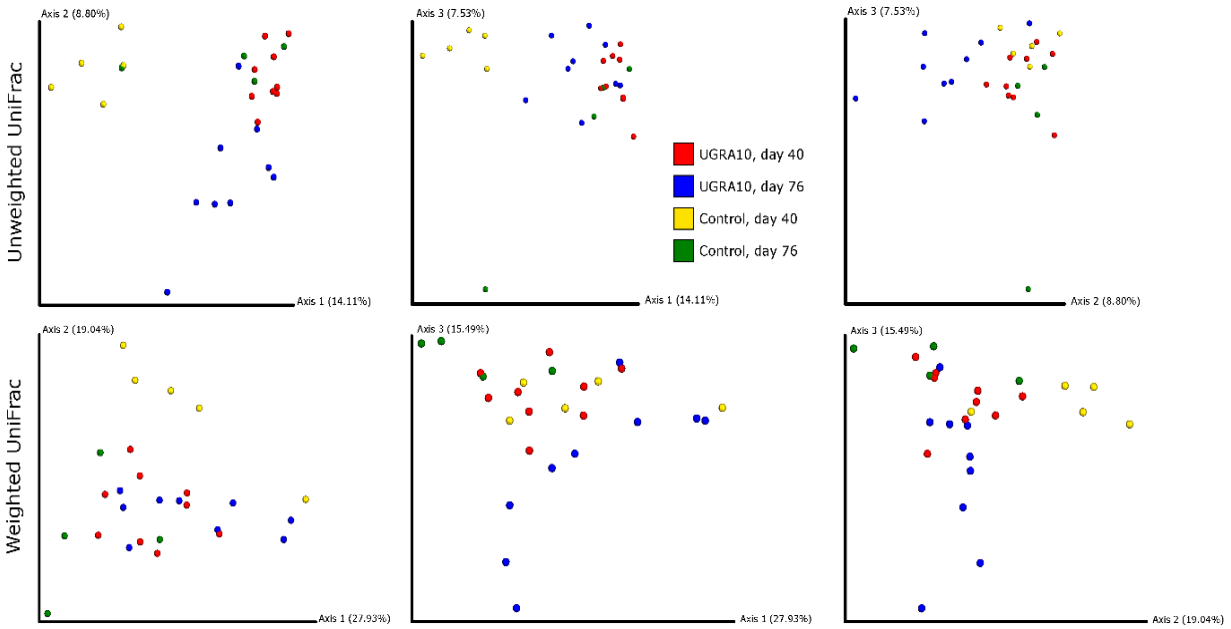

Figure 3. Two-dimensional figures showing three first axes of Principal Coordinate Analysis and representing bacterial communities of ileum of laying hens, using Unweighted and Weighted Unifrac distance matrixes. Proportion of explained variance by each PCo axes is also shown.

Treatment explained a significant proportion of the variance in bacterial community of caecum (both Weighted and Unweighted Unifrac) and its effect depends on the sampling date (see interaction term in Figure 4 and Table 2).

Table 2. PROCRUTES ANOVA exploring the effects of treatment, sampling date and their interaction in the bacterial community of laying hens fed with a control diet or supplemented with *Enterococcus faecalis* UGRA10.

	β-diversity distance matrix	**Explanatory variables**	**d.f.**	***F***	***P***
Ileum	Unweighted Unifrac	Day	1,23	1.47	**0.020**
		Treatment	1,23	2.48	**0.001**
		Day * Treatment	1,23	2.57	**0.001**
	Weighted Unifrac	Day	1,23	1.63	0.060
		Treatment	1,23	2.04	**0.026**
		Day * Treatment	1,23	4.16	**0.001**
Caecum	Unweighted Unifrac	Day	1,23	1.44	**0.029**
		Treatment	1,23	2.12	**0.004**
		Day * Treatment	1,23	2.06	**0.003**
	Weighted Unifrac	Day	1,23	1.54	0.105
		Treatment	1,23	2.32	**0.024**
		Day * Treatment	1,23	2.60	**0.006**

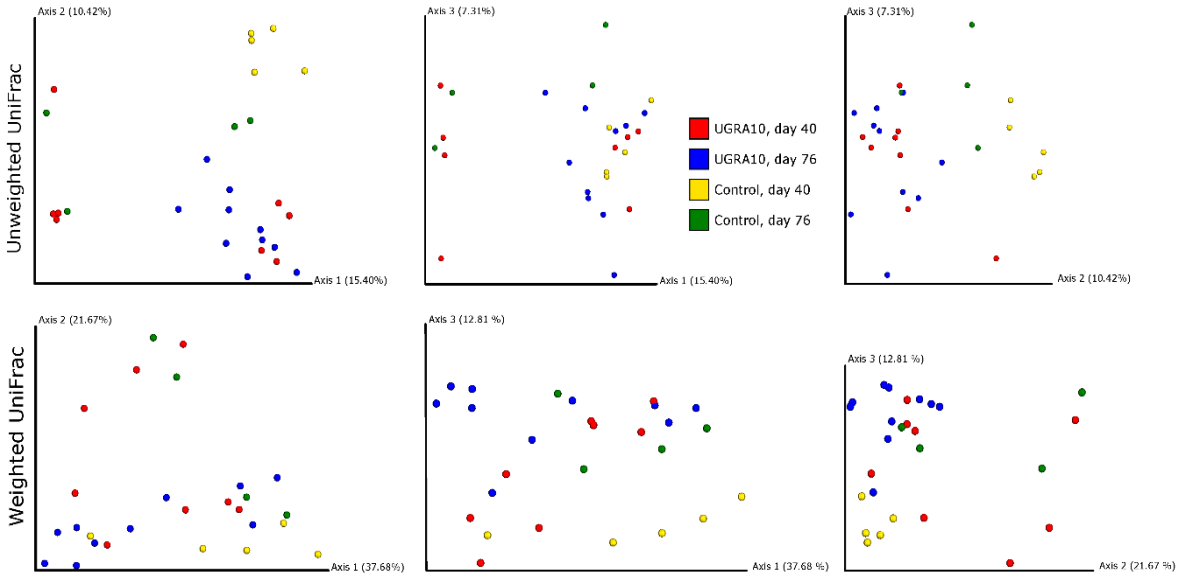

Figure 4. Two-dimensional figures showing three first axes of Principal Coordinate Analysis representing bacterial communities of caecum of laying hens, using Unweighted and Weighted Unifrac distance matrixes. Proportion of explained variance by each PCo axes is also shown.

Presence of *E. faecalis* in Feces

Cultures from each sample were highly and significantly correlated within each triplicate (Kruskal–Wallis, bacterial count as dependent variable, sample as factor, $H_{23,72} = 62.44$, $P < 0.001$), so we calculated the average values for each set of samples within each sample date and each bacterial culture.

Enterococcus sp. was successfully recovered from all Slanetz-Bartley agar plates [control, $N = 72$, log10 CFUs = 6.55 ± 0.16 (average ± SE); *E. faecalis* UGRA10: $N = 72$, 6.67 ± 0.20]. However, treatment, sampling date or their interactions did not significantly explain variation in bacterial counts of *Enterococcus* sp. (GLM, average bacterial count, treatment as factor, $F_{1,20} = 0.01$, $P = 0.915$, sampling time as covariable, $F_{1,20} = 2.15$, $P = 0.158$, interaction, $F_{1,20} = 0.13$, $P = 0.721$).

Following a similar pattern, total anaerobic bacteria (control, $N = 72$, log10 CFUs = 7.27 ± 0.99; *E. faecalis* UGRA10: $N = 72$, 7.40 ± 0.86) did not differ between treatments, sampling date or their interaction (GLM, average bacterial count, treatment as factor, $F_{1,20} = 0.05$, $P = 0.827$, sampling time as covariable, $F_{1,20} = 0.58$, $P = 0.454$, interaction, $F_{1,20} < 0.01$, $P = 0.990$).

E. faecalis UGRA10 Indirect Detection in Feces

The percentage of colonies that showed inhibition properties against E. faecalis S-47 increased along the experiment but only in the treatment group. In the control group, a low percentage of colonies showed antagonism against S-47 (Table 3). Interestingly, most of the colonies that produced antagonism against *E. faecalis* S-47 in the treatment group did not show inhibition against *E. faecalis* UGRA10, except 1 out of 30 colonies on day 76. Most of these colonies may be attributed to *E. faecalis* UGRA10, as this strain is immune against its own bacteriocin (Table 3).

Table 3. Percentages of colonies from fecal samples of laying hens that showed inhibition against indicators strain *E. faecalis* S-47 and percentage of those colonies that inhibition halo disappears against *E. faecalis* UGRA10.

Sampling time	Treatment	% inhibitor colonies against S-47	% of colonies where inhibition halo disappeared against UGRA10
7	Control	0.0	0.0
	UGRA10	0.0	0.0
15	Control	2.5	0. 0
	UGRA10	10.0	100.0
40	Control	0.0	0.0
	UGRA10	37.5	100.0
76	Control	2.5	0.0
	UGRA10	75.0	96.7

Egg Production

As 13 hens were slaughtered on day 40, we analyzed both periods separately, before and after slaughtering. Egg production was maintained in the first half of the experiment, regardless of the treatment (Figure 5A and Table 4), as both slopes of egg production did not differ from 0 (control slope = -0.007, r = -0.02, $P > 0.915$; experimental slope = -0.09; r = -0.24; $P = 0.277$). However, egg production along the second period significantly differed between treatments. While egg production of control hens significantly decreased (slope = -0.15, r = -0.51, $P = 0.020$), hens supplemented with *E. faecalis* UGRA10 maintained egg production along this period (slope = 0.07, r = 0.24, $P = 0.293$; Figure 5B and Table 4).

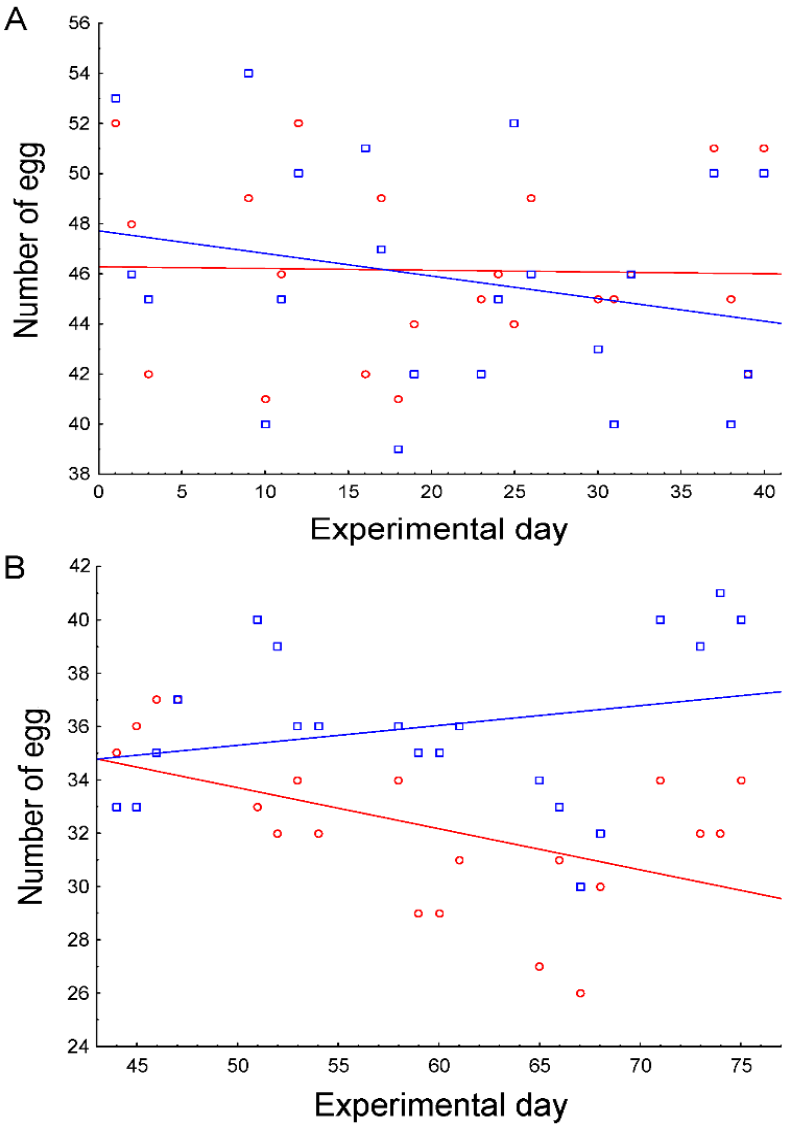

Figure 5. Correlations between number of eggs and sampling date as function of treatment, during the first (A) and second (B) half of the experimental period. Control hens (red) were fed a basal diet while experimental hens (blue) were supplemented with *E. faecalis* UGRA10.

Table 4. General Linear Models explaining egg production per laying hen in both control and diet supplemented with *Enterococcus faecalis* UGRA10, during both halves of the experimental period.

	Explanatory variables	**d.f.**	***F***	***P***
First half	Treatment	1,40	0.65	0.425
	Sampling date	1,40	0.88	0.353
	Treatment* Sampling date	1,40	0.65	0.425
Second half	Treatment	1,36	6.22	**0.017**
	Sampling date	1,36	0.76	0.390
	Treatment* Sampling date	1,36	6.22	**0.017**

Laying hens throughout the experiment period produced significantly more eggs of size L (between 63 and 73 g), regardless of the treatment (GLM, number of eggs as dependent variable, Treatment as factor, $F_{1,47} = 0.04$, $P = 0.848$, Egg size as factor, $F_{2,47} = 370.69$, $P < 0.001$, Sampling date as covariable, $F_{1,47} = 15.03$, $P < 0.001$, Interaction between egg size and treatment, $F_{2,47} = 0.07$, $P = 0.931$; Figure 6). Interestingly, no hen produced any egg of size S (less than 53 g).

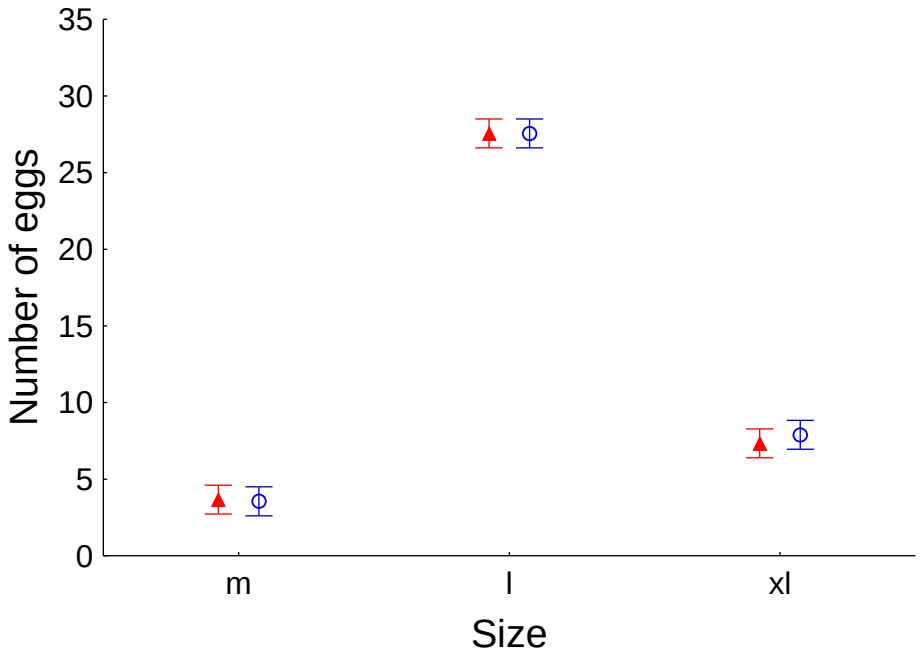

Figure 6. Average ± Standard error of the mean number of eggs produced by laying hens. Once per week, number of eggs was recorded as well as their weight and their size (m: medium; l: large; xl: extra-large). Control values are shown in red while values of laying hens supplemented with *E. faecalis* UGRA10 in the diet are shown in blue.

DISCUSSION

The addition of *E. faecalis* UGRA10 in the diet of laying hens had a positive and significant effect on egg production during the second half of the experiment, allowing these hens to maintain production levels throughout their productive lives. These beneficial productive changes were accompanied by significant changes in the gut microbiota and an increase in the presence of *E. faecalis* UGRA10 along the experimental period in the feces. These results support the use of *E. faecalis* UGRA10 as an enhancer of egg production in laying hens, while modifying bacterial community diversity.

Maintenance of egg production levels throughout laying hens' welfare is essential in poultry and the use of probiotics is one of the most promising strategies to achieve this goal (Patterson & Burkholder, 2003; Kabir, 2009). Our results support this strategy as treated hens maintained egg production, especially during the second half of the experimental period, without altering egg size. In fact, large size eggs (L: 63–73 g; and XL: >73 g) were the most produced eggs. These results are really promising as large size eggs are most demanded in different markets (Araneda Uson, 2006; Souza, 2008; Bejaei, 2009; Reichmann Bellino & Arias Nieto, 2016; Żakowska-Biemans & Tekień, 2017). Different bacterial strains have been studied and their effects tested on animal performance, egg production, enhancement of immune system and changes in gut microbiome. For instance, *Rhodobacter capsulatus* improved hen health and egg quality during the last period of the laying (Lokhande et al., 2013); *Bacillus subtilis* has been tested successfully against Salmonella infection (Oh et al., 2017) and increased egg quality and production (Ribeiro et al., 2014); or *B. licheniformis* acted as an immune system enhancer and a hormone regulator (Wang et al., 2017). The use of *E. faecalis* UGRA10 showed some advantages as enterococci are common bacteria in warm-blood animals (Krieg & Holt, 1984) and have a beneficial interaction with immune system (Franz et al., 2011), hormones and metabolism (Zhao et al., 2013) and gut microbiota (Han et al., 2013; Park et al., 2016). We recovered a high proportion of *Enterococcus sp*. in all fecal samples, around one logarithmic unit smaller than total anaerobic bacteria, so this taxon is well-represented in those fecal samples. Interestingly, the presence of *E. faecalis* UGRA10 increased during the experimental period in the treated group, until it became dominant in fecal samples. This result suggests that this strain may substitute other *Enterococcus* strains/species. This substitution between *Enterococcus* species has been reported before (Sakai et al., 2006; Saelim et al., 2012) and may be caused by the production of antagonistic substances against closely related species (Foulquié-Moreno et al., 2003, 2006; Martín-Platero et al., 2006, 2009; Ruiz-Rodríguez et al., 2013). In this sense, the ability of allochthonous bacteria, such as probiotics, to establish and flourish in complex bacterial ecosystems (as the intestine of vertebrates) is low and depends on taxon (Thomas & Versalovic, 2010). However, the positive effects of these transient bacteria are related to the active and temporary interaction with the host immune system (Are et al., 2008; Thomas & Versalovic, 2010), even though they do not establish as resident members of the bacterial community (Derrien & Vlieg, 2015). For instance, *Enterococcus* as probiotic in poultry seems to induce physical changes in the

gut structure, especially in the development of villus height/crypt depth ratio and villus height in the ileum (Awad et al., 2008), hence the increase in nutrient digestibility (Park et al., 2016). An alternative and non-exclusive hypothesis would point out the role of enterococci as immune system stimulators (Franz et al., 2011) or hormone mediators in avian performance (Zhao et al., 2013). These interactions with the immune system have been examined in model organisms (Foligne et al., 2007; de Vrese & Schrezenmeir, 2008), although our knowledge of this interaction in poultry is still scarce. In broilers, probiotic supplementation in the diet increases serum/plasma immunoglobulin levels and foster change in immune cell numbers and their phagocytosis capacities (Apata, 2008; Lee et al., 2011; Zhang et al., 2012; Salim et al., 2013; Ahmed et al., 2014; Beirão et al., 2018). The use of *E. faecium* as a probiotic produces increases in antibody titers against pathogens are also found (Nayebpor et al., 2007), changes in liver metabolic efficiency (Zheng et al., 2016), modifications in intestinal mucosa proteome (Luo et al., 2013) or increases in leptin levels and hence growth rate (Arslan et al., 2004). In laying hens, a combination of probiotics enhanced antibody response (Zhang et al., 2012). However, the mechanisms that explain the interaction between the use of Enterococcus as probiotic and the immune system and/or hormones in laying hens are still elusive, so further experiments are necessary to elucidate these relationships.

Microbiome of ileum in broilers is dominated by *Lactobacillus*, followed by *Clostridium*, *Streptococcus*, and *Enterococcus* species (Lu et al., 2003). However, *Clostridium* sp. and closely related species became dominant in caecum of laying hens, followed by members of phylum *Bacteroidetes*, especially *Bacteroides*, *Butyricimonas*, and *Prevotella* (Callaway et al., 2009; Nordentoft et al., 2011). Our results are consistent with these previous findings, especially at the phylum level, although differ in the importance of different genera. These differences in bacterial community were found mainly in ileum between treatment and sampling time. Treated hens harbored more bacterial species and a wider range of phylogenetic diversity in the ileum than control ones. Nevertheless, bacterial community in caecum showed similar levels of alpha diversity between treatments. These results of alpha diversity are supported with results of beta diversity. Bacterial communities grouped closely when we compared similarities in Unifrac distance matrixes, showing microbiome change in the gut of treated hens.

The beneficial effects of antibiotics on broilers and hens are related to changes in the microbial community of the gut (Choi et al., 2018) especially toward short-chain fatty

acid producers (Banerjee et al., 2018), but also to the increase of amino acid metabolites, fatty acids, nucleosides, and vitamins (Gadde et al., 2018). Alternatively, antibiotics improve performance through an anti-inflammatory effect mediated by the intestinal epithelium (Niewold, 2007). In spite of the differences in nature of antibiotics and probiotics, the effects of both agents on animals seem to be similar. *Enterococcus* used as a probiotic in poultry induces shifts of fecal microbiota (Han et al., 2013), especially reducing *Salmonella* populations (Waters et al., 2005), although the major phyla abundance (*Bacteroidetes* and *Firmicutes*) remains stable (Zhao et al., 2013). The diet supplemented with *E. faecalis* UGRA10 produced changes in the bacterial community, both in the ileum and the caecum, and supports, at least partially, the hypothesis that the beneficial effect on the maintenance of egg production could be mediated by effects on the gut microbiome. Further experiments should be conceived in order to unveil possible mechanisms in relation to the stimulation of the immune system or the interaction with hormones.

This experiment supports the use of *E. faecalis* UGRA10 in the diet of laying hens for successfully maintaining egg production levels and change microbiome diversity, similar effects to those found with the use of antibiotics (Park et al., 2016). However, the use of a bacterial strain would avoid the use of antibiotics in poultry, since a new member of the bacterial community is introduced instead of high antibiotic doses during prolongated exposition times.

ETHICS STATEMENT

This study was carried out in accordance with the national regulations and the European directive for the protection of animal welfare in research (Directive 2010/63/EU, European Commission, 2010). This study was performed in a regular poultry farm, following Spanish (Law 32/2007, Real Decreto 348/2000, Real Decreto 3/2002, Real Decreto 372/2003) and European regulation for poultry exploitations (Directive 98/58/CE, European Commission, 1998). We also followed the recommendations provided by the National Ministry of Agriculture, Food and Environment (Ministerio de Agricultura, Alimentación, y Medio Ambiente, Guideless of good practices of animal management and welfare in egg producing farms, 2012).

AUTHOR CONTRIBUTIONS

JP-S, AM-P, JA-R, AB, and MM-B conceived and planned the experiments. JA-R and AB carried out the farm experiments. JA-R, MR-R, and MZ-G contributed to sample preparation and performed lab analyses. JP-S, AM-P, JA-R, MR-R, MZ-G, SR-R, MM, EV, and MM-B contributed to the interpretation of the results. JP-S and AM-P took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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REFERENCES

Adams, D. C., & Otárola-Castillo, E. (2013). geomorph: an r package for the collection and analysis of geometric morphometric shape data. *Methods in Ecology and Evolution*, *4*(4), 393–399. https://doi.org/10.1111/2041-210X.12035

Ahmed, S. T., Islam, M. M., Mun, H. S., Sim, H. J., Kim, Y. J., & Yang, C. J. (2014). Effects ofBacillus amyloliquefaciens as a probiotic strain on growth performance, cecal microflora, and fecal noxious gas emissions of broiler chickens. *Poultry Science*, *93*(8), 1963–1971. https://doi.org/10.3382/PS.2013-03718

Alfredson, D. A., & Korolik, V. (2007). Antibiotic resistance and resistance mechanisms in Campylobacter jejuni and Campylobacter coli. *FEMS Microbiology Letters*, *277*(2), 123–132. https://doi.org/10.1111/J.1574-6968.2007.00935.X

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Ananou, S., Muñoz, A., Gálvez, A., Martínez-Bueno, M., Maqueda, M., & Valdivia, E. (2008). Optimization of enterocin AS-48 production on a whey-based substrate. *International Dairy Journal*, *18*(9), 923–927.

https://doi.org/10.1016/J.IDAIRYJ.2008.02.001

Apata, D. (2008). Growth performance, nutrient digestibility and immune response of broiler chicks fed diets supplemented with a culture of Lactobacillus bulgaricus. *Journal of the Science of Food and Agriculture*, *88*(7), 1253–1258. https://doi.org/10.1002/JSFA.3214

Araneda Uson, R. P. (2006). *Percepción de calidad de huevo vista por un grupo de consumidores del Gran Santiago. Bachelor thesis.* Santiago de Chile (Chile): Universidad de Chile, Chile.

Are, A., Aronsson, L., Wang, S., Greicius, G., Lee, Y. K., Gustafsson, J.-Å., … Arulampalam, V. (2008). Enterococcus faecalis from newborn babies regulate endogenous PPARγ activity and IL-10 levels in colonic epithelial cells. *Proceedings of the National Academy of Sciences*, *105*(6), 1943–1948. https://doi.org/10.1073/PNAS.0711734105

Arslan, M., Ozcan, M., Matur, E., Cotelioglu, U., & Ergul, E. (2004). The effects of probiotic on leptin level, body, liver and abdominal fat weights during the rapid growth phase of broilers. *Indian Veterinary Journal*, *81*, 416–420.

Awad, W., Ghareeb, K., & Böhm, J. (2008). Intestinal Structure and Function of Broiler Chickens on Diets Supplemented with a Synbiotic Containing Enterococcus faecium and Oligosaccharides. *International Journal of Molecular Sciences*, *9*(11), 2205–2216. https://doi.org/10.3390/IJMS9112205

Banerjee, S., Sar, A., Misra, A., Pal, S., Chakraborty, A., & Dam, B. (2018). Increased productivity in poultry birds by sub-lethal dose of antibiotics is arbitrated by selective enrichment of gut microbiota, particularly short-chain fatty acid producers. *Microbiology*, *164*(2), 142–153. https://doi.org/10.1099/MIC.0.000597

Baños, A. (2016). *Aplicación de la tecnología de las barreras en el desarrollo de as-48 como bioconservante alimentario. Estudio de probiosis de una cepa productora de as-48*. Tesis Doctoral. Universidad de Granada, University of Granada.

Beirão, B. C. B., Ingberman, M., Jr., C. F., Mesa, D., Bittencourt, L. C., Fascina, V. B., & Caron, L. F. (2018). Effect of an Enterococcus faecium probiotic on specific IgA following live Salmonella Enteritidis vaccination of layer chickens. *Avian Pathology*, *47*(3), 325–333. https://doi.org/10.1080/03079457.2018.1450487

Bejaei, M. (2009). *Attitudes and Preferences of Consumers/Purchasers Toward Different Types of Table Eggs.* Master thesis. Vancouver: University of British Columbia.

Bode, C., Diedrich, B., Muenster, S., Hentschel, V., Weisheit, C., Rommelsheim, K., … Baumgarten, G. (2014). Antibiotics regulate the immune response in both presence and absence of lipopolysaccharide through modulation of Toll-like receptors, cytokine production and phagocytosis in vitro. *International Immunopharmacology*, *18*(1), 27–34. https://doi.org/10.1016/J.INTIMP.2013.10.025

Callaway, T. R., Dowd, S. E., Wolcott, R. D., Sun, Y., McReynolds, J. L., Edrington, T. S., … Nisbet, D. J. (2009). Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. *Poultry Science*, *88*(2), 298–302. https://doi.org/10.3382/PS.2008-00222

Capita, R., & Alonso-Calleja, C. (2013). Antibiotic-Resistant Bacteria: A Challenge for the Food Industry. *Critical Reviews in Food Science and Nutrition*, *53*(1), 11–48. https://doi.org/10.1080/10408398.2010.519837

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Carramiñana, J. J., Rota, C., Agustín, I., & Herrera, A. (2004). High prevalence of multiple resistance to antibiotics in Salmonella serovars isolated from a poultry slaughterhouse in Spain. *Veterinary Microbiology*, *104*(1–2), 133–139. https://doi.org/10.1016/J.VETMIC.2004.08.010

Cebrián, R., Baños, A., Valdivia, E., Pérez-Pulido, R., Martínez-Bueno, M., & Maqueda, M. (2012). Characterization of functional, safety, and probiotic properties of Enterococcus faecalis UGRA10, a new AS-48-producer strain. *Food Microbiology*, *30*(1), 59–67. https://doi.org/10.1016/J.FM.2011.12.002

Chao, A. (1984). Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, *11*(4), 265–270.

Choi, J. H., Lee, K., Kim, D. W., Kil, D. Y., Kim, G. B., & Cha, C. J. (2018). Influence of dietary avilamycin on ileal and cecal microbiota in broiler chickens. *Poultry Science*, *97*(3), 970–979. https://doi.org/10.3382/PS/PEX360

Clemente, J. C., Ursell, L. K., Parfrey, L. W., & Knight, R. (2012). The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell*, *148*(6), 1258–1270. https://doi.org/10.1016/J.CELL.2012.01.035

Collyer, M. L., Sekora, D. J., & Adams, D. C. (2015). A method for analysis of phenotypic change for phenotypes described by high-dimensional data. *Heredity 2015 115:4*, *115*(4), 357–365. https://doi.org/10.1038/hdy.2014.75

De Oliveira, S. D., Siqueira Flores, F., Dos Santos, L. R., & Brandelli, A. (2005). Antimicrobial resistance in Salmonella enteritidis strains isolated from broiler carcasses, food, human and poultry-related samples. *International Journal of Food Microbiology*, *97*(3), 297–305. https://doi.org/10.1016/J.IJFOODMICRO.2004.04.022

de Vrese, M., & Schrezenmeir, J. (2008). Probiotics, Prebiotics, and Synbiotics. *Advances in Biochemical Engineering/Biotechnology*, 1–66. https://doi.org/10.1007/10_2008_097

Del Valle, E. M. M. (2004). Cyclodextrins and their uses: a review. *Process Biochemistry*, *39*(9), 1033–1046. https://doi.org/10.1016/S0032-9592(03)00258-9

Derrien, M., & Vlieg, J. E. T. van H. (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in Microbiology*, *23*(6), 354–366. https://doi.org/10.1016/J.TIM.2015.03.002

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Diarra, M. S., Silversides, F. G., Diarrassouba, F., Pritchard, J., Masson, L., Brousseau, R., … Topp, E. (2007). Impact of Feed Supplementation with Antimicrobial Agents on Growth Performance of Broiler Chickens, Clostridium perfringens and Enterococcus Counts, and Antibiotic Resistance Phenotypes and Distribution of Antimicrobial Resistance Determinants in Escherichia coli Isolates. *Applied and Environmental Microbiology*, *73*(20), 6566–6576. https://doi.org/10.1128/AEM.01086-07

Diarrassouba, F., Diarra, M. S., Bach, S., Delaquis, P., Pritchard, J., Topp, E., & Skura, B. J. (2007). Antibiotic Resistance and Virulence Genes in Commensal Escherichia coli and Salmonella Isolates from Commercial Broiler Chicken Farms. *Journal of Food Protection*, *70*(6), 1316–1327. https://doi.org/10.4315/0362-028X-70.6.1316

Dibner, J. J., & Richards, J. D. (2005). Antibiotic Growth Promoters in Agriculture: History and Mode of Action. *Poultry Science*, *84*(4), 634–643. https://doi.org/10.1093/ps/84.4.634

European Commission. (2003). Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition (Text with EEA relevance). *Brussels: Official Journal of European Union*, 29–43.

European Commission. (2005). Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 Laying Down Requirements for Feed Hygiene (Text with EEA relevance). Brussels: Official Journal of European Union. *Brussels: Official Journal of European Union.*

European Commission. (2008). Commission Regulation (EC) No 589/2008 of 23 June 2008 Laying Down Detailed Rules for Implementing Council Regulation (EC) No 1234/2007 as Regards Marketing Standards for Eggs. *Brussels: Official Journal of European Union*.

European Commission. (2010). Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. *Brussels: Official Journal of European Union.*

Faith, D. P., & Baker, A. M. (2006). Phylogenetic Diversity (PD) and Biodiversity Conservation: Some Bioinformatics Challenges. *Evolutionary Bioinformatics*, *2*, 121–128. https://doi.org/10.1177/117693430600200007

Fenimore, A., Martin, L., & Lappin, M. R. (2017). Evaluation of Metronidazole With and Without Enterococcus Faecium SF68 in Shelter Dogs With Diarrhea. *Topics in Companion Animal Medicine*, *32*(3), 100–103. https://doi.org/10.1053/J.TCAM.2017.11.001

Ferket, P. R. (2004). Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. In T. P. Lyons and K. A. Jacques (Ed.), *Nutritional biotechnology in the feed and food industries. Proceedings of Alltech's 20th Annual Symposium: re-imagining the feed industry, Lexington, Kentucky, USA, 23-26 May 2004* (pp. 57–67). Nottinghan: Nottinghan University Press: Alltech UK.

Ferri, M., Ranucci, E., Romagnoli, P., & Giaccone, V. (2017). Antimicrobial resistance: A global emerging threat to public health systems. *Critical Reviews in Food Science and Nutrition*, *57*(13), 2857–2876. https://doi.org/10.1080/10408398.2015.1077192

Foligne, B., Zoumpopoulou, G., Dewulf, J., Younes, A. Ben, Chareyre, F., Sirard, J.-C., … Grangette, C. (2007). A Key Role of Dendritic Cells in Probiotic Functionality. *PLOS ONE*, *2*(3), e313. https://doi.org/10.1371/JOURNAL.PONE.0000313

Foulquié-Moreno, M. R., Callewaert, R., Devreese, B., Beeumen, J. Van, & Vuyst, L. De. (2003). Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. *Journal of Applied Microbiology*, *94*(2), 214–229. https://doi.org/10.1046/J.1365-2672.2003.01823.X

Foulquié-Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E., & De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology*, *106*(1), 1–24.

https://doi.org/10.1016/J.IJFOODMICRO.2005.06.026

Franz, C. M. A. P., Huch, M., Abriouel, H., Holzapfel, W., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, *151*(2), 125–140. https://doi.org/10.1016/J.IJFOODMICRO.2011.08.014

Franz, C. M. A. P., Stiles, M. E., Schleifer, K. H., & Holzapfel, W. H. (2003). Enterococci in foods—a conundrum for food safety. *International Journal of Food Microbiology*, *88*(2–3), 105–122. https://doi.org/10.1016/S0168-1605(03)00174-0

Fuller, R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology*, *66*(5), 365–378. https://doi.org/10.1111/J.1365-2672.1989.TB05105.X

Gadde, U. D., Oh, S., Lillehoj, H. S., & Lillehoj, E. P. (2018). Antibiotic growth promoters virginiamycin and bacitracin methylene disalicylate alter the chicken intestinal metabolome. *Scientific Reports*, *8*(1), 3592. https://doi.org/10.1038/s41598-018-22004-6

Gaggìa, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology*, *141*(Supplement), S15–S28. https://doi.org/10.1016/J.IJFOODMICRO.2010.02.031

Gilchrist, M. J., Greko, C., Wallinga, D. B., Beran, G. W., Riley, D. G., & Thorne, P. S. (2007). The potential role of concentrated animal feeding operations in infectious disease epidemics and antibiotic resistance. *Environmental Health Perspectives*, *115*(2), 313–316. https://doi.org/10.1289/EHP.8837

Griggs, J. P., & Jacob, J. P. (2005). Alternatives to Antibiotics for Organic Poultry Production. *Journal of Applied Poultry Research*, *14*(4), 750–756. https://doi.org/10.1093/JAPR/14.4.750

Gustafson, R. H., & Bowen, R. E. (1997). Antibiotic use in animal agriculture. *Journal of Applied Microbiology*, *83*(5), 531–541. https://doi.org/10.1046/J.1365-2672.1997.00280.X

Gyles, C. L. (2008). Antimicrobial resistance in selected bacteria from poultry. *Animal Health Research Reviews*, *9*(2), 149–158. https://doi.org/10.1017/S1466252308001552

Habermann, W., Zimmermann, K., Skarabis, H., Kunze, R., & Rusch, V. (2001). Influence of a bacterial immunostimulant (human Enterococcus faecalis bacteria) on the recurrence of relapses in patients with chronic bronchitis. *Arzneimittel-Forsch*, *51*, 931–937. https://doi.org/ 10.1055/s-0031-1300140

Han, W., Zhang, X. L., Wang, D. W., Li, L. Y., Liu, G. L., Li, A. K., & Zhao, Y. X. (2013). Effects of microencapsulated Enterococcus fecalis CG1.0007 on growth performance, antioxidation activity, and intestinal microbiota in broiler chickens. *Journal of Animal Science*, *91*(9), 4374–4382. https://doi.org/10.2527/JAS.2012-5956

Hecker, M. T., Aron, D. C., Patel, N. P., Lehmann, M. K., & Donskey, C. J. (2003). Unnecessary Use of Antimicrobials in Hospitalized Patients: Current Patterns of Misuse With an Emphasis on the Antianaerobic Spectrum of Activity. *Archives of Internal Medicine*, *163*(8), 972–978. https://doi.org/10.1001/ARCHINTE.163.8.972

Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J. H., Chinwalla, A. T., … White, O. (2012). Structure, function and diversity of the healthy human

microbiome. *Nature*, *486*(7402), 207–214. https://doi.org/10.1038/nature11234

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MySistems*, *3*(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Joerger, R. D. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Science*, *82*(4), 640–647. https://doi.org/10.1093/PS/82.4.640

Kabir, S. M. L. (2009). The Role of Probiotics in the Poultry Industry. *International Journal of Molecular Sciences*, *10*(8), 3531–3546. https://doi.org/10.3390/IJMS10083531

Krieg, N. R., & Holt, J. G. (1984). *Bergey's Manual of Systematic Bacteriology.* (MD: Willia). Baltimore.

Lee, K.-W., Li, G., Lillehoj, H. S., Lee, S.-H., Jang, S. I., Babu, U. S., … Siragusa, G. R. (2011). Bacillus subtilis-based direct-fed microbials augment macrophage function in broiler chickens. *Research in Veterinary Science*, *91*(3), e87–e91. https://doi.org/10.1016/j.rvsc.2011.01.018

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine 2004 10:12*, *10*(12), S122–S129. https://doi.org/10.1038/nm1145

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *PNAS*, *102*, 11070–11075. https://doi.org/10.1073/pnas.0504978102

Lokhande, A., Ingale, S. L., Lee, S. H., Kim, J. S., Lohakare, J. D., Chae, B. J., & Kwon, I. K. (2013). The Effects of Rhodobacter capsulatus KCTC-2583 on Cholesterol Metabolism, Egg Production and Quality Parameters during the Late Laying Periods in Hens. *Asian-Australasian Journal of Animal Sciences*, *26*(6), 831–837. https://doi.org/10.5713/AJAS.2012.12559

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Lu, J., Idris, U., Harmon, B., Hofacre, C., Maurer, J. J., & Lee, M. D. (2003). Diversity and Succession of the Intestinal Bacterial Community of the Maturing Broiler Chicken. *Applied and Environmental Microbiology*, *69*(11), 6816–6824. https://doi.org/10.1128/AEM.69.11.6816-6824.2003

Luo, J., Zheng, A., Meng, K., Chang, W., Bai, Y., Li, K., … Yao, B. (2013). Proteome changes in the intestinal mucosa of broiler (Gallus gallus) activated by probiotic Enterococcus faecium. *Journal of Proteomics*, *91*, 226–241. https://doi.org/10.1016/J.JPROT.2013.07.017

Marshall, B. M., & Levy, S. B. (2011). Food Animals and Antimicrobials: Impacts on Human Health. *Clinical Microbiology Reviews*, *24*(4), 718–733. https://doi.org/10.1128/CMR.00002-11

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Analytical Biochemistry*, *366*(1), 102–104. https://doi.org/10.1016/J.AB.2007.03.010

Martín-Platero, Antonio M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2009). Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. *International Journal of Food Microbiology*, *132*(1), 24–32. https://doi.org/10.1016/J.IJFOODMICRO.2009.03.010

Martín-Platero, Antonio M., Valdivia, E., Ruíz-Rodríguez, M., Soler, J. J., Martín-Vivaldi, M., Maqueda, M., & Martínez-Bueno, M. (2006). Characterization of Antimicrobial Substances Produced by Enterococcus faecalis MRR 10-3, Isolated from the Uropygial Gland of the Hoopoe (Upupa epops). *Applied and Environmental Microbiology*, *72*(6), 4245–4249. https://doi.org/10.1128/AEM.02940-05

McEwen, S. A., & Fedorka-Cray, P. J. (2002). Antimicrobial Use and Resistance in Animals. *Clinical Infectious Diseases*, *34*(Supplement_3), S93–S106. https://doi.org/10.1086/340246

Miles, R. D., Butcher, G. D., Henry, P. R., & Littell, R. C. (2006). Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poultry Science*, *85*(3), 476–485. https://doi.org/10.1093/PS/85.3.476

Nayebpor, M., Farhomand, P., & Hashemi, A. (2007). Effects of different levels of direct fed microbial (Primalac) on growth performance and humoral immune response in broiler chickens. *Journal of Animal and Veterinary Advances*, *6*, 1308–1313.

Nelson, J. M., Chiller, T. M., Powers, J. H., & Angulo, F. J. (2007). Fluoroquinolone-Resistant Campylobacter Species and the Withdrawal of Fluoroquinolones from Use in Poultry: A Public Health Success Story. *Clinical Infectious Diseases*, *44*(7), 977–980. https://doi.org/10.1086/512369

Niewold, T. A. (2007). The Nonantibiotic Anti-Inflammatory Effect of Antimicrobial Growth Promoters, the Real Mode of Action? A Hypothesis. *Poultry Science*, *86*(4), 605–609. https://doi.org/10.1093/PS/86.4.605

Nordentoft, S., Mølbak, L., Bjerrum, L., De Vylder, J., Van Immerseel, F., & Pedersen, K. (2011). The influence of the cage system and colonisation of SalmonellaEnteritidis on the microbial gut flora of laying hens studied by T-RFLP and 454 pyrosequencing. *BMC Microbiology*, *11*(1), 1–11. https://doi.org/10.1186/1471-2180-11-187

Ogier, J. C., & Serror, P. (2008). Safety assessment of dairy microorganisms: The Enterococcus genus. *International Journal of Food Microbiology*, *126*(3), 291–301. https://doi.org/10.1016/J.IJFOODMICRO.2007.08.017

Oh, J. K., Pajarillo, E. A. B., Chae, J. P., Kim, I. H., & Kang, D.-K. (2017). Protective effects of *Bacillus subtilis* against *Salmonella* infection in the microbiome of Hy-Line Brown layers. *Asian-Australasian Journal of Animal Sciences*, *30*(9), 1332–1339. https://doi.org/10.5713/AJAS.17.0063

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., & Al, E. (2016). vegan: Community Ecology Package. R Package Version.

Osman, K. M., Kappell, A. D., Elhadidy, M., ElMougy, F., El-Ghany, W. A. A., Orabi, A., … Yousef, H. M. Y. (2018). Poultry hatcheries as potential reservoirs for antimicrobial-resistant Escherichia coli: A risk to public health and food safety. *Scientific Reports*, *8*(1), 1–14. https://doi.org/10.1038/s41598-018-23962-7

Park, J. W., Jeong, J. S., Lee, S. I., & Kim, I. H. (2016). Effect of dietary supplementation with a probiotic (Enterococcus faecium) on production performance, excreta microflora, ammonia emission, and nutrient utilization in ISA brown laying hens.

Poultry Science, *95*(12), 2829–2835. https://doi.org/10.3382/PS/PEW241

Patterson, J. A., & Burkholder, K. M. (2003). Application of prebiotics and probiotics in poultry production. *Poultry Science*, *82*(4), 627–631. https://doi.org/10.1093/PS/82.4.627

Reichmann Bellino, J., & Arias Nieto, W. B. (2016). Caracterización de Mercado Para la Incorporación de Comercialización de Huevo por Peso en Honduras. *Tegucigalpa: Escuela Agrícola Panamericana.*

Ribeiro, V., Albino, L. F. T., Rostagno, H. S., Barreto, S. L. T., Hannas, M. I., Harrington, D., … Ferreira, M. A. (2014). Effects of the dietary supplementation of Bacillus subtilis levels on performance, egg quality and excreta moisture of layers. *Animal Feed Science and Technology*, *195*, 142–146. https://doi.org/10.1016/J.ANIFEEDSCI.2014.06.001

Ruiz-Rodríguez, M., Martínez-Bueno, M., Martín-Vivaldi, M., Valdivia, E., & Soler, J. J. (2013). Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. *FEMS Microbiology Ecology*, *85*(3), 495–502. https://doi.org/10.1111/1574-6941.12138

Saelim, K., Sohsomboon, N., Kaewsuwan, S., & Maneerat, S. (2012). Probiotic properties of Enterococcus faecium CE5-1 producing a bacteriocin-like substance and its antagonistic effect against antibiotic-resistant enterococci in vitro. *Czech Journal of Animal Science*, *57*(11), 529–539. https://doi.org/10.17221/6386-CJAS

Sakai, Y., Tsukahara, T., Bukawa, W., Matsubara, N., & Ushida, K. (2006). Cell Preparation of Enterococcus Faecalis Strain EC-12 Prevents Vancomycin-Resistant Enterococci Colonization in the Cecum of Newly Hatched Chicks. *Poultry Science*, *85*(2), 273–277. https://doi.org/10.1093/PS/85.2.273

Salim, H. M., Kang, H. K., Akter, N., Kim, D. W., Kim, J. H., Kim, M. J., … Kim, W. K. (2013). Supplementation of direct-fed microbials as an alternative to antibiotic on growth performance, immune response, cecal microbial population, and ileal morphology of broiler chickens. *Poultry Science*, *92*(8), 2084–2090. https://doi.org/10.3382/PS.2012-02947

Schleifer, K. H., & Kilpper-Bälz, R. (1984). Transfer of Streptococcus faecalis and Streptococcus faecium to the Genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *34*(1), 31–34. https://doi.org/10.1099/00207713-34-1-31

Singer, R. S., & Hofacre, C. L. (2006). Potential Impacts of Antibiotic Use in Poultry Production. *Avian Diseases*, *50*(2), 161–172. https://doi.org/10.1637/7569-033106R.1

Singh, S., Yadav, A. S., Singh, S. M., & Bharti, P. (2010). Prevalence of Salmonella in chicken eggs collected from poultry farms and marketing channels and their antimicrobial resistance. *Food Research International*, *43*(8), 2027–2030. https://doi.org/10.1016/J.FOODRES.2010.06.001

Souza, R. (2008). La Comercialización de los Huevos. *Selecciones Avicolas: Reus*.

Tagg, J. R., & Mcgiven, A. R. (1971). Assay system for bacteriocins. *Applied Microbiology*, *21*(943).

Teuber, M. (2001). Veterinary use and antibiotic resistance. *Current Opinion in Microbiology*, *4*(5), 493–499. https://doi.org/10.1016/S1369-5274(00)00241-1

Thibodeau, A., Quessy, S., Guévremont, E., Houde, A., Topp, E., Diarra, M. S., & Letellier, A. (2008). Antibiotic resistance in Escherichia coli and Enterococcus spp. isolates from commercial broiler chickens receiving growth-promoting doses of bacitracin or virginiamycin. *Canadian Journal of Veterinary Research*, *72*(2), 129. Retrieved from /pmc/articles/PMC2276897/

Thomas, C. M., & Versalovic, J. (2010). Probiotics-host communication: Modulation of signaling pathways in the intestine. *Gut Microbes*, *1*(3), 148. https://doi.org/10.4161/GMIC.1.3.11712

Torres-Henderson, C., Summers, S., Suchodolski, J., & Lappin, M. R. (2017). Effect of Enterococcus Faecium Strain SF68 on Gastrointestinal Signs and Fecal Microbiome in Cats Administered Amoxicillin-Clavulanate. *Topics in Companion Animal Medicine*, *32*(3), 104–108. https://doi.org/10.1053/J.TCAM.2017.11.002

Trmcic, A., Obermajer, T., Majhenic, A. C., Matijasic, B. B., & Rogelj, I. (2011). Competitive advantage of bacteriocinogenic strains within lactic acid bacteria consortium of raw milk cheese. *Mljekarstvo*, *61*, 26–32.

U.S. FDA. (2017). FDA Reminds Retail Establishments of Upcoming Changes to the Use of Antibiotics in Food Animals. *U.S. Food and Drugs Administration*.

Van Immerseel, F., Buck, J. De, Pasmans, F., Huyghebaert, G., Haesebrouck, F., & Ducatelle, R. (2010). Clostridium perfringens in poultry: an emerging threat for animal and public health. *Avian Pathology*, *33*(6), 537–549. https://doi.org/10.1080/03079450400013162

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

Velasquez, C. G., MacKlin, K. S., Kumar, S., Bailey, M., Ebner, P. E., Oliver, H. F., … Singh, M. (2018). Prevalence and antimicrobial resistance patterns of Salmonella isolated from poultry farms in southeastern United States. *Poultry Science*, *97*(6), 2144–2152. https://doi.org/10.3382/PS/PEX449

Wang, Y., Du, W., Lei, K., Wang, B., Wang, Y., Zhou, Y., & Li, W. (2017). Effects of Dietary Bacillus licheniformis on Gut Physical Barrier, Immunity, and Reproductive Hormones of Laying Hens. *Probiotics and Antimicrobial Proteins*, *9*(3), 292–299. https://doi.org/10.1007/S12602-017-9252-3

Waters, S. M., Murphy, R. A., & Power, R. F. G. (2005). Assessment of the Effects of Nurmi-Type Cultures and a Defined Probiotic Preparation on a Salmonella Typhimurium 29E Challenge In Vivo. *Journal of Food Protection*, *68*(6), 1222–1227. https://doi.org/10.4315/0362-028X-68.6.1222

Wealleans, A. L., Li, W., Romero, L. F., Mathis, G., & Lumpkins, B. (2018). Performance and cost-benefit improvements following supplementation with a combination of direct-fed microbials and enzymes to broiler chickens raised with or without ionophores. *Journal of Applied Poultry Research*, *27*(1), 23–32. https://doi.org/10.3382/JAPR/PFX036

Wegener, H. C. (2003). Antibiotics in animal feed and their role in resistance development. *Current Opinion in Microbiology*, *6*(5), 439–445. https://doi.org/10.1016/J.MIB.2003.09.009

Whittaker, R. H. (1972). Evolution and Measurement of Species Diversity. *Taxon*, *21*(2/3), 213.

World Health Organization. (2018). Antimicrobial resistance. Retrieved February 24, 2019, from http://www.who.int/antimicrobial-resistance/en/

Yang, J. H., Bhargava, P., McCloskey, D., Mao, N., Palsson, B. O., & Collins, J. J. (2017). Antibiotic-Induced Changes to the Host Metabolic Environment Inhibit Drug Efficacy and Alter Immune Function. *Cell Host & Microbe*, *22*(6), 757-765.e3. https://doi.org/10.1016/J.CHOM.2017.10.020

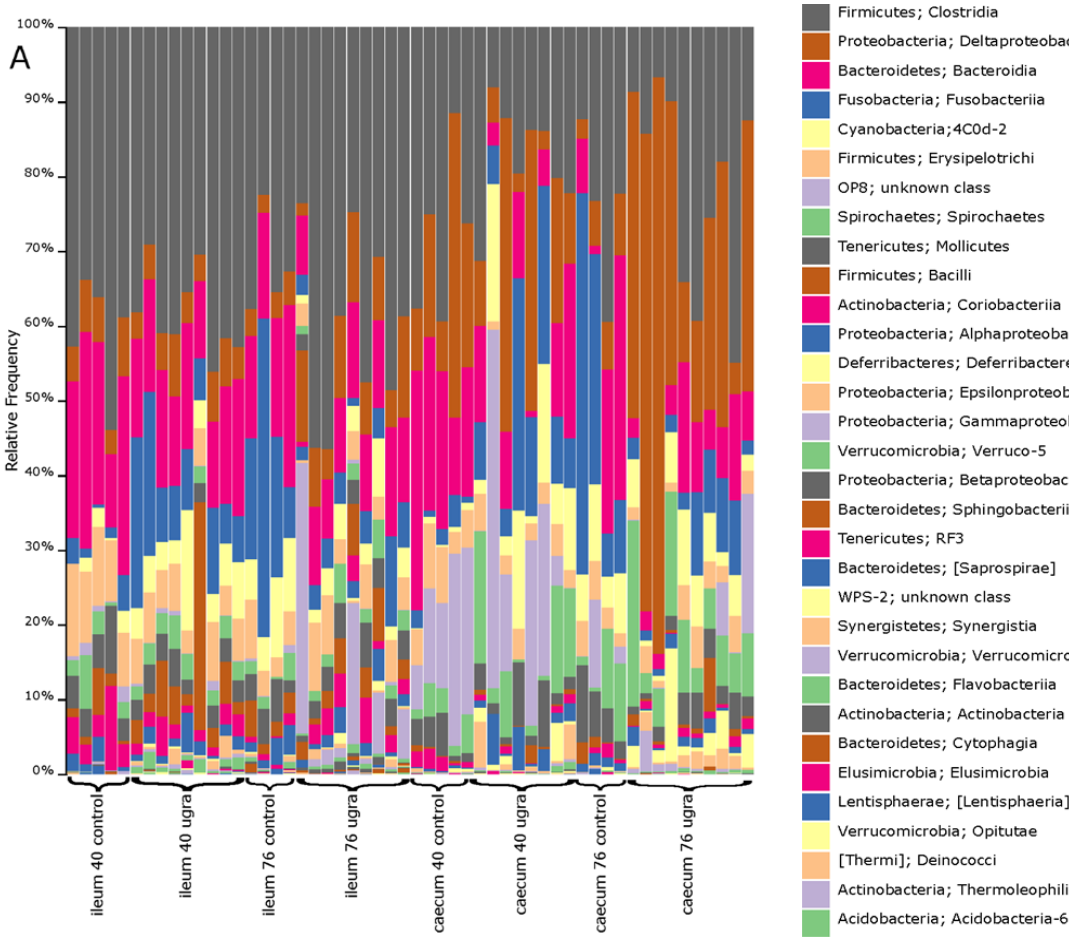
Żakowska-Biemans, S., & Tekień, A. (2017). Free Range, Organic? Polish Consumers Preferences Regarding Information on Farming System and Nutritional Enhancement of Eggs: A Discrete Choice Based Experiment. *Sustainability*, *9*(11), 1999. https://doi.org/10.3390/SU9111999

Zhang, J. L., Xie, Q. M., Ji, J., Yang, W. H., Wu, Y. B., Li, C., … Bi, Y. Z. (2012). Different combinations of probiotics improve the production performance, egg quality, and immune response of layer hens. *Poultry Science*, *91*(11), 2755–2760. https://doi.org/10.3382/PS.2012-02339

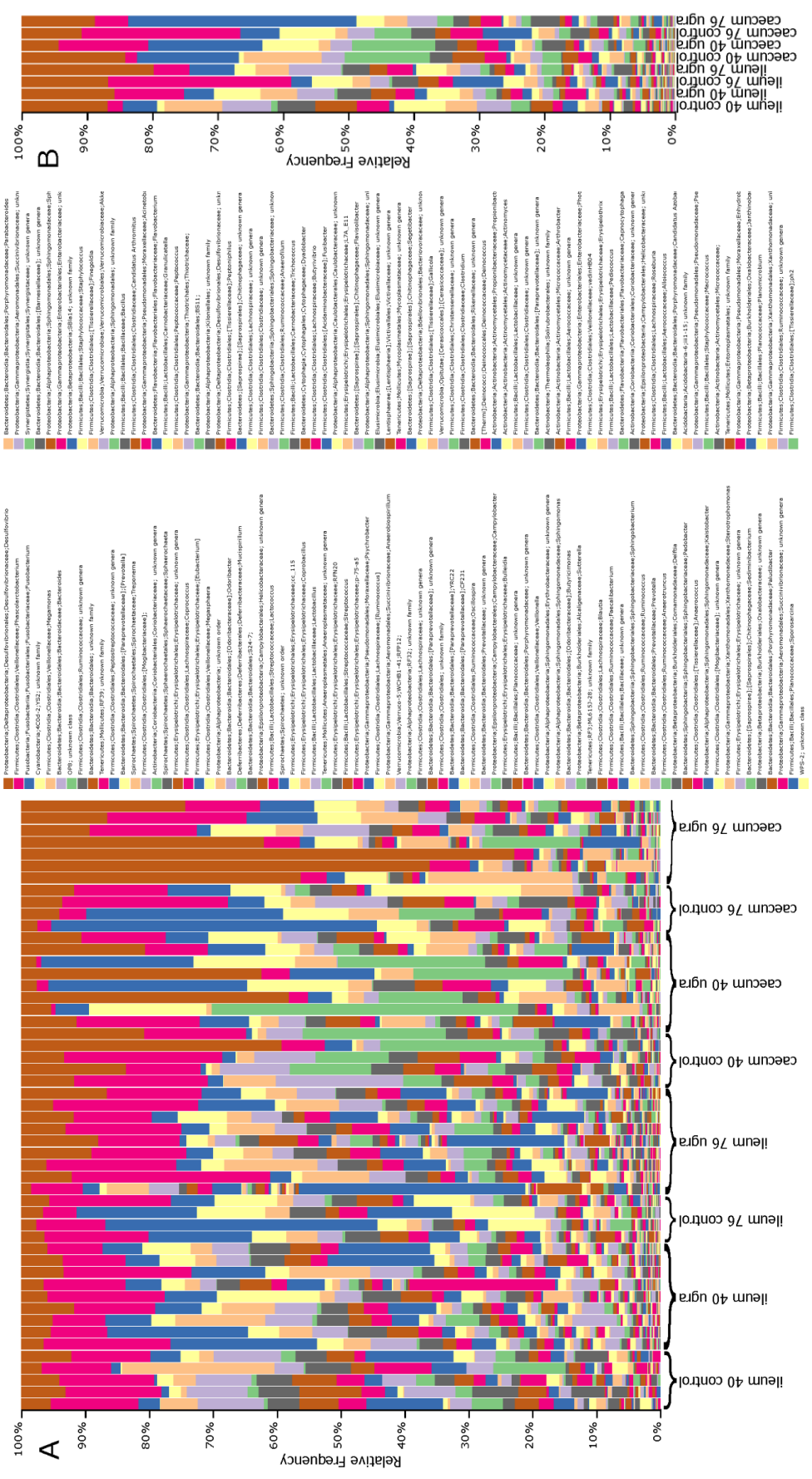
Zhao, X., Guo, Y., Guo, S., & Tan, J. (2013). Effects of Clostridium butyricum and Enterococcus faecium on growth performance, lipid metabolism, and cecal microbiota of broiler chickens. *Applied Microbiology and Biotechnology*, *97*(14), 6477–6488. https://doi.org/10.1007/S00253-013-4970-2

Zheng, A., Luo, J., Meng, K., Li, J., Bryden, W. L., Chang, W., … Yao, B. (2016). Probiotic (Enterococcus faecium) induced responses of the hepatic proteome improves metabolic efficiency of broiler chickens (Gallus gallus). *BMC Genomics*, *17*(1), 1–12. https://doi.org/10.1186/S12864-016-2371-5

SUPPLEMENTARY MATERIAL



Supplementary Figure S1. Bar plots of bacterial relative abundance at the class level in laying hens at different gut region, sampling times and treatments. Classes in the legend are sorted by sequence relative abundance, from most abundant to lowest abundant. UGRA refers to hen fed with *Enterococcus faecalis* UGRA10 at 10^8 Colony Forming Units per grams of fodder; while 40 and 76 refers to the number of days after the experiment started.



Supplementary Figure S2. Bar plots of relative bacterial abundance at the genus level of ileum and caecum in laying hens at different gut region (ileum and caecum), sampling times (40 and 76 days) and treatments (control and UGRA) (A) or grouped by gut region, sampling time and treatment (B). Classes in the legend are sorted by sequence relative abundance. UGRA refers to hen fed with *Enterococcus faecalis* UGRA10 at 10^8 Colony Forming Units per grams of fodder; while 40 and 76 refers to the number of days after the experiment started.

Supplementary Table S1. Sample sizes of bacterial community of ileum and caecum of laying hens, successfully amplified in Illumina MiSeq platform, at different sampling times. In parentheses, sample size before amplification.

	Day 40		Day 76		
	Control	Treatment	Control	Treatment	Total
Ileum	5 (5)	9 (10)	4 (5)	9 (10)	27
Caecum	5 (5)	8 (10)	4 (5)	10 (10)	27

Supplementary Table S2. Number of eggs produced by hens fed with control diets and supplemented with *Enterococcus faecalis* UGRA10 along the experimental period.

Eggs	**Treatment**	**Day**	**Experimental Bout (PRE-FIRST-SECOND)**	**Eggs**	**Treatment**	**Day**	**Experimental Bout (PRE-FIRST-SECOND)**
52	CONTROL	1	First Half	46	CONTROL	32	First Half
53	Experimental	1	First Half	46	Experimental	32	First Half
48	CONTROL	2	First Half	51	CONTROL	37	First Half
46	Experimental	2	First Half	50	Experimental	37	First Half
42	CONTROL	3	First Half	45	CONTROL	38	First Half
45	Experimental	3	First Half	40	Experimental	38	First Half
49	CONTROL	9	First Half	42	CONTROL	39	First Half
54	Experimental	9	First Half	42	Experimental	39	First Half
41	CONTROL	10	First Half	51	CONTROL	40	First Half
40	Experimental	10	First Half	50	Experimental	40	First Half
46	CONTROL	11	First Half	35	CONTROL	44	Second half
45	Experimental	11	First Half	33	Experimental	44	Second half
52	CONTROL	12	First Half	36	CONTROL	45	Second half
50	Experimental	12	First Half	33	Experimental	45	Second half
42	CONTROL	16	First Half	37	CONTROL	46	Second half
51	Experimental	16	First Half	35	Experimental	46	Second half
49	CONTROL	17	First Half	37	CONTROL	47	Second half
47	Experimental	17	First Half	37	Experimental	47	Second half
41	CONTROL	18	First Half	33	CONTROL	51	Second half
39	Experimental	18	First Half	40	Experimental	51	Second half
44	CONTROL	19	First Half	32	CONTROL	52	Second half
42	Experimental	19	First Half	39	Experimental	52	Second half
45	CONTROL	23	First Half	34	CONTROL	53	Second half
42	Experimental	23	First Half	36	Experimental	53	Second half
46	CONTROL	24	First Half	32	CONTROL	54	Second half
45	Experimental	24	First Half	36	Experimental	54	Second half
44	CONTROL	25	First Half	34	CONTROL	58	Second half
52	Experimental	25	First Half	36	Experimental	58	Second half
49	CONTROL	26	First Half	29	CONTROL	59	Second half
46	Experimental	26	First Half	35	Experimental	59	Second half
45	CONTROL	30	First Half	29	CONTROL	60	Second half
43	Experimental	30	First Half	35	Experimental	60	Second half
45	CONTROL	31	First Half	31	CONTROL	61	Second half
40	Experimental	31	First Half	36	Experimental	61	Second half

Eggs	Treatment	Day	Experimental Bout (PRE-FIRST-SECOND)
27	CONTROL	65	Second half
34	Experimental	65	Second half
31	CONTROL	66	Second half
33	Experimental	66	Second half
26	CONTROL	67	Second half
30	Experimental	67	Second half
30	CONTROL	68	Second half
32	Experimental	68	Second half
34	CONTROL	71	Second half
40	Experimental	71	Second half
32	CONTROL	73	Second half
39	Experimental	73	Second half
32	CONTROL	74	Second half
41	Experimental	74	Second half
34	CONTROL	75	Second half
40	Experimental	75	Second half

Supplementary Table S3. Summary of average (±SE) of alpha diversity indexes based in bacterial community of ileum and caecum in laying hens sequenced by Illumina Miseq. Different indexes have been separated by gut region, sampling date and treatment. In treated group, laying hens were supplemented with *Enterococcus faecalis* UGRA10. Sample size is also shown.

Gut region	Sampling date	Experimental group	N	Species richness	Pielou evenness	Faith's diversity index	Shannon's diversity index
Ileum	40 days	Treatment	9	237.00 (8.13)	0.76 (0.01)	27.84 (0.80)	5.98 (0.12)
		Control	5	160.00 (8.24)	0.79 (0.01)	20.25 (1.07)	5.79 (0.10)
	76 days	Treatment	9	224.67 (12.50)	0.74 (0.02)	26.64 (1.13)	5.76 (0.16)
		Control	4	207.50 (22.86)	0.73 (0.03)	24.84 (3.17)	5.56 (0.30)
Caecum	40 days	Treatment	8	167.00 (18.26)	0.67 (0.03)	21.05 (2.00)	4.95 (0.31)
		Control	10	159.40 (13.73)	0.63 (0.04)	20.62 (1.13)	4.61 (0.32)
	76 days	Treatment	5	156.60 (14.28)	0.68 (0.03)	20.19 (1.24)	4.98 (0.30)
		Control	4	147.50 (11.47)	0.69 (0.02)	17.76 (1.33)	4.96 (0.21)

CAPÍTULO II

Allium-Based Phytobiotic Enhances Egg Production in Laying Hens through Microbial Composition Changes in Ileum and Cecum

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Capítulo II

SIMPLE SUMMARY

The misuse of antibiotics has led several countries to ban their use as prophylactics against bacterial diseases or as growth promoters in livestock and poultry. Phytobiotics (bioactive compounds extracted from plants) are one of the alternatives, due to their antimicrobial activity and its modulation of the gut microbiota and the improvement of productive properties. Garlic and onion extracts, rich in antimicrobial compounds, are of the most promising alternative to antibiotics. We supplemented a garlic- and onion-based product in the diet to laying hens at the beginning of their productive life. The group supplied with this product produced in one month more eggs and with bigger size. This increase in production was accompanied by changes in the bacterial community of the gut. These changes in the microbiota suggest an improvement in food digestibility, as the most important changes produced by these compounds occur in the most distal parts of the gut. The relative abundance of beneficial *Lactococcus* in the ileum and *Lactobacillus* in the cecum increased in the experimental group. Both genera are known to have beneficial effects on host. These results are very promising for the use of these compounds in poultry for short periods.

ABSTRACT

Phytobiotics (bioactive compounds extracted from plants) are one of the explored alternatives to antibiotics in poultry and livestock due to their antimicrobial activity and its positive effects on gut microbiota and productive properties. In this study, we supplemented a product based on garlic and onion compounds in the diet to laying hens at the beginning of their productive life (from 16 to 20 weeks post-hatching). The experimental group showed a significant increase in the number of eggs laid and in their size, produced in one month compared to the control. This increase in production was accompanied by microbiota changes in the ileum and cecum by means of high throughput sequencing analyses. These bacterial shifts in the ileum were mainly the result of compositional changes in the rare biosphere (unweighted UniFrac), while in the cecum, treatment affected both majority and minority bacterial groups (weighted and unweighted UniFrac). These changes in the microbiota suggest an improvement in food digestibility. The relative abundance of *Lactococcus* in the ileum and *Lactobacillus* in the cecum increased significantly in the experimental group. The relative abundance of these bacterial genera are known to have positive effects on the hosts. These results are very promising for the use of these compounds in poultry for short periods.

Keywords: *Allium*-based phytobiotic; *Alliaceae* extract; laying hens; gut microbiota; egg production; high-throughput sequencing; Illumina MiSeq platform

INTRODUCTION

The abusive and inappropriate use of antibiotics in the animal production industry and clinical medicine has favored the selection of resistant bacteria and the spread of antibiotic resistance worldwide (Levy & Marshall, 2004; World Health Organization, 2018; Wise, 2008). As a consequence, numerous countries have banned the use of most antibiotics as growth-promoters in livestock and poultry (Bengtsson & Wierup, 2007; Commision, 2003, 2018; Food & Administration, 2013; Wierup, 2004). Some works predicted that these policies will increase production costs and final product prices (Hardy, 2002; Hayes et al., 2001), so the animal production industry is searching for efficient alternatives to the use of antibiotics as growth promoters in livestock, poultry and aquaculture (Allen et al., 2013; Ferket, 2004; Jana et al., 2018). Bacteriocins, bacteriophages, phytobiotics, probiotics, prebiotics and synbiotics have been proposed as the most promising alternatives (Hume, 2010; Joerger, 2003).

Phytobiotics are bioactive compounds supplemented in the diet to improve the health and performance of farm animals (Vidanarachchi et al., 2005; Windisch et al., 2008). Like antibiotics, phytobiotics can directly affect pathogenic bacteria, acting as antimicrobials (El-Ghany & Ismail, 2014; Vidanarachchi et al., 2005) or by blocking some membrane receptors in pathogenic bacteria, making their adhesion to the intestinal mucosa difficult (reviewed in Vidanarachchi et al., 2005). Phytobiotics can also act as prebiotics, supplying specific substrates and stimulating the growth of beneficial bacteria, or acting as growth-promoter metabolites (Allen et al., 2013; Vidanarachchi et al., 2005). Interestingly, phytobiotics may modulate the microbiota-gut-immune system, especially thorough antioxidant and anti-inflammation activities of these compounds (Gheisar & Kim, 2018). Moreover, phytobiotics increase digestive enzyme activity, enhance feed conversion and hence improve the productive parameters of farm animals (Gheisar & Kim, 2018; Windisch et al., 2008). These improvements in digestive function have been related to the growth of beneficial bacteria in the cecum of broilers supplemented with phytobiotics, especially lactic acid bacteria such as lactobacilli and bifidobacteria (Attia et al., 2017; Guo et al., 2004). These bacterial groups improve the host's health by interacting with and training the immune system, allowing the host to allocate resources to production traits (Al-Yasiry et al., 2017; Liu et al., 2013).

Extracts from plants of the Alliaceae family, mainly garlic (*Allium sativum*), onion (*Allium cepa*) and leek (*Allium porrum*) produce a wide variety of compounds showing antimicrobial activity, known of since ancient times (Amagase et al., 2001; Harris et al., 2001; Ye et al., 2013). The supplementation of these compounds has shown promising results in the health and productive parameters of several farm animals such as sheep (Anassori et al., 2017), goats (Zhu et al., 2013), cattle (Abad et al., 2017), pigs (Abad et al., 2018), broilers (Jimoh et al., 2012; Lee et al., 2017) and fishes (Natasya-Ain et al., 2018). These compounds improve intestinal functions such as rumen fermentations or energy-related blood metabolites, contributing to animal health and productivity (Anassori et al., 2017; Busquet et al., 2005). In poultry, allium extracts supplemented in diets produce significant modulatory effects on growth, performance indices, lipid metabolism, gut ecosystem as well as immune responses, especially when poultry are experiencing stress and disease challenge conditions (Kothari et al., 2019). Most of these compounds are secondary metabolites, volatile organosulfur compounds, mainly thiosulfites and thiosulfonates which are responsible for the pungent odor, such as propyl thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) (Amagase et al., 2001; Liu et al., 2012; Ruiz et al., 2010). In in vitro experiments, these compounds showed anti-inflammatory properties in alveolar macrophages from pig lumps (Liu et al., 2012), antimicrobial activity against bacterial strains isolated from pig feces (Ruiz et al., 2010) and against Gram-negative and Gram-positive multidrug-resistant bacteria isolated from human fecal samples (Sorlozano-Puerto et al., 2018).

Previous works showed that experimental supplementation of garlic in the diet did not result in an increase in egg production (Asrat et al., 2018; Ghasemi et al., 2010; Mahmoud et al., 2010; Omer et al., 2019), or even in decrease in egg productivity (Kaya & Macit, 2012). However, in a recent paper, Abad et al., (2020) showed that a commercial *Allium* compound has positive effects on egg production. These differences in productivity effects due to *Allium* additives may be due to dose, duration of feeding or processing techniques (Khan et al., 2019). In this manuscript, we hypothesize that the supplementation of Allium compounds in the diet of laying hens will affect bacterial community composition as well as egg production. We predict that a commercial Alliaceae extract supplemented in the diet of laying hens, similar to that used by Abad et al. (2020) and based on PTS and PTSO, produces beneficial shifts in the gut microbiota and increase productive parameters, i.e., the number of laid eggs and their weights.

MATERIAL AND METHODS

Experimental Animals and Facilities

The experiment was performed in 2014 at an experimental farm (Granja Avícola Gil, SL, Alhendín, Granada, Spain). Laying hens (Hy Line Brown) were placed in cages at the age of 16 weeks with food and water *ad libitum* and kept at 20 ºC ± 2 ºC and 78% ± 3% relative humidity (average ± standard deviation), under a photoperiod of 16 h per day throughout the experimental procedure.

Experimental Design and Sampling Collection

One hundred and eighty experimental laying hens were housed in groups of 6 hens per cage, with treatment groups being randomly distributed between production lines. Control hens (90 hens, 15 cages, 6 animals per cage) received a basal fodder diet (Supplementary Table S1), while experimental hens (90 hens, 15 cages, 6 animals per cage) received the same diet supplemented in feed with a commercial *Alliaceae* extract, Garlicon40 (DOMCA SAU, Granada, Spain) at a final concentration of 150 mg/kg (60 mg of PTSO per kg of feed).

The acclimation period lasted 15 days and then daily egg production (number of eggs) and their weight were recorded every working day (15 days of sampling). On day 30 after experiment started, 5 control and 8 experimental hens selected at random from different cages were euthanized by an intrathoracic injection of 2 mL/hen of T-61 (Intervet, Salamanca, Spain). Immediately after being slaughtered, the hens were dissected and the ileum and cecum were collected using sterile material. Each portion was homogenized in buffered peptone broth and aliquoted with 10% sucrose and finally frozen at -80 ºC. Afterwards, the aliquots were lyophilized (LyoQuest, TELSTAR Technologies SL, Barcelona, Spain). No animal died during the experimental period due to illness or malnutrition.

High-Throughput Sequencing

A total of 20 mg of lyophilized samples were used for bacterial DNA extraction from ileum and cecum samples using the QIAamp DNA Stool Mini Kit (QIAGEN). Amplicon libraries of the V4 region of the 16S rRNA gene were produced from total bacterial DNA by PCR amplification using primer pair 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 786r (5'-GGACTACHVGGGTWTCTAAT-

3') with barcodes on the forward primer following Illumina library preparation (see Supplementary Materials and library preparation details in Peralta-Sánchez et al., 2019). High-throughput sequencing was performed on Illumina MiSeq platform in the Scientific Instrumental Center at the University of Granada (Spain).

Subsequent analyses were performed with QIIME2 v2018.02 (Bolyen et al., 2019). Primer trimming and pair joining were performed using default parameters. Afterwards, quality-filtering and sequence clustering were carried out using the Deblur algorithm, a sub-operational-taxonomic- unit approach that removes low quality sequences as well as sequencing errors (Amir et al., 2017), with a sequence length trimming limit of 252 base pairs. This algorithm allows an Amplicon Sequence Variant (ASV) table to be created. The fragment insertion script implemented in QIIME2 was used to perform a sequence alignment and construct a de-novo phylogenetic tree (Janssen et al., 2018). Taxonomy assignment was based on Greengenes 13_08 with a similarity of 99% (DeSantis et al., 2006). Finally, non-bacterial sequences, i.e., chloroplasts and mitochondria, were removed from the sub-OTU table, although *Cyanobacteria* were retained in subsequent analyses (Ley et al., 2005).

Statistics

We used General Linear Models (GLM) to explore the effect of the treatment, sampling date and their interaction in number of eggs produced per day, mean egg size per day and alpha diversity indexes. For bacterial diversity analyses, we rarefied the ASV table at 2500 sequence depth per sample. We calculated different alpha diversity within sample diversity (Whittaker, 1972), indexes from the ASV table: bacterial OTU richness (or number of observed OTUs), evenness (Pielou, 1966), Faith's phylogenetic diversity index (Faith & Baker, 2006) and Shannon diversity index (Shannon, 1948). Residuals of the number of eggs, mean egg size and alpha diversity indexes after the analyses followed a normal distribution (Kolmogorov-Smirnov normality test; $p > 0.20$), validating the use of parametric statistical tests. These analyses were performed using STATISTICA 12.5 (Statsoft, Tulsa, OK, USA).

Difference in genera and phyla relative abundance between control and treated hens were explored by means of Linear Discriminant Analysis (LDA) Effect Size (LEfSe) (Segata et al., 2011). LEfSe analyses were performed on the Galaxy web platform, run from a public server (Segata et al., 2011).

Weighted and unweighted UniFrac distance (Lozupone et al., 2007; Lozupone & Knight, 2005) were used to calculate beta diversity distance matrixes [differences between sample diversity, (Whittaker, 1972)]. While weighted UniFrac gives more importance to the most abundant bacteria, unweighted UniFrac gives more importance to rare bacteria in the sub-OTUs as it takes into account their presence or absence regardless of abundance (Lozupone et al., 2007). PERMANOVA was performed in order to test these effects on both UniFrac distance matrixes using PRIMER-7 software. Principal Coordinate Analyses were performed in order to visualize the first two PCoA axes using Emperor 2018.2.0 (Vázquez-Baeza et al., 2013).

RESULTS

Effect of *Allium* Supplementation on Egg Productivity of Laying Hens

Laying hens supplemented with the *Alliaceae* extract had higher egg production than the control group (Table 1, Figure 1A). While the control group decreased production throughout the experimental period in just 30 days (average egg number (standard error): 47.07 (0.79), the experimental group increased the number of eggs produced (52.07 (0.79); see interaction term in Table 1, Figure 2A).

The size of the eggs was also significantly affected by our experimental manipulation. Laying hens supplemented with the *Alliaceae* extract laid larger eggs (70.16 (0.18)) than the control group (68.85 (0.18)) (Table 1; Figure 1B). During the experimental period, egg weight in experimental hens increased, while egg size slightly decreased in the control group (see interaction term in Table 1, Figure 2B).

Table 1. General Linear Models exploring the effects of treatment and date in egg productivity (Number of eggs) and size (mean egg size) of laying hens. Treated hens received a basal diet supplemented with a commercial *Alliaceae* extract (Garlicon40 ©). Significant values were set at 0.05, which are in bold. Degrees of freedom (d.f.) are also shown.

	Model	Variables	d.f.	F	*p*
Egg number	1	Treatment	1,27	20.10	< **0.001**
		Date	1,27	1.07	0.310
	2	Treatment	1,26	< 0.01	0.991
		Date	1,26	1.46	0.238
		Treatment *Date	1,26	10.82	**0.003**
Mean egg weight	3	Treatment	1,27	16.93	< **0.001**
		Date	1,27	2.89	0.089
	4	Treatment	1,26	0.04	0.840
		Date	1,26	2.44	0.119
		Treatment *Date	1,26	5.56	**0.019**

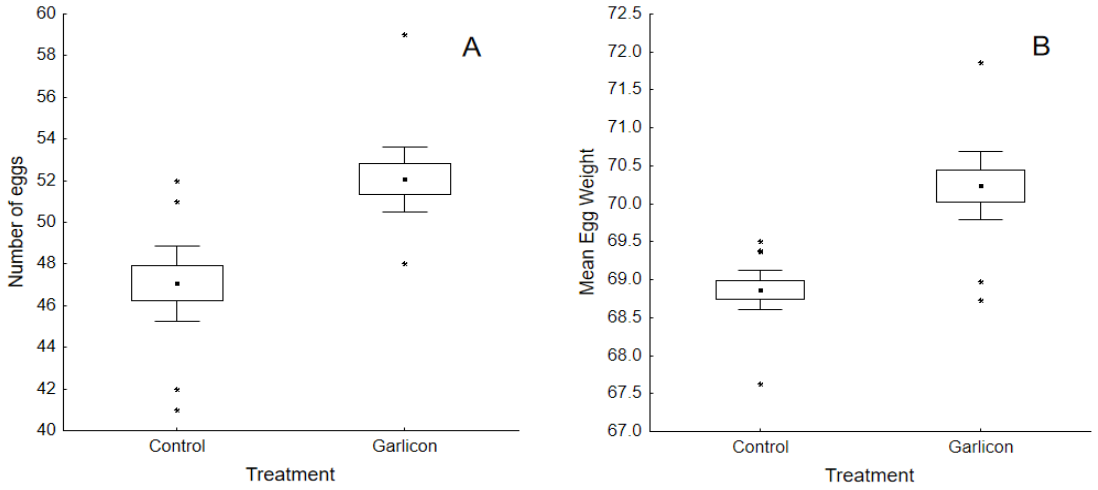

Figure 1. Differences in the mean number of eggs (A) and mean egg weight (B) produced by control and experimental laying hens. Control hens were fed a basal diet while experimental ones were fed a basal diet supplemented with a commercial *Alliaceae* extract (Garlicon40©). In both cases, the number of eggs and egg weight were significantly higher in the experimental group fed the *Alliaceae* extract. Whiskers show 95% confidence interval and asterisks indicate outliers.

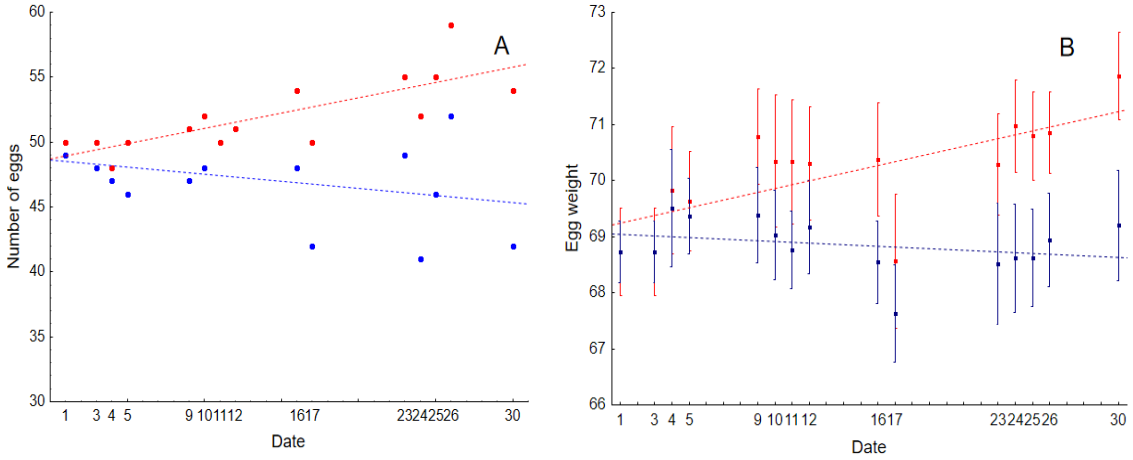

Figure 2. Changes in the number of eggs (A) and egg weight (B) produced by control laying hens (in blue) and laying hens experimentally supplemented with a commercial *Alliaceae* extract (Garlicon40©) (in red) during 30 days of the experimental period. Regression lines and 95% CI are also shown.

Changes in Bacterial Community Composition

The gut microbiota of laying hens is dominated by *Firmicutes*, *Bacteroidetes* and *Proteobacteria*. The relative abundance of these phyla depended on the gut region and treatment group (Figure 3). *Firmicutes* dominated in the ileum while *Proteobacteria* is dominant in the cecum. It is noteworthy that some minority phyla such as *Elusimicrobia* or *Synergistetes* decreased in the cecum of laying hens supplemented with the *Alliaceae*

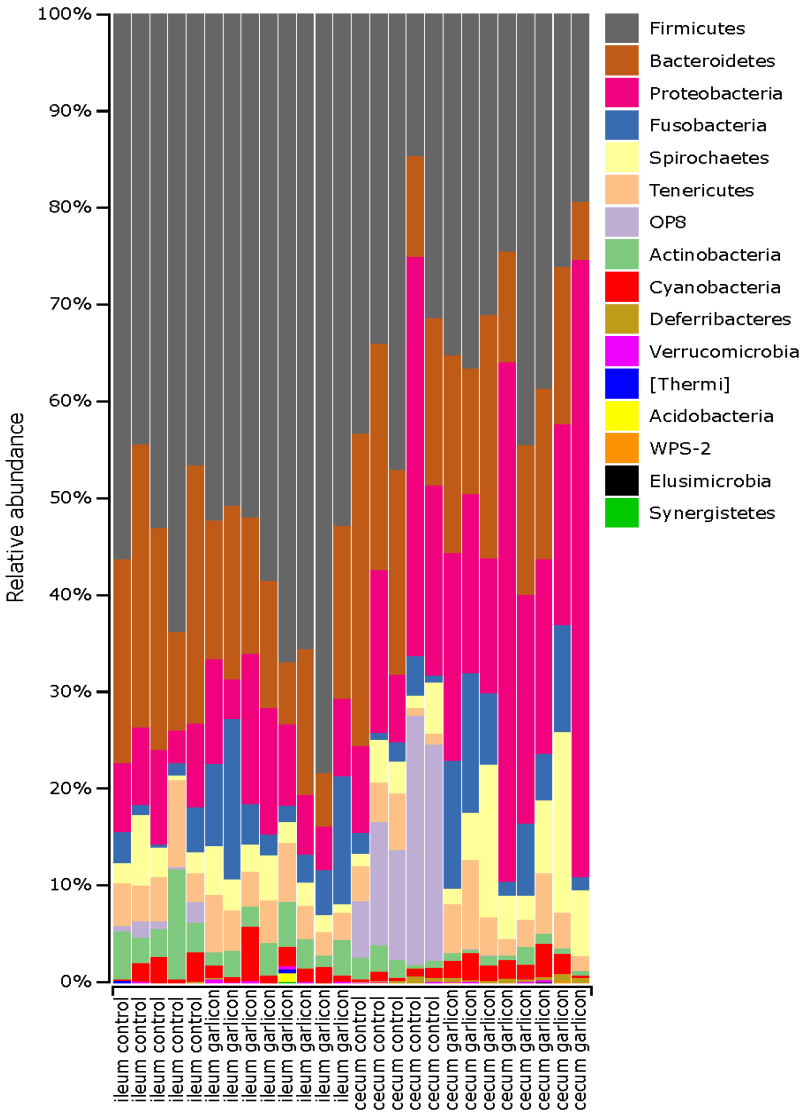

Figure 3. Bar plot of the relative bacterial abundance at the phylum level in different gut regions of laying hens and treatments. Control refers to laying hens fed a basal diet while Garlicon refers to experimental laying hens fed a basal diet supplemented with a commercial *Alliaceae* extract (Garlicon40©).

extract (Figure 3). However, only phylum OP8 differed significantly between treatments in both the ileum and cecum (Figure 4).

At the genus level, *Lactococcus* (*Firmicutes*) and an unidentified genus of *Anaeroplasmataceae* (*Tenericutes*) increased in the ileum while *Bulleidia* (*Firmicutes*), *Bacteroides* (*Bacteroidetes*) and an unknown genus of the phyla OP8 decreased in the supplemented hens (Figure 4, Supplementary Figures S1 and S2). In the cecum, two

unknown genera of α-Proteobacteria and *Lactobacillus* (*Firmicutes*) increased significantly in the supplemented group. *Anaerobiospirillum* and *Acinetobacter* from *Gammaproteobacteria*, the genus *RFN20* (*Firmicutes*), an unknown genus from *Bacteroidetes* and an unknown genus of *OP8* decreased significantly in laying hens supplemented with the *Alliaceae* extract (Figure 4, Supplementary Figures S1 and S3).

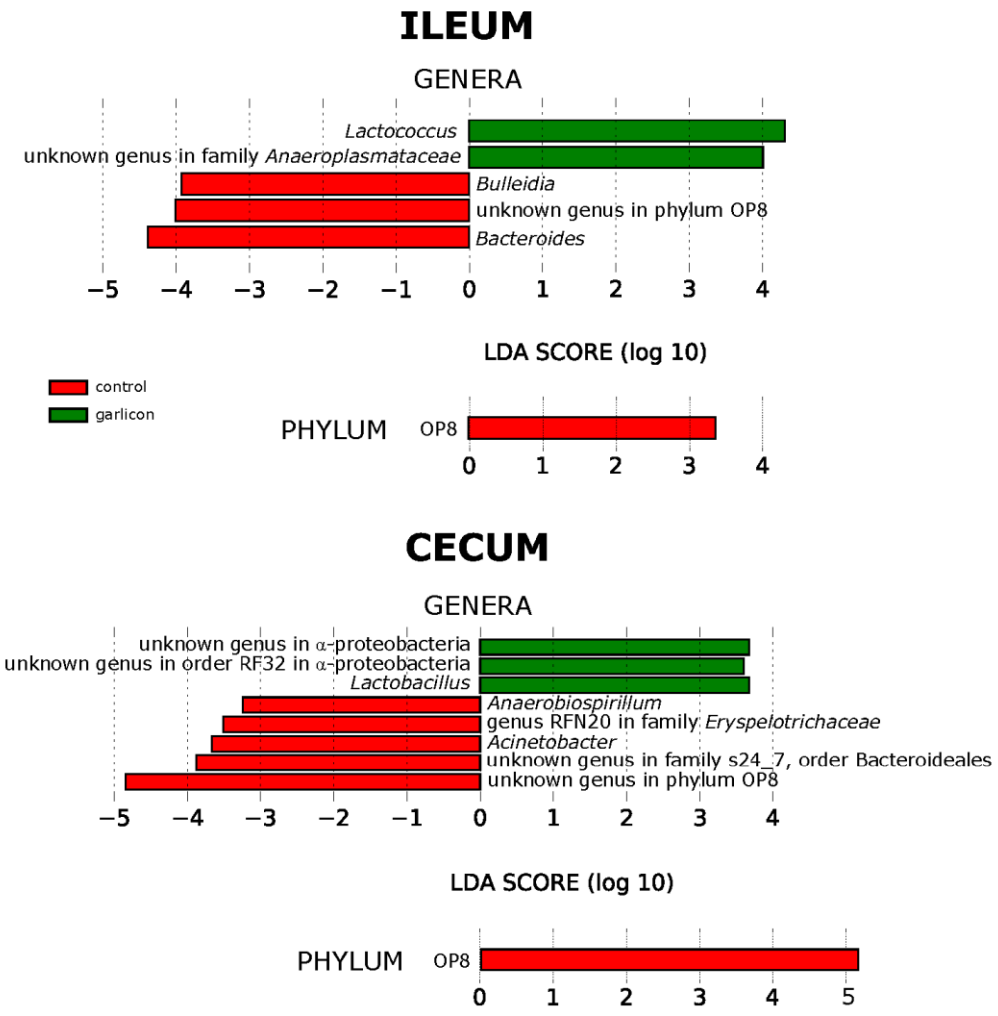

Figure 4. Linear Discriminant Analysis Effect Size (LEfSe) analyses showing bacterial genera (outer circles in the trees) and phyla (inner circles in the tree) that differed significantly between control hens and those supplemented with a commercial *Alliaceae* extract (Garlicon40©), in the ileum and cecum. Green bars and dots indicates a significant increase in relative abundance in the supplemented groups while red bars and dots showed a significant decrease. The cladograms show the position of each of these genera in the bacterial phylogenetic tree.

Effect of *Allium* Compound Supplementation on Alpha and Beta Diversity

Supplementing the diet of laying hens with the *Alliaceae* extract did not affect any of the alpha diversity indexes after 30 days of treatment in any of the gut regions, ileum or cecum (Table 2).

Table 2. General Linear Models exploring the effects of 30-days treatment in alpha diversity indexes in ileum and cecum of laying hens. Treated hens received in their basal diet supplemented with a commercial *Alliaceae* extract (Garlicon40 ©). Significant values were set at 0.05. Degrees of freedom (d.f.) are also shown.

	Alpha diversity index	Control	Experimental	d.f.	F	*p*
Ileum	sub-OTU richness	165.6 (13.12)	162.25 (10.37)	1,11	0.04	0.845
	Faith's diversity index	20.92 (1.51)	20.56 (1.19)	1,11	0.03	0.857
	Evenness	0.79 (0.03)	0.74 (0.02)	1,11	2.20	0.167
	Shannon's diversity index	5.79 (0.26)	5.40 (0.21)	1,11	1.41	0.260
Cecum	sub-OTU richness	173.20 (13.66)	172.12 (10.80)	1,11	< 0.01	0.952
	Faith's diversity index	21.34 (1.09)	20.96 (0.86)	1,11	0.08	0.789
	Evenness	0.67 (0.04)	0.69 (0.03)	1,11	0.18	0.682
	Shannon's diversity index	4.97 (0.33)	5.11 (0.26)	1,11	0.12	0.737

After 30 days of treatment, the bacterial community in laying hens varied significantly between the control and the supplemented hens (Table 3), in both the ileum and cecum samples, forming clear non-overlapping clusters (Figure 5). Interestingly, changes in the bacterial community between the ileum and cecum were similar between control and supplemented hens (see interaction terms in Table 3). Within each gut region, samples from the same treatment level clustered significantly together (Table 3, Figure 5), except for weighted UniFrac metrics in the ileum, showing a marginally significant trend (Table 3). Our experiment affected both, abundant and rare bacterial taxa, as it shows weighted and unweighted UniFrac results, respectively (Table 3, Figure 5).

Table 3. General Linear Models exploring the effects of 30-days treatment in beta diversity indexes in ileum and cecum of laying hens. Treated hens received a basal diet supplemented with a commercial *Alliaceae* extract (Garlicon40 ©). Significant values were set at 0.05, which are in bold. Degrees of freedom (d.f.) are also shown.

	Beta diversity index	Factors	d.f.	F	P
Gut	Unweighted UniFrac	Treatment	1,22	3.53	< **0.001**
		Gut	1,22	1.96	**0.002**
		Gut * Treatment	1,22	0.76	0.898
	Weighted UniFrac	Treatment	1,22	4.27	**0.003**
		Gut	1,22	6.55	< **0.001**
		Gut * Treatment	1,22	1.03	0.391
Ileum	Unweighted UniFrac	Treatment	1,11	1.81	< **0.001**
	Weighted UniFrac	Treatment	1,11	2.21	0.056
Cecum	Unweighted UniFrac	Treatment	1,11	2.63	**0.002**
	Weighted UniFrac	Treatment	1,11	3.11	**0.020**

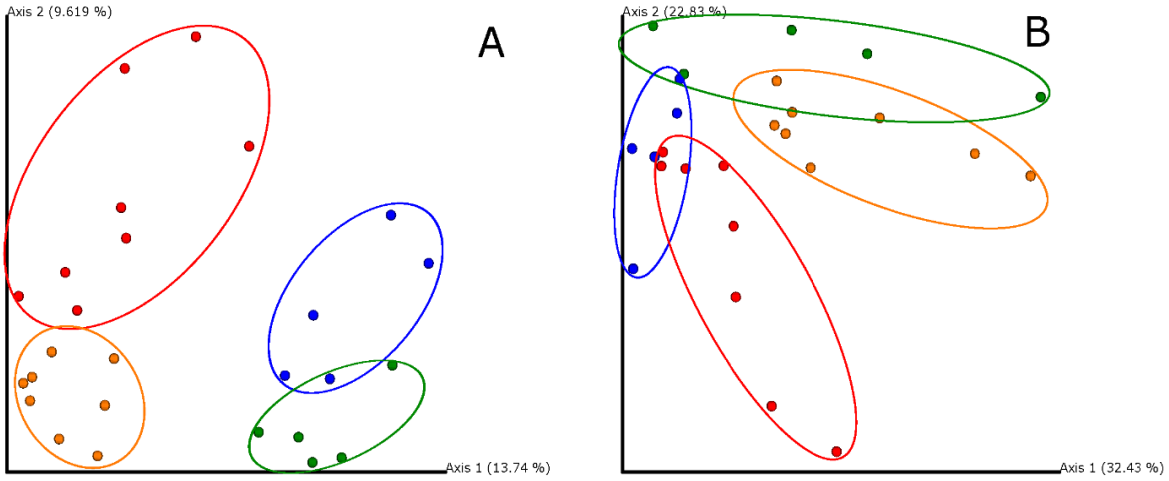

Figure 5. Principal Coordinate Analysis based in Unweighted UniFrac (A) and Weighted UniFrac (B) distance matrixes exploring the effects in the bacterial gut community of the supplementation with a commercial *Alliaceae* extract (Garlicon40©) in laying hens' diet (red: ileum, treated hens; orange: cecum, treated; blue: ileum, control; green: cecum, control). Circles surround samples from similar gut region and treatment. Percentages show the proportion of variance explained by each axis.

DISCUSSION

This study found that laying hens experimentally supplemented with *Allium* by-product compounds, based mainly on PTS and PTSO, significantly increased the number of laid eggs, as well as their size, after only 30 days of treatment. These productive increases in egg production and quality were accompanied by shifts in ileum and cecum

microbiota, where some bacterial groups differed between the supplemented and the control group.

Phytobiotics have shown promising results in the health, performance and productivity of laying hens. Diet supplementation with *Allium* compounds possess beneficial effects: significantly reduces cholesterol levels in the plasma of laying hens (Abdel-Wareth & Lohakare, 2014; Ao et al., 2010; Chowdhury et al., 2002; Yalçın et al., 2006), in egg contents (Yalçın et al., 2006) and even protects against several diseases, including cancer (Omar & Al-Wabel, 2010). Supplementation with different phytobiotics such as black cumin seeds or leaves and extracts from the *Lamiaceae* family (such as peppermint, sage, rosemary, thyme and oregano) increase egg production and egg weight (Abdel-Wareth & Lohakare, 2014; Bölükbasi & Erhan, 2007; Bozkurt et al., 2012; Khan et al., 2013; Park et al., 2016; Radwan et al., 2008). However, results for egg production in laying hens with their diet supplemented by *Allium* compounds are contradictory. Some studies pointed out the lack of effect on egg production or egg weight when hens were provided with different garlic preparations such as garlic paste, oil or powder (Chowdhury et al., 2002; Lanzotti, 2006; Yalçın et al., 2006). However, and in accordance with our results, supplementation of garlic powder shows an increase in egg production (Yalçın et al., 2006). Olobatoke & Mulugeta (2011) found an increase in egg weight and a reduction in laying rate, but in laying hens supplemented with high doses of garlic powder. The differences in the associations between garlic-based compounds supplementation and these variables may be related with breeds of hen and the preparation and presentation of the garlic products (Chowdhury et al., 2002; Lanzotti, 2006; Yalçın et al., 2006), probably related to the composition and quantity of sulfur components (Yalçın et al., 2006).

Garlic, onion and its relatives are plants rich in several volatile organosulfur compounds responsible for the pungent odor and antimicrobial properties (Amagase et al., 2001; Lanzotti, 2006). Allicin was one of the first compounds with antimicrobial activity to be isolated from garlic (Cavallito & Bailey, 2002), although its instability does not allow it to be used in livestock and poultry (Amagase et al., 2001). Allicin has been substituted by other compounds in the use of *Allium*-derived substances in animal production and welfare, such as PTS and PTSO, by-products of the initial compounds present in garlic and onion such as alliin and propiin (Ruiz et al., 2010; Wettenhall, 2020). PTS is quite instable but it converts rapidly into PTSO, a more stable compound (Ruiz et

al., 2010). PTS and PTSO preparations have been shown to increase propionate concentrations in lamb rumen, improve weight gain and reduce non-esterified fatty acids and β-hydroxybutyrate (Anassori et al., 2017). Interestingly, high concentrations of garlic powder (5%) shows stronger garlic flavor in the eggs compared with those eggs laid by control and laying hens supplemented with lower doses (3%) (Olobatoke & Mulugeta, 2011). In spite of this negative effects in organoleptic properties of eggs, PTSO did not seem to alter animal derived products. For instance, milk maintains its organoleptic properties after two months of PTSO supplementation in the diet of cows (Abad et al., 2017).

In broilers, supplementation of PTS and PTSO in the diet produces changes in the morphology and histology of the ileum and increases mucosa complexity in the gut (Peinado et al., 2012). It also produces shifts in the proximal intestinal microbiota of broilers, maintaining mucosal enzyme activity but improving food digestibility (Peinado et al., 2013), with an associated increase in body weight (Peinado et al., 2012). These compounds also reduce *Salmonella* abundance in the ileum and *Escherichia coli* in the cecum of broilers (Peinado et al., 2012). Besides this direct effect of *Allium*-derived compounds, bacterial communities of the gut exclude pathogenic bacteria and enhance the development of the intestinal mucosa, epithelium and lamina propria, resulting in an improvement in farm animals' health (Baurhoo et al., 2009). Reducing pathogenic bacteria brings relief to intestinal challenge and immune stress and hence the host can allocate resources to other traits (Gadde et al., 2017; Thomas & Versalovic, 2010). In this sense, our results showed a significant shift in the bacterial community in the ileum and cecum in laying hens supplemented with *Allium*-derived compounds, as shown by the UniFrac analyses. Our results agree with previous findings where these changes in microbiota are especially important in the most distal parts of the gut of monogastric animals, such as the cecum (Ruiz et al., 2010). The underlying action mechanisms of phytobiotics have not been explored yet, so it would deserve further studies, especially those related with changes in gut mucosa, the immune system and food digestibility.

Our results show that the relative abundance of *Lactococcus* in the ileum and *Lactobacillus* in the cecum increased significantly in laying hens supplemented with PTS and PTSO, while egg production improved. A recent paper based in culture-dependent techniques showed similar increase in egg production and fecal *Lactobacillus* counts in laying hens, supplemented with even lower doses of PTSO

than the present work (Abad et al., 2020). The increase in relative and absolute bacterial abundances of both *Lactococcus* and *Lactobacillus* produce beneficial effects in poultry and farm animals (Gaggìa et al., 2010). Han et al. (2016) found that the relative abundance of *Lactococcus* in the cecum of broilers was positively correlated with body weight. Moreover, supplementation with a phytobiotic in laying hens increased *Lactobacillus* relative abundance in the cecum and simultaneously improved egg production as well as egg weights (Park et al., 2016). Most lactic acid bacteria have an intimate relationship with the health of their animal hosts (Donaldson et al., 2015), so these strains have been widely used as probiotics due to their many beneficial properties (Picard et al., 2005; Song et al., 2017; Zhang et al., 2018). In this sense, these bacteria reduce the intestinal pH by producing lactic acid, and hence, inhibiting the proliferation of pathogenic bacteria (revised in Kiczorowska et al., 2017). The action of *Lactobacillus* may be also related with the reduction in the adhesion ability of *Salmonella* or of pathogenic bacteria as some strains of *Clostridium spp.* or *E. coli* (Gaggìa et al., 2010). Moreover, the levels of *Lactobacillus* could play a major role in promoting and maintaining intestinal inflammation, especially during inflammatory disease (Gaggìa et al., 2010). In this sense, lactic acid bacteria also increase the histological complexity of the gut and stimulate the immune response of the mucosa (Baurhoo et al., 2007; Kiczorowska et al., 2017). Despite not exploring the variables involved in these effects, a net positive effect in both lactic acid bacteria and egg production was found.

In animals, *Bacteroidetes* is present in the small and large intestine, although its relative abundance is much higher in the latter (Donaldson et al., 2015). Our experimental procedure produced a significant reduction in the relative abundance of *Bacteroides* in the ileum and in an unidentified genus of the Order *Bacteroidales* in the cecum. *Bacteroidetes* species are involved in several metabolic activities in the gut, from carbohydrate fermentation to bile acid degradation (Bry et al., 1996; Phillips, 2009). Interestingly, Peinado et al. (2013) found using qPCR that the absolute abundance of *Bacteroides* in Cobb broiler guts negatively correlated with *Lactobacillus* populations. In that study, they found an increase in *Bacteroides* and broiler performance in animals supplemented with PTSO while *Lactobacillus* abundance decreased. We can only speculate that these discrepancies may be due to the use of different molecular techniques (qPCR vs. high-throughput sequencing, absolute vs. relative abundances), different hen

breeds (Cobb vs. Hy Line Brown) and more importantly, differences in age and sex. For instance, ileum microbiota differs significantly between male and female broilers only 3 days after hatching (Lumpkins et al., 2008). Moreover, age, sex and breed has a strong effect on bacterial community in the gut of broilers (Kers et al., 2018). Further research is needed to clarify the effects of these confounding factors and explore new possibilities in broilers and laying hens as shown by the depletion of *Bacteroides* observed in obese children (Rinninella et al., 2019).

Our supplementation with PTS and PTSO depleted other genera that may cause negative effects on their hosts. *Acinetobacter* (*Moraxellaceae, Gammaproteobacteria*) is a common bacterium in soil environments and related with infections in immune-depressed patients (Doughari et al., 2011). *Anaerobiospirillum* (*Succinivibrionaceae, Gammaproteobacteria*) is a strict anaerobic genus causing septicemia and diarrhea in humans (Garrity, 2005). Some other taxa are poorly described so the effect of the reduction in abundance is unknown. These taxa include members of the *Erysipelotrichaceae* family, such as *Bulleida* and the genus *RFN20*, and an unidentified genus in the candidate phylum OP8. In this sense, *Aminicenantes*, the proposed name for this phylum, is poorly characterized and the few described strains cover a wide range of environments (Farag et al., 2014). Similarly, other groups increased their abundance, such as a genus of *Anaeroplasmataceae*, anaerobic obligate commensals in the rumen of some mammals, the role of which has not yet been properly described (Krieg et al., 2010) or the genus belonging to the Order RF32, the abundance of which correlates with histopathology and colonic inflammation in ray challenge with *E. coli*. Due to the lack of available information about the ecology and function of these groups in the gut, we cannot explain the significance of these changes in abundance in most of these strains in the treated hens. Culture-based methods are experiencing a rebirth in order to fill the gap in the knowledge of the huge amount of new microorganisms and diversity that next-generation technology is uncovering (Tamaki, 2019).

CONCLUSIONS

Our experimental supplementation of PTS and PTSO compounds in diet of laying hens increased their egg production and size, while producing shifts in the bacterial communities in the ileum and cecum of the hens. These results are very promising for the use of these phytobiotics in poultry for short periods (4 weeks in this study). Future

research is necessary to understand the underlying mechanisms involved in these improvements, regarding the immune system, food digestibility and for longer exposition periods.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2615/11/2/448/s1; Figure S1. Bar plot of the relative bacterial abundance at the genus level in different gut regions of laying hens and treatments. Control refers to laying hens fed a basal diet while Garlicon refers to experimental laying hens fed a basal diet supplemented with the commercial Alliaceae extract. The sixteen most abundance genera are shown in a unique color set. The color of the rest of genera (less abundant) are repeated every 8 colors; Figure S2. Linear Discriminant Analysis Effect Size (Lefse) showing genera from the ileum that significantly differ between control and experimentally supplemented with a commercial Alliaceae laying hens. Bars showed relative abundance of the genus in each sample. Solid line represents mean relative abundance while dashed line represent the median. k: kingdom; p: phylum; c: class; o: order; f: family; and g: genus; Figure S3. Linear Discriminant Analysis Effect Size (Lefse) showing genera from the cecum (ciego) that significantly differ between control and experimentally supplemented with a commercial Alliaceae laying hens. Bars showed relative abundance of the genus in each sample. Solid line denotes relative abundance while dashed line showed median relative abundance. k: kingdom; p: phylum; c: class; o: order; f: family; and g: genus; Table S1: Nutritional information of the basal feed employed in laying hens.

Author Contributions: Conceptualization, J.J.A.-R., M.J.Z.-G., A.B. and M.M.-B.; methodology, M.R.-R., J.J.A.-R., M.J.Z.-G., A.M.M.-P., A.B., M.M., E.V., M.M.-B. and J.M.P.-S.; validation, A.B., M.M.-B. and J.M.P.-S.; formal analysis, M.R.-R., J.J.A.-R., M.J.Z.-G. and J.M.P.-S.; resources, A.B. and M.M.-B.; data curation, M.R.-R. and J.M.P.-S.; writing—original draft preparation, J.M.P.-S.; writing—review and editing, M.R.-R., J.J.A.-R., M.J.Z.-G., A.M.M.-P., A.B., M.M., E.V., M.M.-B. and J.M.P.-S.; supervision, A.B., M.M.-B. and J.M.P.-S.; project administration, A.B. and M.M.-B.; funding acquisition, A.B. and M.M.-B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, the European directive for the protection of animal welfare in research (Directive 2010/63/EU) and the 'Guide for the Care and Use of Laboratory Animals', as promulgated by the National Institute of Health (Ministerio de Sanidad, Consumo y Bienestar Social) following Spanish legislation (Real Decreto 53/2013). Protocols and procedures were approved by the Ethics Committee of Laboratory Animals of the University of Granada (Spain) (Ref. No. CEEA-2018-227).

Data Availability Statement: Sequences are available in the Sequence Read Archive (SRA) in the GenBank-NCBIwebpage1 under Accession Nos. SAMN09603288 to SAMN9603307 and SAMN09603326 to SAMN 09603341.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

Abad, P., Arroyo-Manzanares, N., Ariza, J. J., Baños, A., & García-Campaña, A. M. (2020). Effect of Allium Extract Supplementation on Egg Quality, Productivity, and Intestinal Microbiota of Laying Hens. *Animals*, *11*(1), 41. https://doi.org/10.3390/ani11010041

Abad, P., Arroyo-Manzanares, N., Gil, L., & García-Campaña, A. M. (2017). Use of onion extract as a dairy cattle feed supplement: Monitoring propyl propane thiosulfonate as a marker of its effect on milk attributes. *Journal of Agricultural and Food Chemistry*, *65*(4), 793–799. https://doi.org/10.1021/acs.jafc.6b04395

Abad, P., Arroyo-Manzanares, N., Rivas-Montoya, E., Ochando-Pulido, J. M., Guillamon, E., García-Campaña, A. M., & Martinez-Ferez, A. (2018). Effects of different vehiculization strategies for the allium derivative propyl propane thiosulfonate during dynamic simulation of the pig gastrointestinal tract. *Canadian Journal of Animal Science*, *99*(2), 244–253. https://doi.org/10.1139/cjas-2018-0063

Abdel-Wareth, A. A. A., & Lohakare, J. D. (2014). Effect of dietary supplementation of peppermint on performance, egg quality, and serum metabolic profile of Hy-Line Brown hens during the late laying period. *Animal Feed Science and Technology*, *197*, 114–120. https://doi.org/10.1016/J.ANIFEEDSCI.2014.07.007

Al-Yasiry, A. R. M., Kiczorowska, B., Samolińska, W., Kowalczuk-Vasilev, E., & Kowalczyk-Pecka, D. (2017). The effect of Boswellia serrata resin diet supplementation on production, hematological, biochemical and immunological parameters in broiler chickens. *Animal*, *11*(11), 1890–1898. https://doi.org/10.1017/S1751731117000817

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., & Casey, T. A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in Microbiology*, *21*(3), 114–119. https://doi.org/10.1016/J.TIM.2012.11.001

Amagase, H., Petesch, B. L., Matsuura, H., Kasuga, S., & Itakura, Y. (2001). Intake of Garlic and Its Bioactive Components. *The Journal of Nutrition*, *131*(3), 955S-962S. https://doi.org/10.1093/JN/131.3.955S

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Xu, Z. Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Anassori, E., Dalir-Naghadeh, B., Valizadeh-Keshmeshtappeh, M., & Jafari, S. (2017). A comparative study on the efficacy of Garlicon and monensin supplementation on blood metabolites and performance of fattening lambs. *Livestock Science*, *199*, 74–78. https://doi.org/10.1016/J.LIVSCI.2017.03.014

Ao, X., Yoo, J. S., Lee, J. H., Jang, H. D., Wang, J. P., Zhou, T. X., & Kim, I. H. (2010). Effects of fermented garlic powder on production performance, egg quality, blood profiles and fatty acids composition of egg yolk in laying hens. *Asian-Australasian Journal of Animal Sciences*, *23*(6), 786–791. https://doi.org/10.5713/AJAS.2010.90543

Asrat, M., Zeryehun, T., Amha, N., & Urge, M. (2018). Effects of supplementation of different levels of garlic (Allium sativum) on egg production, egg quality and hatchability of White Leghorn chicken. *Livest. Res. Rural Dev.*, *30*(37).

Attia, G., El-Eraky, W., Hassanein, E., El-Gamal, M., Farahat, M., & Hernandez-Santana, A. (2017). Effect of dietary inclusion of a plant extract blend on broiler growth performance, nutrient digestibility, caecal microflora and intestinal histomorphology. *International Journal of Poultry Science*, *16*(9), 344–353. https://doi.org/10.3923/ijps.2017.344.353

Baurhoo, B., Ferket, P. R., & Zhao, X. (2009). Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. *Poultry Science*, *88*(11), 2262–2272. https://doi.org/10.3382/PS.2008-00562

Baurhoo, B., Letellier, A., Zhao, X., & Ruiz-Feria, C. A. (2007). Cecal Populations of Lactobacilli and Bifidobacteria and Escherichia coli Populations After In Vivo Escherichia coli Challenge in Birds Fed Diets with Purified Lignin or Mannanoligosaccharides. *Poultry Science*, *86*(12), 2509–2516. https://doi.org/10.3382/PS.2007-00136

Bengtsson, B., & Wierup, M. (2007). Antimicrobial Resistance in Scandinavia after a Ban of Antimicrobial Growth Promoters. *Animal Biotechnology*, *17*(2), 147–156. https://doi.org/10.1080/10495390600956920

Bölükbasi, S. C., & Erhan, M. K. (2007). Effect of dietary thyme (Thymus vulgaris) on laying hens performance and Escherichia coli (E. coli) concentration in feces. *IJNES*, *1*, 55–58.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Bozkurt, M., Küçükyilmaz, K., Çatli, A. U., Çinar, M., Bintaş, E., & Çöven, F. (2012). Performance, egg quality, and immune response of laying hens fed diets supplemented with mannan-oligosaccharide or an essential oil mixture under

moderate and hot environmental conditions. *Poultry Science*, *91*(6), 1379–1386. https://doi.org/10.3382/PS.2011-02023

Bry, L., Falk, P. G., Midtvedt, T., & Gordon, J. I. (1996). A Model of Host-Microbial Interactions in an Open Mammalian Ecosystem. *Science*, *273*(5280), 1380–1383. https://doi.org/10.1126/SCIENCE.273.5280.1380

Busquet, M., Calsamiglia, S., Ferret, A., Carro, M. D., & Kamel, C. (2005). Effect of Garlic Oil and Four of its Compounds on Rumen Microbial Fermentation. *Journal of Dairy Science*, *88*(12), 4393–4404. https://doi.org/10.3168/JDS.S0022-0302(05)73126-X

Cavallito, C. J., & Bailey, J. H. (2002). Allicin, the Antibacterial Principle of Allium sativum. I. Isolation, Physical Properties and Antibacterial Action. *Journal of the American Chemical Society*, *66*(11), 1950–1951. https://doi.org/10.1021/JA01239A048

Chowdhury, S. R., Chowdhury, S. D., & Smith, T. K. (2002). Effects of dietary garlic on cholesterol metabolism in laying hens. *Poultry Science*, *81*(12), 1856–1862. https://doi.org/10.1093/PS/81.12.1856

European Commision. (2003). Commision Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. *Official Journal of the European Union*, *40*, 29–43.

European Commision. (2018). Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending Regulation (EC) No 183/2005 of the European Parliament and of the Council and repealing. *Official Journal of European Union*, *62*, L:2019:004:TOC.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Donaldson, G. P., Lee, S. M., & Mazmanian, S. K. (2015). Gut biogeography of the bacterial microbiota. *Nature Reviews Microbiology*, *14*(1), 20–32. https://doi.org/10.1038/nrmicro3552

Doughari, H. J., Ndakidemi, P. A., Human, I. S., & Benade, S. (2011). The Ecology, Biology and Pathogenesis of Acinetobacter spp.: An Overview. *Microbes and Environments*, *26*(2), 101–112. https://doi.org/10.1264/JSME2.ME10179

El-Ghany, W. A. A., & Ismail, M. (2014). Tackling experimental colisepticaemia in broiler chickens using phytobiotic essential oils and antibiotic alone or in combination. *Iran. J. Vet. Res.*, *15*, 110–115.

Faith, D. P., & Baker, A. M. (2006). Phylogenetic Diversity (PD) and Biodiversity Conservation: Some Bioinformatics Challenges. *Evolutionary Bioinformatics*, *2*, 121–128. https://doi.org/10.1177/117693430600200007

Farag, I. F., Davis, J. P., Youssef, N. H., & Elshahed, M. S. (2014). Global Patterns of Abundance, Diversity and Community Structure of the Aminicenantes (Candidate Phylum OP8). *PLOS ONE*, *9*(3), e92139. https://doi.org/10.1371/JOURNAL.PONE.0092139

Ferket, P. R. (2004). Alternatives to antibiotics in poultry production: responses, practical experience and recommendations. (T. P. Lyons & K. A. Jacques, Eds.), *Nutritional*

Biotechnology in the Feed and Food Industries. Nottinghan University Press: Nottinghan, UK.

Food and Drug Administration. (2013). New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions With GFI #209. *Food and Drug Administration: Silver Spring, MA, USA.*

Gadde, U., Kim, W. H., Oh, S. T., & Lillehoj, H. S. (2017). Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. *Animal Health Research Reviews*, *18*(01), 26–45. https://doi.org/10.1017/S1466252316000207

Gaggìa, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology*, *141*, S15–S28. https://doi.org/10.1016/j.ijfoodmicro.2010.02.031

Garrity, G. (2005). *Bergey's Manual of Systematic Bacteriology*. *2nd ed.; Garrity, G., Ed.; Springer: New York, NY, USA, 2005; Volume 2: The Proteobacteria. Part B: Gammaproteobacteria, 2816p.*

Ghasemi, R., Zarei, M., & Torki, M. (2010). Adding Medicinal Herbs Including Garlic (Allium sativum) and Thyme (Thymus vulgaris) to Diet of Laying Hens and Evaluating Productive Performance and Egg Quality Characteristics. *American Journal of Animal and Veterinary Sciences*, *5*(2), 151–154. https://doi.org/10.3844/AJAVSP.2010.151.154

Gheisar, M. M., & Kim, I. H. (2018). Phytobiotics in poultry and swine nutrition–a review. *Italian Journal of Animal Science*, *17*(1), 92–99. https://doi.org/10.1080/1828051X.2017.1350120

Guo, F. C., Williams, B. A., Kwakkel, R. P., Li, H. S., Li, X. P., Luo, J. Y., … Verstegen, M. W. A. (2004). Effects of mushroom and herb polysaccharides, as alternatives for an antibiotic, on the cecal microbial ecosystem in broiler chickens. *Poultry Science*, *83*(2), 175–182. https://doi.org/10.1093/PS/83.2.175

Han, G. G., Kim, E. B., Lee, J., Lee, J.-Y., Jin, G., Park, J., … Kong, C. (2016). Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. *SpringerPlus*, *5*(1), 1–9. https://doi.org/10.1186/S40064-016-2604-8

Hardy, B. (2002). The issue of antibiotic use in the livestock industry: What have we learned? *Animal Biotechnology*, *13*(1), 129–147. https://doi.org/10.1081/ABIO-120005775

Harris, J. C., Cottrell, S. L., Plummer, S., & Lloyd, D. (2001). Antimicrobial properties of Allium sativum (garlic). *Applied Microbiology and Biotechnology*, *57*(3), 282–286. https://doi.org/10.1007/s002530100722

Hayes, D. J., Jensen, H. H., Backstrom, L., & Fabiosa, J. (2001). Economic impact of a ban on the use of over the counter antibiotics in U.S. swine rations. *The International Food and Agribusiness Management Review*, *4*(1), 81–97. https://doi.org/10.1016/S1096-7508(01)00071-4

Hume, M. E. (2010). Historical perspective: Prebiotics, probiotics, and other alternatives to antibiotics. *Journal of Dairy Science*, *93*, 191–192. https://doi.org/ 10.3382/ps.2010-01030

Jana, P., Karmakar, S., Roy, U., Paul, M., Singh, A.K., & Bera, K.K. (2018). Phytobiotics in aquaculture health management: A review. *J. Entomol. Zool.*, *6*, 1422–1429.

Janssen, S., Mcdonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MSystems*, *3*, e00021-18. https://doi.org/10.1128/mSystems.00021-18

Jimoh, A., Olorede, B., Abubakar, A., Fabiyi, J., Ibitoye, E., Suleiman, N., & Garba, S. (2012). Lipids profile and haematological indices of broiler chickens fed garlic (Allium sativum)-supplemented diets. *Journal of Veterinary Advances*, *2*, 474–480.

Joerger, R. D. (2003). Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poultry Science*, *82*(4), 640–647. https://doi.org/10.1093/PS/82.4.640

Kaya, H., & Macit, M. (2012). Effect of inclusion of garlic (Allium sativum) powder at different levels and copper into diets of hens on performance, egg quality traits and yolk cholesterol content. *International Journal of Poultry Science*, *11*(2), 114–119. https://doi.org/10.3923/IJPS.2012.114.119

Kers, J. G., Velkers, F. C., Fischer, E. A. J., Hermes, G. D. A., Stegeman, J. A., & Smidt, H. (2018). Host and Environmental Factors Affecting the Intestinal Microbiota in Chickens. *Frontiers in Microbiology*, *9*, 235. https://doi.org/10.3389/FMICB.2018.00235

Khan, R. U., Nikousefat, Z., Tufarelli, V., Naz, S., Javdani, M., & Laudadio, V. (2019). Garlic (Allium sativum) supplementation in poultry diets: effect on production and physiology. *World's Poultry Science Journal*, *68*(3), 417–424. https://doi.org/10.1017/S0043933912000530

Khan, S. H., Anjum, M. A., Parveen, A., Khawaja, T., & Ashraf, N. M. (2013). Effects of black cumin seed (Nigella sativa L.) on performance and immune system in newly evolved crossbred laying hens. *Veterinary Quarterly*, *33*(1), 15–21. https://doi.org/10.1080/01652176.2013.782119

Kiczorowska, B., Samolińska, W., Al-Yasiry, A. R. M., Kiczorowski, P., & Winiarska-Mieczan, A. (2017). The natural feed additives as immunostimulants in monogastric animal nutrition - A review. *Annals of Animal Science*, *17*(3), 605–625. https://doi.org/10.1515/AOAS-2016-0076

Kothari, D., Lee, W.-D., Niu, K.-M., & Kim, S.-K. (2019). The Genus Allium as Poultry Feed Additive: A Review. *Animals*, *9*(12), 1032. https://doi.org/10.3390/ANI9121032

Krieg, N. R., Staley, J. T., Brown, D. R., Hedlund, B. P., Paster, B. J., Ward, N. L., … Whitman, W. B. (2010). *Bergey's Manual of Systematic Bacteriology. Volume 4: The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes, 949*. Springer: New York, NY, USA.

Lanzotti, V. (2006). The analysis of onion and garlic. *Journal of Chromatography A*, *1112*(1–2), 3–22. https://doi.org/10.1016/J.CHROMA.2005.12.016

Lee, J. S., Kim, M. J., Park, S. H., Lee, S. B., Wang, T., Jung, U. S., … Lee, H. G. (2017). Effects of dietary mixture of garlic (Allium sativum), coriander (Coriandrum sativum) and probiotics on immune responses and caecal counts in young laying hens. *Journal of Animal Physiology and Animal Nutrition*, *101*(5), e122–e132.

https://doi.org/10.1111/JPN.12573

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine 2004 10:12*, *10*(12), S122–S129. https://doi.org/10.1038/nm1145

Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., & Gordon, J. I. (2005). Obesity alters gut microbial ecology. *PNAS*, *102*, 11070–11075.

Liu, Y., Song, M., Che, T. M., Almeida, J. A. S., Lee, J. J., Bravo, D., … Pettigrew, J. E. (2013). Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. *Journal of Animal Science*, *91*(11), 5294–5306. https://doi.org/10.2527/jas.2012-6194

Liu, Y., Song, M., Che, T. M., Bravo, D., & Pettigrew, J. E. (2012). Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages in vitro. *Journal of Animal Science*, *90*(8), 2774–2783. https://doi.org/10.2527/JAS.2011-4304

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, *73*(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Lumpkins, B. S., Batal, A. B., & Lee, M. (2008). The Effect of Gender on the Bacterial Community in the Gastrointestinal Tract of Broilers. *Poultry Science*, *87*(5), 964–967. https://doi.org/10.3382/PS.2007-00287

Mahmoud, K. Z., Gharaibeh, S. M., Zakaria, H. A., & Qatramiz, A. M. (2010). Garlic (Allium sativum) Supplementation: Influence on Egg Production, Quality, and Yolk Cholesterol Level in Layer Hens. *Asian-Australasian Journal of Animal Sciences*, *23*(11), 1503–1509. https://doi.org/10.5713/AJAS.2010.10124

Natasya-Ain, R., Eirna-Liza, N., Jasmin, M. Y., & Karim, M. (2018). Antibacterial activity of garlic extracts on fish pathogenic bacteria. *Journal of Environmental Biology*, *39*(5), 808–812. https://doi.org/10.22438/jeb/39/5(SI)/25

Olobatoke, R. Y., & Mulugeta, S. D. (2011). Effect of dietary garlic powder on layer performance, fecal bacterial load, and egg quality. *Poultry Science*, *90*(3), 665–670. https://doi.org/10.3382/PS.2010-00736

Omar, S. H., & Al-Wabel, N. A. (2010). Organosulfur compounds and possible mechanism of garlic in cancer. *Saudi Pharmaceutical Journal*, *18*(1), 51–58. https://doi.org/10.1016/J.JSPS.2009.12.007

Omer, H. A. A., Ahmed, S. M., Abdel-Magid, S. S., El-Mallah, G. M. H., Bakr, A. A., & Fattah, M. M. A. (2019). Nutritional impact of inclusion of garlic (Allium sativum) and/or onion (Allium cepa L.) powder in laying hens' diets on their performance, egg quality, and some blood constituents. *Bulletin of the National Research Centre*, *43*(1), 1–9. https://doi.org/10.1186/s42269-019-0061-6

Park, J. H., Song, T. H., & Kim, I. (2016). Egg production, egg quality, and cecal microbial populations of layers fed diets supplemented with fermented phytogenic feed additive. *Turkish Journal of Veterinary and Animal Sciences*, *40*, 660–666. https://doi.org/10.3906/vet-1512-55

Peinado, M. J., Echávarri, A., Ruiz, R., Suárez-Pereira, E., Mellet, C. O., Fernández, J.

M. G., & Rubio, L. A. (2013). Effects of inulin and di-d-fructose dianhydride-enriched caramels on intestinal microbiota composition and performance of broiler chickens. *Animal*, *7*(11), 1779–1788. https://doi.org/10.1017/S1751731113001572

Peinado, M. J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L. A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Animal Feed Science and Technology*, *181*(1–4), 87–92. https://doi.org/10.1016/j.anifeedsci.2013.03.001

Peinado, M. J., Ruiz, R., Echavarri, A., & Rubio, L. A. (2012). Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. *Poultry Science*, *91*(9), 2148–2157. https://doi.org/10.3382/ps.2012-02280

Peralta-Sánchez, J. M., Martín-Platero, A. M., Ariza-Romero, J. J., Rabelo-Ruiz, M., Zurita-González, M. J., Baños, A., … Martínez-Bueno, M. (2019). Egg Production in Poultry Farming Is Improved by Probiotic Bacteria. *Frontiers in Microbiology*, *10*(May), 1–13. https://doi.org/10.3389/fmicb.2019.01042

Phillips, M. L. (2009). Gut reaction: Environmental effects on the human microbiota. *Environmental Health Perspectives*, *117*(5). https://doi.org/10.1289/EHP.117-A198

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F., & Matuchansky, C. (2005). Review article: bifidobacteria as probiotic agents – physiological effects and clinical benefits. *Alimentary Pharmacology & Therapeutics*, *22*(6), 495–512. https://doi.org/10.1111/J.1365-2036.2005.02615.X

Pielou, E. C. (1966). Measurement of diversity in different types of biological collections. *Journal of Theoretical Biology*, *13*(C), 131–144. https://doi.org/10.1016/0022-5193(66)90013-0

Radwan, N., Hassan, R., Qota, E., & Fayek, H. (2008). Effect of natural antioxidant on oxidative stability of eggs and productive and reproductive performance of laying hens. *International Journal of Poultry Science*, *7*, 134–150. https://doi.org/10.3923/IJPS.2008.134.150

Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A., & Mele, M. C. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. *Microorganisms*, *7*(1), 14. https://doi.org/10.3390/MICROORGANISMS7010014

Ruiz, R., García, M. P., Lara, A., & Rubio, L. A. (2010). Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. *Veterinary Microbiology*, *144*(1–2), 110–117. https://doi.org/10.1016/j.vetmic.2009.12.025

Segata, N, Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). LEfSe: Linear Discriminant Analysis Effect Size, *Available online: https://huttenhower.sph.harvard.edu/galaxy/ (accessed on 8 February 2021).*

Segata, Nicola, Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shannon, C. E. (1948). A Mathematical Theory of Communication. *The Bell System Technical Journal*, *27*, 379-423,623-656.

Song, A. A.-L., In, L. L. A., Lim, S. H. E., & Rahim, R. A. (2017). A review on Lactococcus lactis: from food to factory. *Microbial Cell Factoriess*, *16*(1), 1–15. https://doi.org/10.1186/S12934-017-0669-X

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J. J., Baños-Arjona, A., Exposito-Ruiz, M., & Gutierrez-Fernandez, J. (2018). In vitro antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from allium spp. Against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples. *BioMed Research International*, *2018*, 7861207. https://doi.org/10.1155/2018/7861207

Tamaki, H. (2019). Cultivation Renaissance in the Post-Metagenomics Era: Combining the New and Old. *Microbes and Environments*, *34*(2), 117–120. https://doi.org/10.1264/JSME2.ME3402RH

Thomas, C. M., & Versalovic, J. (2010). Probiotics-host communication. *Gut Microbes*, *1*(3), 1–16. https://doi.org/10.4161/GMIC.1.3.11712

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

Vidanarachchi, J., Sims, I. M., & Iji, P. (2005). Phytobiotics: Alternatives to antibiotic growth promoters in monogastric animal feed. *Recent Advances in Animal Nutrition in Australia*, *15*, 131–144.

Wettenhall, C. (2020). *Allium: Ecology, Distribution and Cultivation*. Nova Science Publishers Inc.: Hauppauge, NY, USA, 2020; 282p.

Whittaker, R. H. (1972). Evolution and Measurement of Species Diversity. *Taxon*, *21*(2/3), 213.

Wierup, M. (2004). The Swedish Experience of the 1986 Year Ban of Antimicrobial Growth Promoters, with Special Reference to Animal Health, Disease Prevention, Productivity, and Usage of Antimicrobials. *Microbial Drug Resistance*, *7*(2), 183–190. https://doi.org/10.1089/10766290152045066

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, *86*(14), E140–E148. https://doi.org/10.2527/jas.2007-0459

Wise, R. (2008). The worldwide threat of antimicrobial resistance. *Curr. Sci.*, *95*, 181–187.

World Health Organization. (2018). Antimicrobial resistance. *Available Online: Http://Www.Who.Int/Antimicrobial-Resistance/En/ (Accessed on 17 May 2018)*.

Yalçın, S., Onbaşılar, E. E., Reisli, Z., & Yalçın, S. (2006). Effect of garlic powder on the performance, egg traits and blood parameters of laying hens. *Journal of the Science of Food and Agriculture*, *86*(9), 1336–1339. https://doi.org/10.1002/JSFA.2515

Ye, C. L., Dai, D. H., & Hu, W. L. (2013). Antimicrobial and antioxidant activities of the essential oil from onion (Allium cepa L.). *Food Control*, *30*(1), 48–53. https://doi.org/10.1016/J.FOODCONT.2012.07.033

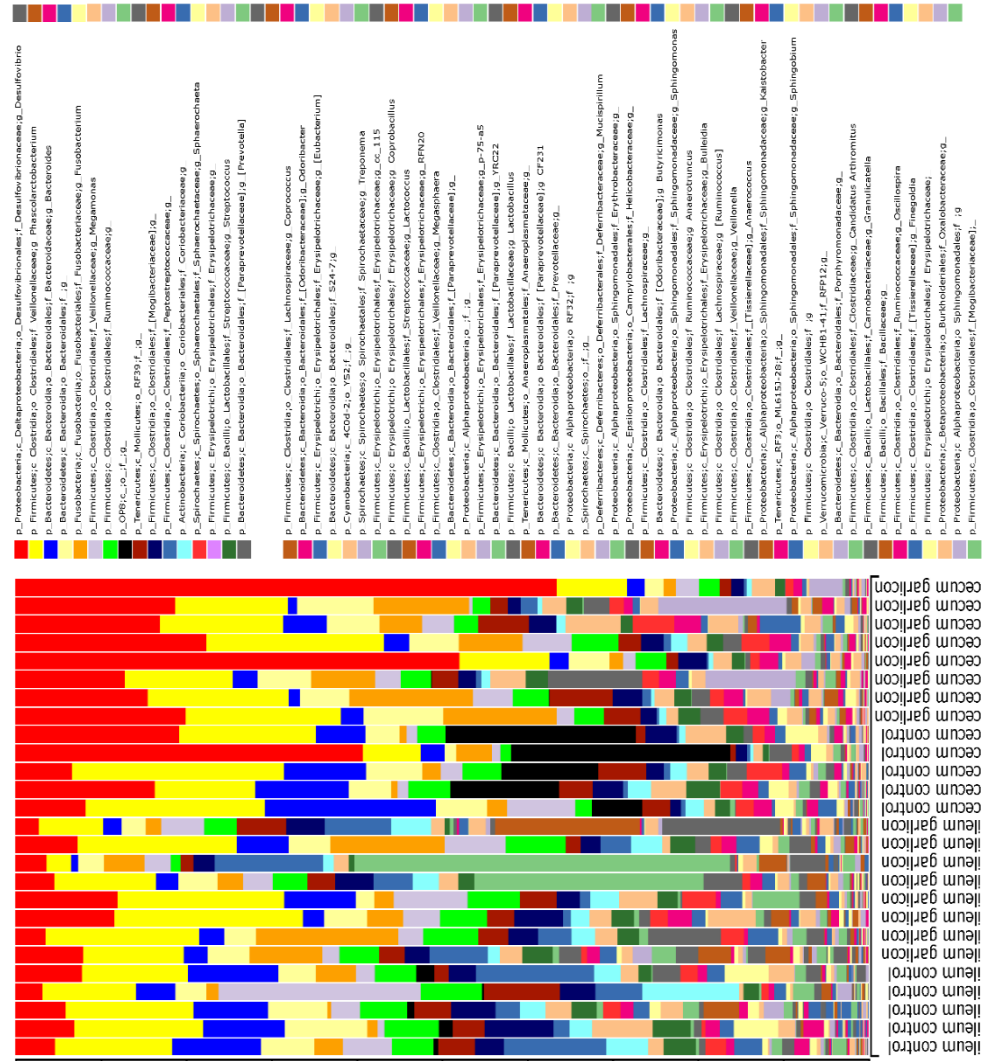
Zhang, Z., Lv, J., Pan, L., & Zhang, Y. (2018). Roles and applications of probiotic Lactobacillus strains. *Applied Microbiology and Biotechnology*, *102*(19), 8135–8143. https://doi.org/10.1007/S00253-018-9217-9

Zhu, Z., Hang, S., Zhu, H., Zhong, S., Mao, S., & Zhu, W. (2013). Effects of garlic oil on milk fatty acid profile and lipogenesis-related gene expression in mammary gland of dairy goats. *Journal of the Science of Food and Agriculture*, *93*(3), 560–567. https://doi.org/10.1002/jsfa.5829

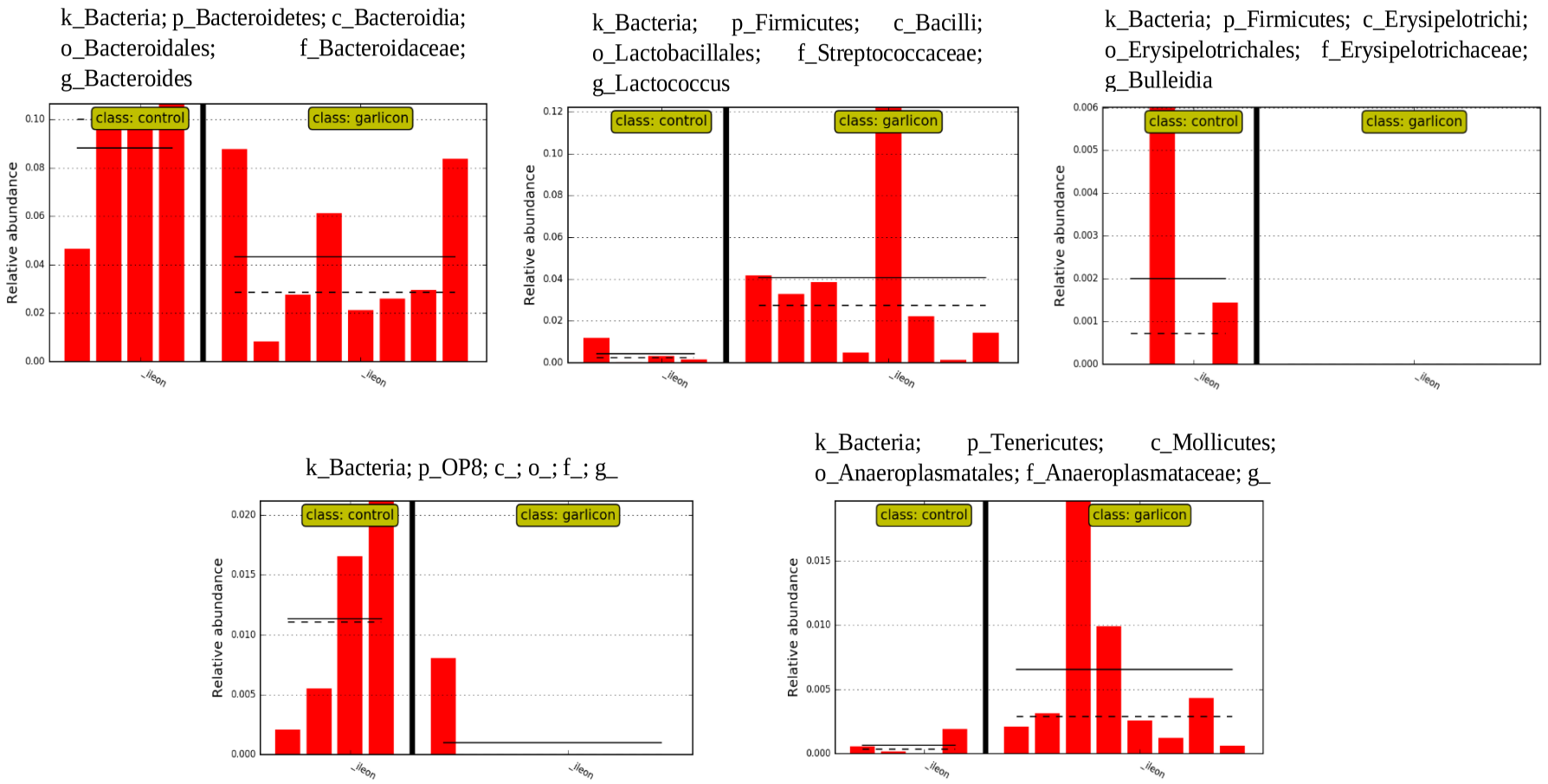
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Nutritional information of the basal feed employed in laying hens.

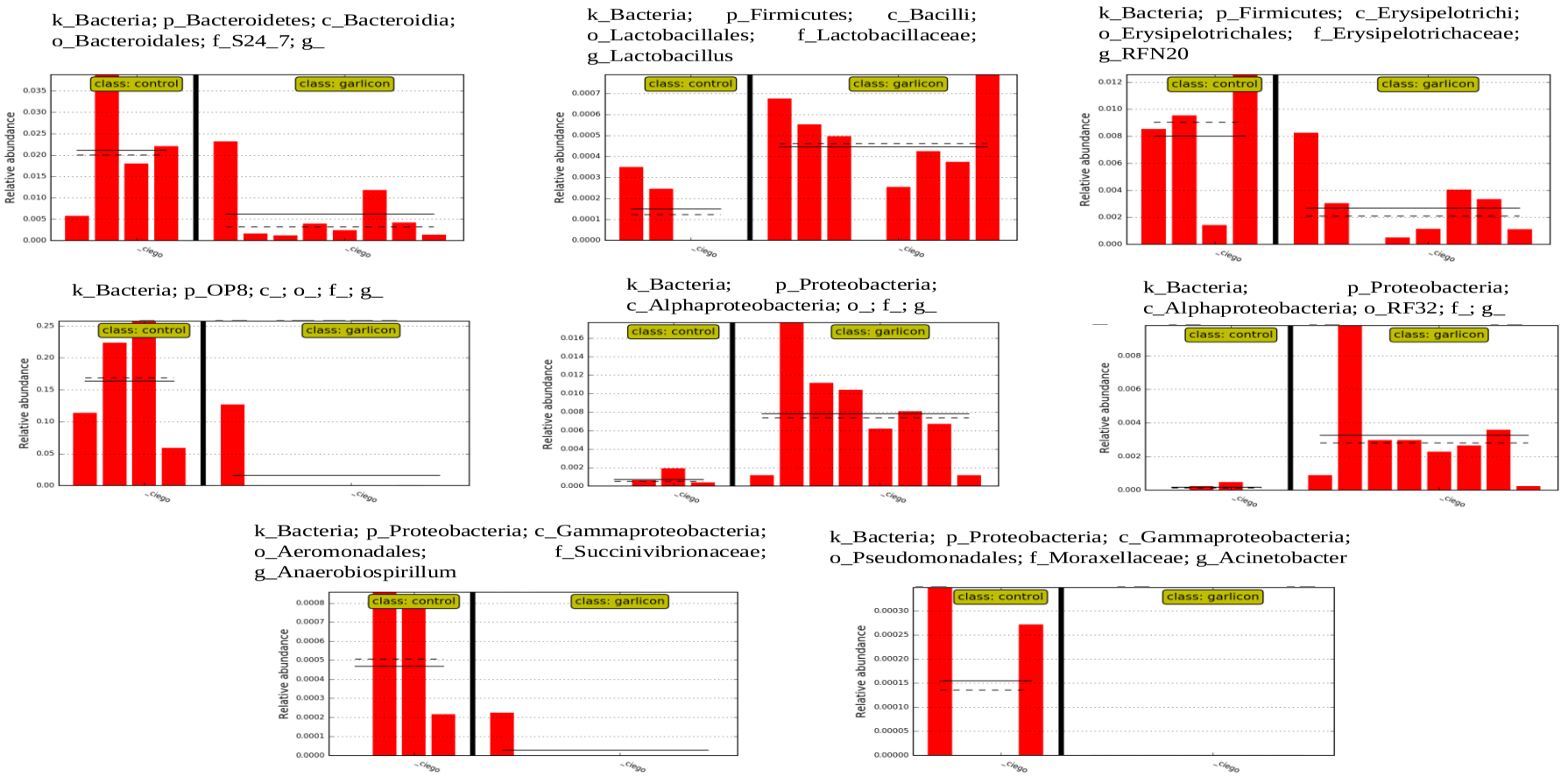
	%
Raw protein	16.00
Carbohydrates	57.57
Raw fiber	3.90
Fat content	3.5
Ashes	13.00
Calcium	3.85
Phosphorus	0.59
Sodium	0.16
Lysine	0.97
Metionine	0.46



Supplementary Figure S1. Bar plot of the relative bacterial abundance at the genus level in different gut regions of laying hens and treatments. Control refers to laying hens fed a basal diet while Garlicon refers to experimental laying hens fed a basal diet supplemented with the commercial *Alliaceae* extract. The sixteen most abundance genera are shown in a unique color set. The color of the rest of genera (less abundant) are repeated every 8 colors.



Supplementary Figure S2. Bar plot of the relative bacterial abundance at the genus level in different gut regions of laying hens and treatments. Control refers to laying hens fed a basal diet while Garlicon refers to experimental laying hens fed a basal diet supplemented with the commercial *Alliaceae* extract. The sixteen most abundance genera are shown in a unique color set. The color of the rest of genera (less abundant) are repeated every 8 colors.



Supplementary Figure S3. Linear Discriminant Analysis Effect Size (Lefse) showing genera from the cecum (*ciego*) that significantly differ between control and experimentally supplemented with a commercial *Alliaceae* (Garlicon40 ©) laying hens. Bars showed relative abundance of the genus in each sample. Solid line in the control group and dashed line in the Garlicon group represent mean relative abundance within each group. Solid line in the Garlicon group showed the grand mean. k: kingdom; p: phylum; c: class; o: order; f: family; and g: genus.

CAPÍTULO III

Allium Extract Implements Weaned Piglet's Productive Parameters by Modulating Distal Gut Microbiota

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ABSTRACT

Antimicrobial resistance (AMR) has risen as a global threat for human health. One of the leading factors for this emergence has been the massive use of antibiotics growth-promoter (AGPs) in livestock, enhancing the spread of AMR among human pathogenic bacteria. Thus, several alter-natives such as probiotics, prebiotics, or phytobiotics have been proposed for using in animal feeding to maintain or improve productive levels while diminishing the negative effects of AGPs. Reducing the use of antibiotics is a key aspect in the pig rearing for production reasons, as well as for the production of high-quality pork, acceptable to consumers. Here we analyze the potential use of *Allium* extract as an alternative. In this study, weaned piglets were fed with *Allium* extract supplementation and compared with control and antibiotic (colistin and zinc oxide) treated piglets. The effects of *Allium* extract were tested by analyzing the gut microbiome and measuring different productive parameters. Alpha diversity indices decreased significantly in *Allium* extract group in caecum and colon. Regarding beta diversity, significant differences between treatments appeared only in caecum and colon. *Allium* extract and antibiotic piglets showed better values of body weight (BW), average daily weight gain (ADG), and feed conversion ratio (FCR) than control group. These results indicate that productive parameters can be implemented by modifying the gut microbiota through phytobiotics such as *Allium* extract, which will drive to drop the use of antibiotics in piglet diet.

Keywords: *Allium* extract; bacterial community; high-throughput sequencing; phytobiotic; piglet microbiome; productive parameters.

INTRODUCTION

Antibiotics have been used to promote growth and production in livestock (Antibiotic Growth Promoters, AGP (Phillips et al., 2003; Thacker, 2013)). However, the inappropriate and indiscriminate use of them contributed to a rising of resistance to antibiotics (Capita & Alonso-Calleja, 2013). This situation drove the World Health Organization (WHO) to call for a global action against Antimicrobial Resistance (AMR; (WHO, 2014)). For this reason, AGPs are banned by the European Union since 2006 (EC Regulation 1831/2003; http://eur-lex.europa.eu/en/index.htm) and by other countries during following years (Maron et al., 2013; U.S. FDA, 2016). However, this ban has produced an increase in mortality, especially at weaning when many stressors affects piglets' health, leading to an increase of post-weaning diarrhea caused by *Escherichia coli* infections (Casewell et al., 2003; Campbell et al., 2013). This increase in mortality directly affects the pork industry, as pork and its derivatives are product highly consumed daily throughout the world (Dietze, 2011). In this sense, reducing the use of antibiotics is a key aspect in the pig rearing for production reasons, but also for the production of high-quality pork, acceptable to consumers.

The mechanisms through which AGPs act are not very clear but it is believed that growth promotion could be associated with changes in the gut microbiota (Kim et al., 2012; Dibner & Richards, 2005). AGPs may favor the reduction of pathogenic bacteria, the reduction of bacterial competition for nutrients, and reduction of microbial compounds, which can decrease animal growth (Gaskins et al., 2002; Niewold, 2007). However, the use of AGPs has undesirable effects such as selection and spread of antibiotic resistance genes (Barton, 2014). Some studies show evidence of the occurrence of AMR in relation to the use of antibiotics in cattle and specifically in the swine industry (Faldynova et al., 2013; Gerzova et al., 2015). Many bacterial strains resistant to a wide variety of antibiotics have been found in the intestinal microbiota of pigs, such as *Campylobacter coli*, *C. jejuni*, *Salmonella*, or the multiresistant *Staphylococcus aureus* (livestock-associated MRSA) (Barton, 2014; Gomes-Neves et al., 2014; Peeters et al., 2015; Thakur & Gebreyes, 2005). Given this problem of AMR and the subsequent ban of AGPs in food animal production, there has been a need to look for alternatives that maintain animal health and increase productive levels of pigs while decreasing the use of antibiotics (Thacker, 2013; Liu et al., 2018).

Different compounds have been proposed as substitutes to AGPs in swine industry improving health and performance of pigs. Probiotics, prebiotics, organic acids, enzymes, or phytobiotics have been widely recognized as promising alternatives to antibiotics in feeds (Thacker, 2013). Phytobiotics are plant-derived products used in animal feed to improve performance of livestock. Some studies had demonstrated their antimicrobial, antioxidants, and immunoregulatory effects in poultry and pigs (Thacker, 2013; Windisch et al., 2008). Given these positive properties of phytobiotics, several researchers have tried to demonstrate that their inclusion in diets can improve pig performance. Some studies have shown positive results using different plant extracts including oregano oil (Li et al., 2012; Ragland et al., 2008), menthol and cinnamon (Li et al., 2012; Maenner et al., 2011), a mixture of different plant extracts (Ahmed et al., 2013; Huang et al., 2010), and garlic (Liu et al., 2013). Garlic had also been used due to its antifungal, antimicrobial, and antioxidant properties (Harris et al., 2001; Tatara et al., 2008). Currently, several active organosulfur compounds extracted from garlic and other Allium plants, such as PTS (propyl propane thiosulfinate) and PTSO (propyl propane thiosulfonate), have been characterized (Ruiz et al., 2010). An *Allium* extract, which includes these compounds, has shown high antimicrobial activity against *Salmonella*, *E. coli*, *Clostridium*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Campylobacter jejuni*, and *Aspergillus pathogens* (Peinado et al., 2012; Sorlozano-Puerto et al., 2018). This product had been mainly used in broiler chickens, modulating intestinal microbiota, improving nutrient digestibility, and reducing pathogens and potentially pathogenic bacteria in the intestinal content (Peinado et al., 2012; Peinado et al., 2013). PTS and PTSO had also been add to pig diet and showed antimicrobial activity against different bacterial groups, decreasing fecal counts of Enterobacteriaceae and coliforms (Ruiz et al., 2010).

The aim of the present study was to evaluate the influence of the *Allium* extract in weaned piglet gut microbiota and how it affects productive parameters such as body weight, daily weight gain, daily feed intake, and feed conversion rate. In this study, we have made a fully randomized experiment using piglets as research animal model supplemented with the *Allium* extract. We have characterized the microbiota in different gut regions by high-throughput sequencing of 16S rRNA gene at 70 days of life. We suggest that this phytobiotic compound improves piglet productive parameters by means of distal gut microbiota modification.

MATERIAL AND METHODS

Piglets and Farm Facilities

The experiment was carried out at IMASDE AGROALIMENTARIA S.L. in Granja La Mata (Experimental Authorization Ref No: B-82334855), a swine experimental farm situated in Mata de Cuellar (Segovia, Spain). A total of 240 piglets (50% female, 50% male) were used in the experiment. Piglets were housed in a non-litter housing system consisting of 2 rooms, using a total of 24 blocks (12 of each room). Ten crossbred piglets of the same sex (50% Pietrain x 25% Landrace* 25% Large White) from commercial genetic breeds were kept per block of 6.05 m^2 (2.16 x 2.80 m). Piglets were from stress-free parents. The rooms had natural and artificial lighting, and the temperature was adjusted according to the piglet age. Piglets were weaned at 28 days of life, with an average weight of 7.34 ± 0.89 kg. The farm fulfilled the national regulations and the European directive for the protection of animal welfare in research (Directive 2010/63/EU, European Commission, 2010).

Experimental Design and Sample Collection

Before starting the experiment, animals were examined and those with signs of illness or injury were removed. Subsequently, groups of 10 piglets of the same sex were as-signed randomly to different blocks (8 blocks per treatment, 4 in each room). Piglets were regularly monitored during rearing. No signs of loss of weight, abnormal behaviors or deaths were detected. Control piglets were fed with a basal diet, while experimental pig-lets received basal diet supplemented with *Allium* extract (equivalent to 20 mg/kg of thiosulfinates and thiosulfonates). This *Allium* extract is commercialized under the trademark of Garlicon (DOMCA S.A.U., Spain), and the applied dose is the recommended by the product leaflet. In addition, another group received basal diet supplemented with 120 mg/kg of the antibiotic colistin (Nipoxyme 100) and 3000 ppm of zinc oxide (ZnO) as positive control (colistin was only used for research purpose because it is prohibited for commercial purpose). Basal diet differed in pre-starter (28 to 42 days) and starter (43 to 70 days) (Supplementary material: Table S1). Both diets and water were supplied ad libitum. Diets were formulated by IMASDE AGROALIMENTARIA S.L. and produced at the factory Gireporc S.A. in Bernuy de Porreros (Segovia, Spain).

Piglets were weighed at weaning (beginning of the experiment—28 days old), at 42 and 70 days old. Other productive parameters were recorded at the end of each experimental stage (42 and 70 days old): Average daily feed intake, ADFI; Average daily weight gain, ADG; and Feed conversion rate, FCR (ADFI divided by ADG). At the end of the experiment (70 days old), one piglet per block (a total of eight piglets of each treatment) was slaughtered by previous electrically stunned and bleed, according to the standardized procedures of slaughterhouse "El cochinillo segoviano" S.L. (Boceguillas, Segovia, Spain). Immediately, pieces of about 10 cm were dissected from different intestinal regions (duodenum and ileum from small intestine; caecum and colon from large intestine) with sterile material. Intestinal pieces were stored in sterile containers and transported to the laboratory, where they were kept at -80ºC until DNA extraction. Intestinal pieces from different gut regions of piglets were dissected using a sterile scalpel and approximately 100 mg of gut content were collected.

DNA Extraction

DNA extraction was carried out using FavorPrep Stool DNA Isolation Mini Kit (Favorgen Biotech Corp., Taiwan), according to manufacturer instructions. DNA extraction was checked by 0.7% agarose gel electrophoresis and DNA concentration was measured using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples were standardized at the same DNA concentration (10 ng/µl) and then stored at -20 °C until DNA amplification.

High-Throughput Sequencing

Amplicon PCR was performed from bacterial total DNA of the V4 region of the 16S rRNA gene using the primer pair U515F (5´ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTA A-3´) and E786R (5´-GTCTCGTGGGCTCG-GAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3´) with overlap partial Illumina primers. This PCR was carried out in a final volume of 25 µL containing 12.5 µL of iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories, Inc.), 0.3 µM of each primer, and 5 µL of template DNA. The amplification program consisted of an initial denaturing step of 98ºC for 1 min followed by an amplification step of 25 cycles of 10 s at 98 °C, 20 s at 52 °C, and 15 s at 72 °C, and a final extension of 5 min at 72 °C. Then, a second PCR was applied to include specific barcodes by adding a unique

combination of a couple of barcodes per sample. This PCR was carried out in a final volume of 25 µL containing 12.5 µL of iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories, Inc.), 0.4 µM of each primer, and 5 µL of purified PCR product from the previous PCR. The amplification pro-gram consisted of an initial denaturing step of 98ºC for 1 min followed by an amplification step of 8 cycles of 10 s at 98 °C, 20 s at 55 °C, and 15 s at 72 °C, and a final extension of 5 min at 72 °C. Purification steps were made using magnetic microparticles with a surface functional group to which DNA can be reversibly linked. Subsequently, the DNA of the magnetic particles were separated by elution (Hawkins, 1998). Then, DNA concentration was measured using Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, CA) and normalized to the same concentration. High-throughput sequencing was carried out on Illumina MiSeq platform in the Scientific Instrumental Center at the University of Granada (CIC-UGR, Spain). Sequences are available in the Genbank-NCBI Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/), BioProject: PRJNA664026, Accession Nos. SAMN16192455 to SAMN16192544.

Sequences Processing and Data Analysis

The processing of the sequences obtained from Illumina MiSeq was carried out with QIIME2 v2018.02 (Quantitative Insights In Microbial Ecology (Bolyen et al., 2019; Caporaso et al., 2010)). First, primers trimming were performed using default parameters using cutadapt plugin (Martin, 2011). Forward reads were selected for the following analysis due to low quality in reverse reads after 120 bp (Phred score < 20). Quality filtering were performed using default parameters. Afterwards, we used Deblur for sequence clustering into sub-OTUs, a sub-operational-taxonimic-unit (sOTU) approach, in order to remove sequencing errors (Amir et al., 2017). Sequences that passed quality filters were truncated to 200 bp, using Phred score of 20 as quality threshold, giving a dataset of 6,548,564 total reads with a mean depth of 70,415 reads per sample. We used fragment insertion script adapted to QIIME2 through the SATé-enabled phylogenetic placement (SEPP) technique, a script that performs the alignment of the sequences and the phylogenetic tree (Janssen et al., 2018). Taxonomy assignation was made with a classifier pretrained on Greengenes 13.08 with a similarity of 99% (DeSantis et al., 2006). Finally, because the primers used are de-signed for bacteria, chloroplasts, mitochondria, and non-bacterial DNA were removed from the sOTU table.

Statistics

To test the effect of treatment on production parameters of pigs, we used Generalized Linear Mixed-Models (GLMM). We used 24 experimental units (2 rooms of 12 experimental units each) with treatment as fixed factor, sex, and room as random factors, and initial body weight as covariate.

For alpha and beta diversity analyses, sOTU table was rarified at 17,000 sequences depth per sample. Samples that did not reach this sequencing depth were excluded for subsequent analyses. Two alpha diversity indices were calculated, i.e., bacterial species richness, as number of observed species; and Faith's phylogenetic diversity index (Faith & Baker, 2006). We used General Linear Models (GLM) to explore the effect of treatment and gut region in different alpha diversity indices. Piglet was used as experimental unit for alpha and beta diversity analysis.

Productive parameters and alpha diversity analyses were performed in Statistica 10.0 (StatSoft).

Beta diversity distance matrixes were calculated using UniFrac distance (C. Lozupone & Knight, 2005). In sub-sequent analysis, we used both Weighted UniFrac and Unweighted UniFrac distance matrixes as we do not have a priori predictions in the effects of the independent variables (gut region and treatment) in the bacterial community. Weighted UniFrac gives more importance to most abundant bacteria as it takes into account the abundance of sequences per sOTU, while Unweighted UniFrac gives the same importance to all bacterial sOTU presents in the samples, giving more importance to minority bacteria as it takes into ac-count the presence or absence of sOTU (Lozupone et al., 2007). Permutational ANOVA (PERMANOVA) based on Type III sums of squares with 999 permutations was used to test treatment and gut region effects on both UniFrac distance matrixes (Collyer et al., 2015) using PRIMER-7 (PRIMER-e). Principal Coordinates Analysis were calculated and visualizations of the first three axes of the PCoA were plotted using Emperor 2018.2.0 (Vázquez-Baeza et al., 2013).

RESULTS

Effects of Treatment on Piglets' Gut Bacterial Alpha Diversity

Duodenum and ileum microbiota of 70 days control piglets were mainly dominated at classes Bacilli and Clostridia, representing more than 90% between both groups. This pattern was similar in *Allium* extract and antibiotic groups, but with a lower proportion of Bacilli and higher proportion of Gammaproteobacteria in duodenum (10.5% and 3.9% in antibiotic and *Allium* extract group respect to 1.9% in control group) and *Clostridia* in ileum (35.3% and 21.1% in antibiotic and *Allium* extract group respect to 13.5% in control group) (Figure 1). At genus level, duodenum and ileum community of piglets was very diverse, dominated by *Lactobacillus* (more than 65%), followed by an unidentified genus of the family *Clostridiaceae* (6.7%), *Sarcina* (5.9%), *Streptococcus* (3%), and an unidentified genus of the family *Peptostreptococcaceae* (2.8%). Duodenum microbiota was very similar in three groups, but in the ileum, more differences appeared, with lower proportion of Lactobacillus in both *Allium* extract and antibiotic group (Supplementary material: Figure S1). However, no statistically significant differences appeared between treatments in duodenum and ileum in neither Species richness (LSD Posthoc test, $p > 0.314$) nor Faith's diversity index (LSD Posthoc test, $p > 0.253$).

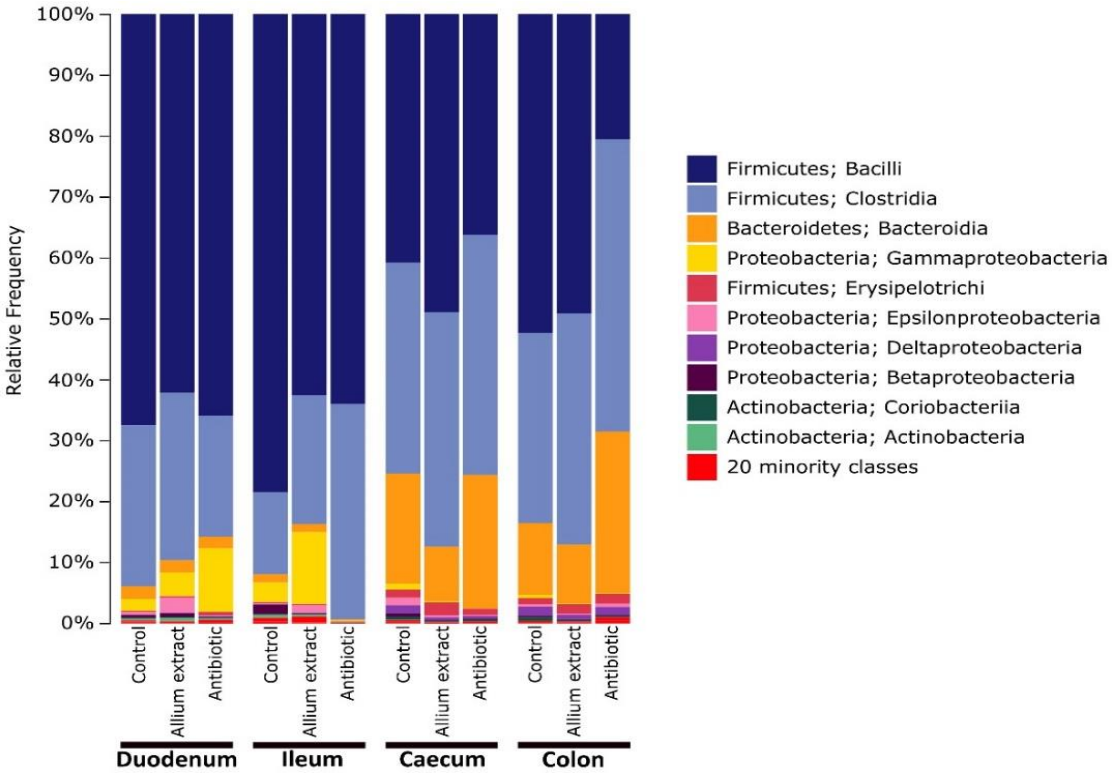

Figure 1. Microbial composition at class level of piglet gut microbiota grouped by gut region and treatment. Classes in the legend are sorted from most abundant to lowest abundant.

Large intestine (caecum and colon) microbiota showed a shift in dominant classes respect to small intestine, with lower proportion of Bacilli and higher proportion of Clostridia and Bacteroidia (Figure 1). Caecum microbiome had a very similar distribution in piglets from different treatments, with a slightly higher proportion in *Allium* extract fed piglets of Bacilli (48.9% respect to 40.8% in control group) and lower proportion of Bacteroidia (9.0% respect to 18.1% in control group). At the class level, colon microbiome of control and *Allium* extract groups were very similar, but the Antibiotic group microbiome showed a lower proportion of Bacilli (20.5% compared to 52.3% in control group) and higher proportion of Clostridia and Bacteroidia (Figure 1). At genus level, caecum microbiome of piglets from different treatments was similar, but in colon region, differences appeared in the antibiotic group, with lower proportion of *Lactobacillus* (14.2%) with respect to control and *Allium* extract groups (48.9 and 46.5%, respectively) and higher proportion of *Prevotella* and the rest of minority genera (Supplementary material: Figure S1). Regarding alpha diversity indices, in caecum, *Allium* extract group showed lower values of Species richness and Faith's diversity index than Control group (LSD Posthoc test, p = 0.007; LSD Posthoc test, p = 0.034 respectively). In colon, *Allium* extract group had lower values of Species richness and Faith's diversity index than antibiotic group (LSD Posthoc test, p = 0.008; LSD Posthoc test, p = 0.019 respectively).

Therefore, none of the small intestine region (duodenum and ileum) showed differences between treatments in Species richness and Faith diversity indices, but significant differences in these alpha diversity indices appeared in large intestine regions (caecum and colon) (Figure 2). Taking into account the whole gut, species richness and Faith's diversity index differed significantly between treatments and between gut regions (Table 1). However, interactions between treatments and gut region were not significant, indicating that alpha diversity indices along the piglets' gut of different treatments changed in the same way (see interaction Gut Region and Treatment in Table 1).

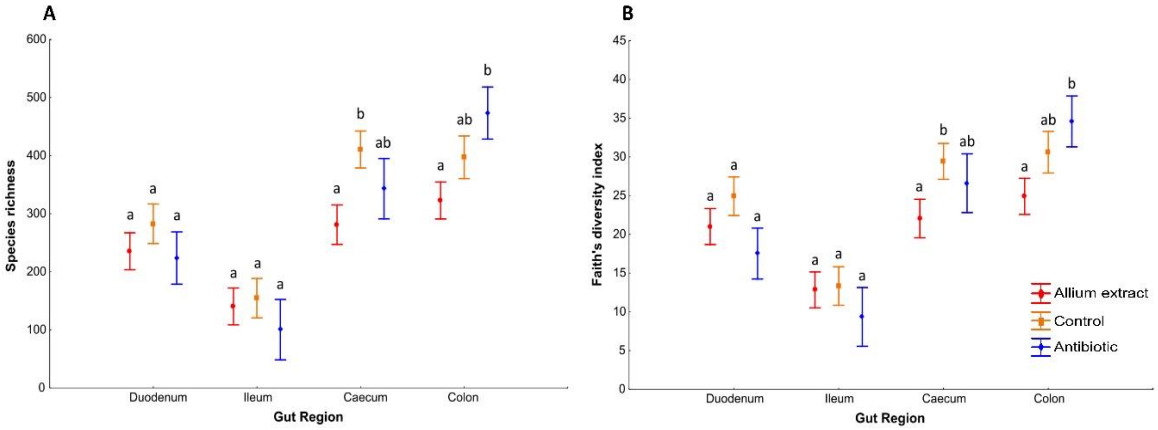

Figure 2. Alpha diversity by gut region. Average ± standard error of the mean of the bacterial species richness and Faith's diversity index of weaned piglets in different gut regions. Bars with different letter within the same gut region denote significant differences in treatment (LSD Posthoc test; $p < 0.05$).

Table 1. General Linear Models exploring the effects of treatment (control, antibiotic and Allium extract) and gut region in the different alpha diversity indices of the bacterial community of weaned piglets. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values ($p < 0.05$) are shown in bold.

Alpha Diversity Index	Control	*Allium* Extract	Antibiotic	Explanatory Variables	D.f.	F	*p*
Species richness	311.75 (27.38)	243.77 (18.71)	294.14 (41.60)	Treatment	2,61	4.03	**0.023**
				Gut Region	3,61	26.41	**<0.001**
				Gut Region*Treatment	6,61	1.57	0.171
Faith's diversity index	24.53 (1.89)	20.14 (1.31)	22.59 (2.89)	Treatment	2,61	3.25	**0.046**
				Gut Region	3,61	22.11	**<0.001**
				Gut Region*Treatment	6,61	1.46	0.208

Effects of Treatment and Gut Region on Beta Diversity

Changes in bacterial communities along different piglets' gut regions were similar in the three experimental groups (see non-significant interaction terms Gut Region*Treatment of both Unweighted and Weighted UniFrac in Table 2). However, Gut Region and Treatment had a significant effect on the intestinal microbiota of the piglets in both UniFrac indices (Table 2). These differences were observed graphically in the Principal Coordinates Analysis (PCoA) when Gut Region, but not Treatment were taken into account (Figure 3). It can also be observed main clustering between small and large intestine samples.

Table 2. General Linear Models exploring the effects of treatment, gut region, and their interaction in beta diversity indices of bacterial community of weaned piglets fed with control diet or supplemented with antibiotic or *Allium* extract. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values are shown in bold.

β-diversity Distance Matrix	Explanatory Variables	D.f.	Pseudo-F	*p*
Unweighted UniFrac	Treatment	2,61	1.84	**0.001**
	Gut Region	3,61	7.88	**0.001**
	Gut Region*Treatment	6,61	1.06	0.303
Weighted UniFrac	Treatment	2,61	2.35	**0.044**
	Gut Region	3,61	9.14	**0.001**
	Gut Region*Treatment	6,61	1.02	0.412

When we studied the effect of Treatment within each gut region, significant differences appeared at the. caecum level with Unweighted UniFrac (Figure 3) and at the colon level with both Unweighted and Weighted UniFrac (Figure 3). Antibiotic samples grouped in a cluster separated from control and *Allium* extract samples. Therefore, our treatment affected mainly to large intestine regions (Table 3).

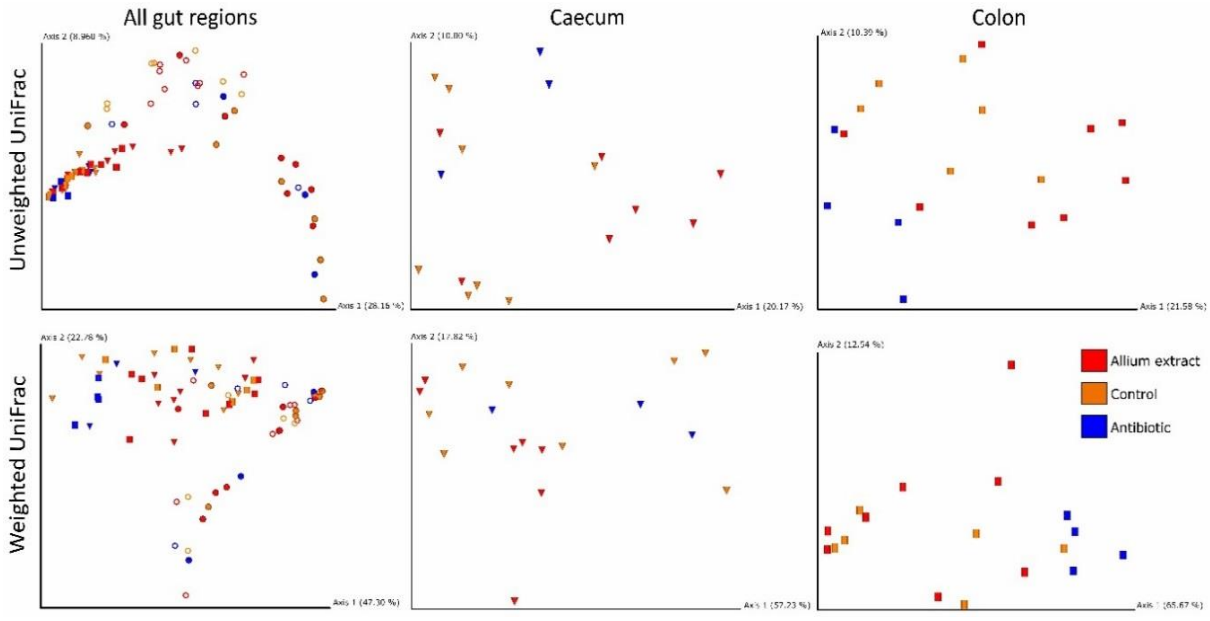

Figure 3. Dimensional figures showing the first two axes of Principal Coordinate Analysis and representing bacterial communities of weaned piglets in all gut regions and taking into account only caecum and colon using Unweighted and Weighted UniFrac distance matrixes. Samples are colored by treatment (Control - yellow; Antibiotic - blue; *Allium* extract - red) and samples from each intestinal region are represented by different shapes (Duodenum - ring; Ileum - sphere; Caecum - cone; Colon - square). Proportion of explained variance by each PCo axes is shown.

Table 3. General Linear Models exploring the effects of treatment in beta diversity indices of bacterial community of weaned piglets fed with control diet or supplemented with antibiotic or *Allium* extract. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values are shown in bold.

	β-diversity Distance Matrix	**D.f.**	**Pseudo-F**	***p***
Duodenum	Unweighted UniFrac	2,16	1.23	0.099
	Weighted UniFrac	2,16	0.25	0.977
Ileum	Unweighted UniFrac	2,15	0.93	0.502
	Weighted UniFrac	2,15	1.08	0.377
Caecum	Unweighted UniFrac	2,15	1.56	**0.007**
	Weighted UniFrac	2,15	1.48	0.191
Colon	Unweighted UniFrac	2,15	1.55	**0.017**
	Weighted UniFrac	2,15	4.18	**0.009**

Effects of Treatment on Piglets' Productive Parameters

Body weight significantly differed between treatments at day 70 (Table 4; Supplementary material: Table S2). Antibiotic and *Allium* extract fed piglets showed higher values of body weight than Control piglets (Table 4; Supplementary material: Table S2; Figure 4A). *Allium* extract group showed lower values of body weight than Antibiotic one, although this difference was marginally significant (LSD Posthoc test; $p = 0.080$).

During pre-starter stage (from 28 to 42 days), Antibiotic piglets had significantly more ADG and showed a better FCR than Control piglets, while *Allium* extract fed piglets showed intermediate values in both parameters. During starter stage (from days 43 to 70) Antibiotic and *Allium* extract showed higher values of ADG than Control piglets (Supplementary material: Table S2). Analyzing global stage (from 28 to 70 days), results showed that Antibiotic and *Allium* extract fed piglets significantly had higher ADG and lower FCR than Control piglets (Table 4; Supplementary material: Table S2; Figure 4B, 4C). No differences were observed between treatments in average daily feed intake (ADFI) or mortality (Table 4; Supplementary material: Table S2).

Table 4. General Linear Models exploring the effects of treatment as factor, sex, and block as random factors and initial body weight as covariate, in weaned piglets fed with control diet or supplemented with antibiotic or *Allium* extract. BW refers to body weight, ADG to average daily gain, FCR to feed conversion rate, and ADFI to average daily feed intake. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values are shown in bold.

Dependent Variable	Control	*Allium* Extract	Antibiotic	Independent Variables	F	D.f.	*p*
Initial BW (28 days), kg	7.34 (0.35)	7.34 (0.33)	7.32 (0.37)	Treatment	<0.01	2,19	0.998
				Sex	0.21	1,19	0.653
				Room	0.93	1,19	0.346
BW 42 days, kg	10.50 (0.49)	10.87 (0.57)	11.40 (0.55)	Treatment	5.69	2,18	**0.012**
				Sex	4.98	1,18	**0.039**
				Room	29.67	1,18	**<0.001**
				Initial BW	116.14	1,18	**<0.001**
BW 70 days, kg	21.01 (0.76)	22.79 (0.98)	23.76 (0.92)	Treatment	14.59	2,18	**<0.001**
				Sex	8.96	1,18	**0.008**
				Room	2.46	1,18	0.134
				Initial BW	86.30	1,18	**<0.001**
ADG 28–70 days, g/d	325.25 (11.01)	367.71 (16.84)	391.53 (15.37)	Treatment	14.59	2,18	**<0.001**
				Sex	8.96	1,18	**0.008**
				Room	2.46	1,18	0.134
				Initial BW	26.13	1,18	**<0.001**
ADFI 28–70 days, g/d	562.73 (30.24)	566.26 (23.25)	583.79 (19.97)	Treatment	0.49	2,18	0.620
				Sex	2.14	1,18	0.161
				Room	18.45	1,18	**<0.001**
				Initial BW	11.22	1,18	**0.004**
FCR 28–70 days, g/g	1.73 (0.06)	1.55 (0.06)	1.50 (0.04)	Treatment	8.27	2,18	**0.003**
				Sex	1.64	1,18	0.216
				Room	11.88	1,18	**0.003**
				Initial BW	1.26	1,18	0.277
Mortality 28–70 days, %	5.00 (2.67)	2.50 (1.64)	1.25 (1.25)	Treatment	0.90	2,18	0.423
				Sex	0.08	1,18	0.787
				Room	1.52	1,18	0.233
				Initial BW	0.66	1,18	0.428

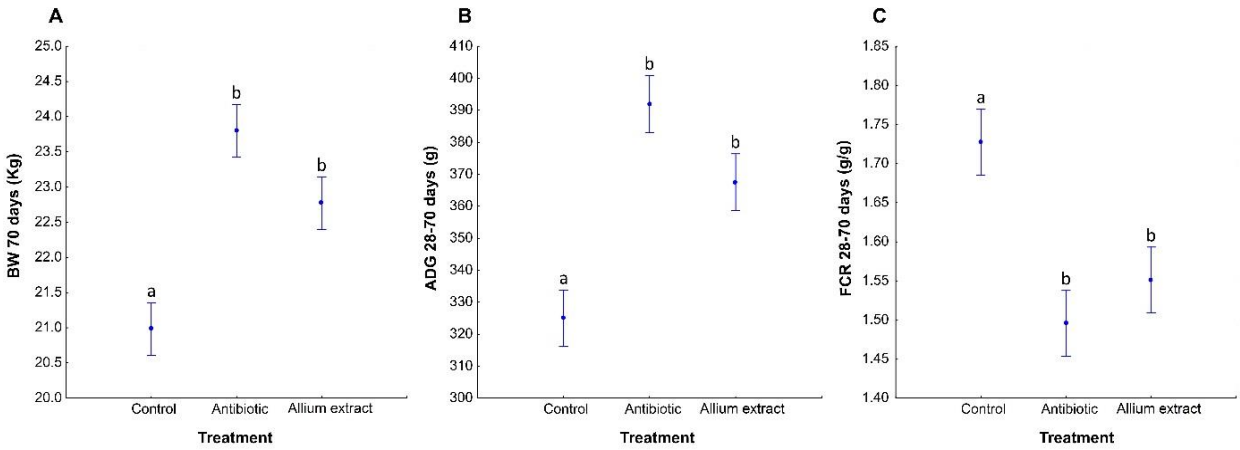

Figure 4. Average ± standard error of the mean of the Body Weight (BW) at 70 days of life (A), Average Daily Gain (ADG) (B), and Feed Conversion Ratio (FCR) (C) from 28 to 70 days of life of weaned piglets fed with control diet or antibiotic or *Allium* extract supplemented diets. Bars with different letter denote significant differences in treatment (LSD Posthoc test; $p < 0.05$).

DISCUSSION

The addition of *Allium* extract in the diet of weaned piglets had a significant increase of body weight (BW) and average daily gain (ADG), and decrease of feed conversion ratio (FCR) respect to control diet. *Allium* extract fed piglets reached similar productive levels to those of antibiotic group (colistin + ZnO), but marginally significant differences appeared in BW and ADG. These beneficial productive changes were accompanied by significant changes in bacterial community as diminution of alpha diversity indices and significant changes in beta diversity in large intestine regions (caecum and colon). These changes in beta diversity only appeared in the caecum and colon but general behavior of gut microbiota was not affected by the treatment (no differences in interaction between Gut and Treatment; Table 2).

Alternatives to antibiotics that maintain productive parameters in pig breeding is essential to fight AMR spreading and improve animal welfare. Several alternatives to antibiotic growth promoters such as probiotics, prebiotics, enzymes, and plant extracts had been proposed to achieve it and also to reduce the probability of AMR appearing (Thacker, 2013; Windisch et al., 2008). From this point of view, plant extracts or phytobiotic, which can modulate microbiota and increase productive parameters, appear to be good and safe alternative to antibiotics (Soler et al., 2018). Different plant extracts improve animal performance, productive parameters, and induce changes in gut microbiome of animals. For instance, oregano oil in growing-finishing pigs improved

growth performance and nutrient digestibility by modulating gut microbiota (Cheng et al., 2018), and oregano oil had been also used together with carbohydrases in piglets, improving feed conversion ratio with respect to control and antibiotic growth promoter diets (Ragland et al., 2008). Other essential oils obtained from thyme and cinnamon improved body weight of weaning pigs and decreased the number of pathogens as *E. coli* in different gut regions (Li et al., 2012); and a mixture of essential oil from mint and cinnamon improved feed efficiency in piglets. *Allium* extract, mainly garlic extract, had also been used in piglets' diet in different studies, reducing diarrhea and inflammation caused by *E. coli* (Liu et al., 2013) and improving piglet performance and body weight (Tatara et al., 2008). In our study, piglet diet was supplemented with *Allium* extract, an extract of onion and garlic, of which the principal active components are propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO). Our results support the use of this phytobiotic compound in piglet diet given that animals showed a performance improvement characterized by an increase of body weight (BW) and average daily gain (ADG), and a decrease of feed conversion ratio (FCR) with respect to control group. Furthermore, in our study, piglets fed with *Allium* extract reached productive levels similar to those obtained using an antibiotic growth promoter (colistin) and ZnO. These results are promising as pork is one of the most consumed meat all over the world (Dietze, 2011; Resano et al., 2011), thus *Allium* extract could be a good alternative to antibiotic growth promoters in pig diet given that improve productive parameters. Results obtained in other studies carried out with piglets suffering from diarrhea fed with plant extracts suggest that the growth promoting effects may be due to their antimicrobial activity (Hanczakowska et al., 2015; Hernández et al., 2004). This conclusion was also obtained in studies of Ruiz et al. (2010) using both PTS and PTSO in swine, which had antimicrobial activity against different bacterial group in pig feces, especially against *Enterobacteriaceae* and other coliforms. Other studies pointed out that plant extracts increase productive parameters stimulating feed consumption (Hanczakowska & Swiatkiewicz, 2012), but other authors found that plant extracts decrease feed consumption (Nowak et al., 2017). Nevertheless, our results shown that piglets fed with *Allium* extract had similar levels of average daily feed intake (ADFI) compared to control and antibiotic groups.

Microbiome of intestine of pigs is dominated by Firmicutes, followed by Proteobacteria in the small intestine and Bacteroidetes in the large intestine (De Rodas et

al., 2018; Xiao et al., 2018; Yang et al., 2016; Zhang et al., 2018). At the class level, dominant classes of each phylum are Bacilli and Clostridia (Firmicutes), Bacteroidia (Bacteroidetes), and Gammaproteobacteria (Proteobacteria). Our results are consistent with these previous findings, especially at the phylum level. Other studies had shown that some *Lactobacillus* species play an important role in intestinal health of piglets by influencing intestinal physiology, regulating the immune system, and balancing the intestinal ecology of the host (Park et al., 2014; Wang et al., 2019). In our experiment, in caecum and colon, piglets supplemented with *Allium* extract showed similar levels of Bacilli versus control group, mainly due to the genus *Lactobacillus*. However, antibiotic group showed lower proportion of *Lactobacillus*, especially in the colon, showing that colistin and ZnO would have an effect on *Lactobacillus* depletion, whereas the genus *Prevotella* had an increase occupying its niche. This decrease in *Lactobacillus* abundance in colistin and ZnO piglets may be related to a depletion in carbohydrate levels in distal parts of the gut. In vitro studies have demonstrated that shifts in pig gut microbiome composition can be produced by changes in substrate structure (Warren et al., 2018). Different *Allium* extracts produce changes in the physiology and histology of the gut of animals. In broilers, onion powder increased length, width, and surface area of intestinal villus (Rahman et al., 2017). In piglets, aged garlic extract improved body weight, the morphology of intestinal villi, and non-specific immune response (Tatara et al., 2008). Other studies using *Allium* extract in growing-finishing pigs showed an increase in productive parameters and an increase of short-chain fatty acids (SCFA) in feces, which is related to high *Lactobacillus* abundance in distal gut (Sánchez et al., 2020). These changes may suggest that *Allium* extracts produce changes in the availability of some substrates necessary for the growth of beneficial bacteria. However, an in vitro study showed that PTSO extracted from *Allium* plants have antimicrobial activity against lactobacilli, bifidobacteria, *Bacteroides*, and Clostridia, and strongly reduce enterobacteria and coliforms in swine microbiota (Ruiz et al., 2010). Whether PTSO and *Allium* extracts affect bacterial community directly or indirectly by change the substrate availability deserve future research.

Changes due to the supplementation of antimicrobials showed that main changes in bacterial community were produced in caecum and colon (Looft et al., 2014). Our results are consistent with these previous findings, showing differences between treatments in large intestine regions (caecum and colon) in both alpha and beta diversity

indices. These changes in bacterial community indices may be due to differential bioavailability of Garlicon in these intestinal regions. In vitro digestion studies of (Abad et al., 2019) showed that Garlicon bioavailability increases as it progresses in the gastrointestinal tract of pigs. Alpha diversity indices in the colon in the Antibiotic group were higher than in the *Allium* extract group. This reduction in alpha diversity levels could be related to the increase of body weight since reduction of alpha diversity has been associated with obesity in several human studies (Menni et al., 2017; Peters et al., 2018; Turnbaugh et al., 2009). Different studies have found evidences that differences in microbial composition could be due to body weight (Han et al., 2017) while other studies showed that changes induced by feed additives in gut microbiota can produce changes in body weight (Angelakis, 2017).

CONCLUSIONS

Our experiment supports the use of *Allium* extract supplemented in the diet of weaning piglets for successfully improving productive parameters such as body weight, average daily gain, or feed conversion ratio levels with respect to control diet. These beneficial effects in productivity correlates with significant changes in the bacterial community of the distal gut. These results are preliminary as further experiments are necessary to untangle whether *Allium* extracts directly affect the gut microbiota and hence the productivity parameters or whether the effects are directly on the bacterial community or on specific bacterial groups related to immune system or piglet's health.

Supplementary Materials: The following are available online at https://www.mdpi.com/2079-6382/10/3/269/s1: Table S1. Calculated composition and analysis (% per Kg of feed) of the diet used for piglets; Table S2. Average ± standard error of the mean of the Body Weight (BW) at 28, 42 and 70 days of life; and Average Daily Gain (ADG), Average Daily Feed Intake (ADFI), Feed Conversion Ratio (FCR) and mortality in different experimental stages and global stage of weaned piglets fed with control diet or Allium extract or antibiotic supplemented diets. Rows with different letter denote significant differences in treatment (LSD Posthoc test; $p < 0.05$); Figure S1. Microbial composition at genus level of piglets gut microbiota grouped by gut region and treatment. Genera in the legend are sorted from most abundant to lowest abundant.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and ethical review and approval were waived for this study. This study was carried out in accordance with the national regulations and the European directive for the protection of animal welfare in research (Directive 2010/63/EU, European Commission, 2010). The experiment was performed at IMASDE AGROALIMENTARIA S.L. in Granja La Mata (Mata de Cuellar, Segovia, Spain). This farm has experimental authorization (Ref No: B-82334855). Gut samples were collected in the course of the regular farm and slaughtering procedures in "El cochinillo segoviano" S.L. (Boceguillas, Segovia, Spain).

Informed Consent Statement: Not applicable.

Data Availability Statement: Sequences are available in the Genbank-NCBI Sequence Read Archive (SRA) (https://www.ncbi.nlm.nih.gov/sra/), BioProject: PRJNA664026, Accession Nos. SAMN16192455 to SAMN16192544.

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REFERENCES

Abad, P., Arroyo-Manzanares, N., Rivas-Montoya, E., Ochando-Pulido, J. M., Guillamon, E., García-Campaña, A. M., & Martinez-Ferez, A. (2019). Effects of different vehiculization strategies for the allium derivative propyl propane thiosulfonate during dynamic simulation of the pig gastrointestinal tract. Canadian Journal of Animal Science, 99(2), 244–253. https://doi.org/10.1139/cjas-2018-0063

Ahmed, S. T., Hossain, M. E., Kim, G. M., Hwang, J. A., Ji, H., & Yang, C. J. (2013). Effects of resveratrol and essential oils on growth performance, immunity, digestibility and fecal microbial shedding in challenged piglets. Asian-Australasian Journal of Animal Sciences, 26(5), 683–690. https://doi.org/10.5713/ajas.2012.12683

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. MSystems, 2(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Angelakis, E. (2017, May 1). Weight gain by gut microbiota manipulation in productive animals. Microbial Pathogenesis. Academic Press. https://doi.org/10.1016/j.micpath.2016.11.002

Barton, M. D. (2014). Impact of antibiotic use in the swine industry. Current Opinion in Microbiology, 19(1), 9–15. https://doi.org/10.1016/j.mib.2014.05.017

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology, 37(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Campbell, J. M., Crenshaw, J. D., & Polo, J. (2013, March 30). The biological stress of early weaned piglets. Journal of Animal Science and Biotechnology. BioMed Central. https://doi.org/10.1186/2049-1891-4-19

Capita, R., & Alonso-Calleja, C. (2013). Antibiotic-Resistant Bacteria: A Challenge for the Food Industry. Critical Reviews in Food Science and Nutrition, 53(1), 11–48. https://doi.org/10.1080/10408398.2010.519837

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nature Methods, 7(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Casewell, M., Friis, C., Marco, E., McMullin, P., & Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy, 52(2), 159–161. https://doi.org/10.1093/jac/dkg313

Cheng, C., Xia, M., Zhang, X., Wang, C., Jiang, S., & Peng, J. (2018). Supplementing oregano essential oil in a reduced-protein diet improves growth performance and nutrient digestibility by modulating intestinal bacteria, intestinal morphology, and antioxidative capacity of growing-finishing pigs. Animals, 8(9), 159. https://doi.org/10.3390/ani8090159

Collyer, M. L., Sekora, D. J., & Adams, D. C. (2015). A method for analysis of

phenotypic change for phenotypes described by high-dimensional data. Heredity, 115(4), 357–365. https://doi.org/10.1038/hdy.2014.75

De Rodas, B., Youmans, B. P., Danzeisen, J. L., Tran, H., & Johnson, T. J. (2018). Microbiome profiling of commercial pigs from farrow to finish. Journal of Animal Science, 96(5), 1778–1794. https://doi.org/10.1093/jas/sky109

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology, 72(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Dibner, J. J., & Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. Poultry Science, 84(4), 634–643. https://doi.org/10.1093/ps/84.4.634

Dietze, K. (2011). Pigs for Prosperity. Food and Agriculture Organization (FAO).

Faith, D. P., & Baker, A. M. (2006). Phylogenetic Diversity (PD) and Biodiversity Conservation: Some Bioinformatics Challenges. Evolutionary Bioinformatics, 2, 121–128. https://doi.org/10.1177/117693430600200007

Faldynova, M., Videnska, P., Havlickova, H., Sisak, F., Juricova, H., Babak, V., … Rychlik, I. (2013). Prevalence of antibiotic resistance genes in faecal samples from cattle, pigs and poultry. Veterinarni Medicina, 58(6), 298–304. https://doi.org/10.17221/6865-VETMED

Gaskins, H. R., Collier, C. T., & Anderson, D. B. (2002). Antibiotics as growth promotants: Mode of action. Animal Biotechnology, 13(1), 29–42. https://doi.org/10.1081/ABIO-120005768

Gerzova, L., Babak, V., Sedlar, K., Faldynova, M., Videnska, P., Cejkova, D., … Rychlik, I. (2015). Characterization of antibiotic resistance gene abundance and microbiota composition in feces of organic and conventional pigs from four EU countries. PLoS ONE, 10(7), e0132892. https://doi.org/10.1371/journal.pone.0132892

Gomes-Neves, E., Antunes, P., Manageiro, V., Gärtner, F., Caniça, M., da Costa, J. M. C., & Peixe, L. (2014). Clinically relevant multidrug resistant Salmonella enterica in swine and meat handlers at the abattoir. Veterinary Microbiology, 168(1), 229–233. https://doi.org/10.1016/j.vetmic.2013.10.017

Han, G. G., Lee, J. Y., Jin, G. D., Park, J., Choi, Y. H., Chae, B. J., … Choi, Y. J. (2017). Evaluating the association between body weight and the intestinal microbiota of weaned piglets via 16S rRNA sequencing. Applied Microbiology and Biotechnology, 101(14), 5903–5911. https://doi.org/10.1007/s00253-017-8304-7

Hanczakowska, E., & Swiatkiewicz, M. (2012). Effect of herbal extracts on piglet performance and small intestinal epithelial villi. Czech Journal of Animal Science, 57(9), 420–429. https://doi.org/10.17221/6316-CJAS

Hanczakowska, Ewa, Świątkiewicz, M., & Grela, E. R. (2015). Effect of dietary inclusion of a herbal extract mixture and different oils on pig performance and meat quality. Meat Science, 108, 61–66. https://doi.org/10.1016/j.meatsci.2015.05.020

Harris, J. C., Cottrell, S. L., Plummer, S., & Lloyd, D. (2001). Antimicrobial properties of Allium sativum (garlic). Applied Microbiology and Biotechnology, 57(3), 282–286. https://doi.org/10.1007/s002530100722

Hawkins, T. (1998). DNA purification and isolation using magnetic particles.

Hernández, F., Madrid, J., García, V., Orengo, J., & Megías, M. D. (2004). Influence of two plant extracts on broilers performance, digestibility, and digestive organ size. Poultry Science, 83(2), 169–174. https://doi.org/10.1093/ps/83.2.169

Huang, Y., Yoo, J. S., Kim, H. J., Wang, Y., Chen, Y. J., Cho, J. H., & Kim, I. H. (2010). Effects of dietary supplementation with blended essential oils on growth performance, nutrient digestibility, blood profiles and fecal characteristics in weanling Pigs. Asian-Australasian Journal of Animal Sciences, 23(5), 607–613. https://doi.org/10.5713/ajas.2010.80120

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. MySistems, 3(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Kim, H. B., Borewicz, K., White, B. A., Singer, R. S., Sreevatsan, S., Tu, Z. J., & Isaacson, R. E. (2012). Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. Proceedings of the National Academy of Sciences of the United States of America, 109(38), 15485–15490. https://doi.org/10.1073/pnas.1205147109

Li, P., Piao, X., Ru, Y., Han, X., Xue, L., & Zhang, H. (2012). Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. Asian-Australasian Journal of Animal Sciences, 25(11), 1617–1626. https://doi.org/10.5713/ajas.2012.12292

Liu, Y., Song, M., Che, T. M., Almeida, J. A. S., Lee, J. J., Bravo, D., … Pettigrew, J. E. (2013). Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli1. Journal of Animal Science, 91(11), 5294–5306.

Liu, Yanhong, Espinosa, C. D., Abelilla, J. J., Casas, G. A., Lagos, L. V., Lee, S. A., … Stein, H. H. (2018). Non-antibiotic feed additives in diets for pigs: A review. Animal Nutrition, 4(2), 113–125. https://doi.org/10.1016/j.aninu.2018.01.007

Looft, T., Allen, H. K., Cantarel, B. L., Levine, U. Y., Bayles, D. O., Alt, D. P., … Stanton, T. B. (2014). Bacteria, phages and pigs: The effects of in-feed antibiotics on the microbiome at different gut locations. ISME Journal, 8(8), 1566–1576. https://doi.org/10.1038/ismej.2014.12

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. Applied and Environmental Microbiology, 73(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. Applied and Environmental Microbiology, 71(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Maenner, K., Vahjen, W., & Simon, O. (2011). Studies on the effects of essential-oil-based feed additives on performance, ileal nutrient digestibility, and selected bacterial groups in the gastrointestinal tract of piglets. Journal of Animal Science, 89(7), 2106–2112. https://doi.org/10.2527/jas.2010-2950

Maron, D. F., Smith, T. J. S., & Nachman, K. E. (2013). Restrictions on antimicrobial use

in food animal production: An international regulatory and economic survey. Globalization and Health, 9(1). https://doi.org/10.1186/1744-8603-9-48

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal, 17(1), 10. https://doi.org/10.14806/ej.17.1.200

Menni, C., Jackson, M. A., Pallister, T., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. International Journal of Obesity, 41, 1099–1105. https://doi.org/10.1038/ijo.2017.66

Niewold, T. A. (2007). The Nonantibiotic Anti-Inflammatory Effect of Antimicrobial Growth Promoters, the Real Mode of Action? A Hypothesis. Poultry Science, 86(4), 605–609. https://doi.org/10.1093/ps/86.4.605

Nowak, P., Kasprowicz-Potocka, M., Zaworska, A., Nowak, W., Stefańska, B., Sip, A., … Frankiewicz, A. (2017). The effect of eubiotic feed additives on the performance of growing pigs and the activity of intestinal microflora. Archives of Animal Nutrition, 71(6), 455–469. https://doi.org/10.1080/1745039X.2017.1390181

Park, S. J., Kim, J., Lee, J. S., Rhee, S. K., & Kim, H. (2014). Characterization of the fecal microbiome in different swine groups by high-throughput sequencing. Anaerobe, 28, 157–162. https://doi.org/10.1016/j.anaerobe.2014.06.002

Peeters, L. E. J., Argudín, M. A., Azadikhah, S., & Butaye, P. (2015). Antimicrobial resistance and population structure of Staphylococcus aureus recovered from pigs farms. Veterinary Microbiology, 180(1–2), 151–156. https://doi.org/10.1016/j.vetmic.2015.08.018

Peinado, M. J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L. A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. Animal Feed Science and Technology, 181(1–4), 87–92. https://doi.org/10.1016/j.anifeedsci.2013.03.001

Peinado, M. J., Ruiz, R., Echavarri, A., & Rubio, L. A. (2012). Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. Poultry Science, 91(9), 2148–2157. https://doi.org/ 10.3382/ps.2012-02280

Peters, B. A., Shapiro, J. A., Church, T. R., Miller, G., Trinh-Shevrin, C., Yuen, E., … Ahn, J. (2018). A taxonomic signature of obesity in a large study of American adults. Scientific Reports, 8(1), 1–13. https://doi.org/10.1038/s41598-018-28126-1

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., … Waddell, J. (2003). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. Journal of Antimicrobial Chemotherapy, 53(1), 28–52. https://doi.org/10.1093/jac/dkg483

Ragland, D., Stevenson, D., & Hill, M. A. (2008). Oregano oil and multi-component carbohydrases as alternatives to antimicrobials in nursery diets. Journal of Swine Health and Production, 16(5), 238–243.

Rahman, S. ur, Khan, S., Chand, N., Sadique, U., & Khan, R. U. (2017). In vivo effects of Allium cepa L. on the selected gut microflora and intestinal histomorphology in broiler. Acta Histochemica, 119(5), 446–450. https://doi.org/10.1016/j.acthis.2017.04.004

Resano, H., Perez-Cueto, F. J. A., de Barcellos, M. D., Veflen-Olsen, N., Grunert, K. G., & Verbeke, W. (2011). Consumer satisfaction with pork meat and derived products

in five European countries. Appetite, 56(1), 167–170. https://doi.org/10.1016/j.appet.2010.10.008

Ruiz, R., García, M. P., Lara, A., & Rubio, L. A. (2010). Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. Veterinary Microbiology, 144(1–2), 110–117. https://doi.org/10.1016/j.vetmic.2009.12.025

Sánchez, C. J., Martínez-Miró, S., Ariza, J. J., Madrid, J., Orengo, J., Aguinaga, M. A., … Hernández, F. (2020). Effect of alliaceae extract supplementation on performance and intestinal microbiota of growing-finishing pig. Animals, 10(9), 1–15. https://doi.org/10.3390/ani10091557

Soler, C., Goossens, T., Bermejo, A., Migura-García, L., Cusco, A., Francino, O., & Fraile, L. (2018). Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. PLoS ONE, 13(5), 1–22. https://doi.org/10.1371/journal.pone.0197353

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J. J., Baños-Arjona, A., Exposito-Ruiz, M., & Gutierrez-Fernandez, J. (2018). In vitro antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from allium spp. Against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples. BioMed Research International, 2018, 7861207. https://doi.org/10.1155/2018/7861207

Tatara, M. R., Sliwa, E., Dudek, K., Gawron, A., Piersiak, T., Dobrowolski, P., … Studziński, T. (2008). Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared in artificial sow. Annals of Agricultural and Environmental Medicine, 15(1), 63–69.

Tatara, M. R., Śliwa, E., Dudek, K., Gawron, A., Piersiak, T., Dobrowolski, P., … Studziński, T. (2008). Aged garlic extract and allicin improve performance and gastrointestinal tract development of piglets reared in artificial sow. Annals of Agricultural and Environmental Medicine, 15(1), 63–69.

Thacker, P. A. (2013). Alternatives to antibiotics as growth promoters for use in swine production: A review. Journal of Animal Science and Biotechnology, 4(1), 1–12. https://doi.org/10.1186/2049-1891-4-35

Thakur, S., & Gebreyes, W. A. (2005). Prevalence and antimicrobial resistance of Campylobacter in antimicrobial-free and conventional pig production systems. Journal of Food Protection, 68(11), 2402–2410. https://doi.org/10.4315/0362-028X-68.11.2402

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., … Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. Nature, 457(7228), 480–484. https://doi.org/10.1038/nature07540

U.S. FDA. (2016). FDA Reminds Retail Establishments of Upcoming Changes to the Use of Antibiotics in Food Animals. U.S. Food and Drugs Administration.

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. GigaScience, 2(1), 16. https://doi.org/10.1186/2047-217X-2-16

Wang, Y., Gong, L., Wu, Y. ping, Cui, Z. wen, Wang, Y. qiang, Huang, Y., … Li, W. fen. (2019). Oral administration of Lactobacillus rhamnosus GG to newborn piglets augments gut barrier function in pre-weaning piglets. Journal of Zhejiang

University: Science B, 20(2), 180–192. https://doi.org/10.1631/jzus.B1800022

Warren, F. J., Fukuma, N. M., Mikkelsen, D., Flanagan, B. M., Williams, B. A., Lisle, A. T., … Gidley, M. J. (2018). Food Starch Structure Impacts Gut Microbiome Composition. MSphere, 3(3). https://doi.org/10.1128/msphere.00086-18

WHO. (2014). Antimicrobial resistance: global report on surveillance 2014. World Health Organization, 1–257. https://doi.org/9789241564748

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. Journal of Animal Science, 86(14), E140–E148. https://doi.org/10.2527/jas.2007-0459

Xiao, Y., Kong, F., Xiang, Y., Zhou, W., Wang, J., Yang, H., … Zhao, J. (2018). Comparative biogeography of the gut microbiome between Jinhua and Landrace pigs. Scientific Reports, 8(1), 1–10. https://doi.org/10.1038/s41598-018-24289-z

Yang, H., Huang, X., Fang, S., Xin, W., Huang, L., & Chen, C. (2016). Uncovering the composition of microbial community structure and metagenomics among three gut locations in pigs with distinct fatness. Scientific Reports, 6(1), 27427. https://doi.org/10.1038/srep27427

Zhang, L., Wu, W., Lee, Y. K., Xie, J., & Zhang, H. (2018). Spatial heterogeneity and co-occurrence of mucosal and luminal microbiome across swine intestinal tract. Frontiers in Microbiology, 9(JAN). https://doi.org/10.3389/fmicb.2018.00048

SUPPLEMENTARY MATERIAL

Table S1. Calculated composition and analysis (% per Kg of feed) of the diet used for piglets.

Ingredients	Prestarter (28-42 d)	Starter (43-70 d)
Barley	15.00	20.00
Corn	0.00	13.39
Cornflakes	10.00	0.00
Wheat	34.24	35.00
Fullfat soybean	7.00	5.00
Soybean meal 47%	9.82	15.51
HP-300 (Soy extract)	8.00	4.00
Acid serum	10.00	0.00
Fat	1.92	2.57
Calcium carbonate	0.86	1.04
Monocalcium phosphate	0.89	1.20
Salt	0.34	0.52
Methionine	0.28	0.24
L-Lysine 50	0.90	0.84
L-Threonine	0.28	0.23
L-Tryptophan	0.06	0.06
Vit & Min Premix	0.40	0.40
Calculated analysis, %		
Ashes	5.57	5.12
Crude protein	19.00	18.50
Ethereal extract	4.92	5.32
Crude fiber	2.93	3.24
Neutral detergent fiber	9.51	10.64
Starch	34.96	40.23
Calcium	0.75	0.72
Total phosphorus	0.63	0.63
Available phosphorus	0.40	0.38
Sodium	0.24	0.22
Net energy, kcal / kg	2.500	2.470
Total lysine	1.39	1.31
Total methionine	0.52	0.48
Total Met+Cys	0.83	0.79
Total threonine	0.95	0.87
Total tryptophan	0.29	0.28
Digestible lysine	1.28	1.20
Digestible methionine	0.49	0.45
Digestible Met+Cys	0.76	0.72
Digestible threonine	0.86	0.78
Digestible tryptophan	0.26	0.25

Table S2. Average ± standard error of the mean of the Body Weight (BW) at 28, 42 and 70 days of life; and Average Daily Gain (ADG), Average Daily Feed Intake (ADFI), Feed Conversion Ratio (FCR) and mortality in different experimental stages and global stage of weaned piglets fed with control diet or *Allium* extract or antibiotic supplemented diets. Rows with different letter denote significant differences in treatment (LSD Posthoc test; $p < 0.05$).

Dependent variable	Control	*Allium* extract	Antibiotic
Initial BW (28 days), kg	7.34 (0.35)^{a}	7.34 (0.33)^{a}	7.32 (0.37)^{a}
BW 42 days, kg	10.50 (0.49)^{a}	10.87 (0.57)^{ab}	11.40 (0.55)^{b}
BW 70 days, kg	21.01 (0.76)^{a}	22.79 (0.98)^{b}	23.76 (0.92)^{b}
ADG 28-42 days, g/d	225.21 (16.71)^{a}	251.93 (29.99)^{ab}	291.25 (20.93)^{b}
ADG 42-70 days. g/d	375.27 (11.27)^{a}	425.60 (23.67)^{b}	441.67 (15.87)^{b}
ADG 28-70 days, g/d	325.25 (11.01)^{a}	367.71 (16.84)^{b}	391.53 (15.37)^{b}
ADFI 28-42 days, g/d	299.02 (29.28)^{a}	248.17 (14.10)^{a}	286.10 (16.40)^{a}
ADFI 42-70 days, g/d	694.59 (38.32)^{a}	725.31 (31.73)^{a}	732.64 (23.50)^{a}
ADFI 28-70 days, g/d	562.73 (30.24)^{a}	566.26 (23.25)^{a}	583.79 (19.97)^{a}
FCR 28-42 days, g/g	1.33 (0.09)^{a}	1.07 (0.12)^{ab}	0.99 (0.04)^{b}
FCR 42-70 days, g/g	1.85 (0.09)^{a}	1.74 (0.12)^{a}	1.67 (0.06)^{a}
FCR 28-70 days, g/g	1.73 (0.06)^{a}	1.55 (0.06)^{b}	1.50 (0.04)^{b}
Mortality 28-42 days, %	1.25 (1.25)^{a}	1.25 (1.25)^{a}	0.00 (0.00)^{a}
Mortality 42-70 days, %	3.88 (1.90)^{a}	1.25 (1.25)^{a}	1.25 (1.25)^{a}
Mortality 28-70 days, %	5.00 (2.67)^{a}	2.50 (1.64)^{a}	1.25 (1.25)^{a}

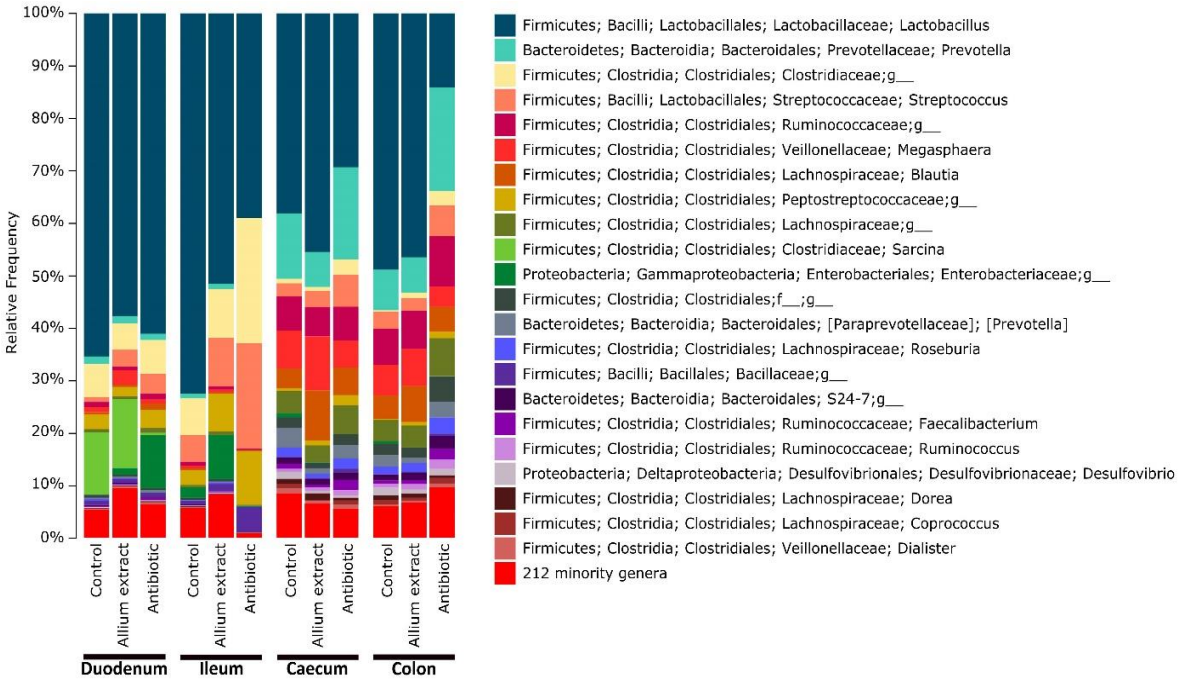

Figure S1. Microbial composition at genus level of piglets gut microbiota grouped by gut region and treatment. Genera in the legend are sorted from most abundant to lowest abundant.

CAPÍTULO IV

Beneficial shifts in the gut bacterial community of juvenile gilthead seabream (*Sparus aurata*) supplemented with *Allium*-Derived Compound Propyl Propane Thiosulfonate (PTSO)

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Microbial Ecology

ABSTRACT

Aquaculture plays an important role in supplying the world food demand and protein sources. High doses of antibiotics are used as prophylactic and growth promoters in the aquaculture industry as a result of high stocking densities and the impossibility of individual treatment of fish, increasing the spread of antimicrobial resistance among pathogenic bacteria. Thus, several alternatives such as probiotics, prebiotics and phytobiotics have been proposed in order to maintain or improve productive levels while diminishing the negative effects of antibiotic growth promoters (AGPs). This study aims to analyze the potential use of an *Allium*-derived compound propyl propane thiosulfonate (PTSO) as an alternative to antibiotics in aquaculture. Gilthead seabream (*Sparus aurata*) juveniles were supplemented in the diet with an *Allium*-derived compound (150 mg/kg of PTSO) and compared with control fish. The effects of this organosulfur compound were tested by measuring the body weight and analyzing the gut microbiota after 12 weeks. The relative abundance of potentially pathogenic *Vibrio* and *Pseudomonas* in the foregut and hindgut of supplemented fish decreased, while potentially beneficial *Lactobacillus* increased compared to control fish. Shannon's alpha diversity index significantly increased in both gut regions of fish fed with *Allium*-derived PTSO supplemented diet. Regarding beta diversity, significant differences between treatments only appeared in the hindgut when rare Operational Taxonomic Units (OTUs) were taken into account. No differences occurred in body weight during the experiment. These results indicate that supplementing the diet with the *Allium*-derived PTSO could produce beneficial changes in intestinal microbiota while maintaining the productive parameters of juvenile gilthead seabream.

Keywords: *Allium*-based phytobiotic; body weight; gilthead seabream; *Sparus aurata*; gut microbiota; propyl propane thiosulfonate.

INTRODUCTION

Aquaculture plays an important role in supplying the world food demand and protein sources, with fish accounting for 17% of the world's animal protein intake. In 2018, world aquaculture production reached 82 million tons, with an estimated value of US$ 250 billion (FAO, 2020). However, economic profits in the industry are affected by fish diseases caused by several pathogenic bacteria. The majority of severe infectious diseases in the industry include furunculosis, caused by *Aeromonas* species; photobacteriosis, caused by *Photobacterium* species; and vibriosis, caused by *Vibrio* species (Yukgehnaish et al., 2020). High doses of antibiotics are used to treat these diseases, given the high stocking densities and the impossibility of individual treatment of fish (Resende et al., 2012). Furthermore, antibiotics have been used for several years for growth promoting purposes in the aquaculture industry. Dietary inclusion of sub-therapeutic doses of antibiotics has been shown to improve feed efficiency and growth in different fish species (He et al., 2017). However, the extensive use of antibiotics in aquaculture, both for therapeutic and growth-promoting purposes, has contributed to a rise in antibiotic resistance in pathogens (Cañada-Cañada et al., 2009). This rise may be further increased by aquaculture systems, which facilitate the accumulation of antibiotic-resistant bacteria in water, sediments, animals and the environment around farms, posing a risk to animal and human health (Baquero et al., 2008). Therefore, a worldwide effort is necessary to minimize or eliminate the use of antibiotics for growth-promoting purposes in livestock and aquaculture. For this reason, the use of antibiotic growth promoters (AGPs) was banned by the European Union in 2006 (European Commision, 2018) and by other countries in the following years (Maron et al., 2013; U.S. FDA, 2016).

The development of non-antibiotic compounds as an alternative to AGPs in the aquaculture industry is of primary importance. Several feed additives have been proposed as promising alternatives for promoting growth performance and increasing immune resistance in fish. Bacteriophages, organic acids, probiotics, prebiotics, synbiotics and phytobiotics or plant extracts have been described as the most promising alternatives (Dawood et al., 2018; Pérez-Sánchez et al., 2018). Phytobiotics are defined as plant-derived bioactive compounds supplemented in the diet to improve the productivity of livestock (Windisch et al., 2008). These include a wide range of plant-derived products such as essential oils, herbs and oleoresins. Phytobiotics are known to exert antimicrobial activity against pathogenic microbes, acting directly as antimicrobials or modulating the

cell membrane, impeding the adhesion of pathogenic microbes to intestinal mucosal cells (Vidanarachchi et al., 2005). Phytobiotics can also act as prebiotics by facilitating a continuous supply of specific substrates for the protective intestinal microbiota or by minimizing the risk of development of pathogens (Vidanarachchi et al., 2005). Furthermore, phytobiotics are known to stimulate digestive secretions such as saliva and bile, and hence improve productive parameters (Gheisar & Kim, 2018). These changes in digestive function have been related to the growth of beneficial bacteria such as *Lactobacillus* or bifidobacteria in the intestine of broiler chickens supplemented with phytobiotics (Attia et al., 2017; Basit et al., 2020).

Allium-species plants, mainly garlic (*Allium sativum*) and onion (*Allium cepa*), produce a wide variety of compounds showing antifungal, antimicrobial and antioxidant activity (Kyung, 2012). Some studies have demonstrated the beneficial effects of *Allium* on growth performance, immune system and control of pathogenic bacteria and fungi in several fish species (Lee & Gao, 2012; Valenzuela-Gutiérrez et al., 2021). Inclusion of onion powder in the diet of beluga juveniles (*Huso huso*) improved growth performance, immune function and blood parameters (Akrami et al., 2015). Inclusion of garlic also showed an increase in weight gain and growth performance of rainbow trout (*Oncorhynchus mykiss*) (Büyükdeveci et al., 2018), an increase in growth performance and immune parameters of guppy fish (*Poecilia reticulata*) (Motlagh et al., 2020a), and increased health status and performance of Nile tilapia (*Oreochromis niloticus*) (Shalaby et al., 2006). Moreover, supplementation of the diet of Asian seabass (*Lates calcarifer*) with garlic showed an improvement in immunological parameters and an increase in body weight, feed conversion and survival after *Vibrio harveyi* challenging (Talpur & Ikhwanuddin, 2012). This ability to control pathogens has also been described in other studies, showing huge potential as antimicrobial agents against fish pathogenic fungi and bacteria, including *Pseudomonas fluorescens*, *Myxococcus piscicola* and *Vibrio anguillarum* (Lee & Gao, 2012; Valenzuela-Gutiérrez et al., 2021).

The activity of these plant compounds in animal feed is related to secondary metabolites, volatile organosulfur compounds such as ajoene, allicin, isoalliin, propyl propane thiosulfinate (PTS) or propyl propane thiosulfonate (PTSO) (Guillamón et al., 2021; Valenzuela-Gutiérrez et al., 2021). PTSO have been widely reported for its antibacterial, antifungal (Sorlozano-Puerto et al., 2018, 2021) and anticoccidial activity (Kim et al., 2013). In addition, it showed beneficial effects on gut health (Guillamón et

al., 2021) and changes in gut microbiota and performance of different species of terrestrial animals, such as mice, broilers chickens, laying hens and pigs (Abad et al., 2020; Peinado et al., 2013; Rabelo-Ruiz et al., 2021a; Rabelo-Ruiz et al., 2021b; Ruiz et al., 2015; Sánchez et al., 2020; Vezza et al., 2021). Furthermore, PTSO have been shown to be toxicologically safe in studies carried out in experimental animals (Lira et al., 2020). However, the potential effects of *Allium*-derived PTSO on the intestinal microbiota and growth performance of fishes have not yet been explored.

Therefore, in this study, we have used juvenile gilthead seabream (*Sparus aurata*) as the animal model in order to evaluate the effects of *Allium*-derived PTSO in foregut and hindgut microbiota by high-throughput sequencing of the 16S rRNA. Our results show how fish supplemented with *Allium*-derived PTSO shifts to a more beneficial bacterial community, by increasing potential probiotic *Lactobacillus* and reducing potential pathogens like *Vibrio*. These changes in microbiota are accompanied by an absence of differences in body weight.

MATERIAL AND METHODS

Allium-based product

The *Allium*-based product used is commercialized under the trademark AquaGarlic® and was supplied by DOMCA (Granada, Spain). This product is standardized in propyl propane thiosulfonate (PTSO) at a concentration of 10%. It is in powder form supported on inert sepiolite.

Animals, experimental design and sample collection

This research was carried out with juvenile gilthead seabream. Animals (n = 780) were randomly assigned to two different experimental groups (390 fish per group), consisting of triplicate tanks (400 L per tank; 130 fish per tank). Fish were kept in a recirculating RAS D-400 water system equipped with physical and biological filters. The temperature was maintained at 21 ± 1 ºC with a photoperiod regime of 12:12 hours (light:dark). All studied specimens were handled in accordance with the European Union Guidelines (Directive 2010/63/UE) for the use of laboratory animals. The Ethical Committee at the University of Granada approved the experiments and they were

endorsed by the Regional Government (Junta de Andalucía, Spain, ref. no. 13/04/2018/048).

The experimental diets were made from commercial fish meal (NUTRAPLUS, Dibaq, Spain) by adding the *Allium* based product (150 mg/kg of PTSO). Once the meal was homogenized, the granulated fish feed was manufactured by SPAROS (Olhão, Portugal). A diet without additive was prepared as a control. In addition, to ensure the concentration of PTSO in feed, UHPLC-ESI-MS/MS analyses were performed, according to the method described by Abad et al. (2016).

At the beginning, fish were randomly housed in different tanks, getting the same initial biomass in each tank. After 2 weeks of acclimatization, they were anesthetized with 80 mg/L of tricaine methanesulfonate (MS-222) and weighed, with average initial body weight (BW) of 7.72 ± 0.61 g. During the experiment (12 weeks), fish were fed ad libitum 3-4 times per day, 6 days per week. All the fish from each tank were collected every 2 weeks until the end of the experiment, anesthetized using MS-222 and weighed in groups of 10 fish. At the end of the experiment (12 weeks), 20 fish per experimental tank were euthanized by an overdose of anesthesia (400 mg/L), followed by spine severing. The fish were immediately dissected and the whole intestine was collected with sterile material. Intestinal pieces were stored in sterile containers and transported to the laboratory, where they were kept at -80ºC until DNA extraction.

DNA extraction

Intestinal pieces from the foregut and hindgut of gilthead seabream were dissected using a sterile scalpel and approximately 100 mg of gut were crushed using a FastPrep FP120 cell disrupter (BIO 101, Thermo Savant). DNA extraction was carried out using FavorPrep™ Stool DNA Isolation Mini Kit (Favorgen Biotech Corp., Taiwan), according to the manufacturer's instructions. DNA extraction was checked by 0.7% agarose gel electrophoresis and DNA concentration was measured using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples were stored at -20ºC until DNA amplification.

16S rRNA gene high-throughput sequencing

Amplicon PCR was performed on the bacterial total DNA of the V4 region of the 16S rRNA gene using the primer pair U515F (5´

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTA A-3´) and E786R (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCT AAT-3´) with Illumina adapter overhang sequences as indicated by the underlined text. Then, a second PCR was performed in order to add two unique Illumina compatible barcodes to each sample, so that the derived sequences can be demultiplexed into their respective samples in downstream analysis. These barcodes overlapped with the sequence of the primers used in the first PCR. Purification steps were made using DNA Purification SPRI Magnetic Beads (Canvax®). PCR amplicons were checked by 1% agarose gel electrophoresis, DNA concentrations were measured using Qubit® 3.0 Fluorometer (Invitrogen™, Carlsbad, CA) and normalized to reach the same concentration per sample. High-throughput sequencing was performed using Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). This sequencing results in paired-end reads of 2 x 300 bp length. Sequencing was carried out on the Illumina MiSeq platform in the Scientific Instrumental Center at the University of Granada (CIC-UGR, Spain). Sequences are available from the Sequence Read Archive (SRA) at the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra), BioProject: PRJNA749674, Accession Nos. SAMN20396460 to SAMN20396707.

Sequences processing and data analysis

The Quantitative Insights Into Microbial Ecology (QIIME2 v2020.11; Bolyen et al., 2019) software was used to analyze the 16S rRNA sequences generated from Illumina MiSeq. First, primer trimmings were performed using *cutadapt* plugin (Martin, 2011). Forward reads were selected for the following analysis due to low quality in reverse reads. Quality filtering was performed using a Phred score of 20 as the threshold. Deblur was used for sequence clustering into a sub-operational-taxonomic-unit (sub-OTU) approach, in order to remove sequencing errors (Amir et al., 2017). Sequences that passed quality filters were trimmed to 200 bp, giving a dataset of 5,568,329 total reads with a mean of 21,667 reads per sample. The fragment insertion script implemented in QIIME2 was used to align the sequences and build a bacterial phylogenetic tree based on a reference phylogenetic tree (SEPP reference Greengenes 13.8; Janssen et al., 2018). Taxonomy assignation was based on a classifier pretrained on Greengenes 13.08 with a similarity of 99% (DeSantis et al., 2006). Finally, reads of chloroplast and mitochondria were excluded by filtering the OTUs table.

Statistics

To test the effect of treatment on the fish's body weight, we used Generalized Linear Mixed-Models (GLMM). We used BW of 10 fish as an experimental unit with treatment as a fixed factor, sampling time as a covariate and tank nested in treatment as a random factor.

For alpha and beta diversity analyses, the OTU table was rarified at 8,000 sequencing depth per sample. Samples that did not reach this sequencing depth were excluded from subsequent analyses. Two alpha diversity indices were calculated, i.e., Shannon diversity index (Shannon, 1948); and bacterial OTU richness (or number of observed OTUs). We used GLMM to explore the effect of treatment and gut region as fixed factors and tank nested in treatment as a random factor in both alpha diversity indices. In these analyses, fish were the experimental unit for alpha and beta diversity analysis.

Body weight and alpha diversity analyses were performed using STATISTICA 10.0 (StatSoft).

Differences in genus and class abundances between control and treated fish were explored by means of Linear Discriminant Analysis Effect Size (LEfSe) (Segata et al., 2011). LEfSe analyses were performed on the Galaxy web platform, implemented on the public server (https://huttenhower.sph.harvard.edu/galaxy/).

Beta diversity distance matrixes were calculated using UniFrac distance. Both Weighted and Unweighted UniFrac indices (Lozupone et al., 2007; Lozupone & Knight, 2005) were used for subsequent analysis. Weighted UniFrac gives more importance to most abundant OTUs, while Unweighted UniFrac gives more importance to low abundant OTUs as it takes their presence or absence irrespective of their abundance. Permutational ANOVA (PERMANOVA) was performed in order to test these effects on both UniFrac distance matrixes using PRIMER-7 software (PRIMER-e), implemented with PERMANOVA plugin. Principal Coordinate Analyses (PCoA) were performed in order to visualize the 2 first axes using EMPeror 2018.2.0 (Vázquez-Baeza et al., 2013).

RESULTS

Changes in bacterial community composition

The gut microbiota of juvenile gilthead seabream is dominated by the classes Gammaproteobacteria, Bacilli and Actinobacteria. The relative abundance of these classes depended on the gut region and treatment. Gammaproteobacteria showed higher abundances in the control group in both gut regions, while Bacilli significantly dominated both gut regions in the *Allium* group (Figure 1, Supplementary Figure S1). Furthermore, significant differences also appeared in minority classes. In the foregut, Coriobacteriia, Bacteroidia, Clostridia and Erysipelotrichi showed higher abundance in *Allium* group. In the hindgut, Clostridia and class WCHB1_64 were higher in *Allium* group while class AT_s54 was higher in the control group (Figure 1).

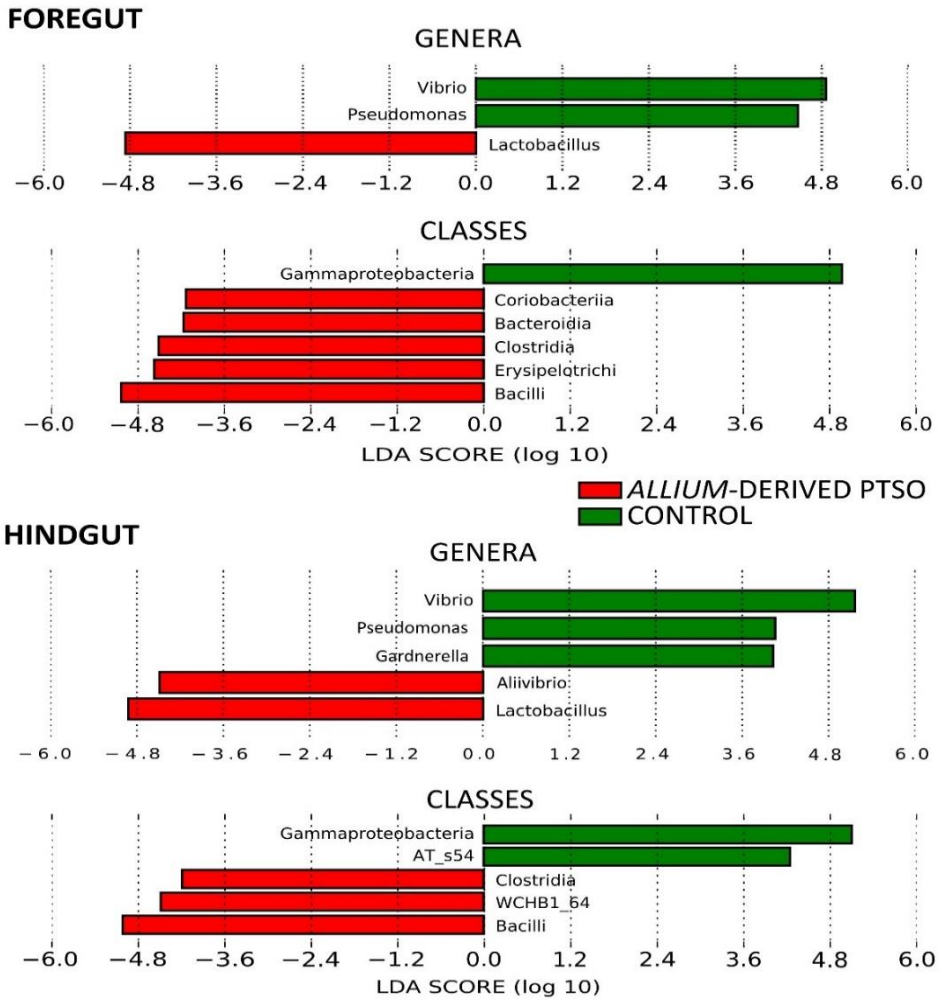

Figure 1. LDA Effect Size (LEfSe) analyses showing bacterial classes and genera that differ significantly between control fishes and those supplemented with *Allium*-derived PTSO, in the foregut and in the hindgut. Significant LDA Score > 4.0.

At the genus level, the foregut and hindgut of control fish were dominated by *Vibrio*, *Pseudomonas*, *Lactobacillus* and *Sphingomonas*. These genera were also the most abundant in *Allium*-derived PTSO supplemented fish, but the relative abundance of these genera changed compared to the control group (Figure 2). While *Vibrio* and *Pseudomonas* showed significantly higher abundances in the foregut of control gilthead seabream, *Lactobacillus* abundance was significantly higher in the foregut of *Allium* supplemented fish (Figure 1). In the hindgut, the genera *Vibrio* and *Pseudomonas* also showed significantly higher abundances in the control group, as well as the minority genus *Gardnerella*, while the *Allium* based product supplemented fish experienced significantly higher abundances of *Lactobacillus* and *Aliivibrio* (Figure 1).

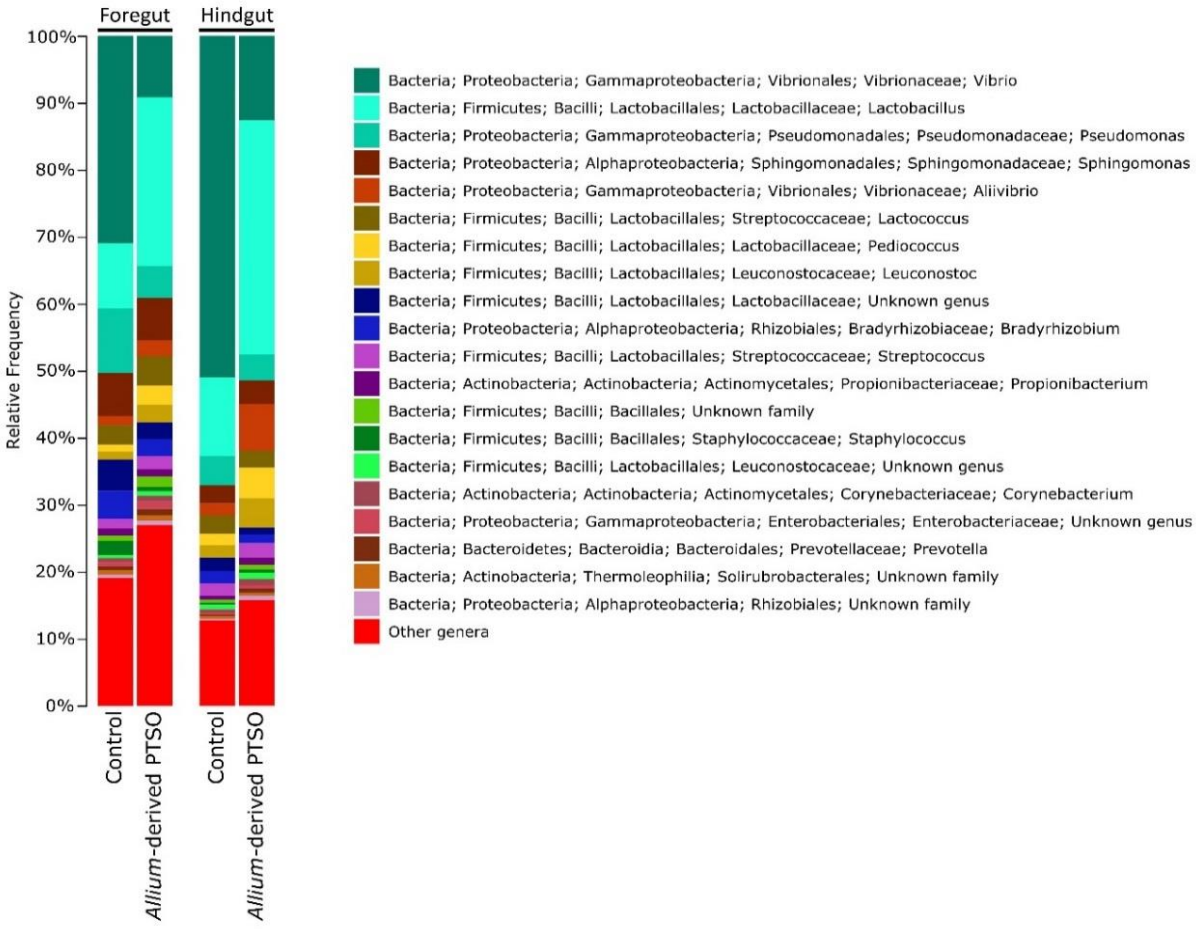

Figure 2. Barplot summarizing the relative bacterial abundance at the genus level in different gut regions (foregut and hindgut) and treatments. Control refers to juvenile gilthead seabream fed with basal diet while *Allium*-derived PTSO refers to experimental juvenile gilthead seabream fed with basal diet supplemented with *Allium*-derived PTSO.

Effect of *Allium* supplementation on alpha and beta diversity indices

Supplementing the diet of juvenile gilthead seabream with *Allium*-derived PTSO affected the Shannon diversity index in both gut regions (Table 1). Supplemented animals showed higher diversity than the control in foregut and hindgut (LSD post-hoc test, $p < 0.012$). However, no differences appeared in number of bacterial OTU richness between the control and *Allium* supplemented fish, either in the foregut or in the hindgut (Table 1; LSD post-hoc test; $p > 0.107$). Shifts in bacterial alpha diversity between foregut and hindgut were similar between the control and *Allium* supplemented gilthead seabream (see Gut Region*Treatment interaction term in both alpha diversity indexes in Table 1).

The bacterial community of gilthead seabream did not vary significantly between the two diets, either when taking into account the most abundant bacterial OTUs (Weighted UniFrac) or minority OTUs (Unweighted UniFrac) (Table 2, Figure 3). In the foregut, marginally significant differences between treatments appeared using weighted UniFrac. Similar non-significant trends were found in the hindgut for both Unweighted and Weighted UniFrac (Table 2). The bacterial community in the foregut significantly differed from hindgut microbiota irrespective of the treatment (Table 2, Supplementary Figure S2). In fact, shifts in the bacterial community between the foregut and hindgut showed similar trends in the control and supplemented animals (non-significant, see Gut Region*Treatment interaction terms in Table 2). Significant differences appeared between tanks in the same treatment group for both UniFrac distance matrixes (Table 2, Supplementary Figure S3). This differences in gut region and tank cannot be observed graphically in the PCoA since it does not take into account the 100% of the variance (74.77% in Weighted UniFrac and 24.01% in Unweighted UniFrac) (Supplementary Figures S2 and S3).

Table 1. General Linear Mixed Models exploring the effects of the treatment (control and *Allium*-derived PTSO), and gut region as factors, and tank nested in treatment in the different alpha diversity indices of the bacterial community of juvenile gilthead seabream. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one is for the error term. Significant *p*-values ($p < 0.05$) are shown in bold.

	Explanatory variables	D.f	F	*p*
Bacterial OTU richness	Treatment	1,188	3.35	0.386
	Gut Region	1,188	3.20	0.075
	Tank (Treatment)	4,188	3.56	**0.008**
	Gut Region*Treatment	1,188	0.18	0.668
Shannon	Treatment	1,188	20.23	**0.009**
	Gut Region	1,188	6.67	**0.011**
	Tank (Treatment)	4,188	0.89	0.469
	Gut Region*Treatment	1,188	0.91	0.341

Effect of *Allium* supplementation on the body weight of juvenile gilthead seabream

No differences appeared in initial body weight between the control and *Allium*-derived PTSO supplemented fish (Table 3). Juvenile gilthead seabream supplemented with PTSO showed production levels similar to those of the control group. Both groups of fish showed a similar trend in body weight throughout the experiment (12 weeks). At the end of the experimental period (12 weeks), no differences in body weight between control and *Allium*-derived PTSO supplemented fish were observed (GLMM, $p = 0.057$) (Figure 4, Table 3).

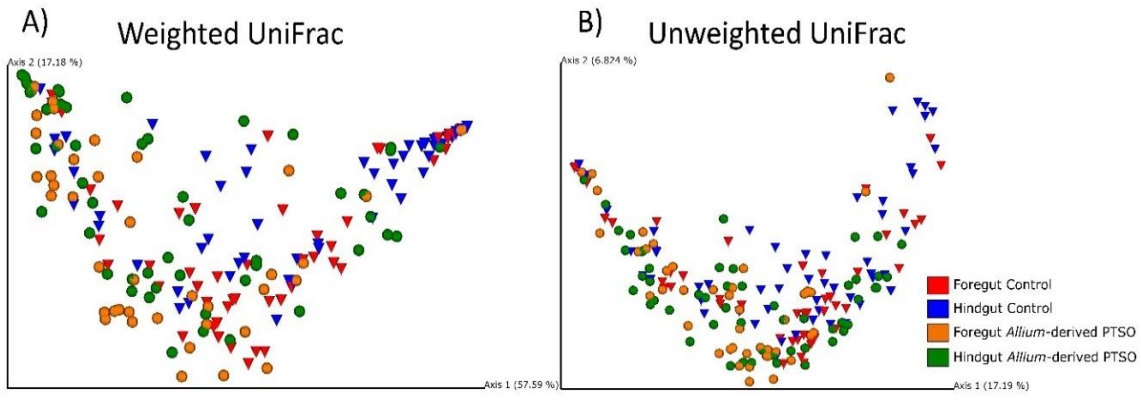

Figure 3. Principal Coordinate Analysis based in Weighted UniFrac (A) and Unweighted UniFrac (B) distance matrixes exploring the effects in the bacterial gut community of the supplementation with *Allium*-derived PTSO in the diet of juvenile gilthead seabream (red: foregut, control fish; blue: hindgut, control; orange: foregut, treated fish; green: hindgut, treated fish). Percentages show the proportion of variance explained by each axis.

Table 2. General Linear Mixed Models exploring the effects of treatment, gut region, tank nested in treatment and the interaction of treatment and gut region in beta diversity indices of bacterial community of juvenile gilthead seabream fed with control diet or supplemented with *Allium*-derived PTSO. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one is for the error term. Significant *p*-values ($p < 0.05$) are shown in bold.

	β-diversity distance matrix	Explanatory variables	D.f.	Pseudo-F	*p*
Gut	Weighted UniFrac	Treatment	1,195	8.13	0.092
		Gut region	1,195	5.13	**0.005**
		Tank (Treatment)	4,195	3.31	**0.002**
		Treatment*Gut region	1,195	0.44	0.702
	Unweighted UniFrac	Treatment	1,195	2.22	0.088
		Gut region	1,195	1.65	**0.031**
		Tank (Treatment)	4,195	1.83	**0.001**
		Treatment*Gut region	1,195	0.83	0.745
Foregut	Weighted UniFrac	Treatment	1,195	5.26	0.082
		Tank (Treatment)	4,195	2.03	**0.016**
	Unweighted UniFrac	Treatment	1,195	1.55	0.189
		Tank (Treatment)	4,195	1.38	**0.011**
Hindgut	Weighted UniFrac	Treatment	1,195	7.82	0.074
		Tank (Treatment)	4,195	2.27	**0.025**
	Unweighted UniFrac	Treatment	1,195	2.15	**0.021**
		Tank (Treatment)	4,195	1.28	**0.041**

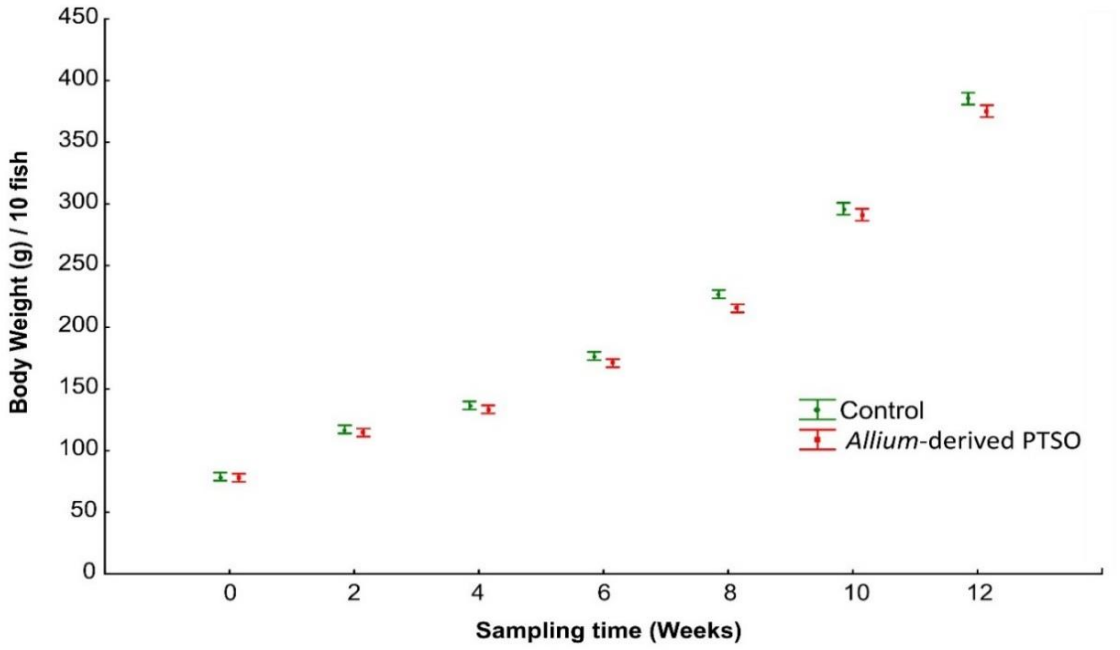

Figure 4. Average ± 95% confidence intervals of the mean of the body weight (g) of control and *Allium*-derived PTSO supplemented fish during the whole experiment (12 weeks).

Table 3. General Linear Mixed Models exploring the effects of treatment as a factor, sampling time as a continuous factor and tank nested in treatment as a random factor in juvenile gilthead seabream fed with control diet or supplemented with *Allium*-derived PTSO (average ± SD). Sample unit is body weight of 10 fish. BW refers to body weight. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one is for the error term. Significant *p*-values are shown in bold.

Sampling time	Control	*Allium*-derived PTSO	Independient variables	F	D.f.	*p*
Mean BW Whole experiment (g/10 fish)	158.33 ± 5.65	153.84 ± 5.46	Treatment	1.74	1,454	0.188
			Tank (Treatment)	1.12	4,454	0.346
			Sampling time	5438.96	1,454	**<0.001**
			Treatment*Sampling time	1.85	1,454	0.174
Initial BW (10 fish)	78.93 ± 0.67	77.96 ± 0.62	Treatment	3.12	1,72	0.268
			Tank (Treatment)	1.29	4,72	0.622
BW Week 12 (10 fish)	385.37 ± 3.49	376.32 ± 3.50	Treatment	3.57	1,30	0.057
			Tank (Treatment)	0.35	4,30	0.772

DISCUSSION

In this study, juvenile gilthead seabream supplemented with an *Allium*-based product rich in the organosulfur compound propyl propane thiosulfonate (PTSO) produced significantly changes in the bacterial abundances of some bacterial groups in the foregut and hindgut after 12 weeks of treatment. Differences in *Allium*-derived PTSO supplemented fish appeared in both gut regions, with a decrease in potentially pathogenic *Vibrio* and *Pseudomonas* and an increase in potentially beneficial *Lactobacillus* in the foregut and hindgut. These results were accompanied by non-significant differences in body weight during this experimental period.

Alternatives to AGPs that maintain productive parameters in aquaculture are essential to stop antimicrobial resistance (AMR) from spreading and to improve animal welfare. Probiotics, prebiotics, phages and plant extracts had been proposed as good alternatives to AGPs in order to reduce the prevalence of AMR (Romero et al., 2012). In this sense, plant extracts or phytobiotics have been reported to modulate gut microbiota and increase productive parameters, appetite stimulation and anti-pathogenic properties in terrestrial and aquatic species, resulting in a good, safe alternative to antibiotics (Reverter et al., 2014; Windisch et al., 2008). Extracts from *Allium* plants, mainly garlic and onion extracts, have been used supplemented in fish diets in different studies, showing beneficial effects on immune system, growth performance and health status

(Chen et al., 2021; Lee & Gao, 2012; Valenzuela-Gutiérrez et al., 2021). The effects of this plant extracts are related to secondary metabolites and organosulfur compounds such as allicin, PTS or PTSO (Guillamón et al., 2021; Valenzuela-Gutiérrez et al., 2021). The addition of allicin on aquafeed has shown beneficial effects on growth performance and survival rate of large yellow croaker (*Larimichthys crocea*) (Huang et al., 2020). Other studies using allicin showed an improve in biochemical, antioxidant and immunological parameters of Nile tilapia (*O. niloticus*) (Hamed et al., 2021) and antibacterial activity in rainbow trout (*O. mykiss*) (Nya et al., 2010). However, no studies have yet explored the effects of *Allium*-derived PTSO on intestinal microbiota and growth performance of fish species. Nevertheless, *Allium*-derived PTSO has been widely used as diet supplement showing beneficial effects in terrestrial animals (reviewed in Guillamón et al. (2021)). This compound has been reported for its antibacterial, antifungal and anticoccidial activity (Kim et al., 2013; Sorlozano-Puerto et al., 2018, 2021). In addition, PTSO have shown beneficial effects on gut microbiota and performance of different species of terrestrial animals, such as mice, broilers chickens, laying hens and pigs (reviewed in Guillamón et al. (2021)). The addition of different doses of PTSO in broiler chickens improved digestibility and productive parameters, and produced changes in intestinal microbiota (Peinado et al., 2012; Rubio et al., 2015; Ruiz et al., 2015). In laying hens, PTSO supplementation produced an increase in the number of eggs laid and in egg size, as well as an increase in potentially beneficial bacterial genera (Abad et al., 2020; Rabelo-Ruiz et al., 2021a). In pig production, PTSO has also shown beneficial effects in gut microbiota and an increase in body weight and productive parameters, both in piglets and growing-finishing pigs (Rabelo-Ruiz et al., 2021b; Sánchez et al., 2020).

Despite the lack of research on the use of PTSO in aquaculture, different extracts from *Allium* plants have been used as diet supplement in different studies. The use of onion (*A. cepa*) powder increased body weight and specific growth rate and improved hematological and immune parameters of juvenile beluga *(H. huso*) (Akrami et al., 2015). Supplementing the diet with garlic (*A. sativum*) showed beneficial effects on growth performance of Asian seabass (*L. calcarifer*) (Abdelwahab et al., 2020; Talpur & Ikhwanuddin, 2012), African catfish (*Clarias gariepinus*) (Gabriel et al., 2019) and rainbow trout (*O. mykiss*) (Büyükdeveci et al., 2018). Other studies using garlic-derived organosulfur compounds, showed its effect as a feed stimulator, growth promoter and antimicrobial agent in several fish species (reviewed in Lee & Gao, 2012). However,

these results are controversial, because other studies noted the lack of effect of *Allium* and their derived organosulfur compounds on different fish species in aquaculture (Motlagh et al., 2020b; Nya et al., 2010). Our results showed no differences in body weight between control and *Allium*-derived PTSO supplemented fish, either during the experiment or at the end of the experimental period (12 weeks). Further studies are needed in order to clarify differences between laboratories, fish species and more importantly, between different active principles and phytobiotic presentation.

Our study showed a significant increase in Shannon's alpha diversity index in the foregut and hindgut of gilthead seabream supplemented with *Allium*-derived PTSO but no correlation with body weight were detected. In a study with largemouth bronze gudgeon (*Coreius guichenoti*), differences in bacterial diversity did not translate into differences in body weight (Li et al., 2016). Alternatively, some studies have demonstrated that the reduction of alpha diversity could be related to increased body weight in birds and obesity in humans (Bae et al., 2017; Menni et al., 2017). Furthermore, a recent study of (Büyükdeveci et al., 2018) with rainbow trout (*O. mykiss*) showed an increase in body weight and a decrease in alpha diversity as the garlic concentration increased. However, another study with rainbow trout (*O. mykiss*) by (Betiku et al., 2018) suggested increased body weight related to higher bacterial diversity. In our study, we observed differences between the foregut and hindgut bacterial communities, independently of supplementation with the *Allium*-derived PTSO. These differences in microbiota are related with differences in gut morphology and functionality, food processing and nutrient intake (Ye et al., 2014). The foregut is the major site of carbohydrate, protein and lipid digestion, while the absorption of undigested compounds continues in the hindgut (Le et al., 2019). The foregut is closer to the fish's mouth and its microbiota could be affected by environmental factors such as diet, water and other external factors, while the microbiota in the hindgut is more affected by host factors, such as health and genotype (Li et al., 2020). In spite of having found significant differences between treatments in some classes and genera, the gut community did not differ in general between the control and *Allium*-derived PTSO treatment, either when considering majority OTUs (Weighted UniFrac) or minority OTUs (Unweighted UniFrac). We only found significant differences in the hindgut when minority OTUs were taken into account.

It is likely that the significant variation between tanks would mask any effects of PTSO supplementation. These differences between tanks have been observed in other

researches. In a recent study, (Minich et al., 2019) found a strong association between tank and Atlantic salmon (*Salmo salar*) microbiota in recirculating aquaculture system (RAS). There may be a continual bacterial exchange between fish microbiota and the water in the tank (Blancheton et al., 2013). Furthermore, an excess of organic matter in the RAS tank, including fish feed and feces, can build up in the tank and promote changes in some bacterial groups (Rurangwa & Verdegem, 2015). Previous research has shown high similarity in fecal microbiota in individuals from the same tank (De Schryver et al., 2011). Fish in the same tank are continuously exchanging excreta and feces, so it is likely a positive feedback between the bacterial community of the gut and the surrounding water, marking strong differences in the gut microbiota between fish from different tanks.

Indeed, our supplement produced changes in the majority genera of the intestinal microbiota. The relative abundance of *Vibrio* and *Pseudomonas* in the foregut and hindgut significantly decreased in juvenile gilthead seabream supplemented with *Allium*-derived PTSO. *Vibrio* spp. are ubiquitous in marine environments and since some species produce clinical diseases, such as vibriosis, this genus is considered to be potentially pathogenic for fish and shellfish species (Abdel-Aziz et al., 2013; Pérez-Sánchez et al., 2018). Some *Vibrio* species which cause devastating impacts on marine fish are *V. anguillarum*, *V. salmonicida*, *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* (Mohamad et al., 2019). In gilthead seabream, (Haldar et al., 2010) showed that *V. harveyi* caused tail rot disease. This fish species is also affected by *V. anguillarum*, which causes external clinical signs, including lethargy, red spots on the ventral and lateral sides of fish and ulcerative injuries (Frans et al., 2011). Remarkably, different plant extracts have demonstrated antimicrobial activity against this pathogenic species in aquaculture. Ginger powder and *A. sativum* powder have shown antimicrobial effects against *V. harveyi* in Asian seabass (*L. calcarifer*). In addition, *Eriobotrya japonica* and *Siegesbeckia glabrescens* showed similar antimicrobial effects against different *Vibrio* species in the kelp grouper (*Epinephelus bruneus*) (reviewed in (Reverter et al., 2014; Stratev et al., 2018)). *Pseudomonas* have been also described as an ubiquitous bacterial genus, but some species are emergent opportunistic fish pathogens (Fadel et al., 2018). *P. anguilliseptica* is the causative agent of winter disease, an induced septicemia characterized by abdominal distension, hemorrhagic kidney, pale liver and congestive intestine with fibrinous yellowish exudate (Fadel et al., 2018). This disease is associated with several farmed fish species such as cod (*Gadus morhua*), seabass and gilthead seabream

(Wiklund, 2016). Other *Pseudomonas* species are considered to be opportunistic pathogens in aquaculture, such as *P. aeruginosa, P. fluorescens* and *P. putida* (Altinok et al., 2006). Several plant extracts have also shown antimicrobial activity against *Pseudomonas* species. A study by Kačániová et al. (2017) using different essential oils extracted from plants demonstrated antimicrobial activity against different *Pseudomonas* species. Another study using garlic extract reduced the mortality of Nile Tilapia (*O. niloticus*) infected with *P. fluorescens* (Diab et al., 2008). The control of these diseases is very relevant due to the reduction in costs derived from fish losses and disease treatment. In this sense, our results highlight the beneficial effect of the *Allium* based compound rich in PTSO in the reduction of pathogens such as *Vibrio* and opportunistic bacteria such as *Pseudomonas*.

The *Allium*-derived PTSO supplemented in the diet of juvenile gilthead seabream also had positive effects, particularly by the increase in potentially beneficial *Lactobacillus* in the foregut and hindgut. This genus is a prevalent constituent of intestinal microbiota of many fish species and is considered a beneficial organism associated with a healthy intestinal epithelium (Estruch et al., 2015). Furthermore, some strains can inhibit the adhesion of fish pathogens to the intestinal epithelium (Balcázar et al., 2008). In addition, different studies using *Lactobacillus* as probiotic in aquaculture showed a positive correlation with fish health and productive parameters. Dietary supplementation of *L. rhamnosus* in red seabream (*Pagrus major*) increased growth, feed utilization and protein and lipid contents (Dawood et al., 2016). Supplementation of probiotic *Lactobacillus spp.* in the live food of gilthead seabream larvae increased digestive enzyme activity, specific growth rate and survival (Suzer et al., 2008). Plant-based diets have been associated with high abundance of bacteria belonging to the phylum Firmicutes (especially lactic acid bacteria, BAL) in the rainbow trout microbiota (Desai et al., 2012). The addition of *Allium*-derived PTSO have also shown an increase in the abundance of different BAL species like *Bifidobacterium, Lactobacillus* and *Lactococcus* in broiler chickens, laying hens and piglets (reviewed in Guillamón et al. (2021)). In our study, PTSO supplementation also increase Firmicutes, especially *Lactobacillus*. However, despite this beneficial effect described in this research (increased *Lactobacillus* and decreased *Vibrio* and *Pseudomonas*), no differences in body weight appeared.

CONCLUSIONS

Our experimental supplementation of the diet of juvenile gilthead seabream with *Allium*-derived PTSO, produces shifts in abundance of the majority bacterial OTUs in the foregut and hindgut, while not affecting growth parameters such as body weight after 12 weeks of experiment. These results are very promising for the use of these phytobiotic compound in aquaculture, given the reduction in potentially pathogenic bacteria such as *Vibrio* and *Pseudomonas* and the increase in potentially beneficial *Lactobacillus*. However, further research is necessary to study how this organosulfur compound from *Allium* affects specific pathogenic strains of these genera. In addition, it would be necessary to study how it affects other parameters related with productivity and health status, such as feed digestibility, intestinal enzyme activity and the immune system.

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REFERENCES

Abad, P., Arroyo-Manzanares, N., Ariza, J. J., Baños, A., & García-Campaña, A. M. (2020). Effect of Allium Extract Supplementation on Egg Quality, Productivity, and Intestinal Microbiota of Laying Hens. *Animals*, *11*(1), 41. https://doi.org/10.3390/ani11010041

Abad, P., Arroyo-Manzanares, N., & García-Campaña, A. M. (2016). A rapid and simple UHPLC-ESI-MS/MS method for the screening of propyl propane thiosulfonate, a new additive for animal feed. *Analytical Methods*, *8*(18), 3730–3739. https://doi.org/10.1039/C6AY00219F

Abdel-Aziz, M., Eissa, A. E., Hanna, M., & Okada, M. A. (2013). Identifying some pathogenic Vibrio/Photobacterium species during mass mortalities of cultured Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) from some Egyptian coastal provinces. *International Journal of Veterinary Science and Medicine*, *1*(2), 87–95. https://doi.org/10.1016/j.ijvsm.2013.10.004

Abdelwahab, A. M., El-Bahr, S. M., & Al-Khamees, S. (2020). Influence of Dietary Garlic (Allium sativum) and/or Ascorbic Acid on Performance, Feed Utilization, Body Composition and Hemato-Biochemical Parameters of Juvenile Asian Sea Bass

(Lates calcarifer). *Animals*, *10*(12), 2396. https://doi.org/10.3390/ani10122396

Akrami, R., Gharaei, A., Mansour, M. R., & Galeshi, A. (2015). Effects of dietary onion (Allium cepa) powder on growth, innate immune response and hemato–biochemical parameters of beluga (Huso huso Linnaeus, 1754) juvenile. *Fish & Shellfish Immunology*, *45*(2), 828–834. https://doi.org/10.1016/J.FSI.2015.06.005

Altinok, I., Kayis, S., & Capkin, E. (2006). Pseudomonas putida infection in rainbow trout. *Aquaculture*, *261*(3), 850–855. https://doi.org/10.1016/j.aquaculture.2006.09.009

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Attia, G., El-Eraky, W., Hassanein, E., El-Gamal, M., Farahat, M., & Hernandez-Santana, A. (2017). Effect of dietary inclusion of a plant extract blend on broiler growth performance, nutrient digestibility, caecal microflora and intestinal histomorphology. *International Journal of Poultry Science*, *16*(9), 344–353. https://doi.org/10.3923/ijps.2017.344.353

Bae, Y., Koo, B., Lee, S., Mo, J., Oh, K., & Mo, I. P. (2017). Bacterial diversity and its relationship to growth performance of broilers. *Korean J. Vet. Res.*, *57*(3), 159–167. https://doi.org/10.14405/kjvr.2017.57.3.159

Balcázar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J. L., & Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*, *278*(1–4), 188–191. https://doi.org/10.1016/j.aquaculture.2008.03.014

Baquero, F., Martínez, J. L., & Cantón, R. (2008). Antibiotics and antibiotic resistance in water environments. *Current Opinion in Biotechnology*, *19*(3), 260–265. https://doi.org/10.1016/j.copbio.2008.05.006

Basit, M. A., Kadir, A. A., Loh, T. C., Abdul Aziz, S., Salleh, A., Zakaria, Z. A., & Banke Idris, S. (2020). Comparative Efficacy of Selected Phytobiotics with Halquinol and Tetracycline on Gut Morphology, Ileal Digestibility, Cecal Microbiota Composition and Growth Performance in Broiler Chickens. *Animals*, *10*(11), 2150. https://doi.org/10.3390/ani10112150

Betiku, O. C., Yeoman, C. J., Gaylord, T. G., Americus, B., Olivo, S., Duff, G. C., & Sealey, W. M. (2018). Water system is a controlling variable modulating bacterial diversity of gastrointestinal tract and performance in rainbow trout. *PLOS ONE*, *13*(4), e0195967. https://doi.org/10.1371/journal.pone.0195967

Blancheton, J. P., Attramadal, K. J. K., Michaud, L., D'Orbcastel, E. R., & Vadstein, O. (2013). Insight into bacterial population in aquaculture systems and its implication. *Aquacultural Engineering*, *53*, 30–39. https://doi.org/10.1016/j.aquaeng.2012.11.009

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(9), 1091. https://doi.org/10.1038/s41587-019-0252-6

Büyükdeveci, M. E., Balcázar, J. L., Demirkale, İ., & Dikel, S. (2018). Effects of garlic-supplemented diet on growth performance and intestinal microbiota of rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *486*, 170–174.

https://doi.org/10.1016/J.AQUACULTURE.2017.12.022

Cañada-Cañada, F., Muñoz De La Peña, A., & Espinosa-Mansilla, A. (2009). Analysis of antibiotics in fish samples. *Analytical and Bioanalytical Chemistry*, *395*(4), 987–1008. https://doi.org/10.1007/s00216-009-2872-z

Chen, J., Wang, F., Yin, Y., & Ma, X. (2021). The nutritional applications of garlic (Allium sativum) as natural feed additives in animals. *PeerJ*, *9*, e11934. https://doi.org/10.7717/PEERJ.11934

Dawood, M. A. O., Koshio, S., & Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Reviews in Aquaculture*, *10*(4), 950–974. https://doi.org/10.1111/raq.12209

Dawood, M. A. O., Koshio, S., Ishikawa, M., Yokoyama, S., El Basuini, M. F., Hossain, M. S., … Moss, A. S. (2016). Effects of dietary supplementation of Lactobacillus rhamnosus or/and Lactococcus lactis on the growth, gut microbiota and immune responses of red sea bream, Pagrus major. *Fish and Shellfish Immunology*, *49*, 275–285. https://doi.org/10.1016/j.fsi.2015.12.047

De Schryver, P., Dierckens, K., Bahn Thi, Q. Q., Amalia, R., Marzorati, M., Bossier, P., … Verstraete, W. (2011). Convergent dynamics of the juvenile European sea bass gut microbiota induced by poly-β-hydroxybutyrate. *Environmental Microbiology*, *13*(4), 1042–1051. https://doi.org/10.1111/j.1462-2920.2010.02410.x

Desai, A. R., Links, M. G., Collins, S. A., Mansfield, G. S., Drew, M. D., Van Kessel, A. G., & Hill, J. E. (2012). Effects of plant-based diets on the distal gut microbiome of rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *350–353*, 134–142. https://doi.org/10.1016/j.aquaculture.2012.04.005

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Diab, A. S., Aly, S. M., John, G., Abde-Hadi, Y., & Mohammed, M. F. (2008). Effect of garlic, black seed and Biogen as immunostimulants on the growth and survival of Nile tilapia, Oreochromis niloticus (Teleostei: Cichlidae), and their response to artificial infection with Pseudomonas fluorescens. *African Journal of Aquatic Science*, *33*(1), 63–68. https://doi.org/10.2989/AJAS.2007.33.1.7.391

Estruch, G., Collado, M. C., Peñaranda, D. S., Tomás Vidal, A., Jover Cerdá, M., Pérez Martínez, G., … Moreau, C. S. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (Sparus aurata) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. *PLoS ONE*, *10*(8), 1–22. https://doi.org/10.1371/journal.pone.0136389

European Commision. (2018). Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending Regulation (EC) No 183/2005 of the European Parliament and of the Council and repealing. *Official Journal of European Union*, *62*, L:2019:004:TOC.

Fadel, A., Mabrok, M., & Aly, S. (2018). Epizootics of Pseudomonas anguilliseptica among cultured seabream (Sparus aurata) populations: Control and treatment strategies. *Microbial Pathogenesis*, *121*, 1–8. https://doi.org/10.1016/j.micpath.2018.04.021

FAO. (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in*

action. Rome: FAO. https://doi.org/10.4060/ca9229en

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. (2011). Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of Fish Diseases*, *34*(9), 643–661. https://doi.org/10.1111/j.1365-2761.2011.01279.x

Gabriel, N. N., Wilhelm, M. R., Habte-Tsion, H. M., Chimwamurombe, P., & Omoregie, E. (2019). Dietary garlic (Allium sativum) crude polysaccharides supplementation on growth, haematological parameters, whole body composition and survival at low water pH challenge in African catfish (Clarias gariepinus) juveniles. *Scientific African*, *5*, e00128. https://doi.org/10.1016/J.SCIAF.2019.E00128

Gheisar, M. M., & Kim, I. H. (2018). Phytobiotics in poultry and swine nutrition–a review. *Italian Journal of Animal Science*, *17*(1), 92–99. https://doi.org/10.1080/1828051X.2017.1350120

Guillamón, E., Andreo-Martínez, P., Mut-Salud, N., Fonollá, J., & Baños, A. (2021). Beneficial Effects of Organosulfur Compounds from Allium cepa on Gut Health: A Systematic Review. *Foods*, *10*(8), 1680. https://doi.org/10.3390/FOODS10081680

Haldar, S., Maharajan, A., Chatterjee, S., Hunter, S. A., Chowdhury, N., Hinenoya, A., … Yamasaki, S. (2010). Identification of Vibrio harveyi as a causative bacterium for a tail rot disease of sea bream Sparus aurata from research hatchery in Malta. *Microbiological Research*, *165*(8), 639–648. https://doi.org/10.1016/j.micres.2009.12.001

Hamed, H. S., Ismal, S. M., & Faggio, C. (2021). Effect of allicin on antioxidant defense system, and immune response after carbofuran exposure in Nile tilapia, Oreochromis niloticus. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *240*, 108919. https://doi.org/10.1016/J.CBPC.2020.108919

He, S., Wang, Q., Li, S., Ran, C., Guo, X., Zhang, Z., & Zhou, Z. (2017). Antibiotic growth promoter olaquindox increases pathogen susceptibility in fish by inducing gut microbiota dysbiosis. *Science China Life Sciences*, *60*(11), 1260–1270. https://doi.org/10.1007/s11427-016-9072-6

Huang, W., Yao, C., Liu, Y., Xu, N., Yin, Z., Xu, W., … Ai, Q. (2020). Dietary Allicin Improved the Survival and Growth of Large Yellow Croaker (Larimichthys crocea) Larvae via Promoting Intestinal Development, Alleviating Inflammation and Enhancing Appetite. *Frontiers in Physiology*, *11*, 587674. https://doi.org/10.3389/FPHYS.2020.587674

Janssen, S., Mcdonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MSystems*, *3*, e00021-18. https://doi.org/10.1128/mSystems.00021-18

Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., … Ivanišová, E. (2017). The antioxidant and antimicrobial activity of essential oils against Pseudomonas spp. isolated from fish. *Saudi Pharmaceutical Journal*, *25*(8), 1108–1116. https://doi.org/10.1016/j.jsps.2017.07.005

Kim, D. K., Lillehoj, H. S., Lee, S. H., Lillehoj, E. P., & Bravo, D. (2013). Improved resistance to Eimeria acervulina infection in chickens due to dietary supplementation with garlic metabolites. *British Journal of Nutrition*, *109*(01), 76–88.

Kyung, K. H. (2012, April 1). Antimicrobial properties of allium species. *Current Opinion in Biotechnology*. Elsevier Current Trends.

https://doi.org/10.1016/j.copbio.2011.08.004

Le, H. T. M. D., Shao, X., Krogdahl, Å., Kortner, T. M., Lein, I., Kousoulaki, K., … Sæle, Ø. (2019). Intestinal function of the stomachless fish, ballan wrasse (Labrus bergylta). *Frontiers in Marine Science*, *6*(MAR), 140. https://doi.org/10.3389/fmars.2019.00140

Lee, J.-Y., & Gao, Y. (2012). Review of the Application of Garlic, Allium sativum, in Aquaculture. *Journal of the World Aquaculture Society*, *43*(4), 447–458. https://doi.org/10.1111/j.1749-7345.2012.00581.x

Li, J. J., Ni, J. J., Wang, C., Yu, Y. H., & Zhang, T. L. (2020). Different response patterns of fish foregut and hindgut microbiota to host habitats and genotypes. *Applied Ecology and Environmental Research*, *18*(5), 7369–7378. https://doi.org/10.15666/aeer/1805_73697378

Li, X., Yan, Q., Ringø, E., Wu, X., He, Y., & Yang, D. (2016). The influence of weight and gender on intestinal bacterial community of wild largemouth bronze gudgeon (Coreius guichenoti, 1874). *BMC Microbiology*, *16*(1), 191. https://doi.org/10.1186/s12866-016-0809-1

Lira, A. C., Prieto, A. I., Baños, A., Guillamón, E., Moyano, R., Jos, A., & Cameán, A. M. (2020). Safety assessment of propyl-propane-thiosulfonate (PTSO): 90-days oral subchronic toxicity study in rats. *Food and Chemical Toxicology*, *144*(111612). https://doi.org/10.1016/j.fct.2020.111612

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, *73*(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Maron, D. F., Smith, T. J. S., & Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: An international regulatory and economic survey. *Globalization and Health*, *9*(1). https://doi.org/10.1186/1744-8603-9-48

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Menni, C., Jackson, M. A., Pallister, T., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. *International Journal of Obesity*, *41*, 1099–1105. https://doi.org/10.1038/ijo.2017.66

Minich, J. J., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., Knight, R., … Allen, E. (2019). Microbial ecology of Atlantic salmon, Salmo salar, hatcheries: Impacts of the built environment on fish mucosal microbiota. *BioRxiv*, (April), 1–19. https://doi.org/10.1101/828749

Mohamad, N., Amal, M. N. A., Yasin, I. S. M., Zamri Saad, M., Nasruddin, N. S., Al-saari, N., … Sawabe, T. (2019). Vibriosis in cultured marine fishes: a review. *Aquaculture*, *512*. https://doi.org/10.1016/j.aquaculture.2019.734289

Motlagh, H. A., Paolucci, M., Lashkarizadeh Bami, M., & Safari, O. (2020a). Sexual parameters, digestive enzyme activities, and growth performance of guppy (Poecilia reticulata) fed garlic (Allium sativum) extract supplemented diets. *Journal of the*

World Aquaculture Society, *51*(5), 1087–1097. https://doi.org/10.1111/jwas.12729

Motlagh, H. A., Safari, O., Selahvarzi, Y., Baghalian, A., & Kia, E. (2020b). Non-specific immunity promotion in response to garlic extract supplemented diets in female Guppy (Poecilia reticulata). *Fish & Shellfish Immunology*, *97*, 96–99. https://doi.org/10.1016/J.FSI.2019.12.007

Nya, E. J., Dawood, Z., & Austin, B. (2010). The garlic component, allicin, prevents disease caused by Aeromonas hydrophila in rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Fish Diseases*, *33*(4), 293–300. https://doi.org/10.1111/j.1365-2761.2009.01121.x

Peinado, M. J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L. A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Animal Feed Science and Technology*, *181*(1–4), 87–92. https://doi.org/10.1016/j.anifeedsci.2013.03.001

Peinado, M. J., Ruiz, R., Echavarri, A., & Rubio, L. A. (2012). Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. *Poultry Science*, *91*(9), 2148–2157. https://doi.org/10.3382/ps.2012-02280

Pérez-Sánchez, T., Mora-Sánchez, B., & Balcázar, J. L. (2018). Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends in Microbiology*, *26*(11), 896–903. https://doi.org/10.1016/j.tim.2018.05.002

Rabelo-Ruiz, M., Ariza-Romero, J. J., Zurita-González, M. J., Martín-Platero, A. M., Baños, A., Maqueda, M., … Peralta-Sánchez, J. M. (2021a). Allium-based phytobiotic enhances egg production in laying hens through microbial composition changes in ileum and cecum. *Animals*, *11*(2), 1–15. https://doi.org/10.3390/ani11020448

Rabelo-Ruiz, M., Teso-Pérez, C., Peralta-Sánchez, J. M., Ariza, J. J., Martín-Platero, A. M., Casabuena-Rincón, Ó., … Martínez-Bueno, M. (2021b). Allium extract implements weaned piglet's productive parameters by modulating distal gut microbiota. *Antibiotics*, *10*(3), 1–15. https://doi.org/10.3390/antibiotics10030269

Resende, J. A., Silva, V. L., Oliveira Fontes, C., Souza-Filho, J. A., Lopes, T., De Oliveira, R., … Diniz, C. G. (2012). Multidrug-Resistance and Toxic Metal Tolerance of Medically Important Bacteria Isolated from an Aquaculture System. *Microbes Environ*, *27*(4), 449–455. https://doi.org/10.1264/jsme2.ME12049

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*, *433*, 50–61. https://doi.org/10.1016/j.aquaculture.2014.05.048

Romero, J., Gloria, C., & Navarrete, P. (2012). Antibiotics in Aquaculture – Use, Abuse and Alternatives. *Health and Environment in Aquaculture*. https://doi.org/10.5772/28157

Rubio, L. A., Peinado, M. J., Ruiz, R., Suárez-Pereira, E., Ortiz Mellet, C., & García Fernández, J. M. (2015). Correlations between changes in intestinal microbiota composition and performance parameters in broiler chickens. *Journal of Animal Physiology and Animal Nutrition*, *99*(3), 418–423. https://doi.org/10.1111/JPN.12256

Ruiz, R., Peinado, M. J., Aranda-Olmedo, I., Abecia, L., Suárez-Pereira, E., Ortiz Melle, C., … Rubio, L. A. (2015). Effects of feed additives on ileal mucosa-associated microbiota composition of broiler chickens. *Journal of Animal Science*, *93*(7), 3410–

3420. https://doi.org/10.2527/jas.2015-8905

Rurangwa, E., & Verdegem, M. C. J. (2015). Microorganisms in recirculating aquaculture systems and their management. *Reviews in Aquaculture*, *7*(2), 117–130. https://doi.org/10.1111/raq.12057

Sánchez, C., Martínez-Miró, S., Ariza, J., Madrid, J., Orengo, J., Aguinaga, M., … Hernández, F. (2020). Effect of Alliaceae Extract Supplementation on Performance and Intestinal Microbiota of Growing-Finishing Pig. *Animals*, *10*(9), 1–15. https://doi.org/10.3390/ANI10091557

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shalaby, A. M., Khattab, Y. A., & Abdel Rahman, A. M. (2006). Effects of Garlic (Allium sativum) and chloramphenicol on growth performance, physiological parameters and survival of Nile tilapia (Oreochromis niloticus). *Journal of Venomous Animals and Toxins Including Tropical Diseases*, *12*(2), 172–201. https://doi.org/10.1590/S1678-91992006000200003

Shannon, C. E. (1948). A Mathematical Theory of Communication. *The Bell System Technical Journal*, *27*, 379–423, 623–656.

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J. J., Baños-Arjona, A., Exposito-Ruiz, M., & Gutierrez-Fernandez, J. (2018). In vitro antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from allium spp. Against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples. *BioMed Research International*, *2018*, 7861207. https://doi.org/10.1155/2018/7861207

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Gil-Martinez, L., Ariza-Romero, J. J., Maroto-Tello, A., … Gutierrez-Fernandez, J. (2021). Antibacterial and antifungal activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate, two organosulfur compounds from allium cepa: In vitro antimicrobial effect via the gas phase. *Pharmaceuticals*, *14*(1), 1–17. https://doi.org/10.3390/ph14010021

Stratev, D., Zhelyazkov, G., Noundou, X. S., & Krause, R. W. M. (2018). Beneficial effects of medicinal plants in fish diseases. *Aquaculture International*, *26*(1), 289–308. https://doi.org/10.1007/s10499-017-0219-x

Suzer, C., Çoban, D., Kamaci, H. O., Saka, Ş., Firat, K., Otgucuoğlu, Ö., & Küçüksari, H. (2008). Lactobacillus spp. bacteria as probiotics in gilthead sea bream (Sparus aurata, L.) larvae: Effects on growth performance and digestive enzyme activities. *Aquaculture*, *280*(1–4), 140–145. https://doi.org/10.1016/j.aquaculture.2008.04.020

Talpur, A. D., & Ikhwanuddin, M. (2012). Dietary effects of garlic (Allium sativum) on haemato-immunological parameters, survival, growth, and disease resistance against Vibrio harveyi infection in Asian sea bass, Lates calcarifer (Bloch). *Aquaculture*, *364–365*, 6–12. https://doi.org/10.1016/j.aquaculture.2012.07.035

U.S. FDA. (2016). FDA Reminds Retail Establishments of Upcoming Changes to the Use of Antibiotics in Food Animals. *U.S. Food and Drugs Administration*.

Valenzuela-Gutiérrez, R., Lago-Lestón, A., Vargas-Albores, F., Cicala, F., & Martínez-Porchas, M. (2021). Exploring the garlic (Allium sativum) properties for fish aquaculture. *Fish Physiology and Biochemistry*, *47*(4), 1179–1198. https://doi.org/10.1007/S10695-021-00952-7

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

Vezza, T., Garrido-Mesa, J., Diez-Echave, P., Hidalgo-García, L., Ruiz-Malagón, A. J., García, F., … Rodríguez-Cabezas, M. E. (2021). Allium-Derived Compound Propyl Propane Thiosulfonate (PTSO) Attenuates Metabolic Alterations in Mice Fed a High-Fat Diet through Its Anti-Inflammatory and Prebiotic Properties. *Nutrients*, *13*(8), 2595. https://doi.org/10.3390/nu13082595

Vidanarachchi, J., Sims, I. M., & Iji, P. (2005). Phytobiotics: Alternatives to antibiotic growth promoters in monogastric animal feed. *Recent Advances in Animal Nutrition in Australia*, *15*, 131–144.

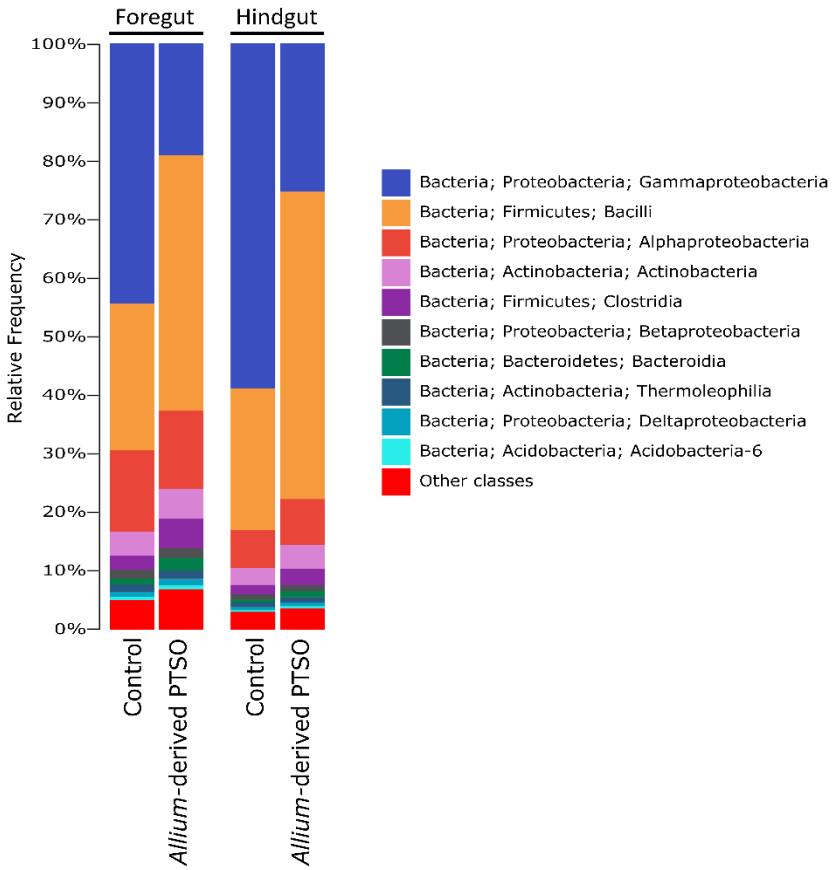
Wiklund, T. (2016). Pseudomonas anguilliseptica infection as a threat to wild and farmed fish in the Baltic Sea. *Microbiology Australia*, *37*(3), 135. https://doi.org/10.1071/MA16046

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, *86*(14), E140–E148. https://doi.org/10.2527/jas.2007-0459

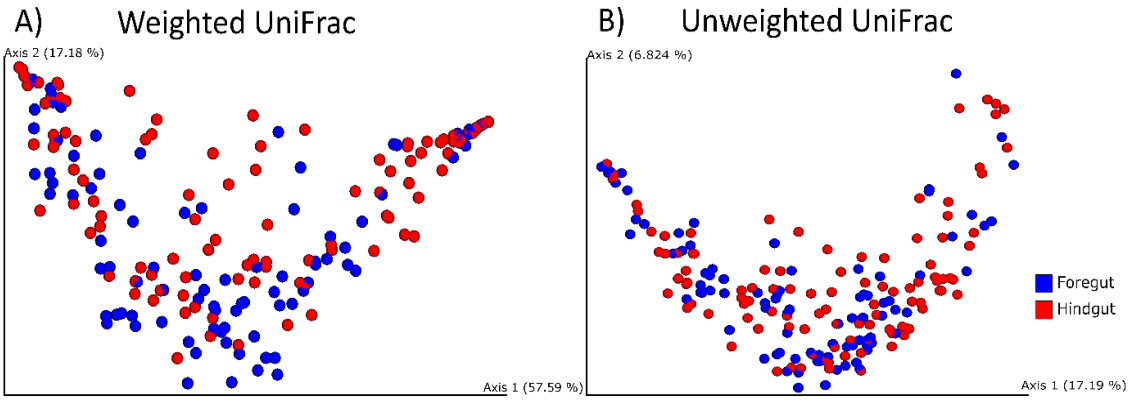
Ye, L., Amberg, J., Chapman, D., Gaikowski, M., & Liu, W. T. (2014). Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. *ISME Journal*, *8*(3), 541–551. https://doi.org/10.1038/ismej.2013.181

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B. A., & Arockiaraj, J. (2020). Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. *Reviews in Aquaculture*, *12*(3), 1903–1927. https://doi.org/10.1111/RAQ.12416

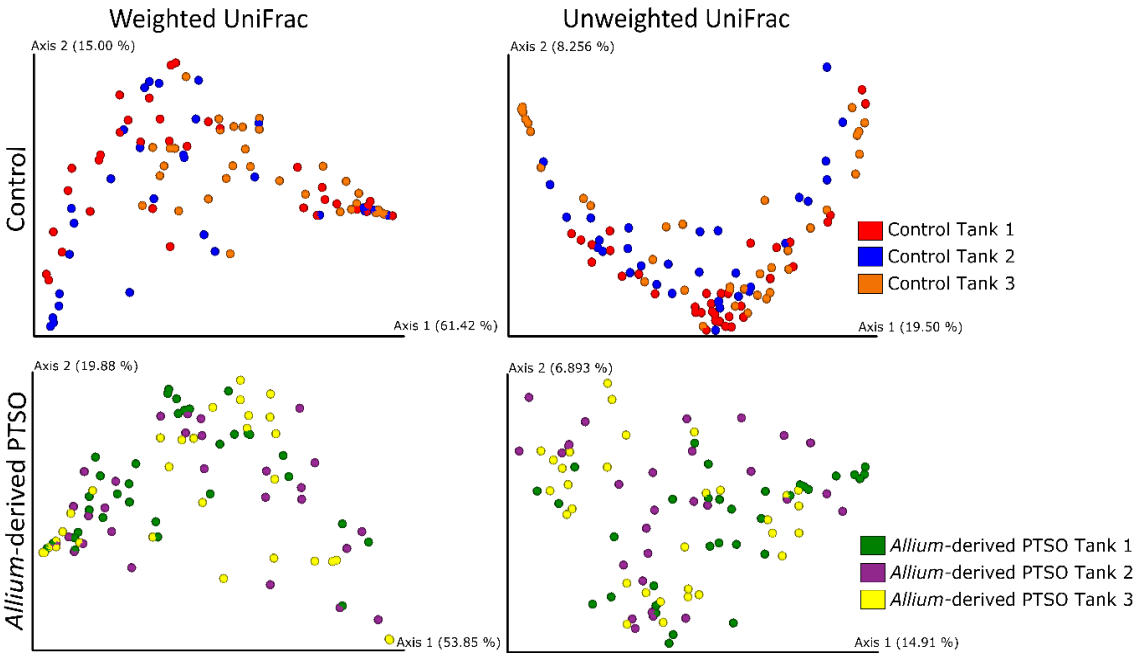
SUPPLEMENTARY MATERIAL



Supplementary Figure S1. Barplot summarizing the relative bacterial abundance at the class level in different gut regions (foregut and hindgut) and treatments. Control refers to juvenile gilthead seabream fed with basal diet while *Allium*-derived PTSO refers to experimental juvenile gilthead seabream fed with basal diet supplemented with *Allium*-derived PTSO.



Supplementary Figure S2. Principal Coordinate Analysis based in Weighted UniFrac (A) and Unweighted UniFrac (B) distance matrixes exploring differences in bacterial community between both gut regions (blue: foregut, red: hindgut). Percentages show the proportion of variance explained by each axis.



Supplementary Figure S3. Principal Coordinate Analysis based in Weighted UniFrac and Unweighted UniFrac distance matrixes exploring differences in bacterial gut community among different tanks of control and *Allium*-derived PTSO (red: control, tank 1, blue: control, tank 2, orange: control, tank 3, green: *Allium*-derived PTSO, tank 1, purple: *Allium*-derived PTSO, tank 2, yellow: *Allium*-derived PTSO, tank 3). Percentages show the proportion of variance explained by each axis.

CAPÍTULO V

Influence of dietary inclusion of *Allium*-Derived Compound Propyl Propane Thiosulfonate (PTSO) on growth performance and intestinal microbiota of European seabass (*Dicentrarchus labrax*) juveniles

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ABSTRACT

The global demand for fish products has increased in recent years and is expected to continue growing significantly in the next years due to the increase in the world population, which is expected to reach 11 billion people by 2100. In this sense, aquaculture plays an important role in supplying the world food demand and protein sources. High doses of antibiotics are used as prophylactic and growth promoters in the aquaculture industry, increasing the spread of antimicrobial resistance among pathogenic bacteria. Therefore, probiotics, prebiotics or phytobiotics have been proposed as alternatives in order to reduce the negative effects of antibiotic while maintaining or improving productive levels. In this study we have analyzed the use of 150 mg/kg of *Allium*-derived propyl propane thiosulfonate (PTSO) in European seabass juveniles and compared with control fish. The effects of PTSO were tested by measuring the body weight and analyzing the gut microbiome of European seabass juveniles after 89 days of feeding trial. The relative abundance of potentially pathogenic *Vibrio* in the foregut and hindgut of supplemented fish decreased, while *Pseudomonas* and *Kocuria* increased compared to control fish. Alpha diversity indices significantly decreased in both gut regions of fish fed with *Allium*-derived PTSO supplemented diet. Studying beta diversity, significant differences between fish fed with different diet appeared in both gut regions with both majority and minority OTUs. These results indicate that supplementing diet with *Allium*-derived PTSO could reduce potentially pathogenic *Vibrio* and increase body weight at the end of the experiment (89 days), producing changes in diversity and composition of microbial communities.

Keywords: *Allium*-derived phytobiotic; body weight; European seabass (*Dicentrarchus labrax*) juveniles; gut microbiota; propyl propane thiosulfonate (PTSO).

INTRODUCTION

In recent years there has been an exponential increase in the world population, which is expected to continue growing in the coming years, reaching almost 11 billion people worldwide in the year 2100 (United Nations, Department of Economic and Social Affairs, Population Division, 2019). This increase in world population will cause an increase in food demand, which can be partially covered by food from aquaculture given the high impact of land-based animal production and the stagnation of wild fishery catches (Merino et al., 2012). This industry currently plays an important role in supplying the world food demand and protein source, with a global aquaculture production of 82 million tons in 2018, which represents a value of US$ 250 billion (FAO, 2020). However, economic profits in the industry are affected by fish diseases caused by several pathogenic bacteria like *Aeromonas*, *Vibrio* or *Photobacterium* (Yukgehnaish et al., 2020). The diseases caused by these pathogenic bacteria are treated by high doses of antibiotics, given the high fish stocking densities and the impossibility of individual treatment (Resende et al., 2012). Furthermore, in aquaculture industry, antibiotics have been used as growth promoters (Antibiotic Growth Promoters, AGP) for several years, showing an improvement in feed efficiency and growth performance in different fish species (He et al., 2017; Lulijwa et al., 2020). However, the extensive use of antibiotics for growth-promoting and therapeutic purposes in aquaculture systems has increase antibiotic resistance in pathogenic bacteria (Cañada-Cañada et al., 2009). Therefore, a worldwide effort is necessary to eliminate or minimize the use of antibiotics in livestock and aquaculture. For this reason, the use of antibiotic growth promoters (AGPs) in animal feed was banned by the European Union in 2006 (European Commision, 2018) and by other countries in the following years (Maron et al., 2013; U.S. FDA, 2016).

Several feed additives have been proposed as alternatives to AGPs in aquaculture industry. The most promising alternatives includes enzymes, bacteriophages, probiotics, prebiotics and plants extracts or phytobiotics (Dawood et al., 2018; Pérez-Sánchez et al., 2018). Phytobiotics are defined as plant-derived bioactive compounds supplemented in the diet to improve animal productivity (Windisch et al., 2008). Phytobiotics are known to have antimicrobial activity against pathogenic bacteria and can act as prebiotics, facilitating a continuous supply of specific substrates for intestinal microbiota or minimizing the risk of pathogenic bacteria development (Vidanarachchi et al., 2005). These products also act as stimulant of saliva and bile secretion, which helps productive

parameters improvement (Gheisar & Kim, 2018). Many potential plants have been identified and used in aquaculture for improvement of fish health, including more than 60 different medicinal-plant species (Bulfon et al., 2015; Van Hai, 2015).

Allium species, mainly garlic (*Allium sativum*) and onion (*Allium cepa*), produce a wide variety of bioactive compounds with antifungal, antimicrobial and antioxidant activity (Kyung, 2012). Dietary supplementation of these compounds has shown promising results, improving health and productive parameters of livestock animals, including goats, cattle, pigs and poultry (Chen et al., 2021). The use of *A. cepa* extract in cattle produced no changes in milk attributes (Abad et al., 2017), while in goats, *A. sativum* oil showed a beneficial effect in the fatty acids profile of the milk (Zhu et al., 2013). The inclusion of *Allium* in growing finishing pigs showed a reduction of *Salmonella,* an increase of *Lactobacillus* and acids levels in feces and an improvement in growth performance (Sánchez et al., 2020; Zivkovic et al., 2019). In poultry industry, *Allium* supplemented in laying hens improved health status, intestinal microbiota and increased egg size and weight (Abad et al., 2020; Omer et al., 2019), and increased growth performance, immunity and antioxidant status of broiler chickens (Ismail et al., 2020; Omar et al., 2020). Application of *Allium* species in fish farming has become popular for promoting growth and improving the activity of defense systems against diseases caused by pathogenic bacteria (Chen et al., 2021; Valenzuela-Gutiérrez et al., 2021). The inclusion of onion (*A. cepa*) powder in the diet of beluga juveniles (*Huso huso*) improved growth performance, immune function and blood parameters (Akrami et al., 2015). Regarding to dietary supplementation of garlic (*A. sativum*) extract, it has been proven that it promotes growth, enhances immune system and improves the control of pathogens (Lee & Gao, 2012; Valenzuela-Gutiérrez et al., 2021). Inclusion of garlic in diet showed an increase in weight gain and growth rate of rainbow trout (*Oncorhynchus mykiss*) (Büyükdeveci et al., 2018), an improvement of food digestibility and biochemical effects of Eurasian perch (*Perca fluviatilis*) juveniles (Zare et al., 2021), and increase immune parameters of skin mucus of guppy fish (*Poecilia reticulata*) (Motlagh et al., 2020b). Many other studies have shown beneficial effects of garlic in aquafeeds. Furthermore, dietary inclusion of garlic has demonstrated its ability to control pathogens, showing antimicrobial activity against fungi and bacteria, including *Pseudomonas fluorescens* or *Vibrio* species (reviewed in Lee and Gao, 2012; Valenzuela-Gutiérrez et al., 2021).

The activity of these plant compounds has usually been related to volatile organosulfur compounds such as ajoene, allicin, isoalliin, methiin, propiin, propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) (Guillamón et al., 2021; Valenzuela-Gutiérrez et al., 2021). PTS and PTSO have shown antibacterial, antifungal (Sorlozano-Puerto et al., 2018, 2021) and anticoccidial activity (Kim et al., 2013). Furthermore, PTSO have demonstrated beneficial effects on intestinal health in several animal species (Guillamón et al., 2021) and has produced changes in gut microbiota and growth performance of different livestock animals, such as mice, broiler chickens, laying hens and pigs (Abad et al., 2020; Peinado et al., 2013; Rabelo-Ruiz et al., 2021a, 2021b; Ruiz et al., 2015; Sánchez et al., 2020; Vezza et al., 2021). In addition, recent studies of Lira et al. (2020) using experimental animals indicated PTSO as a toxicologically safe compound. However, the potential effects of *Allium*-derived PTSO on intestinal microbiota and body weight of European seabass juveniles has not yet been explored.

Therefore, in this study, we have used European seabass (*D. labrax*) juveniles as the animal model in order to evaluate the impact of *Allium*-derived PTSO in foregut and hindgut microbiota by high-throughput sequencing of the V6-V8 region of 16S rRNA gene. We predict that the inclusion of this *Allium*-based product will increase fish growth performance and will induce changes in the gut microbiota after 89 days of feeding trial.

MATERIAL AND METHODS

Animals, experimental design and fish sampling

European seabass (*Dicentrarchus labrax*) juveniles (n = 780) were randomly assigned to two experimental groups (390 fish per group), consisting of triplicate tanks (400 L; 130 fish per tank). Fish were kept in a recirculating RAS D-400 water system equipped with physical and biological filters. 5-10% of the water was renewed daily depending on the quality of water. The temperature was adjusted at 21 ± 1ºC and a photoperiod regime of 12L/12D hours was applied. All studied fish were handled in accordance with the European Union Guidelines (Directive 2010/63/UE) for the use of laboratory animals. The Ethical Committee at the University of Granada approved the experiments and they were endorsed by the Regional Government (Junta de Andalucía, Spain, ref. no. 13/04/2018/048).

The experimental diet was made from commercial fishmeal (NUTRAPLUS, Dibaq, Spain) by adding the *Allium*-based product (150 mg/kg of PTSO). After the meal homogenization, the granulated fish feed was manufactured by SPAROS (Olhão, Portugal). A diet without *Allium*-based additive was prepared as a control. PTSO concentration in feed was checked by UHPLC-ESI-MS/MS analyses, according to the method described by Abad et al. (2016). The *Allium*-based product used is commercialized under the trademark AquaGarlic® and was supplied by DOMCA (Granada, Spain). This product is standardized in propyl propane thiosulfonate (PTSO) at a concentration of 10%. It is presented as a powder on inert sepiolite.

At the beginning of the experiment, fish were randomly housed in different tanks, getting the same initial biomass in each tank. After 2 weeks of acclimatization, fish were anesthetized with 80 mg/L of tricaine methanesulfonate (MS-222) and weighed, with average initial body weight (BW) of 3.78 ± 0.09 g. During the feeding trial (89 days), fish were fed 3-4 times per day, 6 days per week. All the fish from each tank were collected, anesthetized using MS-222 and weighed at days 0, 12, 26, 42, 63 and 89. At the end of the feeding trial (89 days), 20 fish per experimental tank were euthanized by an overdose of anesthesia MS-222 (400 mg/L), followed by spine severing. Fish were immediately dissected and the whole intestine was collected with sterile material. Intestines were stored in sterile 90 mm Petri dishes and transported to the laboratory, where they were kept at -80ºC until DNA extraction.

DNA extraction

Intestinal pieces of approximately 100 mg were dissected from the foregut and hindgut of European seabass (*D. labrax*) juveniles using a sterile scalpel. DNA extraction was carried out following the Modified Salting Out Procedure (MSOP) of Martín-Platero et al. (2007). An initial mechanical lysis step using a cell disrupter FastPrep FP120 (BIO 101, Thermo Savant) was introduced in order to increase cell lysis. In summary, intestine pieces of about 100 mg were introduced in a 2 mL microcentrifuge screw cap tube filled with 100 mg of 2 mm zirconia beads and homogenized by two consecutive pulses of 30 seconds at speed 5 in FastPrep FP120. After that, the MSOP protocol was followed. The yield of the DNA extraction was checked by 0.7% agarose gel electrophoresis. DNA concentration was measured using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and then DNA was stored at -20ºC until PCR amplification.

V6-V8 16S rRNA gene amplification and high-throughput sequencing

V6-V8 region of 16S rRNA gene libraries were constructed using the primers pair B969F (5'-ACGCGHNRAACCTTACC-3') and BA1406R (5'-ACGGGCRGTGWGTRCAA-3') (Comeau et al., 2011) with Illumina adapter overhang sequences. PCR amplification was carried out using the iProof™ High-Fidelity DNA Polymerase (BioRad®). The PCR products were purified and then used as template for a second PCR. In this second PCR amplification, two unique Illumina compatible barcodes were index to each sample. These unique barcodes allow that the derived sequences can be demultiplexed into their respective samples in downstream analysis. The barcodes overlapped with the sequence of the primers used in the first PCR. Purification steps were made using DNA Purification SPRI Magnetic Beads (Canvax®), following the manufacturer's instructions. PCR amplicons were checked by 1% agarose gel electrophoresis and DNA concentrations were measured using Qubit® 3.0 Fluorometer (Invitrogen™, Carlsbad, CA). After that, PCR amplicons were pooled in equimolar concentrations and high-throughput sequencing was carried out with Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). This sequencing results in paired-ends reads of 2 x 300 bp length. Sequencing was carried out in the Illumina MiSeq platform in the Institute of Parasitology and Biomedicine "López-Neyra" (Granada, Spain). Raw sequence files are available in the Sequence Read Archive (SRA) in the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra/) under project number PRJNA809405 and accession numbers SAMN26177506 to SAMN26177705.

Sequences processing and data analysis

16S rRNA reads generated from Illumina MiSeq sequencer were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME2 v2020.11; Bolyen et al., 2019) software. At the beginning, primer trimming was performed using *cutadapt* plugin (Martin, 2011). Quality filtering was performed using a Phred score of 20 as the threshold, removing from the analyses sequences with more than 3 consecutives base pair with a Phred score lower than 20. After that, pair joining was carried out using VSEARCH default parameters (Rognes et al., 2016). Afterward, we use Deblur was used for sequence clustering into a sub-operational-taxonomic-unit (sub-OTU) approach, in order to remove sequencing errors (Amir et al., 2017). Sequences that passed quality filters were trimmed to 400 bp, giving a dataset of 10,832,912 total reads with a mean of 51,098.64 reads per

sample. The fragment insertion script implemented in QIIME2 was used to align the sequences and build a bacterial phylogenetic tree based on a reference phylogenetic tree (SEPP reference Greengenes 13.8; Janssen et al., 2018). The taxonomy was assigned based on a classifier pretrained on Greengenes 13.08 with a similarity of 99% (DeSantis et al., 2006). At the end, sequences of chloroplast, mitochondria and non-bacterial DNA were filtered of the OTU table.

Statistics

To test the effect of different diets on the fish's body weight, we used Generalized Linear Mixed-Models (GLMM). We used mean body weight in the tank as experimental unit with diet as fixed factor.

For alpha and beta diversity analyses, the OTU table was rarified at 10,000 sequencing depth per sample. Samples that did not reach this sequencing depth were excluded from subsequent analyses. Four alpha diversity indices were calculated, i.e., Shannon diversity index (Shannon, 1948); chao1 index (Chao, 1984); Faith phylogenetic diversity index (Faith, 1992); and OTU Richness. We used GLMM to explore the effect of diet and gut region as fixed factors in both alpha diversity indices. In these analyses, fish were the experimental unit for alpha and beta diversity analysis.

Body weight and alpha diversity analyses were performed using STATISTICA 10.0 (StatSoft).

Differences in genera and classes abundances between control and *Allium*-derived PTSO supplemented fish were explored by means of Linear Discriminant Analysis Effect Size (LEfSe) (Segata et al., 2011). LEfSe analyses were performed on the Galaxy web platform, implemented on the public server https://huttenhower.sph.harvard.edu/galaxy/.

Beta diversity distance matrixes were calculated using UniFrac distance. Both Weighted and Unweighted UniFrac indices (Lozupone et al., 2007; Lozupone & Knight, 2005) were used for subsequent analysis. Weighted UniFrac takes into account the relative abundance of bacteria shared between samples, giving more importance to the most abundant bacteria. Unweighted UniFrac gives more importance to rare bacteria in the OTUs as it only considers their presence or absence irrespective of their abundance. Permutational ANOVA (PERMANOVA) was performed in order to test these effects on both UniFrac distance matrixes using PRIMER-7 software (PRIMER-e), implemented

with PERMANOVA plugin. Principal Coordinate Analyses (PCoA) were performed in order to visualize the 2 first axes using EMPeror 2018.2.0 (Vázquez-Baeza et al., 2013, 2017).

RESULTS

Effect of feeding diet on European seabass juvenile growth performance

No differences appeared in the initial body weight of fish fed with control diet and fish fed with *Allium*-derived PSTO supplemented diet (Table 1, Figure 1). No differences in body weight appeared between European seabass juveniles fed with control diet or *Allium*-derived PTSO along the experiment, showing similar body weight at days 12, 26, 42 and 63 (Table 1, Figure 1). However, fish supplemented with *Allium*-derived PTSO showed a significant increase in body weight at the end of the feeding trial (Day 89) (Table 1, Figure 1).

Table 1. General Linear Mixed Models exploring the effects of diet as factor in European seabass juveniles fed with control diet or supplemented with *Allium*-derived PTSO. Significant *p*-values are shown in bold.

	Control	***Allium*-derived PTSO**	***p***
Body Weight Day 0 (g/fish)	3.72 ± 0.05	3.84 ± 0.02	0.110
Body Weight Day 12 (g/fish)	4.64 ± 0.06	4.70 ± 0.04	0.438
Body Weight Day 26 (g/fish)	5.90 ± 0.12	5.87 ± 0.09	0.818
Body Weight Day 42 (g/fish)	8.21 ± 0.12	8.14 ± 0.08	0.640
Body Weight Day 63 (g/fish)	11.96 ± 0.09	12.25 ± 0.10	0.089
Body Weight Day 89 (g/fish)	21.14 ± 0.21	22.08 ± 0.08	**0.013**

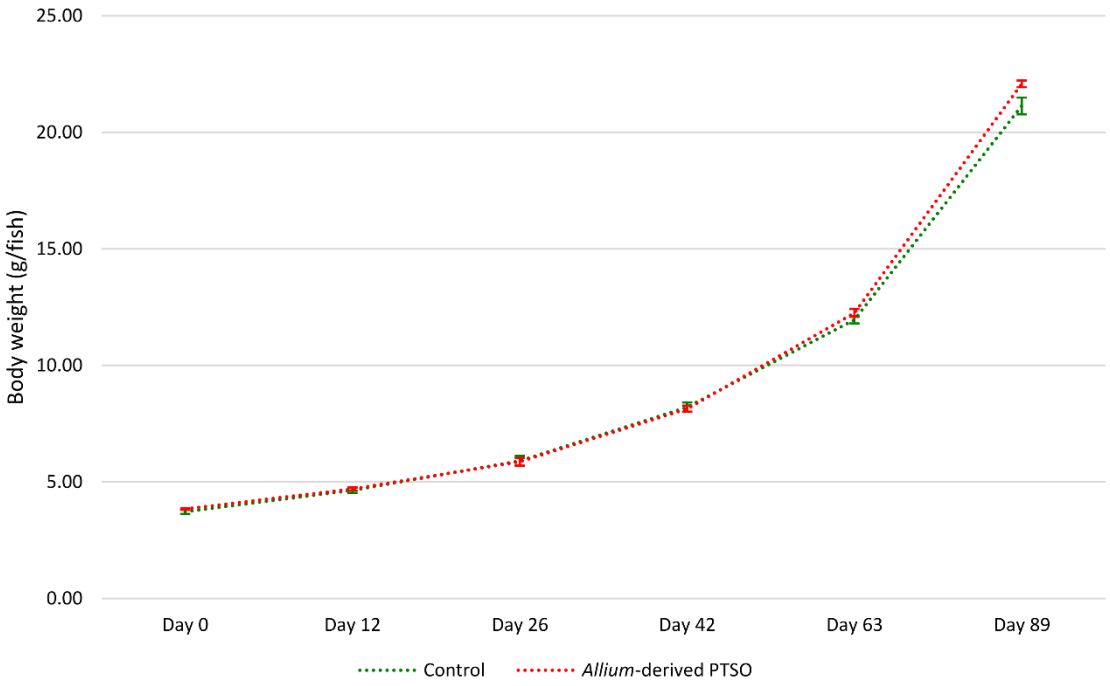

Figure 1. Evolution of growth performance of European seabass (*D. labrax*) juveniles fed with control diet or supplemented with *Allium*-derived PTSO along the feeding trial.

Bacterial community composition

The foregut microbiota of juvenile European seabass was dominated at class level by Gammaproteobacteria (47%), Alphaproteobacteria (25%), Betaproteobacteria (9%), Actinobacteria (8%) and Bacilli (8%) (Figure 2). Fish supplemented with *Allium*-derived PTSO showed a significant decrease in Alphaproteobacteria (13%), Betaproteobacteria (7%) and Bacilli (7%), and an increase of Actinobacteria (21%) (Figure 2, Figure 4). At genus level, the foregut of control fish was dominated by *Ochrobactrum* (22%), *Pseudomonas* (19%) and *Vibrio* (19%). In the foregut of *Allium*-derived PTSO fish appeared an increase in *Pseudomonas* (40%) and *Kocuria* (15%), and a decrease in *Vibrio* (< 1%) and *Ochrobactrum* (11%) (Figure 3, Figure 4).

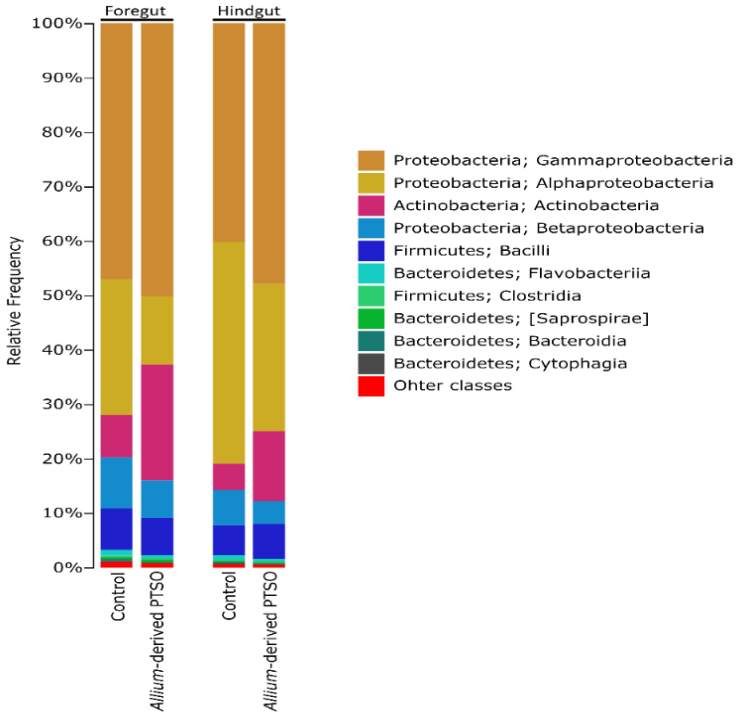

Figure 2. Microbial composition at class level of juvenile European seabass gut microbiota group by experimental diet (control and *Allium*-derived PTSO). Classes in the legend are sorted from most abundant to lowest abundant.

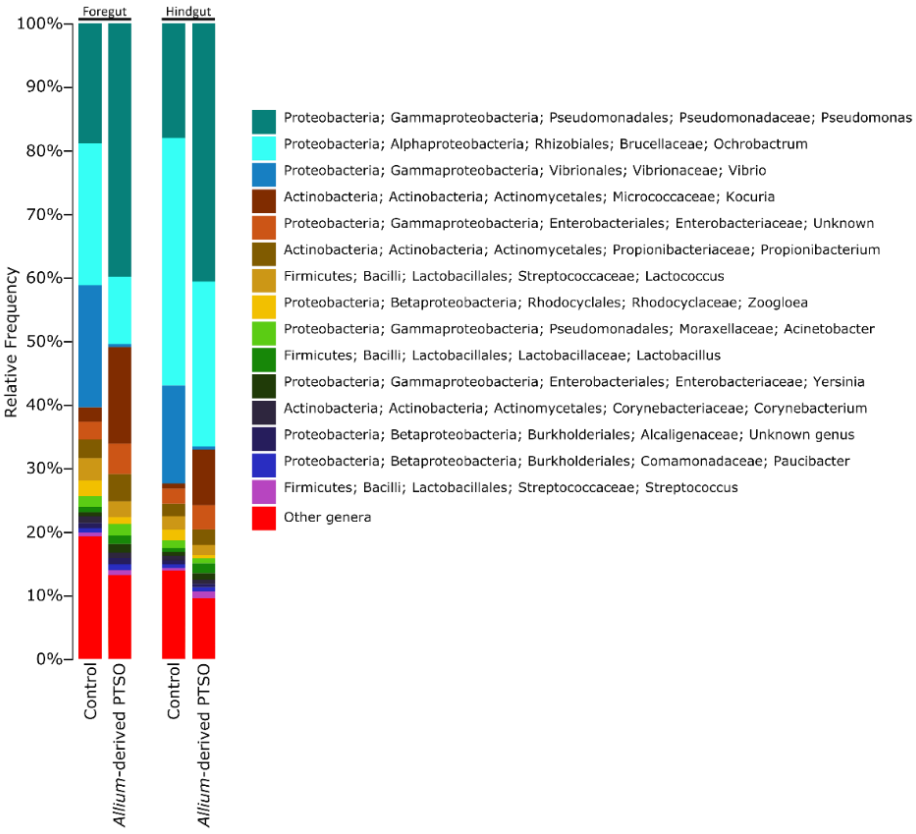

Figure 3. Microbial composition at genus level of juvenile European seabass gut microbiota group by diet (control and *Allium*-derived PTSO). Genera in the legend are sorted from most abundant to lowest abundant.

The bacterial composition of hindgut of control fish was similar to foregut, being dominated at class level by Alphaproteobacteria (41%), Gammaproteobacteria (40%), Betaproteobacteria (7%), Bacilli (5%) and Actinobacteria (5%) (Figure 2). The hindgut microbiota of *Allium*-derived PTSO supplemented fish was similar to control, but with a small but no significant increase of Gammaproteobacteria and a decrease of Alphaproteobacteria (Figure 2). Furthermore, a significant increase in Actinobacteria (13%) and decrease of Betaproteobacteria (4%) and the minority class Mollicutes (Figure 4). At genus level, the foregut of control fish was dominated by *Ochrobactrum* (39%), *Pseudomonas* (18%) and *Vibrio* (15%). In the hindgut of *Allium*-derived PTSO supplemented fish appeared an increase in *Kocuria* (9% respect to 1% in control fish) and *Pseudomonas* (41%), as well as a decrease in *Vibrio* (< 1%) (Figure 3, Figure 4).

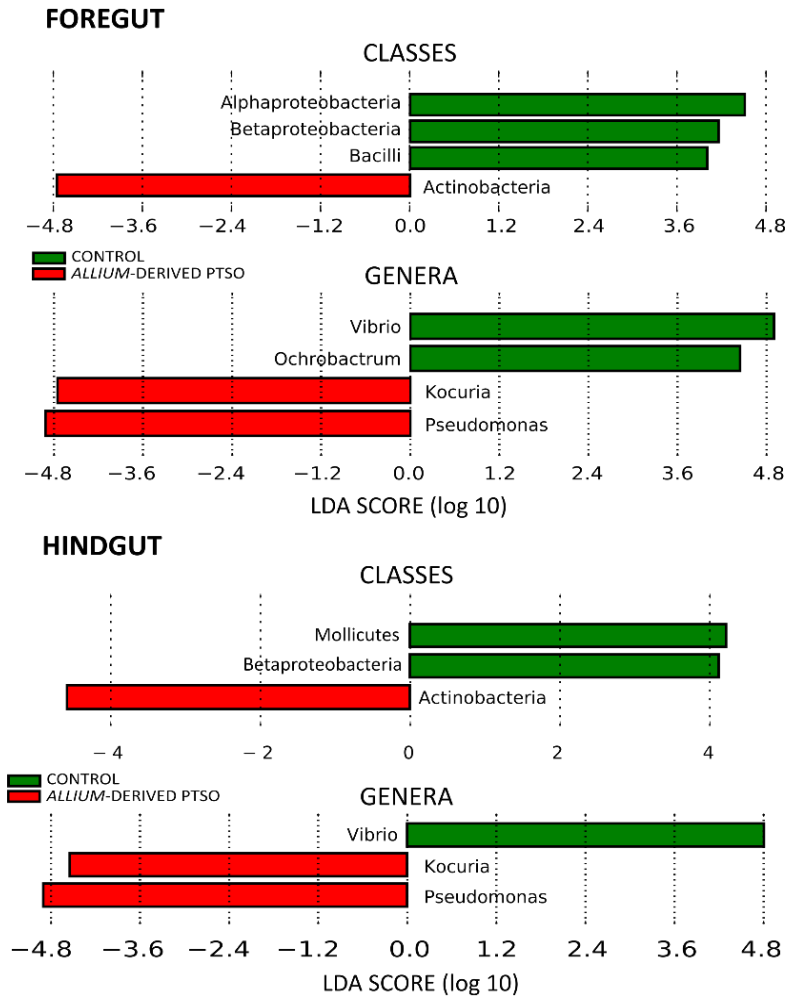

Figure 4. LDA Effect Size (LEfSe) analyses showing bacterial classes and genera that differ significantly between control fish and those supplemented with *Allium*-derived PTSO, in the foregut and in the hindgut of European seabass juveniles. Significant LDA Score > 4.0.

Effect of feeding diet on Alpha and Beta Diversity

Supplementing the diet of European seabass juveniles with *Allium*-derived PTSO affected the alpha diversity indices studied (Table 2). *Allium*-derived PTSO supplemented fish showed a reduction in alpha diversity respect to control fish. However, no differences appeared in gut region, with both foregut and hindgut showing similar levels of alpha diversity. Furthermore, no differences appeared in the interaction of Diet*Gut Region, indicating that the changes in diversity between both gut regions occurs in the same way in both feeding diets (see Diet*Gut Region interaction term in Table 2).

Table 2. General Linear Mixed Models exploring the effects of fish experimental diet (control and *Allium*-derived PTSO) and gut region in the different alpha diversity indices of the bacterial community of juvenile European seabass. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values ($p < 0.05$) are shown in bold.

Alpha Diversity Index	Explanatory variables	D.f.	F	*p*
Chao1 Index	Diet	1,177	83.97	**<0.001**
	Gut Region	1,177	0.02	0.899
	Diet*Gut Region	1,177	1.52	0.220
Faith PD	Diet	1,177	79.90	**<0.001**
	Gut Region	1,177	0.02	0.903
	Diet*Gut Region	1,177	0.36	0.547
OTUs Richness	Diet	1,177	95.10	**<0.001**
	Gut Region	1,177	0.21	0.652
	Diet*Gut Region	1,177	0.55	0.459
Shannon Diversity Index	Diet	1,177	6.51	**0.012**
	Gut Region	1,177	15.23	**<0.001**
	Diet*Gut Region	1,177	0.96	0.330

The bacterial community of European seabass juveniles vary significantly between the two diets, taking into account both the most abundant bacterial OTUs (Weighted UniFrac) and minority OTUs (Unweighted UniFrac) (Table 3, Figure 5). Regarding both gut regions separately, significant differences appeared in both regions. In the foregut, differences between experimental diets appeared with both majority (GLMM, Weighted UniFrac, diet as factor, Pseudo-$F_{1,84} = 18.79$, $p = 0.001$) and minority OTUs (GLMM, Unweighted UniFrac, diet as factor, Pseudo-$F_{1,84} = 4.99$, $p = 0.001$). In the hindgut, results were similar, with differences in diet with both majority (GLMM,

Weighted UniFrac, diet as factor, Pseudo-$F_{1,92}$ = 13.03, p = 0.001) and minority OTUs (GLMM, Unweighted UniFrac, diet as factor, Pseudo-$F_{1,92}$ = 4.89, p = 0.001). This differences in experimental diet and gut region cannot be observed graphically in the PCoA since it does not take into account the 100% of the variance (61.14% in Weighted UniFrac and 15.00% in Unweighted UniFrac) (Figure 5).

Table 3. Permutational ANOVA (PERMANOVA) exploring the effects of Diet, Gut region and the interaction Diet*Gut region in beta diversity indices of bacterial community of European seabass juveniles fed with control diet or supplemented with *Allium*-derived PTSO. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values are shown in bold.

β-diversity distance matrix	Explanatory variables	D.f.	Pseudo-F	*p*
Weighted UniFrac	Diet	1,177	31.51	**0.001**
	Gut region	1,177	14.00	**0.001**
	Diet*Gut region	1,177	0.98	0.409
Unweighted UniFrac	Diet	1,177	8.89	**0.001**
	Gut region	1,177	1.05	0.325
	Diet*Gut region	1,177	0.94	0.595

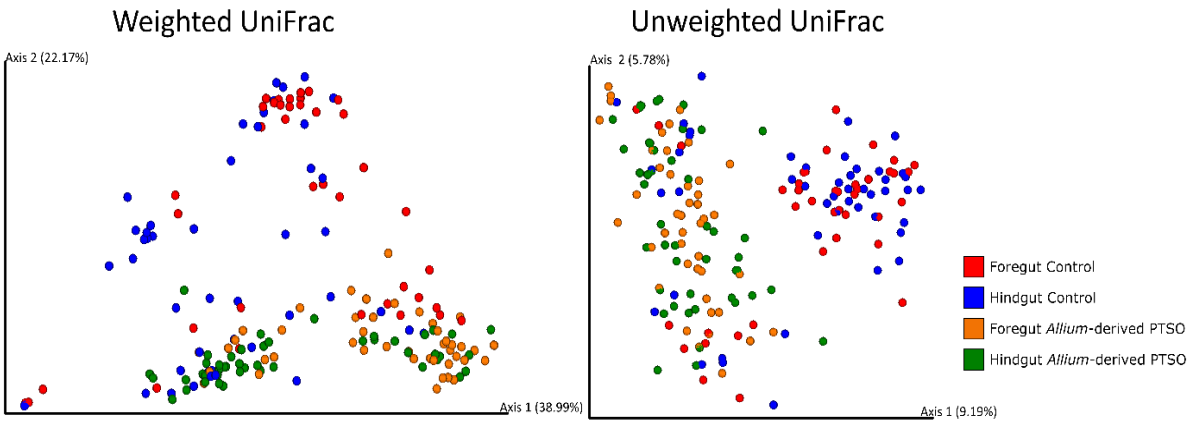

Figure 5. Dimensional figures showing the first two axes of Principal Coordinate Analysis and representing bacterial communities of foregut and hindgut of juvenile European seabass fed with control diet or supplemented with Allium-derived PTSO using Unweighted and Weighted UniFrac distance matrixes. Samples are colored by gut region and experimental diet (Foregut control - red; Hindgut control – blue; Foregut *Allium*-derived PTSO – yellow; Hindgut *Allium*-derived PTSO - green). Proportion of explained variance by each PCo axes is shown.

DISCUSSION

In this study, juvenile European seabass supplemented with an *Allium*-derived organosulfur compound such as propyl propane thiosulfonate (PTSO) produced an increase in body weight at the end of the experiment (89 days). This increase in growth parameters was accompanied by significant changes in the structure of bacterial communities, a reduction of alpha diversity and a decrease in relative abundance of potentially pathogenic *Vibrio* in the foregut and hindgut of PTSO supplemented fish.

The spreading of antimicrobial resistance (AMR) makes it necessary to search for new alternatives to antibiotic growth promoters (AGP) in aquaculture that are capable of stopping the spread of AMR and improving animal welfare. Some compounds have been proposed as good alternatives to AGPs, like probiotics, prebiotics, organic acids and plant extracts (Romero et al., 2012). Plant extracts, also known as phytobiotics, includes a wide range of plant-derived products such as essential oils, herbs and oleoresins (Gheisar & Kim, 2018). Phytobiotics have been proposed as good and safe alternatives to AGPs capable to modulate intestinal microbiota and increase productive parameters, anti-pathogenic properties and appetite stimulation of both terrestrial and aquatic animals (Reverter et al., 2014; Windisch et al., 2008). The phytobiotics used in animal feed come from different plant species, being the products derived from *Allium* plants the most widely used, mainly garlic (*Allium sativum*) and onion (*Allium cepa*) (Guillamón et al., 2021; Kothari et al., 2019). Organosulfur compounds are the most important bioactive compounds derived from *Allium*, showing antibacterial, antifungal, antiviral, anti-inflammatory and antioxidant activities (Kim et al., 2013; Sorlozano-Puerto et al., 2018, 2021). Some of the most commonly used *Allium*-derived organosulfur compounds for animal feed include ajoene, allicin, isoalliin, methiin, propiin, propyl propane thiosulfinate (PTS) and propyl propane thiosulfonate (PTSO) (Guillamón et al., 2021; Valenzuela-Gutiérrez et al., 2021). PTSO addition has shown beneficial effects in different farm animals. In poultry, different doses of PTSO in broiler chickens improved food digestibility and growth performance, and produced changes in gut microbiota (Peinado et al., 2012; Rubio et al., 2015; Ruiz et al., 2015); in laying hens, PTSO increased the number and the size of eggs laid, and produced an increase in potentially beneficial bacteria in the intestinal tract microbiota (Abad et al., 2020; Rabelo-Ruiz et al., 2021a). In pig industry, PTSO has shown beneficial effects in intestinal microbiota and increased growth performance in piglets and growing-finishing pigs (Rabelo-Ruiz et al.,

2021b; Sánchez et al., 2020). The use of *Allium*-derived PTSO in aquaculture has only been studied in gilthead seabream juveniles, showing potentially beneficial changes in gut microbiota and producing no changes in growth performance after 12 weeks of treatment (Rabelo-Ruiz et al., unpublished manuscript).

Despite the little research using PTSO in aquaculture, other *Allium*-based compounds have been used in aquafeeds in different studies using different fish species (Valenzuela-Gutiérrez et al., 2021). Dietary inclusion of onion (*Allium cepa*) powder produced an increase in body weight, specific growth rate (SGR) and immune parameters of beluga juveniles (Akrami et al., 2015). Supplementing the diet with garlic (*Allium sativum*) showed an increase in growth performance in Asian seabass (*Lates calcarifer*) (Abdelwahab et al., 2020; Talpur & Ikhwanuddin, 2012). The use of crude polysaccharides from garlic produced an increase in body weight and SGR in rainbow trout (*Onchorhynchus mykiss*) (Büyükdeveci et al., 2018). Other studies using allicin, a garlic-derived organosulfur compound, showed its benefits as growth promoter, antimicrobial agent and feed stimulator (reviewed in Lee & Gao, 2012). However, these results are controversial, because other studies noted the lack of effect of *Allium* extract and *Allium*-derived compounds on different fish species in aquaculture (Motlagh et al., 2020a; Nya et al., 2010). In fact, in a previous work by our research group using PTSO in gilthead seabream juveniles, the inclusion of this *Allium*-derived compound did not produce changes in growth performance after 12 weeks of treatment (Rabelo-Ruiz et al., unpublished manuscript). The results of this study showed no differences in body weight between control and *Allium*-derived PTSO supplemented European seabass juveniles along the experiment but did evidence a significant increase in body weight at the end of the experimental period (89 days). Further studies are needed to clarify the effects of different phytobiotic presentations on growth performance and health status of different fish species.

Our study showed a significant decrease in all the alpha diversity indices studied in the foregut and hindgut of European seabass juveniles supplemented with PTSO, except in the hindgut with Shannon diversity index. These results were accompanied by no differences in body weight along the experiment and even an increase at the end of the feeding trial (89 days). Some studies have demonstrated that the reduction of alpha diversity could be related to increased body weight in birds and obesity in humans (Bae et al., 2017; Menni et al., 2017). In aquaculture, it is unclear whether microbial diversity

correlates positively or negatively with fish growth. Li et al. (2016) found that differences in bacterial diversity did not translate into differences in body weight of largemouth bronze gudgeon (*Coreius guichenoti*). However, studies with rainbow trout (*Onchorhynchus mykiss*) and Songpu Mirror Carp (*Cyprinus specularis* Songpu) suggested an increase in body weight when appear an increase in bacterial diversity (Betiku et al., 2018; Luo et al., 2020). The studies of Bolnick et al. (2014) showed mixed results, appearing an improve of condition factor (K) of fish related with low microbial diversity of Eurasian perch, and in threespine stickleback (*Gasterosteus aculeatus*) appeared an increase of microbial diversity related with the improve of condition factor. Previous results from our research group (Rabelo-Ruiz et al., unpublished manuscript) showed no differences in body weight accompanied by an increase in alpha diversity indices in gilthead seabream juveniles supplemented with *Allium*-derived PTSO. Despite these differences, it seems clear that changes in microbial diversity are associated with variations in fish condition and growth performance (Bolnick et al., 2014).

Intestinal community differed between the control and *Allium*-derived PTSO diets, either when considering majority OTUs (Weighted UniFrac) or minority OTUs (Unweighted UniFrac). The relative abundance of *Pseudomonas* increased in both the foregut and hindgut of *Allium*-derived PTSO supplemented fish. These results could be negative, since despite the fact that *Pseudomonas* have been described as a ubiquitous bacterial genus, some species are emergent opportunistic fish pathogens (Fadel et al., 2018). *P. anguilliseptica* is considered as pathogen, being the causative agent of winter disease, an illness associated with several farmed fish such as seabass, cod and gilthead seabream (Wiklund, 2016). Other *Pseudomonas* species such *P. aeruginosa, P. putida* or *P. fluorescens* are considered as opportunistic pathogens in aquaculture (Altinok et al., 2006). However, there were also changes in other bacterial groups that may be beneficial. *Kocuria* species are normally found in fish gastrointestinal tract. They have many characteristics for use as potential probiotics in aquaculture, such as the ability to grow in a wide range of pH, salinity and temperature, and their capacity to produce extracellular enzymes that can play an important role in digestion (Piazzon et al., 2019). Furthermore, in rainbow trout, *Kocuria* has been shown to induce the immune system by inhibiting pathogenic bacteria such as *Vibrio anguillarum* (Sharifuzzaman et al., 2011). Therefore, this increase of *Kocuria* found in our experiment could be positive for fish. In addition, we found a significant decreased of relative abundance of *Vibrio* in the foregut and

hindgut of European seabass juveniles supplemented with *Allium*-derived PTSO. *Vibrio* species are ubiquitous in marine environments and some species are considered potentially pathogenic, causing clinical diseases as tail rot disease or vibriosis (Abdel-Aziz et al., 2013; Pérez-Sánchez et al., 2018). *V. anguillarum*, *V. salmonicida*, *V. alginolyticus*, *V. harveyi* or *V. parahaemolyticus* are some of the *Vibrio* species which cause the most devastating effects on marine fish (Mohamad et al., 2019). Some plant extracts have demonstrated antimicrobial activity against different *Vibrio* species in aquaculure. Ginger powder and garlic powder showed antimicrobial effects against *V. harveyi* in Asian seabass (Stratev et al., 2018). The use of garlic has shown antimicrobial effects against *Vibrio* species in aquaculture (Valenzuela-Gutiérrez et al., 2021). An study of Natasya-Ain et al. (2018) showed in vitro inhibitory activity of garlic (*A. sativum*) agaisnt *V. anguillarum*, *V. alginolyticus* and *V. harveyi*. Our results also showed a reduction of potentially pathogenic *Vibrio* in foregut and hingut of *Allium*-derived PTSO supplemented fish, which could be beneficial for animal's health.

CONCLUSIONS

Our experimental supplementation of the diet of European seabass juveniles with *Allium*-derived PTSO, produces an increase in fish body weight at the end of the feeding trial (89 days). This beneficial effects in growth performance were accompanied by significant differences in bacterial communities and a significant reduction of alpha diversity in both foregut and hindgut. In addition, a significant reduction of potentially pathogenic *Vibrio* and an increase of *Kocuria* appeared in the intestine of PTSO supplemented fish. Further research is necessary to unveil how the *Allium*-derived PTSO affects specific pathogenic strains and how this phytobiotic product influence immune system and health status of fish.

ACKNOWLEDGEMENTS

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REFERENCES

Abad, P., Arroyo-Manzanares, N., Ariza, J. J., Baños, A., & García-Campaña, A. M. (2021). Effect of Allium Extract Supplementation on Egg Quality, Productivity, and Intestinal Microbiota of Laying Hens. *Animals*, *11*(1), 41. https://doi.org/10.3390/ani11010041

Abad, P., Arroyo-Manzanares, N., & García-Campaña, A. M. (2016). A rapid and simple UHPLC-ESI-MS/MS method for the screening of propyl propane thiosulfonate, a new additive for animal feed. *Analytical Methods*, *8*(18), 3730–3739. https://doi.org/10.1039/C6AY00219F

Abad, P., Arroyo-Manzanares, N., Gil, L., & García-Campaña, A. M. (2017). Use of onion extract as a dairy cattle feed supplement: Monitoring propyl propane thiosulfonate as a marker of its effect on milk attributes. *Journal of Agricultural and Food Chemistry*, *65*(4), 793–799. https://doi.org/10.1021/acs.jafc.6b04395

Abdel-Aziz, M., Eissa, A. E., Hanna, M., & Okada, M. A. (2013). Identifying some pathogenic Vibrio/Photobacterium species during mass mortalities of cultured Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) from some Egyptian coastal provinces. *International Journal of Veterinary Science and Medicine*, *1*(2), 87–95. https://doi.org/10.1016/j.ijvsm.2013.10.004

Abdelwahab, A. M., El-Bahr, S. M., & Al-Khamees, S. (2020). Influence of Dietary Garlic (Allium sativum) and/or Ascorbic Acid on Performance, Feed Utilization, Body Composition and Hemato-Biochemical Parameters of Juvenile Asian Sea Bass (Lates calcarifer). *Animals*, *10*(12), 2396. https://doi.org/10.3390/ani10122396

Akrami, R., Gharaei, A., Mansour, M. R., & Galeshi, A. (2015). Effects of dietary onion (Allium cepa) powder on growth, innate immune response and hemato–biochemical parameters of beluga (Huso huso Linnaeus, 1754) juvenile. *Fish & Shellfish Immunology*, *45*(2), 828–834. https://doi.org/10.1016/J.FSI.2015.06.005

Altinok, I., Kayis, S., & Capkin, E. (2006). Pseudomonas putida infection in rainbow trout. *Aquaculture*, *261*(3), 850–855. https://doi.org/10.1016/j.aquaculture.2006.09.009

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Xu, Z. Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Bae, Y., Koo, B., Lee, S., Mo, J., Oh, K., & Mo, I. P. (2017). Bacterial diversity and its relationship to growth performance of broilers. *Korean J. Vet. Res.*, *57*(3), 159–167. https://doi.org/10.14405/kjvr.2017.57.3.159

Betiku, O. C., Yeoman, C. J., Gaylord, T. G., Americus, B., Olivo, S., Duff, G. C., & Sealey, W. M. (2018). Water system is a controlling variable modulating bacterial diversity of gastrointestinal tract and performance in rainbow trout. *PLOS ONE*, *13*(4), e0195967. https://doi.org/10.1371/journal.pone.0195967

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*. Nature Publishing Group. https://doi.org/10.1038/s41587-019-0209-9

Bolnick, D. I., Snowberg, L. K., Hirsch, P. E., Lauber, C. L., Knight, R., Caporaso, J. G., & Svanbäck, R. (2014). Individuals' diet diversity influences gut microbial diversity

in two freshwater fish (threespine stickleback and Eurasian perch). *Ecology Letters*, *17*(8), 979–987. https://doi.org/10.1111/ELE.12301

Bulfon, C., Volpatti, D., & Galeotti, M. (2015). Current research on the use of plant-derived products in farmed fish. *Aquaculture Research*, *46*(3), 513–551. https://doi.org/10.1111/ARE.12238

Büyükdeveci, M. E., Balcázar, J. L., Demirkale, İ., & Dikel, S. (2018). Effects of garlic-supplemented diet on growth performance and intestinal microbiota of rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *486*, 170–174. https://doi.org/10.1016/J.AQUACULTURE.2017.12.022

Cañada-Cañada, F., Peña, A. M. D. La, & Espinosa-Mansilla, A. (2009). Analysis of antibiotics in fish samples. *Analytical and Bioanalytical Chemistry*, *395*(4), 987–1008. https://doi.org/10.1007/s00216-009-2872-z

Chao, A. (1984). Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, *11*, 265–270.

Chen, J., Wang, F., Yin, Y., & Ma, X. (2021). The nutritional applications of garlic (Allium sativum) as natural feed additives in animals. *PeerJ*, *9*, e11934. https://doi.org/10.7717/PEERJ.11934

Comeau, A. M., Li, W. K. W., Tremblay, J.-É., Carmack, E. C., & Lovejoy, C. (2011). Arctic Ocean Microbial Community Structure before and after the 2007 Record Sea Ice Minimum. *PLOS ONE*, *6*(11), e27492. https://doi.org/10.1371/JOURNAL.PONE.0027492

Dawood, M. A. O., Koshio, S., & Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Reviews in Aquaculture*, *10*(4), 950–974. https://doi.org/10.1111/raq.12209

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

European Commision. (2018). Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending Regulation (EC) No 183/2005 of the European Parliament and of the Council and repealing. *Official Journal of European Union*, *62*, L:2019:004:TOC.

Fadel, A., Mabrok, M., & Aly, S. (2018). Epizootics of Pseudomonas anguilliseptica among cultured seabream (Sparus aurata) populations: Control and treatment strategies. *Microbial Pathogenesis*, *121*, 1–8. https://doi.org/10.1016/j.micpath.2018.04.021

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *61*(1), 1–10.

FAO. (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in action*. FAO. https://doi.org/10.4060/ca9229en

Gheisar, M. M., & Kim, I. H. (2018). Phytobiotics in poultry and swine nutrition–a review. *Italian Journal of Animal Science*, *17*(1), 92–99. https://doi.org/10.1080/1828051X.2017.1350120

Guillamón, E., Andreo-Martínez, P., Mut-Salud, N., Fonollá, J., & Baños, A. (2021). Beneficial Effects of Organosulfur Compounds from Allium cepa on Gut Health: A

Systematic Review. *Foods*, *10*(8), 1680. https://doi.org/10.3390/FOODS10081680

Hai, N. Van. (2015). The use of medicinal plants as immunostimulants in aquaculture: A review. *Aquaculture*, *446*, 88–96. https://doi.org/10.1016/J.AQUACULTURE.2015.03.014

He, S., Wang, Q., Li, S., Ran, C., Guo, X., Zhang, Z., & Zhou, Z. (2017). Antibiotic growth promoter olaquindox increases pathogen susceptibility in fish by inducing gut microbiota dysbiosis. *Science China Life Sciences*, *60*(11), 1260–1270. https://doi.org/10.1007/s11427-016-9072-6

Ismail, I. E., Alagawany, M., Taha, A. E., Puvača, N., Laudadio, V., & Tufarelli, V. (2020). Effect of dietary supplementation of garlic powder and phenyl acetic acid on productive performance, blood haematology, immunity and antioxidant status of broiler chickens. *Asian-Australasian Journal of Animal Sciences*, *34*(3), 363–370. https://doi.org/10.5713/ajas.20.0140

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MySistems*, *3*(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Kim, D. K., Lillehoj, H. S., Lee, S. H., Lillehoj, E. P., & Bravo, D. (2013). Improved resistance to Eimeria acervulina infection in chickens due to dietary supplementation with garlic metabolites. *British Journal of Nutrition*, *109*(01), 76–88. https://doi.org/10.1017/S0007114512000530

Kothari, D., Lee, W.-D., Niu, K.-M., & Kim, S.-K. (2019). The Genus Allium as Poultry Feed Additive: A Review. *Animals*, *9*(12), 1032. https://doi.org/10.3390/ANI9121032

Kyung, K. H. (2012). Antimicrobial properties of allium species. *Current Opinion in Biotechnology*. Elsevier Current Trends. https://doi.org/10.1016/j.copbio.2011.08.004

Lee, J.-Y., & Gao, Y. (2012). Review of the Application of Garlic, Allium sativum, in Aquaculture. *Journal of the World Aquaculture Society*, *43*(4), 447–458. https://doi.org/10.1111/j.1749-7345.2012.00581.x

Li, X., Yan, Q., Ringø, E., Wu, X., He, Y., & Yang, D. (2016). The influence of weight and gender on intestinal bacterial community of wild largemouth bronze gudgeon (Coreius guichenoti, 1874). *BMC Microbiology*, *16*(1), 191. https://doi.org/10.1186/s12866-016-0809-1

Lira, A. C., Prieto, A. I., Baños, A., Guillamón, E., Moyano, R., Jos, A., & Cameán, A. M. (2020). Safety assessment of propyl-propane-thiosulfonate (PTSO): 90-days oral subchronic toxicity study in rats. *Food and Chemical Toxicology*, *144*(111612). https://doi.org/10.1016/j.fct.2020.111612

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, *73*(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Lulijwa, R., Rupia, E. J., & Alfaro, A. C. (2020). Antibiotic use in aquaculture, policies

and regulation, health and environmental risks: a review of the top 15 major producers. *Reviews in Aquaculture*, *12*(2), 640–663. https://doi.org/10.1111/RAQ.12344

Luo, L., Xu, Q., Xu, W., Li, J., Wang, C., Wang, L., & Zhao, Z. (2020). Effect of Bacillus megaterium-Coated Diets on the Growth, Digestive Enzyme Activity, and Intestinal Microbial Diversity of Songpu Mirror Carp Cyprinus specularis Songpu. *BioMed Research International*, *2020*. https://doi.org/10.1155/2020/8863737

Maron, D. F., Smith, T. J. S., & Nachman, K. E. (2013). Restrictions on antimicrobial use in food animal production: An international regulatory and economic survey. *Globalization and Health*, *9*(1). https://doi.org/10.1186/1744-8603-9-48

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Analytical Biochemistry*, *366*(1), 102–104. https://doi.org/10.1016/j.ab.2007.03.010

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Menni, C., Jackson, M. A., Pallister, T., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. *International Journal of Obesity*, *41*, 1099–1105. https://doi.org/10.1038/ijo.2017.66

Merino, G., Barange, M., Blanchard, J. L., Harle, J., Holmes, R., Allen, I., … Rodwell, L. D. (2012). Can marine fisheries and aquaculture meet fish demand from a growing human population in a changing climate? *Global Environmental Change*, *22*(4), 795–806. https://doi.org/10.1016/J.GLOENVCHA.2012.03.003

Mohamad, N., Amal, M. N. A., Yasin, I. S. M., Saad, M. Z., Nasruddin, N. S., Al-saari, N., … Sawabe, T. (2019). Vibriosis in cultured marine fishes: a review. *Aquaculture*, *512*. https://doi.org/10.1016/j.aquaculture.2019.734289

Motlagh, H. A., Paolucci, M., Bami, M. L., & Safari, O. (2020a). Sexual parameters, digestive enzyme activities, and growth performance of guppy (Poecilia reticulata) fed garlic (Allium sativum) extract supplemented diets. *Journal of the World Aquaculture Society*, *51*(5), 1087–1097. https://doi.org/10.1111/jwas.12729

Motlagh, H. A., Safari, O., Selahvarzi, Y., Baghalian, A., & Kia, E. (2020b). Non-specific immunity promotion in response to garlic extract supplemented diets in female Guppy (Poecilia reticulata). *Fish & Shellfish Immunology*, *97*, 96–99. https://doi.org/10.1016/J.FSI.2019.12.007

Natasya-Ain, R., Eirna-Liza, N., Jasmin, M. Y., & Karim, M. (2018). Antibacterial activity of garlic extracts on fish pathogenic bacteria. *Journal of Environmental Biology*, *39*(5), 808–812. https://doi.org/10.22438/jeb/39/5(SI)/25

Nya, E. J., Dawood, Z., & Austin, B. (2010). The garlic component, allicin, prevents disease caused by Aeromonas hydrophila in rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of Fish Diseases*, *33*(4), 293–300. https://doi.org/10.1111/J.1365-2761.2009.01121.X

Omar, A. E., Al-Khalaifah, H. S., Mohamed, W. A. M., Gharib, H. S. A., Osman, A., Al-Gabri, N. A., & Amer, S. A. (2020). Effects of Phenolic-Rich Onion (Allium cepa L.) Extract on the Growth Performance, Behavior, Intestinal Histology, Amino Acid Digestibility, Antioxidant Activity, and the Immune Status of Broiler Chickens. *Frontiers in Veterinary Science*, *7*, 728.

https://doi.org/10.3389/FVETS.2020.582612

Omer, H. A. A., Ahmed, S. M., Abdel-Magid, S. S., El-Mallah, G. M. H., Bakr, A. A., & Fattah, M. M. A. (2019). Nutritional impact of inclusion of garlic (Allium sativum) and/or onion (Allium cepa L.) powder in laying hens' diets on their performance, egg quality, and some blood constituents. *Bulletin of the National Research Centre*, *43*(1), 1–9. https://doi.org/10.1186/s42269-019-0061-6

Peinado, M. J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L. A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Animal Feed Science and Technology*, *181*(1–4), 87–92. https://doi.org/10.1016/j.anifeedsci.2013.03.001

Peinado, M. J., Ruiz, R., Echavarri, A., & Rubio, L. A. (2012). Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. *Poultry Science*, *91*(9), 2148–2157. https://doi.org/10.3382/ps.2012-02280

Pérez-Sánchez, T., Mora-Sánchez, B., & Balcázar, J. L. (2018). Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends in Microbiology*, *26*(11), 896–903. https://doi.org/10.1016/j.tim.2018.05.002

Piazzon, M. C., Naya-Català, F., Simó-Mirabet, P., Picard-Sánchez, A., Roig, F. J., Calduch-Giner, J. A., … Pérez-Sánchez, J. (2019). Sex, age, and bacteria: How the intestinal microbiota is modulated in a protandrous hermaphrodite fish. *Frontiers in Microbiology*, *10*(OCT), 1–15. https://doi.org/10.3389/fmicb.2019.02512

Rabelo-Ruiz, M., Ariza-Romero, J. J., Zurita-González, M. J., Martín-Platero, A. M., Baños, A., Maqueda, M., … Peralta-Sánchez, J. M. (2021a). Allium-based phytobiotic enhances egg production in laying hens through microbial composition changes in ileum and cecum. *Animals*, *11*(2), 1–15. https://doi.org/10.3390/ani11020448

Rabelo-Ruiz, M., Newman-Portela, A. M., Peralta-Sánchez, J. M., Martín-Platero, A. M., del Mar Agraso, M., Bermúdez, L., … Martínez-Bueno, M. (n.d.). Beneficial shifts in the gut bacterial community of juvenile gilthead seabream (Sparus aurata) supplemented with Allium-Derived Compound Propyl Propane Thiosulfonate (PTSO). *Unpublished Manuscript*.

Rabelo-Ruiz, M., Teso-Pérez, C., Peralta-Sánchez, J. M., Ariza, J. J., Martín-Platero, A. M., Casabuena-Rincón, Ó., … Martínez-Bueno, M. (2021b). Allium extract implements weaned piglet's productive parameters by modulating distal gut microbiota. *Antibiotics*, *10*(3), 1–15. https://doi.org/10.3390/antibiotics10030269

Resende, J. A., Silva, V. L., Fontes, C. O., Souza-Filho, J. A., Lopes, T., Oliveira, R. De, … Diniz, C. G. (2012). Multidrug-Resistance and Toxic Metal Tolerance of Medically Important Bacteria Isolated from an Aquaculture System. *Microbes Environ*, *27*(4), 449–455. https://doi.org/10.1264/jsme2.ME12049

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*, *433*, 50–61. https://doi.org/10.1016/j.aquaculture.2014.05.048

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*(10). https://doi.org/10.7717/PEERJ.2584

Romero, J., Feijoo, C. G., & Navarrete, P. (2012). Antibiotics in Aquaculture – Use, Abuse and Alternatives. *Health and Environment in Aquaculture*. InTech.

https://doi.org/10.5772/28157

Rubio, L. A., Peinado, M. J., Ruiz, R., Suárez-Pereira, E., Mellet, C. O., & Fernández, J. M. G. (2015). Correlations between changes in intestinal microbiota composition and performance parameters in broiler chickens. *Journal of Animal Physiology and Animal Nutrition*, *99*(3), 418–423. https://doi.org/10.1111/JPN.12256

Ruiz, R., Peinado, M. J., Aranda-Olmedo, I., Abecia, L., Suárez-Pereira, E., Melle, C. O., … Rubio, L. A. (2015). Effects of feed additives on ileal mucosa-associated microbiota composition of broiler chickens. *Journal of Animal Science*, *93*(7), 3410–3420. https://doi.org/10.2527/jas.2015-8905

Sánchez, C. J., Martínez-Miró, S., Ariza, J. J., Madrid, J., Orengo, J., Aguinaga, M. A., … Hernández, F. (2020). Effect of Alliaceae Extract Supplementation on Performance and Intestinal Microbiota of Growing-Finishing Pig. *Animals*, *10*(9), 1–15. https://doi.org/10.3390/ANI10091557

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology*, *12*(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Shannon, C. E. (1948). A Mathematical Theory of Communication. *The Bell System Technical Journal*, *27*, 379-423,623-656.

Sharifuzzaman, S. M., Abbass, A., Tinsley, J. W., & Austin, B. (2011). Subcellular components of probiotics Kocuria SM1 and Rhodococcus SM2 induce protective immunity in rainbow trout (Oncorhynchus mykiss, Walbaum) against Vibrio anguillarum. *Fish & Shellfish Immunology*, *30*(1), 347–353. https://doi.org/10.1016/J.FSI.2010.11.005

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J. J., Baños-Arjona, A., Exposito-Ruiz, M., & Gutierrez-Fernandez, J. (2018). In vitro antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from allium spp. Against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples. *BioMed Research International*, *2018*, 7861207. https://doi.org/10.1155/2018/7861207

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Gil-Martinez, L., Ariza-Romero, J. J., Maroto-Tello, A., … Gutierrez-Fernandez, J. (2021). Antibacterial and antifungal activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate, two organosulfur compounds from allium cepa: In vitro antimicrobial effect via the gas phase. *Pharmaceuticals*, *14*(1), 1–17. https://doi.org/10.3390/ph14010021

Stratev, D., Zhelyazkov, G., Noundou, X. S., & Krause, R. W. M. (2018). Beneficial effects of medicinal plants in fish diseases. *Aquaculture International*, *26*(1), 289–308. https://doi.org/10.1007/s10499-017-0219-x

Talpur, A. D., & Ikhwanuddin, M. (2012). Dietary effects of garlic (Allium sativum) on haemato-immunological parameters, survival, growth, and disease resistance against Vibrio harveyi infection in Asian sea bass, Lates calcarifer (Bloch). *Aquaculture*, *364–365*, 6–12. https://doi.org/10.1016/j.aquaculture.2012.07.035

United Nations, Department of Economic and Social Affairs, Population Division. (2019). *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423)*.

U.S. FDA. (2016). FDA Reminds Retail Establishments of Upcoming Changes to the Use of Antibiotics in Food Animals. *U.S. Food and Drugs Administration*.

Valenzuela-Gutiérrez, R., Lago-Lestón, A., Vargas-Albores, F., Cicala, F., & Martínez-Porchas, M. (2021). Exploring the garlic (Allium sativum) properties for fish aquaculture. *Fish Physiology and Biochemistry*, *47*(4), 1179–1198. https://doi.org/10.1007/S10695-021-00952-7

Vázquez-Baeza, Y, Gonzalez, A., Smarr, L., McDonald, D., Morton, J. T., Navas-Molina, J. A., & Knight, R. (2017). Bringing the Dynamic Microbiome to Life with Animations. *Cell Host & Microbe*, *21*(1), 7–10. https://doi.org/10.1016/J.CHOM.2016.12.009

Vázquez-Baeza, Yoshiki, Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

Vezza, T., Garrido-Mesa, J., Diez-Echave, P., Hidalgo-García, L., Ruiz-Malagón, A. J., García, F., … Rodríguez-Cabezas, M. E. (2021). Allium-Derived Compound Propyl Propane Thiosulfonate (PTSO) Attenuates Metabolic Alterations in Mice Fed a High-Fat Diet through Its Anti-Inflammatory and Prebiotic Properties. *Nutrients*, *13*(8), 2595. https://doi.org/10.3390/nu13082595

Vidanarachchi, J., Sims, I. M., & Iji, P. (2005). Phytobiotics: Alternatives to antibiotic growth promoters in monogastric animal feed. *Recent Advances in Animal Nutrition in Australia*, *15*, 131–144.

Wiklund, T. (2016). Pseudomonas anguilliseptica infection as a threat to wild and farmed fish in the Baltic Sea. *Microbiology Australia*, *37*(3), 135. https://doi.org/10.1071/MA16046

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, *86*(14), E140–E148. https://doi.org/10.2527/jas.2007-0459

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B. A., & Arockiaraj, J. (2020). Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. *Reviews in Aquaculture*, *12*(3), 1903–1927. https://doi.org/10.1111/RAQ.12416

Zare, M., Tran, H. Q., Prokešová, M., & Stejskal, V. (2021). Effects of Garlic Allium sativum Powder on Nutrient Digestibility, Haematology, and Immune and Stress Responses in Eurasian Perch Perca fluviatilis Juveniles. *Animals*, *11*(9), 2735. https://doi.org/10.3390/ANI11092735

Zhu, Z., Hang, S., Zhu, H., Zhong, S., Mao, S., & Zhu, W. (2013). Effects of garlic oil on milk fatty acid profile and lipogenesis-related gene expression in mammary gland of dairy goats. *Journal of the Science of Food and Agriculture*, *93*(3), 560–567. https://doi.org/10.1002/jsfa.5829

Zivkovic, V., Stankovic, B., Radovic, C., Gogic, M., Stanojkovic, A., Obradovic, S., & Stojiljkovic, N. (2019). Garlic as alternative for antibiotics in diet for growing pigs. *Biotechnology in Animal Husbandry*, *35*(3), 281–287. https://doi.org/10.2298/bah1903281z

CAPÍTULO VI

Effects of dietary inclusion of crude or hydrolysed *Arthrospira platensis* on intestinal microbiota in gilthead seabream (*Sparus aurata*) fry

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ABSTRACT

Aquaculture production plays an important role in supplying the world demand for food and protein sources. Fish feed has traditionally been made from fishmeal and fish oil, but the industry is currently looking for more sustainable alternatives. In this context, microalgae and cyanobacteria have been proposed as good alternatives for aquafeeds. Therefore, this study evaluates the effect of dietary inclusion of crude or hydrolyzed *Arthrospira platensis* biomass on the growth and intestinal microbiota of gilthead seabream (*Sparus aurata*) fry. A 45-day feeding trial was carried out using four experimental diets containing 5% and 10% (w/w) crude or hydrolyzed *A. platensis* as well as a cyanobacteria-free diet as the control. At the end of the feeding trial, individual body weight and length were recorded to evaluate growth, and the intestines were dissected to analyze the microbiota. None of the dietary treatments evaluated had any effect on fish growth. Diets including 5% crude or hydrolyzed biomass did not produce any changes in either alpha or beta diversity. Fish supplemented with 10% *A. platensis* hydrolysate only showed significant differences with respect to control fish in minority OTUs of the intestinal microbiota (Unweighted UniFrac). However, the inclusion of 10% crude biomass of this cyanobacterium showed a decrease in intestinal alpha diversity and produced significant changes in majority OTUs of the bacterial community (Weighted UniFrac), with diet showing the greatest difference with respect to control fish. These results suggest that 5% crude biomass or 5% or 10% hydrolyzed *A. platensis* biomass supplemented in the diet of gilthead seabream fry could be useful in order to reduce the use of fishmeal and fish oil in starter feeds while maintaining the fish's overall condition.

Keywords: *Arthrospira platensis*, fish nutrition, gut microbiota, microalgae hydrolysate, *Sparus aurata*, spirulina.

INTRODUCTION

Global fish production reached 179 million t in 2018, where 82 million t came from aquaculture production (FAO, 2020). In the same year, the production of aquaculture had an estimated value of US$ 250 billion. Aquaculture accounts for half of the fish for human consumption, and plays an important role in supplying the world demand for food and protein sources. Regarding fish aquaculture, some of the most produced species in the European Union include Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*) (APROMAR, 2020).

In fish production, the early life-cycle stages constitute a critical phase in fish development. During this period, fish undergo drastic morphological and physiological changes that determine their viability in later stages (Torres et al., 2020). Weaning is one of the most critical stages in marine fish rearing, where optimum quality inert diets should be used. These diets must be attractive, tasty, economical, suitable fit for fish mouth size, and provide necessary nutrients for larval or fry growth and development (Vizcaíno et al., 2016). From a nutritional point of view, these diets must contain an adequate fatty acid profile, especially for n-3 and n-6 polyunsaturated fatty acids and high protein levels (Vizcaíno et al., 2016). These nutritional requirements are currently covered by fishmeal and fish oil as the main sources of protein and fatty acids, as well as by plant ingredients such as soybean meal (Camacho-Rodríguez et al., 2018; Oliva-Teles et al., 2015). In recent years, there has been great interest in using microalgae as a sustainable alternative to reduce or replace the use of unsustainable feedstuff for preparing aquafeeds such as fishmeal or fish oil (Shah et al., 2018). Several species of microalgae (including some species of cyanobacteria) have been used in weaning diets, being a promising feed ingredient due to its high micronutrient and macronutrient content, such as polyunsaturated fatty acids and protein (Roy & Pal, 2014). Species of the genus *Arthrospira* present balanced amino acid and fatty acid profiles and high contents of proteins, vitamins, minerals and bioactive compounds (Niccolai et al., 2019). These *Arthrospira* species, mainly *A. platensis,* have been successfully used as ingredients in aquafeeds for several fish species such as rainbow trout (*Oncorhynchus mykiss*) (Güroy et al., 2019), catfish (*Ompok pabda*) (Akter et al., 2021; Liu et al., 2019) and gilthead seabream (*S.aurata*) (Galafat et al., 2020). However, including microalgae in the diet of early life stages of fish is difficult because the fish larvae cannot digest dietary compounds

like juvenile or adult fish, given that fish larvae lack the enzymatic activity necessary for digesting the formulated diets (Khoa et al., 2019; Zambonino Infante & Cahu, 2001).

In this sense, protein hydrolysates in fish diets during early life stages could help improve the maturation of the digestive organs, as well as nutrient digestion and assimilation (Srichanun et al., 2014). These benefits could be related to the mixture of free amino acids, di-, tri-, and oligopeptides obtained after enzymatic hydrolysis, which are more easily digestible by intestinal cells (Galafat et al., 2020). Several studies have shown the positive effects on survival, health status and productive parameters of dietary inclusion of protein hydrolysates during early life stages of marine and freshwater fish (Egerton et al., 2020; Refstie et al., 2004; Srichanun et al., 2014; Zheng et al., 2012). However, most of the protein hydrolysates used in the research are of animal origin, mainly from fish, although some are of plant origin. In this regard, and taking into account the aforementioned interest in using microalgae as dietary ingredient for fish diets, protein hydrolysates obtained from microalgae could be used as an alternative in order to reduce or replace fishmeal in weaning diets for marine fish larvae, while maintaining growth parameters. Some recent studies have demonstrated the beneficial effects of microalgae hydrolysates in aquafeeds. A study by Valente et al. (2019) demonstrated that the use of defatted biomass from the microalga *Nannochloropsis* sp. did not affect the gut morphology or growth of European seabass (*Dicentrarchus labrax*). Recent research by Ayala et al. (2020) supplementing juvenile gilthead seabream (*S. aurata*) with 2.5% and 5% crude and hydrolyzed *Nannochloropsis gaditana* showed similar levels of feed conversion rate, specific growth, daily feed intake and survival. The beneficial effects of *Arthrospira* sp. (Cyanophyceae) have previously been demonstrated in a recent study by Galafat et al. (2020) using low doses of this microalgae species hydrolysate as an additive in juvenile gilthead seabream (*S. aurata*) diet. However, no research has studied the use of this microalga, crude or hydrolyzed, in early life stages of fish and related it to growth parameters and gut microbiota.

The results obtained in this research indicate that the inclusion of 5% and 10% of crude or hydrolyzed *A. platensis* biomass did not affect the growth performance of gilthead seabream fry after a 45-day feeding trial. Furthermore, 5% crude or hydrolyzed microalgae biomass did not produce changes in microbial diversity and composition, and 10% hydrolyzed biomass produced changes only in minority OTUs, so these diets could be suitable for use in starter feed for aquaculture species.

MATERIAL AND METHODS

Microalgae biomass

Crude *Arthrospira platensis* biomass was provided by Biorizon Biotech (Almería, Spain). *A. platensis* and *Spirulina platensis* are considered to be synonyms (Guiry & Guiry, 2021). The term microalgae refers to unicellular photosynthetic microorganisms of microscopic size, including the cyanobacterium *A. platensis* (Cyanophyceae). To obtain the microalgal protein hydrolysate from crude biomass, enzymatic hydrolysis was performed following the method described by Saadaoui et al. (2019) and modified by Galafat et al. (2020). In summary, a sludge containing 150 g/L of microalgae biomass was incubated with 0.2% w/w mixture of commercial proteases (Alcalase 2.4L® and Flavourzyme 1000L® from Novozymes A/S, Bagsvaerd, Denmark) under controlled conditions (pH 8.0 and 50 ºC with continuous stirring) for 4 h. After hydrolysis, the reaction mixture was immediately heated to 80 °C for 15 min in order to inactivate the proteolytic enzymes. The hydrolysate was kept at 4 ºC until use.

Experimental diets

Five isonitrogenous and isolipidic experimental diets were formulated. C-5 and C-10 included 5 and 10% crude microalgae biomass, respectively, and H-5 and H-10 included 5% and 10% microalgae protein hydrolysate, respectively. Additionally, a microalgae-free diet was used as control (CT). The formulation and proximate composition of these experimental diets are shown in Table 1. The diets were designed and manufactured by Ceimar-University of Almería (Service of Experimental Diets, Almería, Spain) following standard aquafeed procedures.

Fish and experimental design

Fertilized gilthead seabream eggs *(Sparus aurata*) were stocked in an incubator until hatching. Then, hatched larvae were transferred to a 5,000 L tank. Larval rearing took place at the Planta Experimental de Cultivos Marinos facility at the Instituto Español de Oceanografía (IEO, Mazarrón, Murcia, Spain). At the beginning, hatched larvae began to feed with rotifers (*Brachionus plicatilis*) from when mouth opening occurred (5 days post-hatching, dph) until 27 dph. The rotifer density was adjusted to 20 rotifers per mL. From 20 to 27 dph, *Artemia nauplii* was introduced in the tank at a concentration of 1-3 per mL, and *Artemia metanauplii* was introduced in the tank at 26 dph until the end of the

weaning period (50 dph). From 40 to 50 dph, larvae were progressively weaned by a feeding regime based on *A. nauplii* and inert diet (Skretting commercial feed). *Artemia* was progressively reduced from 3 to 0.5 *Artemia* per mL, while inert feed was progressively increased. After weaning, the gilthead seabream larvae were transferred to 170 L tanks (510 larvae per tank, with a density of 3 larvae per L) in an open circulation system five days before the feeding trial. The water was sterilized by UV, adjusted to 20-23 ºC and provided to the tank system at 150 L/hour. The photoperiod was fixed at a 14:10 (light:dark) cycle (450 lx). Dissolved oxygen was maintained above 6.5 mg/L by supplemental aeration. Nitrate (<50 mg/L), nitrite (<0.2 mg/L) and ammonia (<0.1 mg/L) levels were determined once weekly.

Table 1. Ingredients and proximate composition (g/kg on dry matter) of the experimental diets.

	CT	C-5	C-10	H-5	H-10
Ingredients					
Fishmeal LT94[1]	685.0	641.0	597.0	641.0	597.0
***A. platensis* meal**[2]		50.0	100.0		
***A. platensis* protein hydrolysate**[3]				50.0	100.0
Attractant premix[4]	80.0	80.0	80.0	80.0	80.0
Wheat gluten[5]	50.0	50.0	50.0	50.0	50.0
Soybean protein concentrate[6]	20.0	20.0	20.0	20.0	20.0
Fish oil	53.0	55.0	58.0	55.0	58.0
Soybean lecithin[7]	40.0	40.0	40.0	40.0	40.0
Choline chloride	2.0	2.0	2.0	2.0	2.0
Wheat meal[8]	17.0	9.0		9.0	
Betaine	2.0	2.0	2.0	2.0	2.0
Vitamins and minerals premix[9]	30.0	30.0	30.0	30.0	30.0
Vitamin C	1.0	1.0	1.0	1.0	1.0
Binder (alginate)	20.0	20.0	20..0	20..0	20.0
***Proximate composition* (% dry weight)**					
Crude protein	58.9	59.1	59.3	59.3	59.4
Crude lipid	18.1	18.0	17.9	18.4	18.3
Ash	16.0	16.0	15.7	15.8	16.1
Crude fiber	2.1	1.9	2.3	2.0	2.2
NfE[10]	4.9	5.0	4.8	4.5	4.0

Dietary treatment codes are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* protein hydrolysate - supplemented diet; H-10: 10% *A. platensis* protein hydrolysate-supplemented diet. [1](protein: 69.4%; lipid: 12.3%), Norsildemel (Bergen, Norway); [2](protein: 60.5%; lipid: 5.6%); [3]Liquid product containing 150 g microalgae meal L^{-1}; [4](50% squid meal, 25% shrimp meal, 25% krill meal); [5](protein: 76.0%; lipid: 1.9%); [6](protein: 50.0%; lipid: 1.0%); [7]Lecico P700 IP (Lecico GmbH, Germany) [8](protein: 12.0%; lipid: 2.0%); [9]Vitamin & Mineral Premix: Vitamins (IU or mg/kg premix): vitamin A (retinyl acetate), 2,000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E , 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2,500 mg; vitamin B1(thiamine hydrochloride), 3,000 mg; vitamin B2 (riboflavin), 3,000 mg; calcium

pantothenate, 10,000 mg; nicotinic acid, 20,000 mg; vitamin B6 (pyridoxine hydrochloride), 2,000 mg; vitamin B9 (folic acid), 1,500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (Lifebioencapsulation SL, Almería Spain); ^{10}NfE: Nitrogen free extract calculated as 100 – (% crude protein + % ether extract + % ash + % crude fiber).

From 55 dph, fry were fed *ad libitum* exclusively with the experimental diets six times daily. The different dietary treatments (CT, C-5, C-10, H-5, H-10) were randomly assigned to the experimental tanks. Each dietary treatment was tested for 45 days (until 100 dph). Feeding rate and feed size were adjusted according to fish age, larval weight and water temperature, following the recommendations for gilthead seabream fry provided by Skretting España (Burgos, Spain).

Fish sampling

At the beginning of the experimental trial (55 dph), larvae were individually weighed and measured after 12 h fasting. At the end of the feeding trial (100 dph), fasted fish from each tank were randomly sampled and 24 fasted fish per treatment were euthanized by overdose of anesthesia (50 ppm clove oil) according to the requirements of the Council Directive 2010/63/UE. The fish were dissected and the whole intestine was collected with sterile material. The intestines were stored in sterile tubes and transported to the laboratory, where they were kept at -80 ºC until DNA extraction.

DNA extraction

DNA extraction of the whole intestine from the gilthead seabream fry was carried out following the Modified Salting Out Procedure (MSOP) by Martín-Platero et al. (2007), modified with an initial mechanical lysis step using the cell disrupter FastPrep FP120 (BIO 101, Thermo Savant). Briefly, intestines were introduced into a 2 mL microcentrifuge tube filled with approximately 100 mg of 2 mm zirconia ceramic beads and homogenized by two 30-second pulses at speed 5 in FastPrep FP120. Then, the MSOP protocol was followed. DNA extraction was checked by 0.7% agarose gel electrophoresis and DNA concentration was measured using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). Samples were stored at -20 ºC until DNA amplification.

V4 16S rRNA gene amplification and high-throughput sequencing

V4 region of 16S rRNA gene libraries were constructed using the primer pair U515F (5´TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3´) and E786R (5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3´) with Illumina adapter overhang sequences indicated by the underlining. This PCR was carried out with 12.5 µL of iProof™ High-Fidelity DNA Polymerase (BioRad®), 0.3 µM of each primer and 5 µL of template DNA in a final volume of 25 µL. The amplification program consisted of an initial denaturing step of 98 ºC for 1 min followed by an amplification step of 25 cycles of 10 s at 98 ºC, 20 s at 52 ºC and 15 s at 72 ºC, and a final extension step of 5 min at 72 ºC. After that, the purified PCR amplicons were used as template for a second PCR in order to index two unique Illumina compatible barcodes to each sample, so that the derived sequences could be demultiplexed into their respective samples in downstream analysis. These barcodes overlapped with the sequence of the primers used in the first PCR. This second PCR was carried out in a final volume of 25 µL, containing 12.5 µL of iProof™ High-Fidelity DNA Polymerase (BioRad®), 0.4 µM of each primer, 5 µL of DNA and water to a final volume of 25 µL. The amplification program consisted of an initial denaturing step of 98 ºC for 1 min followed by an amplification step of 8 cycles of 10 s at 98 ºC, 20 s at 55 ºC, and 15 s at 72 ºC, and a final extension step of 5 min at 72 ºC. Purification steps were made using DNA Purification SPRI Magnetic Beads (Canvax®) following the manufacturer's instructions. PCR amplicons were checked by 1% agarose gel electrophoresis, DNA concentrations were measured using Qubit® 3.0 Fluorometer (Invitrogen™, Carlsbad, CA) and pooled in equimolar concentrations. High-throughput sequencing was carried out with Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), resulting in paired-end reads of 2 x 300 bp length. Sequencing was carried out on the Illumina MiSeq platform at the Institute of Parasitology and Biomedicine "López-Neyra" (Granada, Spain). Sequences are available in the Sequence Read Archive (SRA) in the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra/), BioProject: PRJNA795922, Accession Nos. SAMN24803029 to SAMN24803111.

Sequences processing and data analysis

The Quantitative Insights Into Microbial Ecology (QIIME2 v2021.4; Bolyen et al., 2019) software was used to analyze the 16S rRNA sequences generated from Illumina MiSeq. First, primer trimming was performed using Cutadapt plugin default parameters (Martin, 2011). Pair joining was performed using VSEARCH default parameters (Rognes et al., 2016). Sequences with more than 3 consecutives Phred scores lower than 20 were filtered out from the analyses in the quality filtering step. Then Deblur, a sub-operational-taxonomic-unit (sub-OTU) approach, was used in order to remove sequencing errors (Amir et al., 2017). The fragment insertion script implemented in QIIME2 was used in order to align the sequences and build a de-novo bacterial phylogenetic tree (Janssen et al., 2018). Taxonomy assignation was performed using the Greengenes 13.8 database with a similarity of 99% (DeSantis et al., 2006). Finally, reads of chloroplast, mitochondria or eukaryotes were also excluded by filtering the OTU table.

Statistics

For alpha diversity analysis, the OTU table was rarified at 10,000 sequences depth per sample. Samples that did not reach this sequencing depth were excluded from subsequent analyses. We calculated four alpha diversity indexes from the OTU table: Shannon diversity index (Shannon, 1948), Faith's phylogenetic diversity index (Faith & Baker, 2006), Chao1 index (Chao, 1984) and bacterial OTU richness (or number of observed species). These analyses were performed using STATISTICA 10.0 (StatSoft).

Beta diversity distance matrixes were calculated using UniFrac distance based on the rarified OTU table at 10,000 sequence depth per sample. Both Weighted and Unweighted UniFrac indices (Lozupone et al., 2007; Lozupone & Knight, 2005) were used for subsequent analysis. Weighted UniFrac gives more importance to the most abundant OTUs, while Unweighted UniFrac gives more importance to minority OTUs as it takes into account their presence or absence independently of their abundance. Permutational ANOVA (PERMANOVA) was performed in order to test the effect of different diets on both UniFrac distance matrixes, using PRIMER-7 software (PRIMER-e), implemented with PERMANOVA plugin. Principal Coordinate Analyses (PCoA) were performed in order to visualize the 2 first axes using EMPeror 2021.4.0 (Vázquez-Baeza et al., 2017; Vázquez-Baeza et al., 2013).

RESULTS

Effect of diet on growth performance

The inclusion of 5% and 10% crude and hydrolyzed *A. platensis* did not affect gilthead seabream fry growth. In fact, no differences appeared among the different experimental groups in any of the growth parameters studied, such as final body weight (GLMM, treatment as factor, p = 0.239) and final body length (GLMM, treatment as factor, p = 0.373) (Figure 1, Table 2).

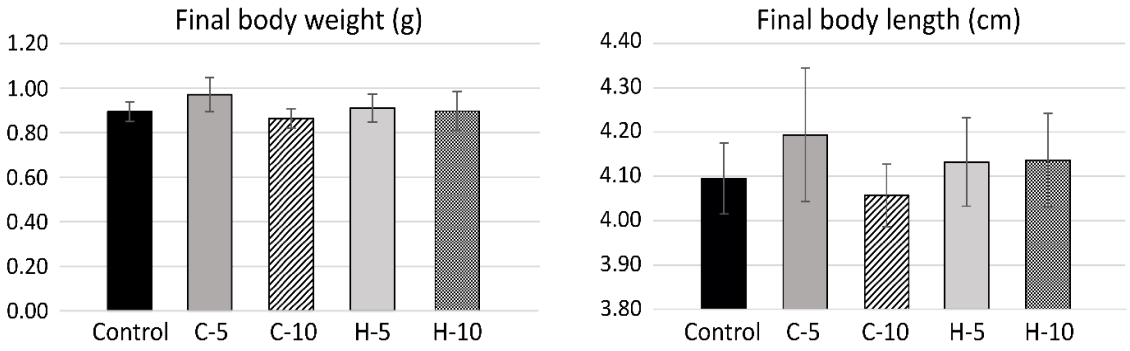

Figure 1. Final fish body weight and body length of European seabass fry fed with control diet or supplemented with 5% crude microalgae (C-5), 10% crude microalgae (C-10), 5% microalgae hydrolysate (H-5) and 10% microalgae hydrolysate (H-10) (mean ± standard deviation).

Table 2. Weight and length of gilthead seabream fry fed experimental diets at the end of the 45-day feeding trial. Dietary treatments are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet. Values are mean ± Standard Error of triplicate tanks. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one is for the error term. Values in the same row with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

	CT	C-5	C-10	H-5	H-10	D.f.	F	*p*
Final body weight (g)	0.90 ± 0.03[a]	0.97 ± 0.04[a]	0.87 ± 0.03[a]	0.91 ± 0.04[a]	0.90 ± 0.05[a]	4,10	1.08	0.416
Final body length (cm)	4.10 ± 0.05[a]	4.19 ± 0.09[a]	4.06 ± 0.04[a]	4.13 ± 0.06[a]	4.14 ± 0.06[a]	4,10	0.70	0.608

Bacterial community composition

The gut microbiota of gilthead seabream fry is dominated at class level by Alphaproteobacteria (71%), followed by Gammaproteobacteria (21%) and Bacilli (5%). The relative abundance of these phyla was similar in fish feed diets supplemented with microalgae, but small differences in gut composition appeared between the different treatments (Figure 2). However, the microbiota of fish supplemented with 10% crude biomass (C-10) was the one that differed the most with respect to control fish, showing an increase in Alphaproteobacteria (83%) and a decrease in Gammaproteobacteria (14%) and Bacilli (1%).

At the genus level, the intestines of gilthead seabream fry were dominated by *Ochrobactrum*, *Pseudomonas* and *Vibrio*. The relative abundance of these genera was similar in all experimental groups, with the microbiota of fish fed with diet C-10 being the one that differed most with respect to control fish (Figure 3). These fish showed an increase of the majority genus *Ochrobactrum* and a small decrease in minority genus such as *Vibrio* and an unknown genus of Bacillales (Figure 3).

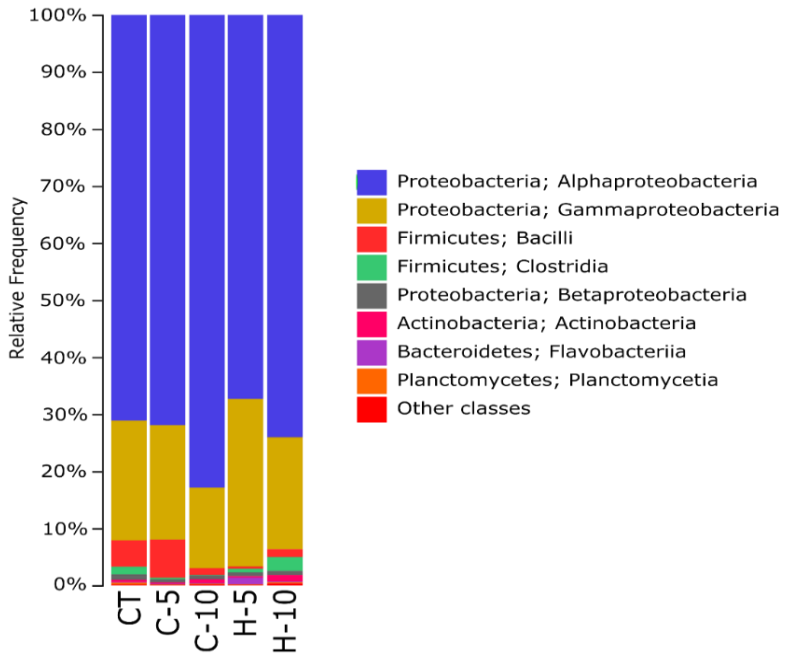

Figure 2. Barplot of the relative bacterial abundance at the class level in fish fed on the different dietary treatments. Each bar represents the mean of the relative abundance for the different bacterial classes in the samples from each treatment. Dietary treatments are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

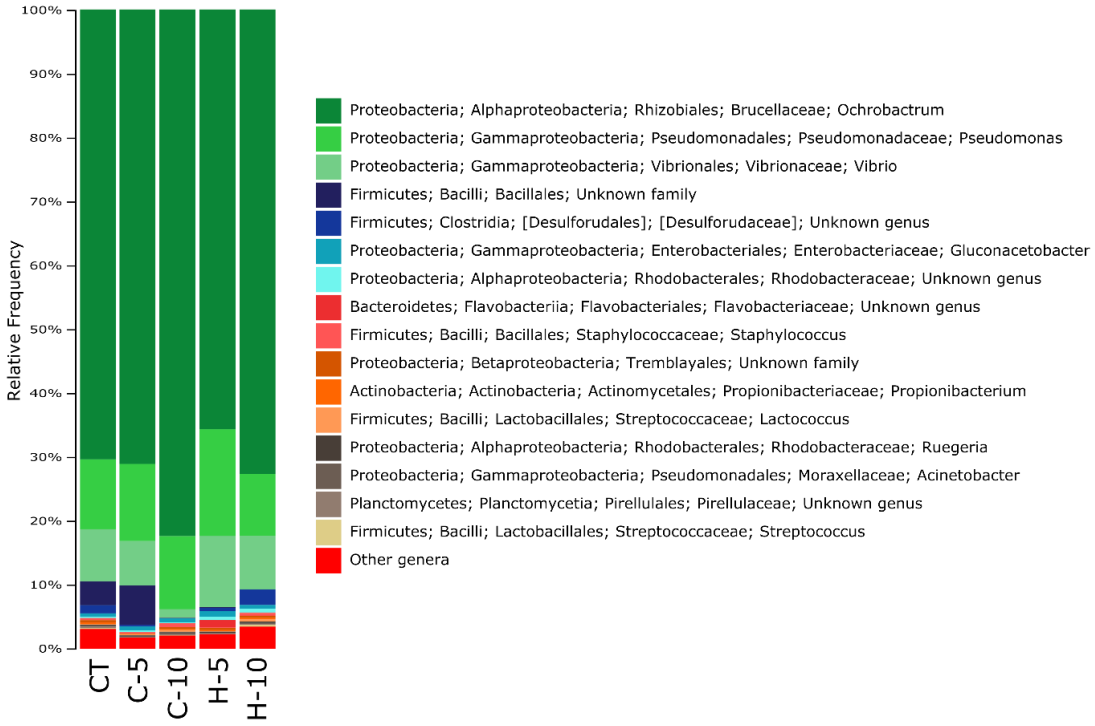

Figure 3. Barplot of the relative bacterial abundance at the genus level in fish fed on the different dietary treatments. Each bar represents the mean of the relative abundance of the different bacterial genera in the samples from each treatment. Dietary treatments are CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet.

Effect of diet on Alpha and Beta Diversity

The supplementation of crude and hydrolyzed microalgae in the diet of gilthead seabream fry did not affect bacterial OTU richness (Table 3). Regarding Shannon's alpha diversity index, C-10-fed fish showed a decrease in this index with respect to the control and the rest of experimental groups after the 45-day feeding trial (Table 3).

At the end of the feeding trial, the bacterial community of gilthead seabream fry varied significantly between different dietary treatments, taking into account both the most abundant (Weighted UniFrac) and minority OTUs (Unweighted UniFrac) (Table 4). Regarding the most abundant OTUs, fish supplemented with 10% crude biomass (C-10) were the only ones showing changes in the bacterial community with respect to control fish (LSD Post-hoc, $p = 0.002$). Fish fed on other experimental diets did not differ with respect to the control (LSD Post-hoc, $p > 0.231$). Regarding minority OTUs, the only diet that produced changes in gut bacterial communities with respect to the control was that including 10% hydrolyzed biomass (H-10) (LSD Post-hoc, $p = 0.022$). No differences

appeared between the control and fish fed on the other experimental diets (LSD Post-hoc, $p > 0.183$). These differences in diet cannot be observed graphically in the PCoA since it does not take into account 100% of the variance (69.35% in Weighted UniFrac and 22.35% in Unweighted UniFrac) (Figure 4).

Table 3. General Linear Mixed Models exploring the effects of the treatment (CT, C-5, C-10, H-5 and H-10) as factor in the different alpha diversity indices of the bacterial intestinal community in gilthead seabream fry. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one is for the error term. Significant *p*-values ($p < 0.05$) are shown in bold.

Alpha Diversity Index	Explanatory Variables	D.f.	F	*p*
Shannon's Diversity Index	Treatment	4,77	4.25	**0.004**
Bacterial OTU richness	Treatment	4,77	1.10	0.363

Table 4. General Linear Mixed Models exploring the effects of treatment in beta diversity indices of bacterial community in juvenile gilthead seabream fry fed on control or 5% and 10% of crude or hydrolysed *A. platensis*-supplemented diets. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one is for the error term. Significant *p*-values ($p < 0.05$) are shown in bold.

β-Diversity Distance Matrix	Explanatory Variables	D.f.	Pseudo-F	*p*
Weighted UniFrac	Treatment	4,77	3.22	**0.003**
Unweighted UniFrac	Treatment	4,77	1.28	**0.035**

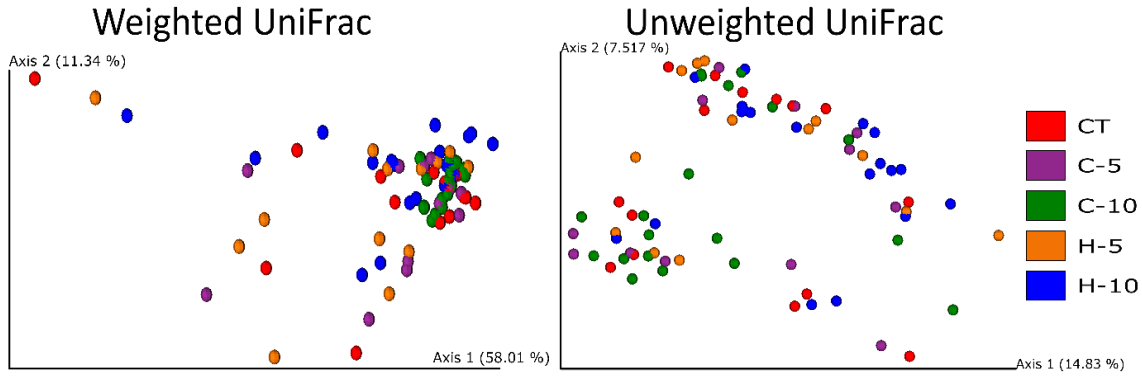

Figure 4. Principal Coordinate Analysis based in Weighted UniFrac (A) and Unweighted UniFrac (B) distance matrixes exploring the effects in the bacterial gut community of the supplementation with *A. platensis* in gilthead seabream fry' diet (CT: control diet, C-5: 5% crude *A. platensis*-supplemented diet, C-10: 10% crude *A. platensis*-supplemented diet; H-5: 5% *A. platensis* hydrolysate-supplemented diet; H-10: 10% *A. platensis* hydrolysate-supplemented diet). Percentages show the proportion of variance explained by each axis.

Capítulo VI

DISCUSSION

This study found that dietary inclusion of 5% and 10% of crude and hydrolyzed *A. platensis* in gilthead seabream fry did not affect growth performance. Furthermore, inclusion of 5% crude and hydrolyzed *A. platensis* biomass did not produce changes in intestinal microbial diversity and composition, and 10% hydrolyzed biomass only produced changes in minority OTUs of the bacterial communities. However, the inclusion of 10% crude biomass caused a reduction in alpha diversity and changes in majority OTUs.

Total fish production has increased in the last few decades, with aquaculture being of great importance and reaching worldwide production of 82 million t in 2018 (FAO, 2020). Furthermore, the global demand for fish products is expected to continue increasing significantly in the years to come, due to the increase in world population, which is expected to reach almost 11 billion in 2100 (FAO, 2020). This increase in aquaculture production together with finite supplies and the unsustainability of fishmeal makes it necessary to find sustainable alternative protein sources for the aquafeed sector to be able to maintain its productive parameters (Rimoldi et al., 2020). Various sources of protein have been proposed as alternatives, with plant feedstuffs being the most commonly used in aquaculture as they are cheap and available in large quantities compared to fishmeal (Daniel, 2018; Gasco et al., 2018). However, single cell proteins, including bacteria, microalgae and yeasts, represent alternative non-conventional nitrogen sources that can be used as dietary ingredients for aquafeeds with a high content of valuable and bioactive compounds (Rimoldi et al., 2020). The inclusion of different microalgae and cyanobacteria species in feed have been successfully evaluated as fish dietary supplements, noting positive effects on fish health and performance in different life stages (Roy & Pal, 2014). However, few studies have considered the effect of dietary microalgae inclusion in starter diets in marine fish, obtaining mixed results. Dietary supplementation of *A. platensis* showed no differences in growth performance but enhanced immune activity in yellow catfish (*Pelteobagrus fulvidraco*) (Liu et al., 2020). Another study by Vizcaíno et al. (2016) showed increased growth performance, nutrient absorption and utilization, and survival of gilthead seabream fry fed a diet supplemented with 5% *Tetraselmis suecica*, but 5% inclusion of *Tisochrysis lutea* induced a significant reduction in fish growth and nutrient utilization. Including 3% *Phaeodactylum* sp., *Nannochloropsis* sp., *and Chlorella* sp. in aquafeed improves the growth performance of

Senegalese sole (*Solea senegalensis*) fry (Peixoto et al., 2021). Our results found that dietary inclusion of 5% and 10% crude and hydrolyzed *A. platensis* did not affect fish body weight and body length after a 45-day feeding trial.

Enzymatic hydrolysis techniques have been developed to improve the functional and nutritional properties of by-products, by degrading native proteins into smaller biologically active peptides (Wu et al., 2021). The enzymatic hydrolysis treatment has demonstrated some advantages in nutrient digestibility and bioavailability of fish proteins (Siddik et al., 2021). As well as the positive effects of including the protein hydrolysate in aquafeeds, an improvement in growth parameters could be expected. Recent studies have indicated that protein hydrolysates in aquatic animals' diet act as growth promoters, improving animals' health and enhancing the utilization efficiency of nutrients, making them a good alternative to fishmeal (Leduc et al., 2018). Given the results obtained with fish protein hydrolysates, enzymatic treatment of microalgae biomass has arisen as a novel strategy for aquafeeds. Some studies have demonstrated an increase in nutrient quality and bioavailability after hydrolysis of different microalgae species (Agboola et al., 2019; Teuling et al., 2019). In a recent study by Galafat et al. (2020), *Arthrospira* sp. protein hydrolysate increased intestinal enzyme activity and improved the intestinal health of gilthead seabream juveniles, but no differences appeared in growth performance. In addition, Ayala et al. (2020) did not observe changes in growth parameters and feed intake in gilthead seabream juveniles influenced by dietary inclusion of crude or hydrolyzed *Nannochloropsis gaditana* either. Our results agree with these previous studies, with no differences in body growth of gilthead seabream fry appearing after a 45-day feeding trial, either with 5 or 10% crude or hydrolyzed *A. platensis* biomass.

Gut microbiota is a complex community of microorganisms, which is dynamically altered depending on several biotic (e.g., genotype, genetics, sex, age and diet) and abiotic (e.g., environmental factors like water composition and temperature) factors (Butt & Volkoff, 2019). Diet is one of the most important factors influencing the diversity and composition of the gut microbiota. The inclusion of microalgae in the fish diet has shown the influence of this supplement on intestinal microbiota (Sagaram et al., 2021). Lyons et al. (2017) investigated the effects of 5% *Schizochytrium limacinum* meal on rainbow trout (*Oncorhynchus mykiss*) distal intestinal microbiota. In this study, similar microbial taxa were observed in algae-fed and control fish, however, the algae-fed group showed higher

microbial diversity levels. Another study by Jorge et al. (2019) also showed an increase in the microbial diversity of gilthead seabream juveniles supplemented with *N. gaditana* meal. Carballo et al. (2019) investigated the effects of microalgae *Phaeodactylum tricornutum* cells and polysaccharide-enriched extract on Senegalese sole (*Solea senegalensis*) gut microbiome. The polysaccharide-enriched extract decreased microbial diversity, whereas *P. tricornutum* cells increased microbial diversity and produced a reduction in the relative proportion of *Vibrio*. Research by Rico et al. (2015) showed an increased number of species when 15% *Ulva rigida* and *Gracilaria cornea* was included, while a large reduction in microbial diversity was observed when 25% *U. rigida* was added. Results from Rosenau et al. (2021) showed that total fishmeal replacement with *A. platensis* in African catfish (*Clarias gariepinus*) produced an increase in the different alpha diversity indices studied. However, our results showed no differences in the microbial diversity of gilthead seabream fry supplemented with 5% crude *A. platensis,* or 5 or 10% hydrolyzed *A. platensis.* A reduction in alpha diversity was observed in fish fed with a diet supplemented with 10% crude microalgae biomass. Therefore, these disparate results suggest that microbial diversity depends on fish species, development stage and microalgae presentation and concentration.

Regarding the intestinal microbial composition of gilthead seabream fry, in our experiment this was mainly dominated by Alphaproteobacteria and Gammaproteobacteria. These results corroborate the findings of another study with early embryonic stages of gilthead seabream (Nikouli et al., 2019). At the genus level, the gut microbiota was dominated by *Ochrobactrum*, *Pseudomonas* and *Vibrio.* Species of the genus *Ochrobactrum* have previously been described as opportunistic pathogens in humans (Brady & Leber, 2018; Hernández-Torres et al., 2014; Ryan & Pembroke, 2020). *Ochrobactrum* spp. have been detected in gut microbiota of different fish species such as Atlantic salmon (*S. salar*), European seabass (*D. labrax*), yellow catfish (*P. fulvidraco*) and zebrafish (*Danio rerio*) (Cantas et al., 2012; Gajardo et al., 2016; Lokesh & Kiron, 2016; Nayak, 2010; Ringø et al., 2016; Wu et al., 2010), but the pathogenic effects of species of this genus on fish have not yet been described. The relative abundance of *Ochrobactrum* in the gut microbiota of gilthead seabream fry supplemented with different experimental diets was quite similar to the control, except for fish fed on the C-10 diet which showed an increase in the most abundant genus *Ochrobactrum*. A study by Ringø et al. (2006) suggested that the abundance of this bacteria in the microbiota of the

gastrointestinal tract of fish species is easily affected by dietary manipulation. In turn, *Pseudomonas* and *Vibrio* are diverse genera and among the most important bacterial genera in aquaculture (Egerton et al., 2018). Species of *Vibrio* are ubiquitous in fish species but some of them are considered to be fish pathogens and opportunistic pathogens (Abdel-Aziz et al., 2013; Pérez-Sánchez et al., 2018). The reduction of *Vibrio* in gut microbiota of fish supplemented with 10% crude biomass could be positive, but further studies are necessary to find out whether the *Vibrio* that appeared in fish microbiota correspond to pathogenic species or not.

Therefore, further research would be necessary to discover whether changes in 10% crude *A. platensis* supplemented diet on microbial diversity and changes in majority species such as *Ochrobactrum* or *Vibrio* could be beneficial or detrimental to fish health and growth. Unlike the C-10 diet, the other experimental diets did not produce major changes in the composition and diversity of the microbial communities, nor changes in gilthead seabream fry body growth. The results obtained in this study indicate the possible use of diets including 5% crude or 5% or 10% hydrolyzed *A. platensis* biomass in aquafeed for gilthead seabream fry.

CONCLUSIONS

The inclusion of crude or hydrolyzed *A. platensis* did not affect gilthead seabream fry growth compared to fish fed with the control diet. The inclusion of 5% crude or hydrolyzed biomass did not affect gut microbiota, while 10% hydrolyzed biomass only produced changes in minority OTUs. However, 10% crude biomass reduced microbial diversity and affected the most abundant intestinal bacterial OTUs. Based on the results obtained in this work, 5% crude and 5% and 10% hydrolyzed *A. platensis* biomass could be recommended as dietary ingredients in starter feeds for gilthead seabream fry. Still, further studies should be conducted in order to ascertain the optimum utilization of this microalga or its hydrolysate as well as the feasibility of incorporating it into commercial diets.

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REFERENCES

Abdel-Aziz, M., Eissa, A. E., Hanna, M., & Okada, M. A. (2013). Identifying some pathogenic Vibrio/Photobacterium species during mass mortalities of cultured Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) from some Egyptian coastal provinces. *International Journal of Veterinary Science and Medicine*, *1*(2), 87–95. https://doi.org/10.1016/j.ijvsm.2013.10.004

Agboola, J. O., Teuling, E., Wierenga, P. A., Gruppen, H., & Schrama, J. W. (2019). Cell wall disruption: An effective strategy to improve the nutritive quality of microalgae in African catfish (Clarias gariepinus). *Aquaculture Nutrition*, *25*(4), 783–797. https://doi.org/10.1111/ANU.12896

Akter, T., Hossain, A., Rabiul Islam, M., Hossain, M. A., Das, M., Rahman, M. M., … Abdel-Tawwab, M. (2021). Effects of spirulina (Arthrospira platensis) as a fishmeal replacer in practical diets on growth performance, proximate composition, and amino acids profile of pabda catfish (Ompok pabda). *Journal of Applied Aquaculture*. https://doi.org/10.1080/10454438.2021.1936740

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

APROMAR. (2020). La Acuicultura en España 2020. *Asociación Empresarial de Acuicultura de España (APROMAR)*. Retrieved from http://www.apromar.es/sites/default/files/2020/Informe Acuicultura España 2020_APROMARv1.2.pdf

Ayala, M. D., Galián, C., Fernández, V., Chaves-Pozo, E., García de la Serrana, D., Sáez, M. I., … Arizcun, M. (2020). Influence of low dietary inclusion of the microalga

nannochloropsis gaditana (Lubián 1982) on performance, fish morphology, and muscle growth in juvenile gilthead seabream (sparus aurata). *Animals*, *10*(12), 1–18. https://doi.org/10.3390/ani10122270

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(9), 1091. https://doi.org/10.1038/s41587-019-0252-6

Brady, M. T., & Leber, A. (2018). Less Commonly Encountered Nonenteric Gram-Negative Bacilli. *Principles and Practice of Pediatric Infectious Diseases*, 855-859.e3. https://doi.org/10.1016/B978-0-323-40181-4.00151-1

Butt, R. L., & Volkoff, H. (2019). Gut Microbiota and Energy Homeostasis in Fish. *Frontiers in Endocrinology*, *0*(JAN), 9. https://doi.org/10.3389/FENDO.2019.00009

Camacho-Rodríguez, J., Macías-Sánchez, M. D., Cerón-García, M. C., Alarcón, F. J., & Molina-Grima, E. (2018). Microalgae as a potential ingredient for partial fish meal replacement in aquafeeds: nutrient stability under different storage conditions. *Journal of Applied Phycology*, *30*(2), 1049–1059. https://doi.org/10.1007/s10811-017-1281-5

Cantas, L., Sørby, J. R. T., Aleström, P., & Sørum, H. (2012). Culturable Gut Microbiota Diversity in Zebrafish. *Zebrafish*, *9*(1), 26. https://doi.org/10.1089/ZEB.2011.0712

Carballo, C., Pinto, P. I. S., Mateus, A. P., Berbel, C., Guerreiro, C. C., Martinez-Blanch, J. F., … Manchado, M. (2019). Yeast β-glucans and microalgal extracts modulate the immune response and gut microbiome in Senegalese sole (Solea senegalensis). *Fish & Shellfish Immunology*, *92*, 31–39. https://doi.org/10.1016/J.FSI.2019.05.044

Chao, A. (1984). Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics*, *11*, 265–270.

Daniel, N. (2018). A review on replacing fish meal in aqua feeds using plant protein sources. *International Journal of Fisheries and Aquatic Studies*, *6*(2), 164–179.

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Egerton, S., Wan, A., Murphy, K., Collins, F., Ahern, G., Sugrue, I., … Stanton, C. (2020). Replacing fishmeal with plant protein in Atlantic salmon (Salmo salar) diets by supplementation with fish protein hydrolysate. *Scientific Reports*, *10*(1), 1–16. https://doi.org/10.1038/s41598-020-60325-7

Egerton, Sian, Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The gut microbiota of marine fish. *Frontiers in Microbiology*, *9*(MAY), 1–17. https://doi.org/10.3389/fmicb.2018.00873

Faith, D. P., & Baker, A. M. (2006). Phylogenetic Diversity (PD) and Biodiversity Conservation: Some Bioinformatics Challenges. *Evolutionary Bioinformatics*, *2*, 121–128. https://doi.org/10.1177/117693430600200007

FAO. (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in action*. Rome: FAO. https://doi.org/10.4060/ca9229en

Gajardo, K., Rodiles, A., Kortner, T. M., Krogdahl, Å., Bakke, A. M., Merrifield, D. L., & Sørum, H. (2016). A high-resolution map of the gut microbiota in Atlantic salmon

(Salmo salar): A basis for comparative gut microbial research. *Scientific Reports*, *6*(1), 1–10. https://doi.org/10.1038/srep30893

Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Jérez-Cepa, I., Mancera, J. M., & Alarcón, F. J. (2020). Evaluation of Arthrospira sp. enzyme hydrolysate as dietary additive in gilthead seabream (Sparus aurata) juveniles. *Journal of Applied Phycology*, *32*(5), 3089–3100. https://doi.org/10.1007/s10811-020-02141-0

Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., & Caruso, G. (2018). *Feeds for the Aquaculture Sector - Current Situation and Alternative Sources* (1st ed.). Springer International Publishing. https://doi.org/10.1007/978-3-319-77941-6

Guiry, M. D., & Guiry, G. M. (2021). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. Retrieved July 1, 2021, from https://ww.algaebase.org

Güroy, B., Güroy, D., Mantoğlu, S., Çelebi, K., Şahin, O. I., Kayalı, S., & Canan, B. (2019). Dietary Spirulina (Arthrospira platensis, Gomont, 1892) improved the flesh quality and shelf life of rainbow trout (Oncorhynchus mykiss, Walbaum, 1792) fed fish meal or plant-based diet. *Aquaculture Research*, *50*(9), 2519–2527. https://doi.org/10.1111/are.14206

Hernández-Torres, A., Ruiz Gómez, J., García-Vázquez, E., & Gómez-Gómez, J. (2014). Internal Medicine: Open Access Open Access Case Report Ochrobactrum Anthropi Bacteraemia: Report of Six Cases and Review of the Literature. *Intern Med*, *4*, 1. https://doi.org/10.4172/2165-8048.1000134

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., … Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MySistems*, *3*(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Jorge, S. S., Enes, P., Serra, C. R., Castro, C., Iglesias, P., Teles, A. O., & Couto, A. (2019). Short-term supplementation of gilthead seabream (Sparus aurata) diets with Nannochloropsis gaditana modulates intestinal microbiota without affecting intestinal morphology and function. *Aquaculture Nutrition*, *25*(6), 1388–1398. https://doi.org/10.1111/ANU.12959

Khoa, T. N. D., Waqalevu, V., Honda, A., Shiozaki, K., & Kotani, T. (2019). Early ontogenetic development, digestive enzymatic activity and gene expression in red sea bream (Pagrus major). *Aquaculture*, *512*, 734283. https://doi.org/10.1016/j.aquaculture.2019.734283

Leduc, A., Zatylny-Gaudin, C., Robert, M., Corre, E., Corguille, G. Le, Castel, H., … Henry, J. (2018). Dietary aquaculture by-product hydrolysates: impact on the transcriptomic response of the intestinal mucosa of European seabass (Dicentrarchus labrax) fed low fish meal diets. *BMC Genomics 2018 19:1*, *19*(1), 1–20. https://doi.org/10.1186/S12864-018-4780-0

Liu, C., Liu, H., Han, D., Xie, S., Jin, J., Yang, Y., & Zhu, X. (2020). Effects of dietary Arthrospira platensis supplementation on the growth performance, antioxidation and immune related-gene expression in yellow catfish (Pelteobagrus fulvidraco). *Aquaculture Reports*, *17*, 100297. https://doi.org/10.1016/J.AQREP.2020.100297

Liu, C., Liu, H., Xu, W., Han, D., Xie, S., Jin, J., … Zhu, X. (2019). Effects of dietary Arthrospira platensis supplementation on the growth, pigmentation, and antioxidation in yellow catfish (Pelteobagrus fulvidraco). *Aquaculture*, *510*, 267–

275. https://doi.org/10.1016/j.aquaculture.2019.05.067

Lokesh, J., & Kiron, V. (2016). Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Scientific Reports*, *6*(19707). https://doi.org/10.1038/srep19707

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, *73*(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Lyons, P. P., Turnbull, J. F., Dawson, K. A., & Crumlish, M. (2017). Effects of low-level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout Oncorhynchus mykiss (Walbaum). *Aquaculture Research*, *48*(5), 2438–2452. https://doi.org/10.1111/are.13080

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Analytical Biochemistry*, *366*(1), 102–104. https://doi.org/10.1016/j.ab.2007.03.010

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Nayak, S. K. (2010). Role of gastrointestinal microbiota in fish. *Aquaculture Research*, *41*(11), 1553–1573. https://doi.org/10.1111/J.1365-2109.2010.02546.X

Niccolai, A., Chini Zittelli, G., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Research*, *42*(April). https://doi.org/10.1016/j.algal.2019.101617

Nikouli, E., Meziti, A., Antonopoulou, E., Mente, E., & Kormas, K. A. (2019). Host-Associated Bacterial Succession during the Early Embryonic Stages and First Feeding in Farmed Gilthead Sea Bream (Sparus aurata). *Genes*, *10*(7), 483. https://doi.org/10.3390/GENES10070483

Oliva-Teles, A., Enes, P., & Peres, H. (2015). Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. Feed and Feeding Practices in Aquaculture. *Elsevier Ltd*. https://doi.org/10.1016/b978-0-08-100506-4.00008-8

Peixoto, D., Pinto, W., Gonçalves, A. T., Machado, M., Reis, B., Silva, J., … Costas, B. (2021). Microalgal biomasses have potential as ingredients in microdiets for Senegalese sole (Solea senegalensis) post-larvae. *Journal of Applied Phycology*, *33*(4), 2241–2250. https://doi.org/10.1007/S10811-021-02431-1

Pérez-Sánchez, T., Mora-Sánchez, B., & Balcázar, J. L. (2018). Biological Approaches for Disease Control in Aquaculture: Advantages, Limitations and Challenges. *Trends in Microbiology*, *26*(11), 896–903. https://doi.org/10.1016/j.tim.2018.05.002

Refstie, S., Olli, J. J., & Standal, H. (2004). Feed intake, growth, and protein utilisation by post-smolt Atlantic salmon (Salmo salar) in response to graded levels of fish protein hydrolysate in the diet. *Aquaculture*, *239*(1–4), 331–349. https://doi.org/10.1016/j.aquaculture.2004.06.015

Rico, R. M., Tejedor-Junco, M. T., Tapia-Paniagua, S. T., Alarcón, F. J., Mancera, J. M., López-Figueroa, F., … Moriñigo, M. A. (2015). Influence of the dietary inclusion

of Gracilaria cornea and Ulva rigida on the biodiversity of the intestinal microbiota of Sparus aurata juveniles. *Aquaculture International*, *24*(4), 965–984. https://doi.org/10.1007/S10499-015-9964-X

Rimoldi, S., Gini, E., Koch, J. F. A., Iannini, F., Brambilla, F., & Terova, G. (2020). Effects of hydrolyzed fish protein and autolyzed yeast as substitutes of fishmeal in the gilthead sea bream (Sparus aurata) diet, on fish intestinal microbiome. *BMC Veterinary Research*, *16*(1), 1–13. https://doi.org/10.1186/s12917-020-02416-1

Ringø, E., Zhou, Z., Vecino, J. L. G., Wadsworth, S., Romero, J., Krogdahl, … Merrifield, D. L. (2016). Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition*, *22*(2), 219–282. https://doi.org/10.1111/ANU.12346

Ringø, Einar, Sperstad, S., Myklebust, R., Refstie, S., & Krogdahl, Å. (2006). Characterisation of the microbiota associated with intestine of Atlantic cod (Gadus morhua L.): The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture*, *261*(3), 829–841. https://doi.org/10.1016/J.AQUACULTURE.2006.06.030

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, *4*(10). https://doi.org/10.7717/PEERJ.2584

Rosenau, S., Oertel, E., Mott, A. C., & Tetens, J. (2021). The Effect of a Total Fishmeal Replacement by Arthrospira platensis on the Microbiome of African Catfish (Clarias gariepinus). *Life*, *11*(6), 558. https://doi.org/10.3390/LIFE11060558

Roy, S. Sen, & Pal, R. (2014). Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics. *Proceedings of the Zoological Society*, *68*(1). https://doi.org/10.1007/s12595-013-0089-9

Ryan, M. P., & Pembroke, J. T. (2020). The Genus Ochrobactrum as Major Opportunistic Pathogens. *Microorganisms*, *8*(11), 1–30. https://doi.org/10.3390/MICROORGANISMS8111797

Saadaoui, H., Espejo-Carpio, F. J., Guadix, E. M., Amar, R. Ben, & Pérez-Gálvez, R. (2019). Bi-objective optimization of tuna protein hydrolysis to produce aquaculture feed ingredients. *Food and Bioproducts Processing*, *115*, 26–35. https://doi.org/10.1016/j.fbp.2019.03.001

Sagaram, U. S., Gaikwad, M. S., Nandru, R., & Dasgupta, S. (2021). Microalgae as feed ingredients: recent developments on their role in immunomodulation and gut microbiota of aquaculture species. *FEMS Microbiology Letters*, *368*(11). https://doi.org/10.1093/FEMSLE/FNAB071

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Kabir Chowdhury, M. A., Parsaeimehr, A., … Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology*, *30*(1), 197–213. https://doi.org/10.1007/s10811-017-1234-z

Shannon, C. E. (1948). A Mathematical Theory of Communication. *The Bell System Technical Journal*, *27*, 379–423, 623–656.

Siddik, M. A. B., Howieson, J., Fotedar, R., & Partridge, G. J. (2021). Enzymatic fish protein hydrolysates in finfish aquaculture: a review. *Reviews in Aquaculture*, *13*(1), 406–430. https://doi.org/10.1111/RAQ.12481

Srichanun, M., Tantikitti, C., Kortner, T. M., Krogdahl, Å., & Chotikachinda, R. (2014).

Effects of different protein hydrolysate products and levels on growth, survival rate and digestive capacity in Asian seabass (Lates calcarifer Bloch) larvae. *Aquaculture*, *428–429*, 195–202. https://doi.org/10.1016/j.aquaculture.2014.03.004

Teuling, E., Wierenga, P. A., Agboola, J. O., Gruppen, H., & Schrama, J. W. (2019). Cell wall disruption increases bioavailability of Nannochloropsis gaditana nutrients for juvenile Nile tilapia (Oreochromis niloticus). *Aquaculture*, *499*, 269–282. https://doi.org/10.1016/J.AQUACULTURE.2018.09.047

Torres, M., Navarro, J. C., Varó, I., Agulleiro, M. J., Morais, S., Monroig, & Hontoria, F. (2020). Expression of genes related to long-chain (C18–22) and very long-chain (>C24) fatty acid biosynthesis in gilthead seabream (Sparus aurata) and Senegalese sole (Solea senegalensis) larvae: investigating early ontogeny and nutritional regulation. *Aquaculture*, *520*, 734949. https://doi.org/10.1016/j.aquaculture.2020.734949

Valente, L. M. P., Custódio, M., Batista, S., Fernandes, H., & Kiron, V. (2019). Defatted microalgae (Nannochloropsis sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets. *Fish Physiology and Biochemistry*, *45*(3), 1067–1081. https://doi.org/10.1007/s10695-019-00621-w

Vázquez-Baeza, Y., Gonzalez, A., Smarr, L., McDonald, D., Morton, J. T., Navas-Molina, J. A., & Knight, R. (2017). Bringing the Dynamic Microbiome to Life with Animations. *Cell Host & Microbe*, *21*(1), 7–10. https://doi.org/10.1016/J.CHOM.2016.12.009

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

Vizcaíno, A. J., Saéz, M. I., López, G., Arizcun, M., Abellán, E., Martínez, T. F., … Alarcón, F. J. (2016). Tetraselmis suecia and Tisochrysis lutea meal as dietary ingredients for gilthead sea bream (Sparus aurata L.) fry. *Journal of Applied Phycology*, *28*(5), 2843–2855. https://doi.org/10.1007/s10811-016-0845-0

Wu, J. ping, Liu, W., Wen, H., Zhou, Y., & Wu, J. jun. (2021). Animal by-products with or without enzymatic hydrolysis completely replacement of fish meal in genetically improved farmed tilapia diets (Oreochromis niloticus). *Aquaculture Research*, *52*(1), 291–301. https://doi.org/10.1111/ARE.14892

Wu, S., Gao, T., Zheng, Y., Wang, W., Cheng, Y., & Wang, G. (2010). Microbial diversity of intestinal contents and mucus in yellow catfish (Pelteobagrus fulvidraco). *Aquaculture*, *303*(1–4), 1–7. https://doi.org/10.1016/J.AQUACULTURE.2009.12.025

Zambonino Infante, J. L., & Cahu, C. L. (2001). Ontogeny of the gastrointestinal tract of marine fish larvae. In *Comparative Biochemistry and Physiology - C Toxicology and Pharmacology* (Vol. 130, pp. 477–487). Elsevier Inc. https://doi.org/10.1016/S1532-0456(01)00274-5

Zheng, K., Liang, M., Yao, H., Wang, J., & Chang, Q. (2012). Effect of dietary fish protein hydrolysate on growth, feed utilization and IGF-I levels of Japanese flounder (Paralichthys olivaceus). *Aquaculture Nutrition*, *18*(3), 297–303. https://doi.org/10.1111/j.1365-2095.2011.00896.x

CAPÍTULO VII

Impact of dietary inclusion of combined use of microalgae biomass and phytase on growth performance and gut microbiota in juvenile European seabass (*Dicentrarchus labrax*)

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ABSTRACT

This work evaluates the effect of dietary inclusion of crude microalgae formulation (*Arthrospira platensis* and *Nannochloropsis gaditana*) and phytase on growth performance and intestinal microbiota of European seabass (*Dicentrarchus labrax*) juveniles. An 83-day feeding trial was carried out using four experimental diets containing 2.5% of microalgae formulation and 500, 1,000, 2,000 and 10,000 phytase units (FTU) per kg of feed as well as a microalgae and phytase free diet as control. At the end of the feeding trial, body weight was recorded for evaluation of growth performance, and intestines were dissected for microbiota analysis. Fish fed with diet including microalgae and the higher concentrations of phytase (2,000 and 10,000 FTU/kg feed) increased significantly their body weight respect to control, while fish fed with lower phytase concentrations showed intermediate body weight. Furthermore, the inclusion of 2.5% of microalgae and 10,000 FTU/kg showed a similar bacterial composition to control group, not appearing differences in alpha and beta diversity, nor with majority OTUs (Weighted UniFrac) nor minority OTUs (Unweighted UniFrac). The rest of experimental diets showed differences respect to control diet in alpha diversity (1,000 and 2,000 FTU/kg diets decreased Faith PD index) or beta diversity (500 FTU/kg diet in majority and minority OTUs, and 1,000 and 2,000 FTU/kg diets in minority OTUs). These results confirm the usefulness of the diet including 2.5% of microalgae formulation and 10,000 FTU/kg in European seabass juveniles producing no changes in the general structure of gut bacterial communities and significantly increasing body weight after 83 days of feeding trial. Therefore, this supplement could be useful to increase aquafeed quality and maintain performance and health status of European seabass juveniles.

Keywords: *Arthrospira platensis*, *Dicentrarchus labrax*, fish nutrition, gut microbiota, microalgae, *Nannochloropsis gaditana***,** phytase.

INTRODUCTION

As the world human population looks to reach about 9.7 billion people by 2050 and almost 11 billion people in 2100 (United Nations, Department of Economic and Social Affairs, Population Division, 2019), our food systems need to produce large amounts of animal protein for a growing population. Given the high impacts of land-based animal production and the stagnation of wild fishery catches, researches are focused on aquaculture to meet this growing protein demand (Merino et al., 2012). Furthermore, the human health benefits of diets rich in fish make it even more pressing, so aquaculture would potentially cover this demand (Tilami & Sampels, 2017). In 2018, global fish production was estimated to reach about 179 million tons, of which 82 million tons correspond to aquaculture. This production had an estimated value of US$ 250 billion (FAO, 2020). Furthermore, aquaculture production accounted for the 52% of fish for human consumption (FAO, 2020).

Traditionally, nutritional requirements of fish have been covered by fishmeal and fish oil as the main protein and fatty acid sources, as well as by plant ingredients (Camacho-Rodríguez et al., 2018; Oliva-Teles et al., 2015). However, the high dependence of aquaculture on fishmeal and fish oil, as well as its increasing price and its low sustainability, have led the aquafeed production industry to look for alternatives (Ghamkhar & Hicks, 2020). Various alternative ingredients derived from plants and animals have been proposed to fully or partially substitute fishmeal and fish oil in aquafeeds. In recent years, great interest has arisen for the use of microalgae and specific strains of cyanobacteria as a sustainable alternative to cover the nutritional requirements of animals and to improve aquafeed quality and animals' health and performance (Shah et al., 2018). Several microalgae species have been used in fish diets due to their high micronutrient and macronutrient contents, as protein and polyunsaturated fatty acids (Roy & Pal, 2014). However, the nutritional value of microalgae can vary substantially between different species, production methods or processing into functional aquafeeds (FAO, 2019).

Several studies have demonstrated the advantages of the application of microalgae biomass as feed for aquaculture (Shah et al., 2018). The use of microalgae could have beneficial effects because of their nutritional quality and their positive effects on growth performance of fish species. Furthermore, the inclusion of microalgae produces an

increase of protein and triglycerides in muscle, omega-3 content, physiological activity and carcass quality (Becker, 2004). Some of the most commonly used microalgae genera as feed for aquaculture include *Tetraselmis, Schizochytrium, Chlorella, Nannochloropsis* and *Arthrospira* (reviewed in Shah et al., 2018 and Yarnold et al., 2019). Species of the genus *Arthrospira* present high content of proteins, vitamins, minerals and active compounds, as well as a balanced amino acid and fatty acid profiles (Niccolai et al., 2019). This genus, and specially the species *A. platensis*, have been successfully used in aquafeeds for several fish species such as gilthead seabream (*Sparus aurata*), rainbow trout (*Onchorhynchus mykiss*) or pabda catfish (*Ompok pabda*) (Akter et al., 2021; Galafat et al., 2020; Güroy et al., 2019). Regarding *Nannochloropsis* sp., this is one of the most used microalgae in marine aquaculture nutrition, particularly during larval stages. This microalga presents high protein, vitamin, pigment and fatty acid contents (Jorge et al., 2019). Dietary inclusion of *N. gaditana* on gilthead seabream (*S. aurata*) showed an increase in body length and body weight (Ayala et al., 2020) and increased bacterial species richness (Jorge et al., 2019). However, despite the good results obtained in previous researches, the inclusion of microalgae in aquafeeds for larvae, fry and juveniles becomes difficult as they have lower enzymatic activity than adults' fish, having less ability to digest the formulated diets (Khoa et al., 2019).

Phytase is an enzyme that hydrolyze indigestible phytate rendering available phosphorous for absorption (Cao et al., 2007). Phytase supplementation also leads to increase bioavailability of other minerals and trace elements (Cao et al., 2007). The incorporation of commercial enzymes such as phytase in aquafeeds could improve nutrients digestibility and bioavailability (Kumar et al., 2012). A study of Cheng & Hardy (2002) showed an increase in nutrient digestibility of barley, canola meal and wheat diets supplemented with microbial phytase in rainbow trout (*O. mykiss*). The inclusion of microbial phytase in plant-based diets increased apparent nutrient digestibility but not influenced growth performance and body composition of juvenile olive flounder (*Paralichthys olivaceus*) and juvenile Korean rockfish (*Sebastes schlegeli*) (Pham et al., 2008; Yoo et al., 2005). Another study supplementing the diet with soybean and 2,000 units of phytase (FTU) per kg of feed showed an increase in productive parameters of red seabream (*Pagrus major*) (Biswas et al., 2007). Nevertheless, the potential effects of phytase together with microalgae on intestinal microbiota and growth performance of fish species has not been explored yet.

Therefore, we have used juvenile European seabass (*D. labrax*) as animal model in order to evaluate the effects of 2.5% of microalgae formulation (*A. platensis* and *N. gaditana*) together with different phytase concentrations (500, 1,000, 2,000 and 10,000 FTU/kg feed) on growth performance and on intestinal microbiota by high-throughput sequencing of the V6-V8 region of 16S rRNA gene. We predict that the inclusion of phytase together with microalgae will increase nutrients digestibility and bioavailability. These changes might increase final body weight and productive parameters of fish, as well as induce changes in gut microbiota.

MATERIAL AND METHODS

Animals, experimental design and fish sampling

Juvenile European seabass (*Dicentrarchus labrax*) were provided by commercial sources (Cultivos Piscícolas de Barbate S.L., Cádiz, Spain). Fish (n = 375) were randomly distributed in 400-L tanks (25 fish per tank with initial stocking density of ~2.5 kg of fish/m^3) in an open system circuit, and acclimated for one month to seawater (38 ‰ salinity), natural photoperiod (September-December) for our latitude (36º 31’ 44” N) and constant temperature (18–19 ºC) at the indoor wet laboratories located in the Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cádiz, Cádiz, Spain; Operational Code REGA ES11028000312).

Fish were kept and handled following the guidelines for experimental procedures in animal research of the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. The Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía, reference number 23/10/2019/176).

Microalgae cultures were up-scaled from Erlenmeyer flasks with f/2 nutrient medium for seawater strains and BG11 medium for freshwater strains, mean light intensity at 240 µmol photons m^{-2} s^{-1}, photoperiod 12:12 (L:D), temperature 25 ± 2°C and 1.5% CO_2 enriched air continuously supplied during the light period. Crude biomass of *Arthrospira platensis* was produced in 100 L bubble columns located inside a greenhouse at the Biorizon Biotech facilities (Almería, Spain) while *Nannochloropsis gaditana* was obtained from tubular photobioreactors at the pilot plant (EU H2020 SABANA facilities) of the Universidad de Almería (Spain). The culture was harvested daily by centrifugation (at a dilution rate of 0.3 d^{-1}), and then the concentrated biomass was freeze-dried. Raw

microalgae biomass (approx. 15% dry matter) was freeze-dried and stored at -20 °C until further use. The chemical composition of crude biomass of *A. platensis* was 65% crude protein and 5% crude lipid while *N. gaditana* meal yielded 44.5% crude protein, and 17.7% crude lipid, all values on dry matter basis.

Five isolipid and isoenergetic experimental diets (46% crude protein, 15% crude lipid) were designed and formulated by the Experimental Diet Service of the University of Almería (Spain). General proximal composition of the diets is shown in Table 1. The four experimental diets contain 2.5 % of crude biomass of *A. platensis* (Cyanobacteria) and *N. gaditana* and different concentration of phytase (500, 1,000, 2,000 and 10,000 phytase units, FTU) per kg of feed. One unit of phytase activity (FTU) is expressed as the amount of enzyme that releases 1 μmol of inorganic phosphate from phytate per minute under the reaction conditions specified in the International Standard ISO 30024:2009. Additionally, a free-microalgae and phytase diet was used as control (CT). The enzyme phytase used in the diets was ePhyt®, which is produced by Global Feed S.L. (Tervalis Group, Huelva, Spain). ePhyt® is a bacterial 3-phytase (EC 3.1.3.8.) preparation produced by fermentation of a strain of *Komagataella phaffii*, belonging to the additive category 'zootechnical additives' and to the functional group 'digestibility enhancers', authorised as an additive in animal nutrition, according to Regulation (EC) 1831/2003 (additive number 4a25).

At the beginning of the experiment, fish were randomly housed in different tanks, getting the same initial biomass within each tank. Before starting the feeding trial, fish were anesthetized with 30 ppm of clove oil and weighed, with an average initial body weight (BW) of 29.1 ± 0.2 g. The volume of each tank was adjusted to maintain a similar fish density throughout the experiment (3.5 kg of fish/m^3). During the experimental period (83 days), fish were fed ad libitum two times per day (10:00 AM and 14:00 PM). At the end of the feeding trial (day 83^{th}), fish were fasted for one day and afterwards, 4 fish per tank (n = 12 fish per experimental group) were euthanized by a lethal dose (1 mL/L of marine water) of 2-phenoxyethanol (Sigma-Aldrich, Ref. 77699). Fish were immediately dissected, the whole intestine was collected with sterile material and stored in sterile containers. Intestinal pieces were kept at -80ºC until DNA extraction.

At the end of the feeding trial the following growth parameters were calculated: i) specific growth rate (SGR) = (100 × (ln final body weight - ln initial body weight)/days;

ii) weight gain (WG) = (100 × (body weigh increase)/initial body weight; and iii) feed efficiency (FE) = weight gain/total feed intake.

DNA extraction

DNA extraction of the whole intestine of European seabass juveniles was carried out following the Modified Salting Out Procedure (MSOP) by Martín-Platero et al. (2007). The modification of this procedure includes an initial mechanical lysis step using a cell disrupter FastPrep FP120 (BIO 101, Thermo Savant) to increase cell lysis. In short, intestines were introduced in a 2 mL microcentrifuge screw cap tube filled with 100 mg of 2 mm zirconia beads and homogenized by two consecutive pulses of 30 seconds at speed 5 in FastPrep FP120. Afterwards, we followed the MSOP protocol. DNA extraction yield was checked by 0.7% agarose gel electrophoresis and DNA concentration was measured using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA was stored at -20ºC until PCR amplification.

Table 1. Ingredients (g/kg on dry matter) of the experimental diets.

Ingredients (g/kg dry matter)	Control	2.5% microalgae + phytase Diets
LT94 fishmeal	100.0	100.0
Lysine	12.00	12.0
Methionine	6.0	6.0
Squid meal	20.0	20.0
Fishmeal hydrolysate CPSP90	10.0	10.0
Krill meal	20.0	20.0
Microalgae formulation	0	25.0
Wheat gluten	76.0	80.0
Soy protein concentrate	340.0	330.0
Pea protein concentrate	90.0	80.0
Sunflower seed	130.0	125.0
Fish oil	120.0	120.0
Soy lecithin	10.0	10.0
Wheat flour	15.0	11.0
Choline chloride	5.0	5.0
Betaine	5.0	5.0
Vitamins y minerals[1]	20.0	20.0
Vitamin C	1.0	1.0
Guar gum	20.0	20.0

[1]Vitamin & Mineral Premix: Vitamins (IU or mg/kg premix): vitamin A (retinyl acetate), 2,000,000 IU; vitamin D3 (DL-cholecalciferol), 200,000 IU; vitamin E , 10,000 mg; vitamin K3 (menadione sodium bisulphite), 2,500 mg; vitamin B1(thiamine hydrochloride), 3,000 mg; vitamin B2 (riboflavin), 3,000 mg; calcium pantothenate, 10,000 mg; nicotinic acid, 20,000 mg;

vitamin B6 (pyridoxine hydrochloride), 2,000 mg; vitamin B9 (folic acid), 1,500 mg; vitamin B12 (cyanocobalamin), 10 mg vitamin H (biotin), 300 mg; inositol, 50,000 mg; betaine, 50,000 mg; vitamin C (ascorbic acid), 50,000 mg. Minerals (mg kg^{-1} premix): Co (cobalt carbonate), 65 mg; Cu (cupric sulphate), 900 mg; Fe (iron sulphate), 600 mg; I (potassium iodide), 50 mg; Mn (manganese oxide), 960 mg; Se (sodium selenite), 1 mg; Zn (zinc sulphate) 750 mg; Ca (calcium carbonate), 186,000 mg; KCl, 24,100 mg; NaCl 40,000 mg; excipient sepiolite, colloidal silica (Lifebioencapsulation SL, Almería Spain).

V6-V8 16S rRNA gene amplification and high-throughput sequencing

V6-V8 region of 16S rRNA gene libraries were constructed using the primers pair B969F (5'-ACGCGHNRAACCTTACC-3') and BA1406R (5'-ACGGGCRGTGWGTRCAA-3') (Comeau et al., 2011) with Illumina adapter overhang sequences. Amplicons were generated using the iProof™ High-Fidelity DNA Polymerase (BioRad®). The purified PCR products were used as template for a second PCR in which two unique Illumina compatible barcodes were indexed to each sample. These barcodes allow that the derived sequences can be demultiplexed into their respective samples in downstream analysis. The barcodes overlapped with the sequence of the primers used in the first PCR. All purification steps were made using DNA Purification SPRI Magnetic Beads (Canvax®) following the manufacturer's instructions. PCR amplicons were checked by 1% agarose gel electrophoresis and DNA concentrations were measured using Qubit® 3.0 Fluorometer (Invitrogen™, Carlsbad, CA). Then, PCR amplicons were pooled in equimolar concentrations. High-throughput sequencing was carried out with Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), resulting in paired-ends reads of 2 x 300 bp length. Sequencing was carried out in the Illumina MiSeq platform in the Institute of Parasitology and Biomedicine "López-Neyra" (Granada, Spain). Sequences are available in the Sequence Read Archive (SRA) in the Genbank - NCBI webpage (https://www.ncbi.nlm.nih.gov/sra/), BioProject: PRJNA749674, Accession Nos. SAMN20396460 to SAMN20396707.

Sequences processing and data analysis

In order to analyze the 16S rRNA sequences generated from Illumina MiSeq, we used the Quantitative Insights Into Microbial Ecology (QIIME2 v2021.4; Bolyen et al., 2019) software. First, primers trimming was performed using *cutadapt* plugin default parameters (Martin, 2011). Pair joining was performed using default parameters. Then, a quality filter was carried out to remove from the analyses the sequences with more than 3 consecutives base pairs with a Phred score lower than 20. Afterwards, we used Deblur,

a sub-operational-taxonomic-unit (sub-OTU) approach, in order to remove sequencing errors (Amir et al., 2017). We performed fragment insertion of 16S sequences using the SEPP algorithm implemented in QIIME2 in order to align the sequences and build a de-novo bacterial phylogenetic tree (Janssen et al., 2018). Taxonomy assignation was based on Greengenes 13.8 with a similarity of 99% (DeSantis et al., 2006). Finally, reads of chloroplast, mitochondria or non-bacterial reads were also filtered out.

Statistics

Growth performance results are shown as average ± standard error of the mean (mean ± SEM). After assessing homogeneity of variance and normality, statistical analysis of the data was carried out using one-way ANOVA followed by Fisher LSD Post-hoc tests. A comparison of triplicate tanks for all parameters was also performed with one-way ANOVA.

For alpha diversity analysis, OTU table was rarified at 10,000 sequences depth per sample. Samples that did not reach this sequencing depth were excluded for subsequent analyses. We calculated three alpha diversity indices from the OTU table: Shannon diversity index (Shannon, 1948), Faith Phylogenetic Diversity index (Faith and Baker, 2006) and bacterial OTU richness (or Species richness). We used Generalized Linear Mixed Models (GLMM) to explore the effect of diet as fixed factor and tank nested in diet as a random factor in all the alpha diversity indices studied. In these analyses, individual fish was the experimental unit. Post-hoc comparisons were performed using Fisher LSD test. These analyses were performed using STATISTICA 10.0 (StatSoft).

Beta diversity distance matrixes were calculated using UniFrac distance based on the rarified OTU table at 10,000 sequences depth per sample. We used both Weighted and Unweighted UniFrac indices (Lozupone et al., 2007; Lozupone & Knight, 2005) for subsequent analysis. Weighted UniFrac gives more importance to the most abundant OTUs, while Unweighted UniFrac gives more importance to rare OTUs as it takes into account their presence or absence independently of their abundance. Permutational ANOVA (PERMANOVA) was performed in order to test the effect of diet as fixed factor and tank nested in diet as random factor in both UniFrac distance matrixes. We used PRIMER-7 software (PRIMER-e) implemented with PERMANOVA plugin. Principal Coordinate Analyses (PCoA) were performed in order to visualize the 2 first axes using EMPeror 2021.4.0 (Vázquez-Baeza et al., 2017; Vázquez-Baeza et al., 2013).

RESULTS

Effect of feeding diet on European seabass juveniles growth performance

No differences appeared in initial mean body weight (IMBW) and initial biomass in the tank (IB) between different experimental diets at the beginning of the feeding trial (Table 2). The inclusion of 2.5% of microalgae and 2,000 or 10,000 FTU/kg significantly increased final mean body weight (FMBW) respect to control diet. Dietary inclusion of 2.5% of microalgae together with lower levels of phytase (500 and 1,000 FTU/kg) showed intermediate levels of FMBW, not appearing significant differences between fish fed with these experimental diets and control fish (Table 2, Figure 1).

Other growth performance parameters showed similar results. Percentaje of weight gain (WG%), final biomass in the tank (FB), total biomass gain per tank (TBG), specific growth rate (SGR) and feed efficiency (FE) were also affected by inclusion 2.5% of microalgae and 2,000 or 10,000 FTU/kg, showing higher values of these productive parameters than control fish (Table 2). Furthermore, differences appeared between 2,000 and 10,000 FTU/kg, showing the most phytase concentrated diet the higher values of FMBW, FB, TBG, WG% and FE (Table 2). No differences in survival rate occurred bewteen any of the different experimental diets ($p = 0.913$).

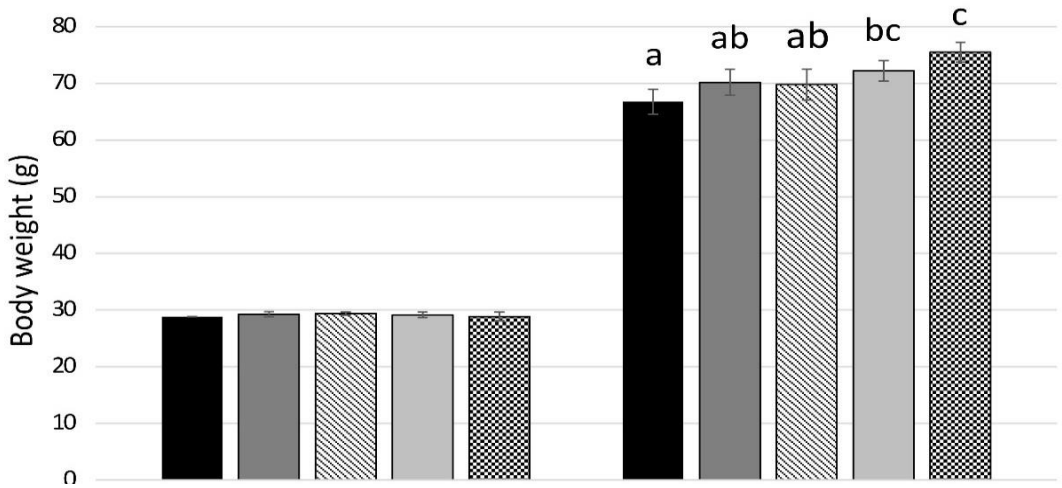

Figure 1. Evolution of fish growth of European seabass juveniles fed with control diet or supplemented with 2.5% of microalgae (*A. platensis* and *N. gaditana*) and different phytase units (500, 1,000, 2,000 and 10,000 FTU) (mean ± standard deviation). Bars with different lowercase letter indicate significant differences among dietary treatments ($p < 0.05$).

Bacterial Community Composition

The gut microbiota of juvenile European seabass is dominated by Gammaproteobacteria (>60%), followed by Alphaproteobacteria, Bacilli, Actinobacteria and Betaproteobacteria. The relative abundance of these classes varied in the experimental diets, especially in fish supplemented with 2.5% of microalgae and low levels of phytase (500, 1,000 and 2,000 FTU/kg). The gut microbiota of European seabass juveniles supplemented with 2.5% of microalgae and 10,000 FTU/kg showed a microbial composition similar to control fish (Figure 2).

At genus level, gut microbiota of European seabass juveniles fed with control diet was dominated by *Pseudomonas, Vibrio* and *Ochrobactrum*. Fish fed with experimental diets containing 2.5% of microalgae and 500, 1,000 and 2,000 FTU/kg respectively showed an increase in *Vibrio* and *Ochrobactrum* and a decrease in minority genera (Figure 3). However, fish fed with the experimental diet containing 2.5% of microalgae and the higher phytase concentration (10,000 FTU/kg) showed the most similar bacterial composition to the control, following similar trend as at the class level.

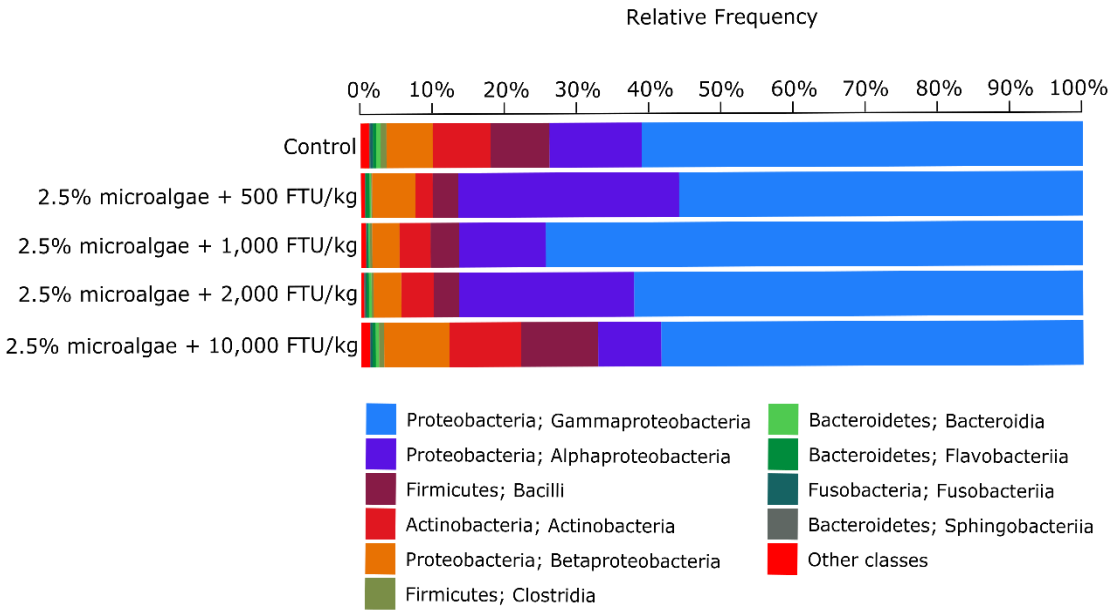

Figure 2. Microbial composition at class level of juvenile European seabass gut microbiota group by experimental diet (control, 500, 1,000, 2,000 and 10,000 FTU/kg). Each bar represents the mean of the relative abundance of the different bacterial classes in the samples from each treatment. Classes in the legend are sorted from most abundant to lowest abundant.

Table 2. One-way ANOVA exploring the effects of diet as factor in European seabass juveniles fed with control diet or supplemented with 2.5% of microalgae (*A. platensis and N. gaditana*) and different phytase units (500, 1,000, 2,000 and 10,000 FTU/kg). IMW refers to initial mean body weight, FMBW to final mean body weight, IB to initial biomass, FB to final biomass, TBG to total biomass gain per tank, WG to percentage of weight gain, SGR to specific growth rate and FE to feed efficiency. Tank was used as experimental unit. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant p-values are shown in bold. Letters with different superscript in a same row denote significant differences (Fisher LSD Post-hoc test; $p < 0.05$).

	Control	**2.5% microalgae + 500 FTU/kg**	**2.5% microalgae + 1,000 FTU/kg**	**2.5% microalgae + 2,000 FTU/kg**	**2.5% microalgae + 10,000 FTU/kg**	**D.f.**	**F**	***p***
IMBW (g/fish)	28.84 ± 0.03^{a}	29.26 ± 0.26^{a}	29.38 ± 0.15^{a}	29.16 ± 0.29^{a}	28.86 ± 0.43^{a}	4,10	0.82	0.543
FMBW (g/fish)	66.76 ± 1.26^{a}	70.18 ± 1.30^{ab}	69.80 ± 1.58^{ab}	72.19 ± 1.04^{bc}	75.51 ± 1.01^{c}	4,10	6.65	**0.007**
IB (g/tank)	720.9 ± 0.6^{a}	731.5 ± 6.5^{a}	734.6 ± 3.7^{a}	729.1 ± 7.3^{a}	721.5 ± 10.8^{a}	4,10	0.82	0.543
FB (g/tank)	1623.9 ± 19.4^{a}	1707.2 ± 25.7^{b}	1697.0 ± 34.1^{ab}	1731.7 ± 25.2^{b}	1862.0 ± 5.1^{c}	4,10	13.16	**0.001**
TBG (g/tank)	935.2 ± 25.9^{a}	1009.2 ± 23.0^{ab}	996.1 ± 31.3^{ab}	1053.9 ± 14.9^{b}	1158.2 ± 20.2^{c}	4,10	12.27	**0.001**
WG (%)	131.5 ± 4.2^{a}	139.8 ± 2.4^{ab}	137.6 ± 5.9^{ab}	147.6 ± 2.2^{b}	161.8 ± 5.4^{c}	4,10	7.34	**0.005**
SGR	1.00 ± 0.02^{a}	1.04 ± 0.01^{ab}	1.03 ± 0.03^{ab}	1.08 ± 0.01^{bc}	1.15 ± 0.02^{c}	4,10	7.15	**0.006**
FE	0.67 ± 0.01^{a}	0.70 ± 0.02^{a}	0.71 ± 0.03^{a}	0.72 ± 0.02^{a}	0.80 ± 0.02^{b}	4,10	5.61	**0.012**
Survival (%)	97.3 ± 1.3^{a}	97.3 ± 1.3^{a}	97.3 ± 2.7^{a}	96.0 ± 2.3^{a}	98.7 ± 1.3^{a}	4,10	0.25	0.903

Effect of feeding diet on Alpha and Beta Diversity

Supplementing the diet of European seabass juveniles with 2.5% of microalgae formulation and different phytase concentrations affected Shannon and Faith PD indices after 83 days of feeding trial (Table 3). These differences in both alpha diversity indices were characterized by a reduction on microbial diversity in fish with 1,000 and 2,000 FTU/kg diets respect to fish fed with control diet (Fisher LSD Post-hoc, $p \leq 0.006$ for Shannon index and Fisher LSD Post-hoc, $p \leq 0.033$ for Faith PD index). Fish supplemented with diets containing 2.5% of microalgae and 500 or 10,000 FTU/kg showed similar levels in Shannon (Fisher LSD Post-hoc, $p \geq 0.052$) and Faith PD indices (Fisher LSD Post-hoc, $p \geq 0.440$) than control fish. However, bacterial OTU richness of fish with control diets did not differ for fish with experimental diets (Table 3).

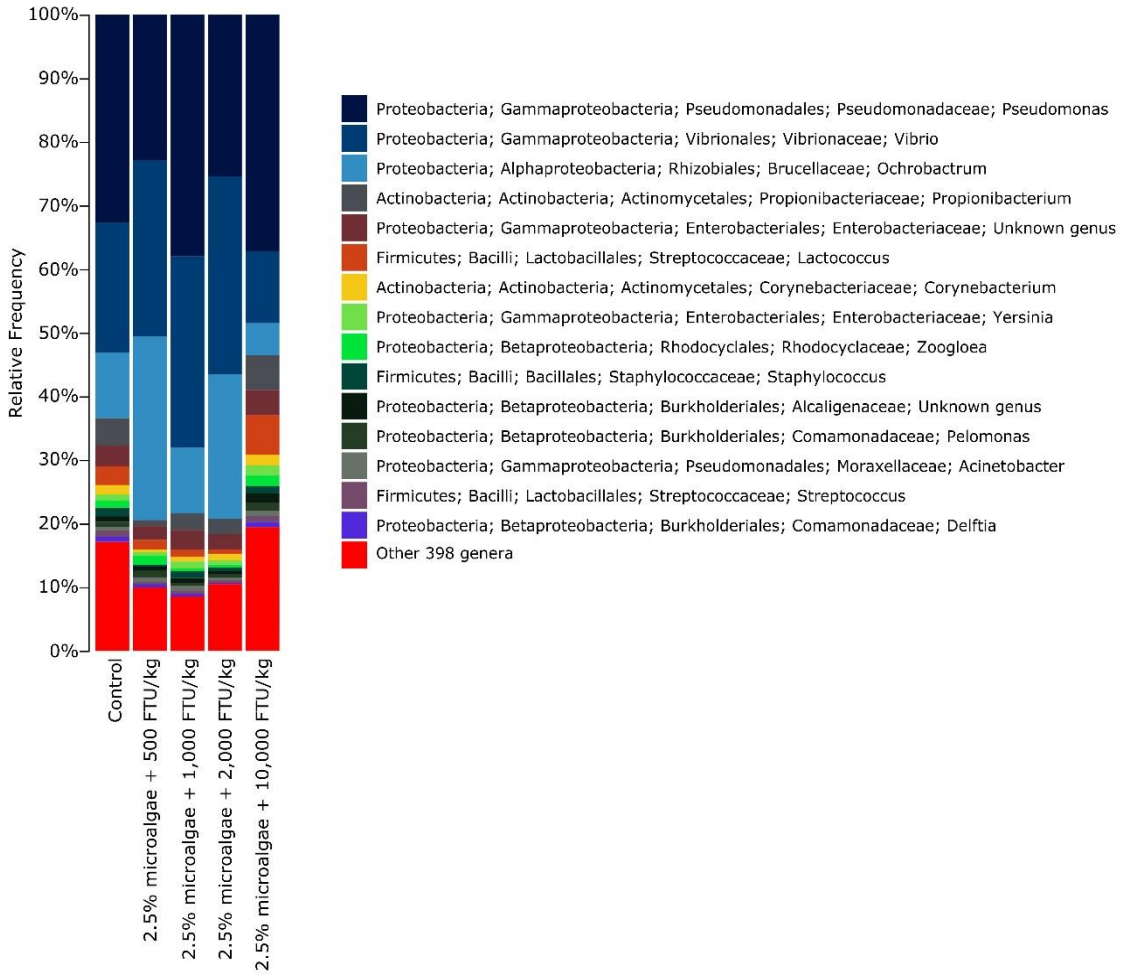

Figure 3. Microbial composition at genus level of juvenile European seabass gut microbiota group by diet (control, 500, 1,000, 2,000 and 10,000 FTU/kg). Each bar represents the mean of the relative abundance of the different bacterial genera in the samples from each treatment. Genera in the legend are sorted from most abundant to lowest abundant.

Table 3. General Linear Mixed Models exploring the effects of fish diet (control, 500, 1,000, 2,000 and 10,000 FTU/kg) and tank nested in diet in the different alpha diversity indices of the bacterial community of juvenile European seabass. D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant p-values ($p < 0.05$) are shown in bold.

Alpha Diversity Index	Explanatory variables	D.f	F	*p*
Shannon	Diet	4,95	5.75	< **0.001**
	Tank (Diet)	10,95	0.83	0.598
Faith PD	Diet	4,95	2.77	**0.031**
	Tank (Diet)	10,95	1.50	0.152
Bacterial OTU Richness	Diet	4,95	2.26	0.068
	Tank (Diet)	10,95	1.36	0.212

After 83 days of feeding trial, the bacterial community in the gut of European seabass juveniles varied significantly between the different diets, taking into account both majority (Weighted UniFrac) and minority (Unweighted UniFrac) bacterial OTUs (Figure 4, Table 4). Taking into account majority OTUs, fish with experimental diets differed from control ones except the diet including 10,000 FTU/kg (Pair-wise test, t = 0.979, p = 0.368). Interestingly, results based in Unweighted UniFrac distance matrix showed that diets supplemented with 2.5% of microalgae and 500 FTU/kg differed significantly from the control one (Pair-wise test, t = 1.449, p = 0.049). Therefore, the only diet that did not affect either majority or minority OTUs of the bacterial community was the diet including 2.5% of microalgae and 10,000 FTU/kg.

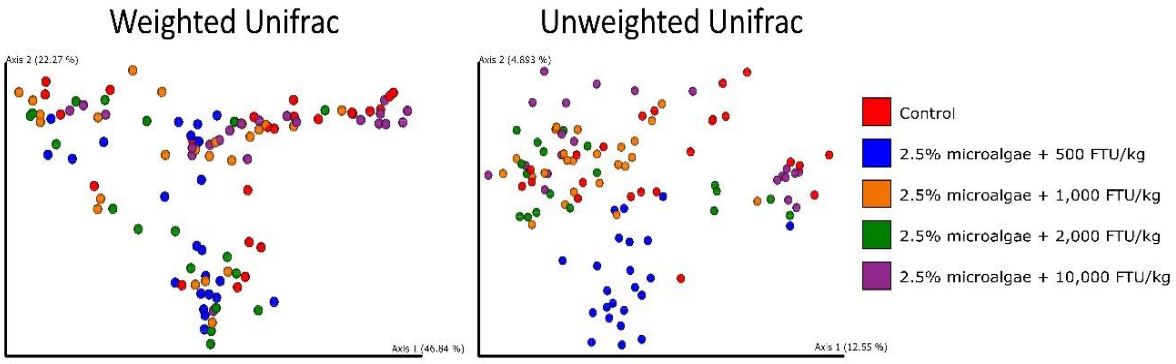

Figure 4. Dimensional figures showing the first two axes of Principal Coordinate Analysis and representing bacterial communities of juvenile European seabass fed with control diet or supplemented with 2.5% of microalgae (*A. platensis* and *N. gaditana*) and different levels of phytase (500, 1,000, 2,000 and 10,000 FTU/kg) using Weighted and Unweighted UniFrac distance matrixes. Samples are colored by experimental diet (Control - red; 500 FTU/kg - blue; 1,000 FTU/kg – orange; 2,000 FTU/kg – green; 10,000 FTU/kg – purple). Proportion of explained variance by each PCo axes is shown.

Table 4. Permutational ANOVA (PERMANOVA) exploring the effects of diet and tank nested in diet in beta diversity indices of bacterial community of European seabass juveniles fed with control diet or supplemented with 2.5% of microalgae formulation (*A. platensis and N. gaditana*) and different phytase units (500, 1,000, 2,000 and 10,000 FTU/kg). D.f. refers to degree of freedom. The first number is the degree of freedom of the independent variable and the second one for the error term. Significant *p*-values are shown in bold.

β-Diversity Distance Matrix	Explanatory variables	D.f.	Pseudo-F	*p*
Weighted UniFrac	Diet	4,109	7.25	**0.001**
	Tank (Diet)	10,109	0.65	0.929
Unweighted UniFrac	Diet	4,109	1.96	**0.001**
	Tank (Diet)	10,109	1.20	**0.006**

DISCUSSION

Supplementation of European seabass juveniles with 2.5% of microalgae (*A. platensis* and *N. gaditana*) and 10,000 FTU/kg showed a significant increase in body weight respect to control diet and did not affect microbial composition and diversity. The inclusion of microalgae and 2,000 FTU/kg also increased body weight of fish, but changes in bacterial diversity and community composition appeared. Finally, lower levels of phytase (500 and 1,000 FTU/kg) and 2.5% of microalgae produce a non-significant increase in body weight as well as changes in bacterial communities' composition and diversity. These results support the use of 2.5% of microalgae and 10,000 FTU/kg of phytase as a promoting of growth performance, while not modifying bacterial communities' diversity and composition.

The current increase in aquaculture production together with the unsustainability of fishmeal and fish oil derived diets makes it necessary to look for sustainable alternatives for aquaculture sector that can improve or maintain productive parameters (Rimoldi et al., 2020). Dietary inclusion of different species of microalgae and cyanobacteria have been successfully evaluated as fish dietary supplements, pointing out positive effects on fish health and performance in different life stages (Roy & Pal, 2014). The inclusion of the species used in our experiment (*N. gaditana* and *A. platensis*) in fish diet have shown disparate results in previous studies. A recent study of Ayala et al. (2020) showed an increase in body weight and body length of gilthead seabream (*S. aurata*) supplemented with 2.5 and 5% of *N. gaditana*. Another research studying the inclusion of *N. gaditana* extract in juvenile gilthead seabream (*S. aurata*) showed no differences in

body weight but produces benefits in fish muscle composition and skin pigmentation (Sales et al., 2021). Senegalese sole (*Solea senegalensis*) juveniles fed with 15% of *N. gaditana* showed a better growth performance and nutrient utilization than commercial diets, as well as an increase in microvilli length and surface (Vizcaíno et al., 2018). Regarding the use of *A. platensis*, dietary inclusion of 30% this cyanobacterium increased growth performance, feed efficiency and health status of Nile tilapia juveniles (*Oreochromis niloticus*) (Velasquez et al., 2016). Another research of Galafat et al. (2020) showed higher enzyme activities and increased microvilli length and absorptive capacity in gilthead seabream juveniles supplemented 2 and 4% of *Arthrospira* sp. Finally, another study of Goda et al. (2018) evaluated the effect of dietary inclusion of 5% of *A. platensis* during weaning of European seabass (*D. labrax*), producing these cyanobacteria an increase in body weight, average daily gain, SGR, survival rate and proximate body composition. Our results showed a significant increase in body weight, percentage of weight gain and SGR in fish supplemented with 2.5% of microalgae formulation (*A. platensis* and *N. gaditana*) and high levels of microbial phytase (2,000 and 10,000 FTU/kg).

Supplementing animal diets with exogenous enzymes such as phytases, xylanases, carbohydrases or proteases has been extensively investigated in poultry and swine (Adeola & Cowieson, 2011). Phytases can increase feed digestibility by increasing the bioavailability of phosphorous from phytate (Cao et al., 2007; Greiner & Konietzny, 2006). In aquaculture, the use of phytase to improve mineral and nutrient digestibility has emerged quite rapidly. Several studies have demonstrated the benefits of phytase in utilization and nutrient digestibility of nutrients in aquaculture diets (reviewed in Cao et al., 2007 and Kumar et al., 2012). This increase in nutrient digestibility has not always been related with an improvement in fish growth performance, as reported results have been inconsistent. Microbial phytase inclusion in plant-based diets increased nutrient digestibility but not affected growth performance of olive flounder (*P. olivaceus*) and Korean rockfish juveniles (*S. schlegelii*) (Pham et al., 2008; Yoo et al., 2005). The inclusion of 3,000 FTU/kg in Nile tilapia (*O. niloticus*) did not produced changes in body weight respect to control diet (Green et al., 2021). However, other studies has demonstrated an increase in productive parameters of red seabream (*P. major*) (Biswas et al., 2007) and rainbow trout (*O. mykiss*) (Çantaş & Yildirim, 2020) supplemented with 2,000 FTU/kg, and Nile tilapia (*O. niloticus*) supplemented with 500 or 1,000 FTU/kg

(Abo Norag et al., 2018). Nevertheless, to our modest knowledge, the effects of phytase inclusion together with microalgae biomass on fish growth performance has not been explored previously.

The effects of the inclusion of microalgae on microbial diversity of fish intestine has shown disparate results. Dietary inclusion of microalgae (*Tetraselmis chuii* and *Phaeodactylum tricornutum*) in gilthead seabream (*S. aurata*) caused a reduction in alpha diversity indices (Cerezuela et al., 2012). Other study with gilthead seabream (*S. aurata*) fed with hydrolysed *N. gaditana* supplemented diet produced no changes in microbial diversity (Cerezo-Ortega et al., 2021). However, total fishmeal replacement by *A. platensis* on African catfish (*Clarias gariepinus*) indicated an increase in the different alpha diversity indices studied (Rosenau et al., 2021). With regards to dietary phytase inclusion on intestinal microbial diversity of fish species, it has been little studied, being most of the studies related with Nile tilapia (*O. niloticus*). The inclusion of 300 mg/kg of phytase showed no differences in species diversity of Nile tilapia (*O. niloticus*) intestinal microbiota (Adeoye et al., 2016a). A blend of enzymes including phytase (together with protease and xylanase) also produced no differences in species richness and Shannon diversity index in Nile tilapia (Adeoye et al., 2016b). Another recent research of Maas et al. (2021) showed similar results, with no differences in species richness and diversity of Nile tilapia supplemented with 1,000 FTU/kg. In our experiment, the addition of 2.5% of microalgae and 1,000 and 2,000 FTU/kg produced a decrease in Shannon and Faith PD alpha diversity indices, whereas no differences in alpha diversity appeared when adding fish diet with 2.5% of microalgae and the lower (500 FTU/kg) or higher concentration of phytase (10,000 FTU/kg). These results point out that the effect of phytase addition to microalgae could be dose dependent and that these high and low doses could be the most efficient in fish diet, since intermediate doses produced a reduction in diversity.

Nevertheless, our results showed that the inclusion of 2.5% of microalgae and 500 FTU/kg produced changes in the majority and minority OTUs of intestinal microbial communities. Intermediate concentrations of phytase (1,000 and 2,000 FTU/kg) also showed differences in majority OTUs of intestinal microbiota respect to control group. However, dietary supplementation with 2.5% of microalgae and 10,000 FTU/kg did not affect microbial communities' composition. This absence of differences together with the increase in growth performance make the higher dose the most appropriate for use in aquaculture. These results are promising given that the effects on microbial communities'

composition of the inclusion of microalgae together with phytase in animal diets have not been previously explored either in terrestrial or aquatic animals. On the other hand, the use of microalgae and phytase independently has been previously studied showing disparate results. The inclusion of 15% of *A. platensis* on Sabah giant grouper diet produced changes in gut microbiome (Man et al., 2020). In other study, total fishmeal replacement by *A. platensis* did not affect the overall microbiome structure of African catfish (*C. gariepinus*) (Rosenau et al., 2021). Dietary inclusion of *N. gaditana* in gilthead seabream (*S. aurata*) did not showed differences in microbial community composition respect to control diet (Cerezo-Ortega et al., 2021). The effects of phytase inclusion on beta diversity of fish species has hardly been studied. Adeoye et al. (2016b) demonstrated that the inclusion of a blend of enzymes including phytase in Nile tilapia (*O. niloticus*) produced changes in beta diversity of the intestinal microbial communities. However, the effects of this dietary supplement on the composition of microbial communities has been extensively studied previously in terrestrial animals. The inclusion of 500 U/kg of microbial phytase in broiler chickens' diet did not affect microbial communities of the caecum (Tang et al., 2020). However, the inclusion of 1,500 FTU/kg produced changes in microbial communities of crop and ileum of broiler chickens (Künzel et al., 2019). A recent study of Metzler-Zebeli et al. (2020) with fattening pigs also noted differences in microbial communities of pigs supplemented with 650 FTU/kg. Therefore, further experiments are needed in the industry to unveil the effect of different phytase levels on microalgae degradation and digestibility and to see if an increase in microalgae concentration could affect growth performance and gut microbiota of fish.

CONCLUSIONS

The results obtained in this study show that 2.5% of crude microalgae formulation (*A. platensis* and *N. gaditana*) and 10,000 FTU/kg of phytase inclusion in juvenile European seabass diet increased body weight and productive parameters while not affected intestinal microbial diversity and microbial communities' composition of fish intestine. Therefore, this experimental diet could be a good supplement in aquafeed in order to improve aquafeed quality and increase fish body growth. Still, future research should be conducted in order to study the possibility of including higher levels of microalgae and reduce fishmeal and fish oil levels without affecting growth performance and gut microbiota.

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REFERENCES

Abo Norag, M. A., El-Shenawy, A. M., Fadl, S. E., Abdo, W. S., Gad, D. M., Rashed, M. A., & Prince, A. M. (2018). Effect of phytase enzyme on growth performance, serum biochemical alteration, immune response and gene expression in Nile tilapia. *Fish & Shellfish Immunology*, *80*, 97–108. https://doi.org/10.1016/J.FSI.2018.05.051

Adeola, O., & Cowieson, A. J. (2011). BOARD-INVITED REVIEW: Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *Journal of Animal Science*, *89*(10), 3189–3218. https://doi.org/10.2527/JAS.2010-3715

Adeoye, A. A., Jaramillo-Torres, A., Fox, S. W., Merrifield, D. L., & Davies, S. J. (2016). Supplementation of formulated diets for tilapia (Oreochromis niloticus) with selected exogenous enzymes: Overall performance and effects on intestinal histology and microbiota. *Animal Feed Science and Technology*, *215*, 133–143. https://doi.org/10.1016/J.ANIFEEDSCI.2016.03.002

Adeoye, A. A., Yomla, R., Jaramillo-Torres, A., Rodiles, A., Merrifield, D. L., & Davies, S. J. (2016). Combined effects of exogenous enzymes and probiotic on Nile tilapia

(Oreochromis niloticus) growth, intestinal morphology and microbiome. *Aquaculture*, *463*, 61–70. https://doi.org/10.1016/J.AQUACULTURE.2016.05.028

Akter, T., Hossain, A., Rabiul Islam, M., Hossain, M. A., Das, M., Rahman, M. M., … Abdel-Tawwab, M. (2021). Effects of spirulina (Arthrospira platensis) as a fishmeal replacer in practical diets on growth performance, proximate composition, and amino acids profile of pabda catfish (Ompok pabda). *Journal of Applied Aquaculture*. https://doi.org/10.1080/10454438.2021.1936740

Amir, A., McDonald, D., Navas-Molina, J. A., Kopylova, E., Morton, J. T., Zech Xu, Z., … Knight, R. (2017). Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns. *MSystems*, *2*(2), e00191-16. https://doi.org/10.1128/mSystems.00191-16

Ayala, M. D., Galián, C., Fernández, V., Chaves-Pozo, E., Serrana, D. G. de la, Sáez, M. I., … Arizcun, M. (2020). Influence of Low Dietary Inclusion of the Microalga Nannochloropsis gaditana (Lubián 1982) on Performance, Fish Morphology, and Muscle Growth in Juvenile Gilthead Seabream (Sparus aurata). *Animals*, *10*(12), 1–18. https://doi.org/10.3390/ANI10122270

Becker, W. (2004). Microalgae in human and animal nutrition. In *Richmond A (ed) Handbook of microalgal culture: biotechnology and applied phycology. Blackwell Science Ltd, Cambridge* (pp. 312–351).

Biswas, A. K., Kaku, H., Ji, S. C., Seoka, M., & Takii, K. (2007). Use of soybean meal and phytase for partial replacement of fish meal in the diet of red sea bream, Pagrus major. *Aquaculture*, *267*(1–4), 284–291. https://doi.org/10.1016/J.AQUACULTURE.2007.01.014

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(9), 1091. https://doi.org/10.1038/s41587-019-0252-6

Camacho-Rodríguez, J., Macías-Sánchez, M. D., Cerón-García, M. C., Alarcón, F. J., & Molina-Grima, E. (2018). Microalgae as a potential ingredient for partial fish meal replacement in aquafeeds: nutrient stability under different storage conditions. *Journal of Applied Phycology*, *30*(2), 1049–1059. https://doi.org/10.1007/s10811-017-1281-5

Çantaş, İ. B., & Yildirim, Ö. (2020). Supplementation of Microbial Phytase with Safflower Meal in Rainbow Trout (Oncorhynchus mykiss): The Effects on Growth, Digestibility, Environmental, and Serum Biological Parameters. *Journal of Chemistry, Article ID 4634796*, 8. https://doi.org/10.1155/2020/4634796

Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., … Li, D. (2007). Application of microbial phytase in fish feed. *Enzyme and Microbial Technology*, *40*(4), 497–507. https://doi.org/10.1016/J.ENZMICTEC.2007.01.007

Cerezo-Ortega, I. M., Di Zeo-Sánchez, D. E., García-Márquez, J., Ruiz-Jarabo, I., Sáez-Casado, M. I., Balebona, M. C., … Tapia-Paniagua, S. T. (2021). Microbiota composition and intestinal integrity remain unaltered after the inclusion of hydrolysed Nannochloropsis gaditana in Sparus aurata diet. *Scientific Reports*, *11*(1), 1–16. https://doi.org/10.1038/s41598-021-98087-5

Cerezuela, R., Fumanal, M., Tapia-Paniagua, S. T., Meseguer, J., Morinigo, M. Á., & Esteban, M. Á. (2012). Histological alterations and microbial ecology of the intestine in gilthead seabream (Sparus aurata L.) fed dietary probiotics and

microalgae. *Cell and Tissue Research*, *350*(3), 477–489. https://doi.org/10.1007/s00441-012-1495-4

Cheng, Z. J., & Hardy, R. W. (2002). Effect of microbial phytase on apparent nutrient digestibility of barley, canola meal, wheat and wheat middlings, measured in vivo using rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition*, *8*(4), 271–277. https://doi.org/10.1046/j.1365-2095.2002.00219.x

Comeau, A. M., Li, W. K. W., Tremblay, J.-É., Carmack, E. C., & Lovejoy, C. (2011). Arctic Ocean Microbial Community Structure before and after the 2007 Record Sea Ice Minimum. *PLOS ONE*, *6*(11), e27492. https://doi.org/10.1371/JOURNAL.PONE.0027492

DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., … Andersen, G. L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology*, *72*(7), 5069–5072. https://doi.org/10.1128/AEM.03006-05

Faith, D. P., & Baker, A. M. (2006). Phylogenetic Diversity (PD) and Biodiversity Conservation: Some Bioinformatics Challenges. *Evolutionary Bioinformatics*, *2*, 121–128. https://doi.org/10.1177/117693430600200007

FAO. (2019). Microalgae in aquatic animal feeds. *FAO Aquaculture Newsletter*, *No. 60*(August. Rome).

FAO. (2020). *The State of World Fisheries and Aquaculture 2020. Sustainability in action*. Rome: FAO. https://doi.org/10.4060/ca9229en

Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Jérez-Cepa, I., Mancera, J. M., & Alarcón, F. J. (2020). Evaluation of Arthrospira sp. enzyme hydrolysate as dietary additive in gilthead seabream (Sparus aurata) juveniles. *Journal of Applied Phycology*, *32*(5), 3089–3100. https://doi.org/10.1007/s10811-020-02141-0

Ghamkhar, R., & Hicks, A. (2020). Comparative environmental impact assessment of aquafeed production: Sustainability implications of forage fish meal and oil free diets. *Resources, Conservation and Recycling*, *161*, 104849. https://doi.org/10.1016/J.RESCONREC.2020.104849

Goda, A. A., Omar, E. A., Srour, T. M., Nour, A. M., Ismail, M. M., Baromh, M. Z., & Kotit, A. M. (2018). Dietary Spirulina platensis and Chlorella marina Microalgae as Growth Promoters During Weaning Post Larvae of European Seabass, Dicentrarchus labrax. *Journal of the Advances in Agricultural Researches*, *23*(3), 542–557.

Green, B. W., Rawles, S. D., Schrader, K. K., McEntire, M. E., Abernathy, J., Ray, C. L., … Webster, C. D. (2021). Impact of dietary phytase on tilapia performance and biofloc water quality. *Aquaculture*, *541*, 736845. https://doi.org/10.1016/J.AQUACULTURE.2021.736845

Greiner, R., & Konietzny, U. (2006). Phytase for Food Application. *Food Technology and Biotechnology*, *44*(2), 125–140.

Güroy, B., Güroy, D., Mantoğlu, S., Çelebi, K., Şahin, O. I., Kayalı, S., & Canan, B. (2019). Dietary Spirulina (Arthrospira platensis, Gomont, 1892) improved the flesh quality and shelf life of rainbow trout (Oncorhynchus mykiss, Walbaum, 1792) fed fish meal or plant-based diet. *Aquaculture Research*, *50*(9), 2519–2527. https://doi.org/10.1111/are.14206

Janssen, S., McDonald, D., Gonzalez, A., Navas-Molina, J. A., Jiang, L., Xu, Z. Z., …

Knight, R. (2018). Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information. *MySistems*, *3*(3), e00021-18. https://doi.org/10.1128/mSystems.00021-18

Jorge, S. S., Enes, P., Serra, C. R., Castro, C., Iglesias, P., Teles, A. O., & Couto, A. (2019). Short-term supplementation of gilthead seabream (Sparus aurata) diets with Nannochloropsis gaditana modulates intestinal microbiota without affecting intestinal morphology and function. *Aquaculture Nutrition*, *25*(6), 1388–1398. https://doi.org/10.1111/ANU.12959

Khoa, T. N. D., Waqalevu, V., Honda, A., Shiozaki, K., & Kotani, T. (2019). Early ontogenetic development, digestive enzymatic activity and gene expression in red sea bream (Pagrus major). *Aquaculture*, *512*, 734283. https://doi.org/10.1016/j.aquaculture.2019.734283

Kumar, V., Sinha, A., Makkar, H., De Boeck, G., & Becker, K. (2012). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, *96*(3), 335–364. https://doi.org/10.1111/J.1439-0396.2011.01169.X

Künzel, S., Borda-Molina, D., Kraft, R., Sommerfeld, V., Kühn, I., Camarinha-Silva, A., & Rodehutscord, M. (2019). Impact of coccidiostat and phytase supplementation on gut microbiota composition and phytate degradation in broiler chickens. *Animal Microbiome*, *1*(1), 1–14. https://doi.org/10.1186/S42523-019-0006-2

Lozupone, C. A., Hamady, M., Kelley, S. T., & Knight, R. (2007). Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology*, *73*(5), 1576–1585. https://doi.org/10.1128/AEM.01996-06

Lozupone, C., & Knight, R. (2005). UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*, *71*(12), 8228–8235. https://doi.org/10.1128/AEM.71.12.8228-8235.2005

Maas, R. M., Deng, Y., Dersjant-Li, Y., Petit, J., Verdegem, M. C. J., Schrama, J. W., & Kokou, F. (2021). Exogenous enzymes and probiotics alter digestion kinetics, volatile fatty acid content and microbial interactions in the gut of Nile tilapia. *Scientific Reports 2021 11:1*, *11*(1), 1–16. https://doi.org/10.1038/s41598-021-87408-3

Man, Y. B., Zhang, F., Ma, K. L., Mo, W. Y., Kwan, H. S., Chow, K. L., … Wong, M. H. (2020). Growth and intestinal microbiota of Sabah giant grouper reared on food waste-based pellets supplemented with spirulina as a growth promoter and alternative protein source. *Aquaculture Reports*, *18*, 100553. https://doi.org/10.1016/J.AQREP.2020.100553

Martín-Platero, A. M., Valdivia, E., Maqueda, M., & Martínez-Bueno, M. (2007). Fast, convenient, and economical method for isolating genomic DNA from lactic acid bacteria using a modification of the protein "salting-out" procedure. *Analytical Biochemistry*, *366*(1), 102–104. https://doi.org/10.1016/j.ab.2007.03.010

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Merino, G., Barange, M., Blanchard, J. L., Harle, J., Holmes, R., Allen, I., … Rodwell, L. D. (2012). Can marine fisheries and aquaculture meet fish demand from a growing human population in a changing climate? *Global Environmental Change*, *22*(4), 795–806. https://doi.org/10.1016/J.GLOENVCHA.2012.03.003

Metzler-Zebeli, B. U., Klinsoda, J., Vötterl, J. C., & Verhovsek, D. (2020). Maturational

Changes Alter Effects of Dietary Phytase Supplementation on the Fecal Microbiome in Fattening Pigs. *Microorganisms*, *8*(7), 1073. https://doi.org/10.3390/MICROORGANISMS8071073

Niccolai, A., Chini Zittelli, G., Rodolfi, L., Biondi, N., & Tredici, M. R. (2019). Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Research*, *42*(April). https://doi.org/10.1016/j.algal.2019.101617

Oliva-Teles, A., Enes, P., & Peres, H. (2015). *Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish*. *Feed and Feeding Practices in Aquaculture*. Elsevier Ltd. https://doi.org/10.1016/b978-0-08-100506-4.00008-8

Pham, M. A., Lee, K.-J., Dang, T. M., Lim, S.-J., Ko, G.-Y., Eo, J., & Oh, D.-H. (2008). Improved Apparent Digestibility Coefficient of Protein and Phosphorus by Supplementation of Microbial Phytase in Diets Containing Cottonseed and Soybean Meal for Juvenile Olive Flounder (Paralichthys olivaceus). *Asian-Australasian Journal of Animal Sciences*, *21*(9), 1367–1375. https://doi.org/10.5713/AJAS.2008.80053

Rimoldi, S., Gini, E., Koch, J. F. A., Iannini, F., Brambilla, F., & Terova, G. (2020). Effects of hydrolyzed fish protein and autolyzed yeast as substitutes of fishmeal in the gilthead sea bream (Sparus aurata) diet, on fish intestinal microbiome. *BMC Veterinary Research*, *16*(1), 1–13. https://doi.org/10.1186/s12917-020-02416-1

Rosenau, S., Oertel, E., Mott, A. C., & Tetens, J. (2021). The Effect of a Total Fishmeal Replacement by Arthrospira platensis on the Microbiome of African Catfish (Clarias gariepinus). *Life*, *11*(6), 558. https://doi.org/10.3390/LIFE11060558

Roy, S. Sen, & Pal, R. (2014). Microalgae in Aquaculture: A Review with Special References to Nutritional Value and Fish Dietetics. *Proceedings of the Zoological Society*, *68*(1). https://doi.org/10.1007/s12595-013-0089-9

Sales, R., Galafat, A., Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Cerón-García, M. C., … Alarcón, F. J. (2021). Effects of dietary use of two lipid extracts from the microalga Nannochloropsis gaditana (Lubián, 1982) alone and in combination on growth and muscle composition in juvenile gilthead seabream, Sparus aurata. *Algal Research*, *53*, 102162. https://doi.org/10.1016/J.ALGAL.2020.102162

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Kabir Chowdhury, M. A., Parsaeimehr, A., … Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology*, *30*(1), 197–213. https://doi.org/10.1007/s10811-017-1234-z

Shannon, C. E. (1948). A Mathematical Theory of Communication. *The Bell System Technical Journal*, *27*, 379–423, 623–656.

Tang, H. C., Sieo, C. C., Abdullah, N., Chong, C. W., Gan, H. M., Mohd Asrore, M. S., … Ho, Y. W. (2020). Effects of supplementing freeze-dried Mitsuokella jalaludinii phytase on the growth performance and gut microbial diversity of broiler chickens. *Journal of Animal Physiology and Animal Nutrition*, *104*(1), 116–125. https://doi.org/10.1111/JPN.13208

Tilami, S. K., & Sampels, S. (2017). Nutritional Value of Fish: Lipids, Proteins, Vitamins, and Minerals. *Reviews in Fisheries Science & Aquaculture*, *26*(2), 243–253. https://doi.org/10.1080/23308249.2017.1399104

United Nations, Department of Economic and Social Affairs, Population Division. (2019). *World Population Prospects 2019: Highlights (ST/ESA/SER.A/423)*.

Vázquez-Baeza, Y., Gonzalez, A., Smarr, L., McDonald, D., Morton, J. T., Navas-Molina, J. A., & Knight, R. (2017). Bringing the Dynamic Microbiome to Life with Animations. *Cell Host & Microbe*, *21*(1), 7–10. https://doi.org/10.1016/J.CHOM.2016.12.009

Vázquez-Baeza, Y., Pirrung, M., Gonzalez, A., & Knight, R. (2013). EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience*, *2*(1), 16. https://doi.org/10.1186/2047-217X-2-16

Velasquez, S. F., Chan, M. A., Abisado, R. G., Traifalgar, R. F. M., Tayamen, M. M., Maliwat, G. C. F., & Ragaza, J. A. (2016). Dietary Spirulina (Arthrospira platensis) replacement enhances performance of juvenile Nile tilapia (Oreochromis niloticus). *Journal of Applied Phycology*, *28*(2), 1023–1030. https://doi.org/10.1007/S10811-015-0661-Y

Vizcaíno, A. J., Rodiles, A., López, G., Sáez, M. I., Herrera, M., Hachero, I., … Alarcón, F. J. (2018). Growth performance, body composition, and digestive functionality of Senegalese sole (Solea senegalensis Kaup, 1858) juveniles fed diets including microalgae freeze-dried biomass. *Fish Physiology and Biochemistry*, *44*(2), 661–677. https://doi.org/10.1007/S10695-018-0462-8

Yarnold, J., Karan, H., Oey, M., & Hankamer, B. (2019). Microalgal Aquafeeds As Part of a Circular Bioeconomy. *Trends in Plant Science*, *24*(10), 959–970. https://doi.org/10.1016/j.tplants.2019.06.005

Yoo, G. Y., Wang, X., Choi, S., Han, K., Kang, J. C., & Bai, S. C. (2005). Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean rockfish Sebastes schlegeli fed diets containing soybean meal. *Aquaculture*, *243*(1–4), 315–322. https://doi.org/10.1016/J.AQUACULTURE.2004.10.025

DISCUSIÓN GENERAL Y CONCLUSIONES

1. DISCUSIÓN GENERAL

El aumento exponencial que ha experimentado la población mundial en los últimos años, junto con los problemas asociados a la alimentación de animales, como la prohibición de utilizar APCs o el empleo de compuestos poco sostenibles en la elaboración de dietas, ha hecho necesaria la búsqueda de nuevos compuestos capaces de: (1) mejorar la calidad nutricional de las dietas y aumentar su sostenibilidad, (2) mantener o aumentar el crecimiento y los niveles productivos de los animales, y (3) por último reemplazar en la medida de lo posible el uso de APCs. Para ello, en esta Tesis se han evaluado los efectos de algunas dietas enriquecidas bien con microorganismos como la cepa potencialmente probiótica *Enterococcus faecalis* UGRA10 (Capítulo I), o bien mediante la utilización de extractos derivados de plantas del género *Allium* (Capítulos II a V), microalgas crudas e hidrolizadas (Capítulo VI) o microalgas junto con preparados enzimáticos (Capítulo VII) sobre el crecimiento, la productividad y la microbiota intestinal de animales del sector avícola, porcino y piscícola.

La utilización de probióticos en producción animal ha mostrado resultados positivos en diferentes animales de granja, como pollos, gallinas, cerdos, ovejas o vacas. Estos compuestos han conseguido aumentar el crecimiento, la tasa de conversión del alimento en carne o la productividad de los animales, además de mejorar la actividad enzimática del intestino y en la actividad del sistema inmunitario, así como una estabilización de la microbiota del tracto gastrointestinal y una reducción de microorganismos potencialmente patógenos como *Escherichia coli, Salmonella*, *Eimeria acervulina* o *Clostridium perfringens* (revisado en Anadón et al., 2019; Mahesh et al., 2021; Markowiak & Ślizewska, 2018). La mayoría de los probióticos empleados actualmente pertenecen al grupo de bacterias del ácido láctico (BAL), destacando especies de los géneros anteriormente englobados dentro de *Lactobacillus,* así como *Lactococcus, Bifidobacterium*, o *Enterococcus,* o también algunas especies del género *Bacillus*. En el caso del género *Enterococcus*, sin embargo, su uso sigue siendo controvertido pues no contiene el calificativo de GRAS ("*Generally Recognized As Safe*"), ya que incluye algunas especies y cepas potencialmente patógenas. Por ello, los estudios con este género como potencial probiótico son escasos. Sin embargo, los que se han llevado a cabo han mostrado resultados positivos no solo para la salud sino también para el crecimiento de los animales (Franz et al., 2011). Específicamente, la utilización de algunas cepas de *E. faecalis* como potencial probiótico en lechones, peces y aves ha

mostrado efectos positivos para la salud, el crecimiento y la composición de su microbiota intestinal, como el aumento de bacterias beneficiosas y la reducción de patógenos. En lechones, la cepa LAB31 de *E. faecalis* ha conseguido aumentar el crecimiento y reducir los procesos diarreicos, además de producir un aumento de especies del género *Lactobacillus* en las heces (Hu et al., 2015; Wei, 2014); en peces, en un trabajo realizado por Baños et al. (2019) con truchas arcoíris se observó el efecto protector de la cepa *E. faecalis* UGRA10 frente al patógeno *Lactococcus garviae*; mientras que en gallinas, la suplementación de la dieta con *E. faecalis* ha producido mejoras en la forma de los huevos y ha conseguido aumentar la dureza de su corteza (Song et al., 2016). En otro trabajo de Song et al. (2019) realizado con gallinas, la producción de huevos no se vio afectada por la presencia de *E. faecalis* en la dieta, pero se produjo una mejora en la calidad de los huevos, apareciendo mayor dureza de la corteza y mayor calidad proteínica de la clara. Además, se observó un aumento de la diversidad microbiana, así como un ligero incremento en las poblaciones del filo *Bacteroidetes* y una reducción del filo *Firmicutes*, lo cual se ha asociado con un aumento del peso de los animales durante la producción de huevos (Videnska et al., 2014). En otro estudio, la suplementación de la dieta de gallinas ponedoras con una cepa de *E. faecalis* ha aumentado la tasa de puesta, sin afectar al peso ni a la calidad de los huevos (Zhang et al., 2019). Teniendo en cuenta todo lo comentado anteriormente, y dado que el uso de la cepa *E. faecalis* UGRA10 como suplemento para la dieta de gallinas ponedoras no se ha estudiado previamente, en esta Tesis se ha evaluado el efecto de su incorporación en la dieta sobre las comunidades microbianas del intestino y la producción de huevos de gallinas ponedoras (Capítulo I). *E. faecalis* UGRA10 fue inicialmente seleccionada por sus propiedades tecnológicas, funcionales y potencialmente probióticas, además de por su capacidad de producir la enterocina AS-48, una bacteriocina de amplio espectro de acción, y por último por su capacidad para estimular la respuesta inmunitaria en animales (Baños, 2016; Cebrián et al., 2012). Los resultados obtenidos en el Capítulo I han mostrado mejoras en la producción de huevos en gallinas ponedoras, manteniendo su producción a largo plazo, mientras que en el grupo control empieza a caer la puesta a partir de los 40 días de experimento. Además, no se observaron diferencias en el tamaño de los huevos, obteniéndose en su mayoría huevos de tamaño L y XL, lo cual podría resultar bastante positivo dado que los huevos de gran tamaño son los más demandados por el consumidor (Żakowska-Biemans & Tekień, 2017). Resultados similares en cuanto al número, tamaño y peso de los huevos se han obtenido en otros estudios utilizando otras bacterias potencialmente probióticas. Por

ejemplo, la suplementación de la dieta de gallinas con un probiótico formulado con distintas especies bacterianas (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, *Enterococcus faecium*) y/o con el hongo *Aspergillus oryzae* también se relacionó con el incremento en la puesta de huevos de talla L y XL (Tang et al., 2017). La cepa Gallipro de *Bacillus subtilis* provocó igualmente un incremento de la producción, del peso y de la calidad de los huevos (Ribeiro et al., 2014). Resultados similares se obtuvieron al utilizar un preparado probiótico de *B. subtilis* KATMIRA1933 y *Bacillus amyloliquefaciens* B-1895 (Mazanko et al., 2018). Por su parte, la utilización de *Pediococcus acidilactici* en la dieta de gallinas ponedoras también ha supuesto un incremento en la producción y en el peso de los huevos (Mikulski et al., 2020).

Los resultados de este capítulo sugieren que el mantenimiento de la producción de huevos podría estar asociado a cambios en la composición de las comunidades microbianas del íleon y del ciego de las gallinas suplementadas con *E. faecalis* UGRA10. Estas variaciones en la microbiota intestinal podrían estar mediando en la producción de huevos, como sugiere Kraimi et al. (2019). En un trabajo realizado por Shang et al. (2020) utilizando el polisacárido inulina como suplemento de la dieta de gallinas ponedoras también se observó que la función de la dieta en la producción de huevos puede estar asociada con una modulación selectiva de las comunidades microbianas del intestino. Algunos de los principales cambios observados en nuestro estudio incluyen un aumento de la abundancia relativa de *Fusobacterium* y una reducción del género *Phascolarctobacterium* en las gallinas suplementadas con *E. faecalis* UGRA10. Este incremento en la abundancia relativa de *Fusobacterium* en el íleon y el ciego se observó principalmente al final del experimento (76 días), y podría estar relacionado con beneficios en el hospedador, dado que el género *Fusobacterium* contribuye a la producción de butirato y al metabolismo de los ácidos biliares, además también se ha relacionado con una reducción de los niveles de colesterol en sangre (Tzeng et al., 2021). En un trabajo de Zhang et al. (2021) también se ha observado un aumento de este género asociado con la utilización de probióticos como *Bacillus subtilis* en la dieta de gallinas. Por su parte, la reducción en el género *Phascolarctobacterium* podría ser negativa para las gallinas puesto que la presencia de este género se ha asociado con la producción de propionato, el cual puede pasar a la sangre y llegar al cerebro, aumentando la producción de neurotransmisores; y además se ha relacionado con el metabolismo de los ácidos grasos (Borrelli et al., 2017). Por otra parte, los resultados obtenidos en nuestro estudio

mediante técnicas dependientes de cultivo han demostrado una mayor presencia de la cepa *E. faecalis* UGRA10 en las heces a medida que avanza el experimento, sugiriendo que esta cepa podría estar sustituyendo a otras bacterias o enterococos autóctonos de la microbiota intestinal, entre las que se podrían incluir a especies o cepas potencialmente patógenas. En trabajos anteriores se ha demostrado que la suplementación de la dieta con probióticos puede llevar asociada una reducción de patógenos. Por ejemplo, la adición en la dieta de una cepa probiótica de *B. subtilis* produjo una reducción en los niveles de bacterias patógenas como *E. coli* y *C. perfringens*, y un aumento de las poblaciones de bacterias beneficiosas, destacando *Lactobacillus* y *Bifidobacterium*, lo que podría estar asociado con la mejora en la producción y la calidad de los huevos (Guo et al., 2017). Por ello, la mejora en la producción de huevos encontrada en el Capítulo I también podría estar asociada a la reducción de bacterias patógenas en el intestino de gallinas. Sin embargo, hay otros motivos por los cuales este potencial probiótico puede conllevar a un incremento en la producción de huevos, como las modificaciones en la morfología intestinal o la producción de ácidos grasos de cadena corta (SCFAs). Estudios anteriores han mostrado cambios en la estructura intestinal producidos por cepas de *Enterococcus* empleados como probióticos, ocasionando esta bacteria un aumento de la altura de las vellosidades del íleon (Awad et al., 2008). También se ha observado que el uso de *E. faecalis* aumenta los niveles de SCFAs como propionato o butirato en el intestino de lechones y de peces, los cuales están relacionados con el crecimiento y la salud de los animales (Allameh et al., 2017; Wang et al., 2019). Por tanto, sería necesario completar con más estudios que permitan determinar la causa por la cual la cepa *E. faecalis* UGRA10 actúa sobre la estructura y morfología de la mucosa del tracto digestivo de los animales, sobre la producción de SFCAs, o su influencia directa sobre la composición de bacterias potencialmente patógenas que estén presentes en este ambiente.

Los extractos de plantas o fitobióticos también han sido ampliamente utilizados en los últimos años como aditivos que permiten mejorar la calidad de las dietas e incluso para eliminar o reducir en la medida de lo posible la utilización de los antibióticos como promotores del crecimiento (Hashemi & Davoodi, 2011; Vondruskova et al., 2010). La presencia de estos compuestos en dietas, se ha relacionado con mejoras en la salud y el rendimiento de animales de granja, pudiendo actuar adicionalmente como agentes antimicrobianos, antioxidantes, antiinflamatorios o prebióticos (Allen et al., 2013; Gheisar & Kim, 2018; Windisch et al., 2008). Entre los fitobióticos utilizados en

alimentación animal, son de gran importancia los derivados del género *Allium*, cuyos efectos suelen estar relacionados con metabolitos secundarios, como los compuestos bioactivos organosulfurados, entre los que destacan la alicina, el propil propano tiosulfinato (PTS) o el propil propano tiosulfonato (PTSO) (Gheisar & Kim, 2018; Guillamón et al., 2021). El PTSO presenta actividad antimicrobiana, antifúngica y anticoccidia (Kim et al., 2013; Sorlozano-Puerto et al., 2018, 2021), y ha mostrado efectos beneficiosos en la salud, la microbiota y el crecimiento de ratones, pollos de engorde y cerdos (Peinado et al., 2013; Peinado et al., 2012; Sánchez et al., 2020; Vezza et al., 2021). Sin embargo, son pocos los trabajos que relacionan los parámetros productivos de los animales suplementados con extractos de *Allium* ricos en PTSO con la composición y la diversidad de la microbiota intestinal mediante estudios de secuenciación masiva. En esta Tesis se han evaluado los efectos de estos compuestos sobre las comunidades microbianas del intestino y sobre el crecimiento y los parámetros productivos de gallinas ponedoras (Capítulo II), lechones destetados (Capítulo III), juveniles de dorada (Capítulo IV) y juveniles de lubina (Capítulo V).

Para determinar la posibilidad de emplear el extracto de *Allium* rico en PTSO como suplemento para el sector avícola, en el Capítulo II se ha utilizado este compuesto en la dieta de gallinas ponedoras y se han estudiado sus efectos sobre la producción de huevos y la composición de la microbiota del tracto intestinal. Los resultados en cuanto a producción obtenidos en estudios previos utilizando extractos de *Allium* en la dieta de gallinas son dispares. En algunos casos no se han observado mejoras en la producción de huevos en gallinas suplementadas con extractos de ajo y cebolla (Asrat et al., 2018); en otros trabajos se ha observado un incremento en el peso de los huevos pero no un aumento de la producción (Abad et al., 2021; Mahmoud et al., 2010); mientras que en otros se ha observado un aumento de la producción de huevos en gallinas suplementadas con extractos de ajo y cebolla, pero no se ha producido un incremento en el peso y la talla de los mismos (Omer et al., 2019). Probablemente la diferencia en los resultados de estos trabajos esté relacionada con el porcentaje o la concentración del principio activo en la dieta, o incluso con la forma de administración. Los resultados obtenidos en nuestro estudio han evidenciado un incremento significativo en el peso de los huevos, así como un aumento de la producción de huevos en las gallinas alimentadas durante 30 días con la dieta fitobiótica, mientras que la producción del grupo control tiene una tendencia negativa, produciéndose una disminución en la puesta a medida que avanza el

experimento. Comparando nuestros resultados con los del estudio de Abad et al. (2021), se puede determinar que el PTSO tiene efectos similares en el peso de los huevos utilizando diferentes concentraciones (30 mg/kg en el estudio de Abad et al. (2021) frente a los 60 mg/kg utilizados en este trabajo). La producción de huevos sí parece estar relacionada con la dosis de PTSO empleada, ya que la dosis de 60 mg/kg produjo un aumento de la puesta de huevos (Capítulo II) frente a la dosis de 30 mg/kg de PTSO, que no se relacionó con una mejora en la producción de huevos por parte de las gallinas respecto al grupo control (Abad et al., 2021). Sin embargo, estas diferencias también podrían ser debidas a otros factores, como la utilización de diferentes variedades de gallinas (Hy Line Brown vs Lohmann Brown) o también a la propia edad de las gallinas al inicio del experimento (16 semanas vs 36 semanas).

El incremento en la puesta y el peso de los huevos estuvo acompañado con cambios en la diversidad y en la composición de la microbiota intestinal en el íleon y en el ciego de los animales. Los mayores cambios en la estructura de las comunidades microbianas aparecen en la parte posterior del tracto digestivo intestinal, lo cual se podría explicar por la mayor disponibilidad del principio activo (PTSO) en esta parte del digestivo. En relación con este resultado, Abad et al. (2018) demostraron un incremento en la concentración de este compuesto a medida que avanza el intestino en un modelo de simulación *in vitro* de intestino de cerdos, utilizando varios tanques y reactores conectados en serie a los cuales se añadían soluciones gástricas, simulando el funcionamiento del intestino de cerdo. Sin embargo, y a pesar de las diferencias morfológicas y funcionales del tracto gastrointestinal de gallinas y cerdos, como la mayor compartimentación o la presencia de microvellosidades más alargadas en el intestino de gallinas, es posible que el PTSO actúe de forma similar, resistiendo al efecto de las enzimas digestivas en las regiones anteriores y liberándose mayoritariamente en el ciego de los animales. El PTSO produjo un incremento de ciertos grupos de bacterias potencialmente beneficiosas en el intestino de las gallinas, como por ejemplo las del género *Lactococcus* en el íleon y *Lactobacillus* en el ciego. Este incremento en la abundancia relativa de las poblaciones de *Lactobacillus* también fue observado en los estudios realizados por Abad et al. (2021), donde encontraron mayores niveles de este género en heces de gallinas suplementadas con PTSO. Otra investigación realizada en gallinas ponedoras también mostró una relación entre el incremento de las poblaciones de *Lactobacillus* en el ciego y un aumento de la puesta y el peso de los huevos (Park et

al., 2016). La mayor producción de huevos podría estar relacionada con otros efectos positivos ejercidos por *Lactobacillus* en el intestino de gallinas, como la secreción de metabolitos y enzimas que facilitan la digestión y absorción de nutrientes (Alaqil et al., 2020). En el trabajo de Pan & Yu (2013) también se ha relacionado una mayor absorción de nutrientes con un incremento en las poblaciones de *Lactobacillus*, lo cual podría estar mediando en la productividad de las gallinas. Además, la presencia de *Lactobacillus* juega un papel importante en la hidrólisis de lactosa, generando ácido láctico y SCFAs que reducen el pH intestinal y contribuyen a producir un efecto de barrera intestinal contra algunos patógenos como por ejemplo *Salmonella* (Cesari et al., 2014). Por otra parte, Han et al. (2016) encontraron una correlación entre el aumento en las proporciones de *Lactococcus* en el ciego de pollos y el incremento del peso de los animales. Además, la utilización del extracto de PTSO en nuestro estudio también produjo una reducción de algunos grupos bacterianos que podrían estar asociados con efectos negativos en el hospedador, como el género *Acinetobacter*, un potencial patógeno de gallinas (Zhu et al., 2020) o el género *Anaerobiospirillum*, en el cual se incluyen algunas especies patógenas de mamíferos y aves (Elokil et al., 2020). Por ello, estos resultados parecen indicar los posibles beneficios de la ingesta de PTSO en la producción de huevos probablemente equilibrando o modificando parte de la composición de la microbiota intestinal de gallinas ponedoras.

Con respecto a la industria porcina, como se ha descrito ampliamente en el apartado de Introducción, durante años se han conseguido mantener los niveles de producción gracias en parte a la mejora genética de las razas destinadas a la producción de carne, a la mejora en las dietas, y también por la utilización de APCs, entre otros factores. Sin embargo, su impacto negativo por el incremento de multirresistencias a antibióticos ha favorecido el uso de ciertos aditivos alimentarios como alternativa, entre los que se encuentran algunos derivados de plantas, como los fitobióticos (Gheisar & Kim, 2018; Thacker, 2013). Algunos de estos compuestos han mostrado resultados positivos al ser incorporados a la dieta de animales del sector porcino (Windisch et al., 2008). En este sentido, se ha propuesto el uso de un extracto derivado de plantas del género *Allium* como alternativa a los APCs en la industria porcina. Para comprobar la posibilidad de utilizar este compuesto en lechones, en el Capítulo III se han evaluado sus efectos sobre la composición y la diversidad de la microbiota de diferentes regiones del tracto intestinal y los parámetros productivos de lechones destetados a los 28 días en

comparación con una dieta control y otra dieta antibiótica (colistina y óxido de zinc). Los resultados obtenidos en este capítulo apuntan a que el uso de este extracto puede resultar beneficioso en la industria porcina, dado que su adición durante 42 días permitió obtener unos valores de peso corporal, ganancia de peso diaria (ADG) y tasa de conversión del alimento (FCR) de los lechones similares a los obtenidos con el tratamiento antibiótico. Por tanto, su aplicación permitiría, por ejemplo, reducir el uso de colistina, un antibiótico ampliamente utilizado durante años para tratar la diarrea post-destete en lechones y también como promotor del crecimiento. Entre las consecuencias negativas de su uso destaca el aumento creciente de microorganismos resistentes a este antibiótico (Rhouma et al., 2017). Algunos extractos obtenidos de plantas como tomillo, orégano, cúrcuma, menta, canela o ajo, entre otros, también produjeron resultados similares a los obtenidos en este trabajo, provocando un mayor crecimiento en los cerdos suplementados con estas dietas experimentales (revisado en Gheisar & Kim, 2018; Omonijo et al., 2018; Thacker, 2013). Sin embargo, los resultados obtenidos varían en función de la dosis, la especie o el principio activo empleado. Los trabajos realizados por Ogbuewu et al. (2019) y Sánchez et al. (2020) con extractos de ajo, también mostraron mayor ganancia de peso y mejoras en otros parámetros productivos relacionados con el crecimiento en cerdos, como el índice de consumo del alimento, la tasa de conversión del alimento, la eficiencia alimenticia o la tasa de crecimiento específico (SGR). Además, la suplementación de la dieta con extracto de ajo estimula la secreción de enzimas intestinales y mejora el sistema inmunitario (Ogbuewu et al., 2019).

En lo referente a la microbiota intestinal, los resultados obtenidos en el Capítulo III de esta Tesis han mostrado cambios en la composición microbiana del ciego y colon, reduciéndose la diversidad en ambas regiones en lechones suplementados con la dieta experimental. Este efecto, se ha relacionado con obesidad en algunos trabajos realizados con humanos (Menni et al., 2017). Además, en el ciego y el colon de los lechones suplementados con PTSO se obtiene una abundancia relativa de *Lactobacillus* similar a la del control, mientras que el tratamiento antibiótico redujo las poblaciones de esta bacteria. Este hecho puede resultar positivo para los lechones, dado que el aumento de *Lactobacillus* se ha asociado con un incremento en la eficiencia alimenticia, así como una mayor concentración de SCFAs como acetato y butirato en el colon, los cuales pueden actuar como fuente de energía para las células del colon de los cerdos (Valeriano et al., 2017). El butirato no es un metabolito propio de los lactobacilos, pero se ha observado

que este grupo bacteriano puede mejorar la capacidad de producción de las bacterias productoras de butirato en el tracto intestinal (Chu et al., 2020). Además, una alta abundancia de lactobacilos en la parte distal del intestino se ha relacionado con un aumento de los SCFAs y un incremento de los parámetros productivos en cerdos (Sánchez et al., 2020). También encontramos cambios en otros géneros menos abundantes, como *Blautia* y *Megasphaera*, las cuales incrementaron ligeramente su abundancia relativa en el ciego y el colon de los lechones suplementados con *Allium*. *Blautia* se ha relacionado con la descomposición de fibras y almidón, convirtiéndolos en SCFAs (principalmente acetato), además de producir una mejora en la eficiencia alimenticia de los cerdos (Bergamaschi et al., 2020; Yang et al., 2018). Por su parte, especies de *Megasphaera* están involucradas en la degradación de ácido láctico, produciendo diferentes SFCAs cómo acetato, propionato y butirato (Kajihara et al., 2017). La aparición de estos cambios principalmente en las partes finales del intestino podría estar asociada con la disponibilidad del extracto de *Allium* en algunas de las regiones intestinales. Así, en el estudio llevado a cabo por Abad et al. (2018) en el cual utilizaron una simulación *in vitro* del tracto gastrointestinal de cerdo, demostraron que el PTSO (en forma capsulada) es resistente a las secreciones del estómago y de las regiones anteriores del intestino, alcanzando concentraciones superiores de este compuesto en las regiones finales del tracto intestinal. Este hecho podría explicar los efectos inducidos por la dieta experimental en las regiones posteriores del intestino de lechones.

Para conocer los efectos de la utilización de PTSO sobre el crecimiento y las comunidades microbianas del intestino de especies piscícolas, en los Capítulos IV y V se ha empleado este compuesto como aditivo en la dieta de juveniles de doradas y lubinas. En este sentido, algunos compuestos derivados plantas como romero, eucalipto, orégano o ajo, entre otras, han mostrado efectos positivos en la salud y el crecimiento de peces (Reverter et al., 2014). Los extractos de ajo (*Allium*), al igual que en mamíferos y aves, también parecen conferir propiedades biológicas interesantes para su utilización en acuicultura, pudiendo actuar igualmente como promotores del crecimiento, estimulantes del apetito, inmunoestimulantes, o como potentes antimicrobianos, antivirales, antioxidantes o antiparasitarios (Lee & Gao, 2012; Valenzuela-Gutiérrez et al., 2021). Las propiedades de estos compuestos, como se ha comentado anteriormente, son debidas a la presencia de compuestos organosulfurados como la alicina, el PTS o el PTSO. Sin embargo, la adición de PTSO en la dieta de animales acuáticos como doradas y lubinas

no se había estudiado hasta ahora, por lo que los resultados obtenidos los Capítulos IV y V de esta Tesis resultan especialmente novedosos.

Con respecto a los parámetros productivos, los resultados obtenidos en ambos estudios fueron similares, no encontrándose diferencias en lo que respecta al crecimiento de los peces con respecto a la dieta control a lo largo del experimento. No obstante, en las lubinas suplementadas con PTSO sí que se produjo un aumento significativo del peso al final del experimento (Día 89). Otros trabajos en los que han utilizado extractos del género *Allium* como suplementos en la dieta de peces han mostrado resultados dispares con respecto al crecimiento. Así, por ejemplo, en el caso de la trucha arcoíris (Büyükdeveci et al., 2018), del pez gato africano (Gabriel et al., 2019) o de la tilapia del Nilo (Shalaby et al., 2006) la administración de estos compuestos en la dieta logró un aumento del crecimiento de los animales, en cambio en el pez guppy el tratamiento no consiguió ningún efecto sobre el crecimiento de los animales (Motlagh et al., 2020a, 2020b). Las diferencias descritas entre estos estudios podrían estar asociadas a la forma en la que se administró la dieta, ya que en los estudios en los cuales se observaron mejoras en el crecimiento el extracto de ajo se administró en forma de polvo, mientras que en los estudios con pez guppy, en los cuales no aparecieron mejoras en el crecimiento, se utilizó un extracto acuoso de ajo. Sin embargo, en nuestro estudio la suplementación de extracto de PTSO en polvo no supuso un aumento en el crecimiento de los peces, salvo en el último muestreo de lubinas. No obstante, hay otros factores que podrían estar relacionados con estas diferencias, como la calidad o los niveles de inclusión de los compuestos activos, la duración del experimento, el tamaño y la edad de los peces, el sistema de cultivo o la utilización de diferentes especies de peces (Sales, 2009). Estas diferencias en el crecimiento de las distintas especies de peces pueden estar asociadas a los propios requerimientos nutricionales de cada especie.

En lo que se refiere a la composición del microbioma intestinal, los cambios producidos por el PTSO en la diversidad alfa y beta no fueron iguales en ambas especies de peces. En el caso de las doradas (Capítulo IV), la adición de PTSO produjo un aumento del índice de diversidad de Shannon, mientras que en las lubinas (Capítulo V) se observó una disminución de la diversidad con los distintos índices estudiados, a pesar que en ambos casos se utilizó la misma concentración del principio activo (PTSO). Con los datos actuales, no está claro si la diversidad microbiana se correlaciona de forma positiva o negativa con el crecimiento de los peces. Por ejemplo, en la perca, la diversidad

microbiana más baja se ha relacionado con una mejor condición del animal; el espinoso de laboratorio mostró una tendencia opuesta, apareciendo una correlación positiva entre la diversidad microbiana y la condición de los animales; mientras que el espinoso macho salvaje no mostró ningún efecto de la diversidad microbiana sobre la condición (Bolnick et al., 2014). El factor de condición se basa en que los peces de mayor peso a una determinada longitud presentan una mejor condición o un mejor bienestar (Froese, 2006). En estudios realizados con otras especies de peces también se observaron resultados dispares en la relación entre crecimiento y diversidad microbiana. En un trabajo realizado con *Coreius guichenoti*, un pez perteneciente a la familia de las carpas, no se encontró relación entre el peso corporal y la diversidad microbiana (Li et al., 2016); mientras que en carpa común se relacionó un aumento de la diversidad con un mayor crecimiento de los peces (Luo et al., 2020). A pesar de estas diferencias, parece claro que los cambios en la diversidad microbiana se asocian con variaciones en la condición y el crecimiento de los peces (Bolnick et al., 2014). La diferencia que hemos obtenido en la diversidad entre ambos estudios podría estar relacionada con la diferencia en la composición microbiana autóctona de cada una de las especies más que por factores externos, como la temperatura, la salinidad o la composición del agua, ya que estos factores estaban controlados, y en las etapas tempranas del desarrollo la microbiota intestinal parece estar muy relacionada con la propia comunidad bacteriana del ambiente en el que se encuentran los animales (Stephens et al., 2015).

A pesar de estas diferencias en los índices de diversidad, en ambos estudios han aparecido algunos cambios similares en la composición microbiana en ambas especies de peces, especialmente en lo que respecta a los géneros mayoritarios como *Vibrio*. En ambos casos se ha observado una reducción en su abundancia relativa tanto en la parte anterior y como posterior del intestino de los peces suplementados con PTSO. Este hecho podría resultar positivo para la salud de los peces, dado que algunas especies de *Vibrio* son potencialmente patógenas para especies piscícolas (Abdel-Aziz et al., 2013; Yukgehnaish et al., 2020), como por ejemplo *V. parahaemolyticus*, *V. harveyi, V. anguillarum, V. cholerae* y *V. vulnificus*, entre otras (Yukgehnaish et al., 2020). En el caso de *V. harveyi*, es el principal responsable de enfermedades en doradas como la podredumbre de la cola (Haldar et al., 2010). Por su parte, *V. anguillarum* es uno de los principales causantes de vibriosis, una enfermedad caracterizada por producir úlceras en la piel y por la aparición de manchas rojas en los laterales y el vientre de diferentes

especies de peces, incluyendo doradas y lubinas (Frans et al., 2011; Torrecillas et al., 2017). Por tanto, la reducción de especies de *Vibrio* causada por el consumo de PTSO podría resultar positiva para la salud de juveniles de dorada y lubina o para el tratamiento de enfermedades ligadas a estas especies de peces. Sin embargo, la adición de PTSO también produjo cambios diferentes en la composición de algunos géneros bacterianos. Por ejemplo, el género *Pseudomonas* redujo su abundancia relativa en las doradas y aumentó en las lubinas suplementadas con PTSO. Este género se ha descrito como ubicuo, siendo común en la microbiota intestinal de peces, y algunas especies se han considerado patógenos oportunistas (Yukgehnaish et al., 2020). *P. anguilliseptica* es el agente causante de la enfermedad de invierno, una septicemia caracterizada por distensión abdominal, hemorragias en riñón, hígado pálido e intestino con exudado amarillento, y que afecta a diferentes especies de peces como el bacalao, la lubina y la dorada (Wiklund, 2016). Otras especies consideradas como oportunistas son *P. aeruginosa*, *P. fluorescens* y *P. putida*. En doradas también se encontró un aumento en la abundancia relativa de *Lactobacillus*, un género considerado como beneficioso ya que está asociado a un epitelio intestinal sano en algunas especies de peces (Estruch et al., 2015). Además, los lactobacilos pueden inhibir la adhesión al epitelio intestinal de bacterias patógenas de peces como *Vibrio anguillarum*, *Aeromonas hydrophila*, *Aeromonas salmonicida* y *Yersinia ruckeri* (Balcázar et al., 2008). En lubinas no se han observado cambios en las poblaciones de lactobacilos, pero si se ha visto un incremento en la abundancia relativa de *Kocuria*, género que ha sido relacionado con la inducción del sistema inmunitario, pudiendo inhibir el crecimiento de bacterias patógenas como *Vibrio anguillarum* (Sharifuzzaman et al., 2011). Sin embargo, sería necesario estudiar en profundidad mediante PCR cuantitativa (qPCR) o PCR digital (ddPCR) si estos cambios en la abundancia relativa se corresponden con cambios en la abundancia absoluta de especies potencialmente patógenas o potencialmente beneficiosas.

En resumen, los resultados obtenidos utilizando compuestos derivados del ajo, como el PTSO, aunque han sido diferentes según el modelo de animal elegido, parecen ser en conjunto positivos, bien en cuanto a alguno de los parámetros productivos medidos (puesta de huevos en aves o peso en lechones) o en cuanto a la reducción en potenciales patógenos, como sucede en peces como doradas y lubinas. En cualquier caso, no parecen inducir efectos negativos en el crecimiento de los animales.

Por otra parte, la insostenibilidad de las dietas por el uso excesivo de ingredientes como la harina y el aceite de pescado es uno de los principales problemas asociados con la alimentación de animales de acuicultura. Estos compuestos, y principalmente la harina de pescado, se han utilizado de forma masiva durante años, siendo uno de los componentes mayoritarios de las dietas empleadas en alimentación de peces (Shepherd & Jackson, 2013). Para elaborar la harina de pescado se suelen utilizar peces forrajeros obtenidos en pesca de captura, los cuales se consideran adecuados para consumo humano, y por ello no se considera sostenible su uso en la elaboración de dietas de peces (Cashion et al., 2017; Gasco et al., 2018; Shepherd & Jackson, 2013). Por tanto, ha surgido la necesidad de utilizar compuestos que puedan satisfacer los requerimientos nutricionales de los animales de acuicultura, y con ello reducir o eliminar en lo posible el uso de productos derivados del pescado. Entre las posibles alternativas se han descrito compuestos de origen vegetal o proteínas de origen animal, como harina de hueso o de pluma (Gasco et al., 2018), entre otros. Además de estos compuestos, se ha propuesto en los últimos años el uso de especies de microalgas para la elaboración de dietas de peces dado su alto contenido en proteínas, lípidos, vitaminas, minerales, y pigmentos, entre otros nutrientes (Shah et al., 2018). Por este motivo, en el Capítulo VI se ha utilizado biomasa cruda e hidrolizada de la microalga *Arthrospira platensis*, y en el Capítulo VII se ha utilizado una mezcla de *A. platensis* y *Nannochloropsis gaditana* junto con un preparado de la enzima fitasa a diferentes concentraciones, con el fin de mejorar la calidad nutricional de los piensos y con ello reducir el impacto del empleo de harina de pescado en la dieta de doradas y lubinas en etapas tempranas del desarrollo.

Para comprobar la posibilidad de utilizar biomasa cruda e hidrolizada de microalgas en la dieta de larvas de dorada como alternativa a la harina de pescado, en el Capítulo VI se ha suplementado el pienso con un 5% y/o 10% de *A. platensis* tanto cruda como hidrolizada. La utilización de hidrolizados de proteínas en la dieta de peces en etapas tempranas del desarrollo parece permitir una mejora en la digestión y asimilación de los nutrientes, de acuerdo con los estudios de Egerton et al. (2020) y Srichanun et al. (2014), que favorecen tanto la maduración de los órganos digestivos como el crecimiento de los peces. La utilización de hidrolizados de microalgas en la dieta de peces ha mostrado resultados beneficiosos para los animales (Ayala et al., 2020; Shah et al., 2018; Valente et al., 2019). La inclusión de un hidrolizado *N. gaditana* en la dieta de esturiones siberianos supuso un aumento del peso corporal de los peces (Bongiorno et al., 2022). En

doradas, un 5% de biomasa hidrolizada de *N. gaditana* ha mostrado una mejora en parámetros relacionados con la morfología del animal, así como un incremento de las fibras musculares (Ayala et al., 2020). Otro estudio realizado por Galafat et al. (2020) ha mostrado una mejora en el epitelio intestinal, la capacidad antioxidante y la pigmentación del músculo de juveniles de dorada suplementados con *Arthrospira* spp., sin llegar a afectar a su crecimiento. Sin embargo, el uso de *A. platensis* cruda e hidrolizada en las etapas larvarias ha sido poco estudiado hasta la fecha. Los resultados obtenidos han demostrado la posibilidad de utilizar estas dietas suplementadas con el 5% de *A. platensis* cruda, 5% de *A. platensis* hidrolizada o 10% de *A. platensis* hidrolizada, ya que mantuvieron en todos los casos el crecimiento de las larvas de dorada alcanzando niveles similares al control, sin ningún efecto negativo en los animales y sin provocar alteraciones en la composición de las comunidades microbianas del tracto intestinal. Además, estos resultados se pueden complementar con los obtenidos en un estudio reciente realizado por Galafat et al. (2022), que demostraron una mejora en la actividad de algunas enzimas intestinales relacionadas con los procesos de digestión, como la tripsina, la quimotripsina y la leucina aminopeptidasa, así como un aumento de la longitud y el diámetro de las microvellosidades intestinales, y un incremento de la capacidad antioxidante del hígado de larvas de dorada alimentadas con las mismas dietas suplementadas con *A. platensis* cruda e hidrolizada. Resultados similares fueron obtenidos por Galafat et al. (2020), donde no encontraron ningún cambio en relación con el crecimiento de juveniles de dorada alimentados con hidrolizados de *Arhtrospira* sp., pero sí se produjeron mejoras del epitelio intestinal (mayor longitud de las microvellosidades y mayor capacidad de absorción), además de incrementar la actividad de algunas enzimas intestinales (tripsina, quimotripsina y leucina aminopeptidasa) y la capacidad antioxidante. Por tanto, estos resultados en conjunto parecen indicar la posibilidad de utilizar biomasa cruda e hidrolizada de microalgas como alternativa al uso de harinas de pescado en la dieta, ya que, aunque no influyen en el crecimiento del animal, sin embargo, no inducen cambios en la composición de la microbiota intestinal, pero si mejoras en otros parámetros relacionados con la salud intestinal y el bienestar animal.

Por último y para evaluar el efecto de la enzima fitasa junto con microalgas en la dieta de especies acuáticas, en el Capítulo VII de esta Tesis se ha incorporado un 2,5% de biomasa cruda de *A. platensis* y *N. gaditana* y diferentes concentraciones de fitasa en el pienso de juveniles de lubina. La fitasa es una enzima que hidroliza el ácido fítico no

digerible y hace que la concentración de fósforo esté disponible para su absorción por parte de los animales, además de aumentar la disponibilidad de otros minerales que pueden encontrarse asociados al ácido fítico, como calcio, hierro o potasio (Kumar et al., 2012). El uso de fitasa ha sido ampliamente estudiado, mostrando mejoras en la salud y en los niveles productivos de animales de granja (Romano & Kumar, 2018). Aunque tradicionalmente en acuicultura se ha utilizado menos esta enzima, desde hace unos años diversos estudios han añadido fitasa en la dieta de algunas especies acuáticas, mostrando resultados positivos en la digestibilidad de nutrientes y en algunos casos mejorando el crecimiento de los peces (Kumar et al., 2012; Lemos & Tacon, 2017). Sin embargo, hasta la fecha no se han realizado estudios que combinen la fitasa junto con biomasa de microalgas. Por ello, en nuestro trabajo hemos evaluado cómo afecta la inclusión de biomasa cruda de *A. platensis* y *N. gaditana* junto con 500, 1.000, 2.000 o 10.000 FTU/kg de fitasa al crecimiento y a la composición y la diversidad de las comunidades microbianas del intestino de juveniles de lubina. Los mejores resultados en cuanto a crecimiento y a microbiota se obtuvieron con la dieta que incluye 2,5% de biomasa de microalgas y 10.000 FTU/kg de fitasa. Los peces suplementados con esta dieta aumentaron de forma significativa su peso corporal, el porcentaje de ganancia de peso, la tasa de crecimiento específico (SGR) y la eficiencia alimenticia, mientras que presentaron una composición de la microbiota intestinal similar a la de los peces control. Además, los resultados obtenidos por Galafat et al. (2021) mostraron una mayor altura de las vellosidades intestinales, así como un aumento de la actividad de las enzimas tripsina, quimotripsina, L-aminopeptidasa y fosfatasa alcalina en las lubinas suplementadas con 2,5% de microalgas y 10.000 FTU/kg de fitasa. Estos resultados obtenidos en cuanto al crecimiento no son muy comunes con dosis altas de fitasa, ya que las concentraciones de esta enzima utilizadas en la mayor parte de trabajos son de 500 a 2.000 FTU/kg en especies terrestres, y de 500 a 4.000 FTU/kg en especies acuáticas (Lemos & Tacon, 2017; Romano & Kumar, 2018). Sin embargo, algunos estudios realizados con pollos, gallinas y peces sí que han mostrado resultados similares, demostrando sus beneficios a dosis elevadas de fitasa sobre la salud, el crecimiento o la productividad de los animales (Boney & Moritz, 2017; Kim et al., 2017; Yan & Reigh, 2002). Con respecto a la microbiota intestinal, pocos trabajos han estudiado los efectos de la fitasa sobre las comunidades microbianas de peces, aunque algunas investigaciones realizadas por Adeoye et al. (2016a, 2016b) con tilapias del Nilo no han mostrado alteraciones en la diversidad y en la estructura de las comunidades microbianas de las peces suplementados

con esta enzima. Por tanto, la utilización de una dosis elevada de fitasa podría afectar positivamente a la digestibilidad de los diferentes componentes de la dieta, lo cual se podría relacionar con la mejora en los procesos de digestión y por tanto un aumento de los distintos parámetros de crecimiento estudiados en el Capítulo VII.

En resumen, los resultados obtenidos en esta Tesis muestran la importancia de suplementar las dietas de animales con aditivos, en algunos casos potenciales probióticos, en otros compuestos derivados de plantas, y en otros microalgas solas o junto con preparados enzimáticos, permitiendo mejorar la calidad nutricional de estas dietas y mantener o aumentar el crecimiento y los parámetros productivos de animales del sector avícola, porcino y piscícola. El uso de la cepa UGRA10 de *Enterococcus faecalis* parece confirmar y completar los estudios sobre su efecto potencial probiótico en animales y sus propiedades tecnológicas y funcionales (Capítulo I); el consumo de los compuestos fitobióticos derivados de plantas del género *Allium* (Capítulos II a V) también parece ejercer un efecto beneficioso, en algunos casos modificando la composición de la microbiota como es el caso del PTSO principalmente en peces, y también un efecto positivo en la producción y el tamaño de huevos en gallinas o un incremento del peso y otros parámetros de crecimiento en lechones; por último la biomasa de microalgas cruda e hidrolizada parece ser una buena alternativa a las dietas basadas en harinas de pescado (Capítulo VI), y la administración de microalgas junto con preparados enzimáticos como la fitasa incrementa la mejora productiva de juveniles de lubina sin alterar la microbiota intestinal (Capítulo VII). Además, la mayoría de estas dietas inducen algunos cambios en la composición de la microbiota del tracto intestinal de los animales, como el incremento de bacterias beneficiosas o en algunos casos la reducción de géneros potencialmente patógenos. Estos resultados abren la posibilidad de nuevas investigaciones que permitan explorar otros parámetros relacionados con aspectos importantes para la salud y el bienestar de los animales, como la respuesta de su sistema inmunitario, su resistencia frente a patógenos, la morfología intestinal, la actividad de las enzimas intestinales o la composición de la microbiota intestinal, entre otros. Esto último se ha estudiado en esta Tesis mediante secuenciación masiva, mostrando en algunos casos un aumento relativo de géneros bacterianos beneficiosos o una disminución relativa de géneros bacterianos potencialmente patógenos. Sin embargo, habría que corroborar estos resultados mediante cuantificación absoluta por ejemplo mediante PCR cuantitativa o PCR digital, pudiendo confirmar los efectos que ejercen estos tratamientos sobre bacterias beneficiosas y

patógenas de cada especie animal. Por otra parte, también se podría estudiar la aplicación de diferentes dosis de estos compuestos, así como la posibilidad de emplearlos como aditivos para la dieta de otras especies de animales tanto terrestres como acuáticos. Todo este conocimiento permitirá un diseño racional de compuestos aditivos y microorganismos probióticos para elaboración de dietas de distintos animales.

2. CONCLUSIONES

1. La dieta suplementada con *Enterococcus faecalis* UGRA10 en gallinas ponedoras incrementa la producción de huevos y aumenta la diversidad microbiana en íleon y ciego.

2. El extracto del género *Allium* (PTSO) utilizado como aditivo para la dieta de gallinas ponedoras aumenta la cantidad y el peso de los huevos hasta los 30 días de tratamiento, junto con un incremento de la proporción relativa de cepas potencialmente probióticas como *Lactococcus* en el íleon y *Lactobacillus* en el colon.

3. La adición de extracto del género *Allium* aumenta el crecimiento de lechones destetados tras 42 días de tratamiento, con peso corporal, ganancia de peso y tasa de conversión del alimento similares a los obtenidos con un tratamiento antibiótico. Esta mejora se asocia con cambios en la estructura de las comunidades microbianas del ciego y del colon.

4. El uso de PTSO en la dieta de juveniles de doradas aumenta la proporción relativa del género *Lactobacillus* y disminuye otros géneros potencialmente patógenos de peces como *Vibrio* o *Pseudomonas*, sin afectar a su crecimiento.

5. La suplementación de la dieta de juveniles de lubinas con PTSO reduce la proporción relativa del género *Vibrio* y produce un aumento significativo del peso corporal de los peces a los 89 días de tratamiento.

6. Las dietas de larvas de doradas adicionadas con un 5% de biomasa cruda, 5% de biomasa hidrolizada o 10% de biomasa hidrolizada de *Arthrospira platensis* permiten mejorar la calidad nutricional del pienso sin alterar la composición de la microbiota intestinal ni inducir efectos negativos en el crecimiento de los animales.

7. El uso de 2,5% de *Arthrospira platensis* y *Nannochloropsis gaditana* y altos niveles de fitasa (10.000 FTU/kg) en la dieta de juveniles de lubinas se asocia con un incremento del peso corporal y de otros parámetros productivos como el porcentaje de ganancia de peso, la tasa de crecimiento específica o la eficiencia alimenticia, sin alterar la composición de sus comunidades microbianas intestinales.

3. REFERENCIAS

Abad, P., Arroyo-Manzanares, N., Ariza, J. J., Baños, A., & García-Campaña, A. M. (2021). Effect of Allium Extract Supplementation on Egg Quality, Productivity, and Intestinal Microbiota of Laying Hens. *Animals*, *11*(1), 41. https://doi.org/10.3390/ani11010041

Abad, P., Arroyo-Manzanares, N., Rivas-Montoya, E., Ochando-Pulido, J. M., Guillamon, E., García-Campaña, A. M., & Martinez-Ferez, A. (2018). Effects of different vehiculization strategies for the allium derivative propyl propane thiosulfonate during dynamic simulation of the pig gastrointestinal tract. *Canadian Journal of Animal Science*, *99*(2), 244–253. https://doi.org/10.1139/cjas-2018-0063

Abdel-Aziz, M., Eissa, A. E., Hanna, M., & Okada, M. A. (2013). Identifying some pathogenic Vibrio/Photobacterium species during mass mortalities of cultured Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) from some Egyptian coastal provinces. *International Journal of Veterinary Science and Medicine*, *1*(2), 87–95. https://doi.org/10.1016/j.ijvsm.2013.10.004

Adeoye, A. A., Jaramillo-Torres, A., Fox, S. W., Merrifield, D. L., & Davies, S. J. (2016). Supplementation of formulated diets for tilapia (Oreochromis niloticus) with selected exogenous enzymes: Overall performance and effects on intestinal histology and microbiota. *Animal Feed Science and Technology*, *215*, 133–143. https://doi.org/10.1016/J.ANIFEEDSCI.2016.03.002

Adeoye, A. A., Yomla, R., Jaramillo-Torres, A., Rodiles, A., Merrifield, D. L., & Davies, S. J. (2016). Combined effects of exogenous enzymes and probiotic on Nile tilapia (Oreochromis niloticus) growth, intestinal morphology and microbiome. *Aquaculture*, *463*, 61–70. https://doi.org/10.1016/J.AQUACULTURE.2016.05.028

Alaqil, A. A., Abbas, A. O., El-Beltagi, H. S., Abd El-Atty, H. K., Mehaisen, G. M. K., & Moustafa, E. S. (2020). Dietary Supplementation of Probiotic Lactobacillus acidophilus Modulates Cholesterol Levels, Immune Response, and Productive Performance of Laying Hens. *Animals*, *10*(9), 1588. https://doi.org/10.3390/ANI10091588

Allameh, S. K., Ringø, E., Yusoff, F. M., Daud, H. M., & Ideris, A. (2017). Dietary supplement of Enterococcus faecalis on digestive enzyme activities, short-chain fatty acid production, immune system response and disease resistance of Javanese carp (Puntius gonionotus, Bleeker 1850). *Aquaculture Nutrition*, *23*(2), 331–338. https://doi.org/10.1111/ANU.12397

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., & Casey, T. A. (2013). Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends in Microbiology*, *21*(3), 114–119. https://doi.org/10.1016/J.TIM.2012.11.001

Anadón, A., Ares, I., Martínez-Larrañaga, M. R., & Martínez, M. A. (2019). Prebiotics and Probiotics in Feed and Animal Health. In R. Gupta, A. Srivastava, & R. Lall (Eds.), *Nutraceuticals in Veterinary Medicine* (pp. 261–285). Springer, Cham. https://doi.org/10.1007/978-3-030-04624-8_19

Asrat, M., Zeryehun, T., Amha, N., & Urge, M. (2018). Effects of supplementation of different levels of garlic (Allium sativum) on egg production, egg quality and hatchability of White Leghorn chicken. *Livest. Res. Rural Dev.*, *30*(37).

Awad, W., Ghareeb, K., & Böhm, J. (2008). Intestinal structure and function of broiler chickens on diets supplemented with a synbiotic containing Enterococcus faecium

and oligosaccharides. *International Journal of Molecular Sciences*, *9*(11), 2205–2216. https://doi.org/10.3390/IJMS9112205

Ayala, M. D., Galián, C., Fernández, V., Chaves-Pozo, E., de la Serrana, D. G., Sáez, M. I., … Arizcun, M. (2020). Influence of Low Dietary Inclusion of the Microalga Nannochloropsis gaditana (Lubián 1982) on Performance, Fish Morphology, and Muscle Growth in Juvenile Gilthead Seabream (Sparus aurata). *Animals*, *10*(12), 1–18. https://doi.org/10.3390/ANI10122270

Balcázar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J. L., & Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture*, *278*(1–4), 188–191. https://doi.org/10.1016/j.aquaculture.2008.03.014

Baños, A. (2016). Aplicación de la tecnología de las barreras en el desarrollo de as-48 como bioconservante alimentario. Estudio de probiosis de una cepa productora de as-48. Tesis Doctoral, Universidad de Granada.

Baños, A., Ariza, J. J., Nuñez, C., Gil-Martínez, L., García-López, J. D., Martínez-Bueno, M., & Valdivia, E. (2019). Effects of Enterococcus faecalis UGRA10 and the enterocin AS-48 against the fish pathogen Lactococcus garvieae. Studies in vitro and in vivo. *Food Microbiology*, *77*, 69–77. https://doi.org/10.1016/j.fm.2018.08.002

Bergamaschi, M., Tiezzi, F., Howard, J., Huang, Y. J., Gray, K. A., Schillebeeckx, C., … Maltecca, C. (2020). Gut microbiome composition differences among breeds impact feed efficiency in swine. *Microbiome*, *8*(1), 1–15. https://doi.org/10.1186/S40168-020-00888-9

Bolnick, D. I., Snowberg, L. K., Hirsch, P. E., Lauber, C. L., Knight, R., Caporaso, J. G., & Svanbäck, R. (2014). Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). *Ecology Letters*, *17*(8), 979–987. https://doi.org/10.1111/ELE.12301

Boney, J. W., & Moritz, J. S. (2017). Phytase dose effects in practically formulated diets that vary in ingredient composition on feed manufacturing and broiler performance. *Journal of Applied Poultry Research*, *26*(2), 273–285. https://doi.org/10.3382/JAPR/PFW071

Bongiorno, T., Foglio, L., Proietti, L., Vasconi, M., Moretti, V. M., Lopez, A., … Parati, K. (2022). Hydrolyzed microalgae from biorefinery as a potential functional ingredient in Siberian sturgeon (A. baerii Brandt) aquafeed. *Algal Research*, *62*, 102592. https://doi.org/10.1016/J.ALGAL.2021.102592

Borrelli, L., Coretti, L., Dipineto, L., Bovera, F., Menna, F., Chiariotti, L., … Fioretti, A. (2017). Insect-based diet, a promising nutritional source, modulates gut microbiota composition and SCFAs production in laying hens. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-16560-6

Büyükdeveci, M. E., Balcázar, J. L., Demirkale, İ., & Dikel, S. (2018). Effects of garlic-supplemented diet on growth performance and intestinal microbiota of rainbow trout (Oncorhynchus mykiss). *Aquaculture*, *486*, 170–174. https://doi.org/10.1016/J.AQUACULTURE.2017.12.022

Cashion, T., Le Manach, F., Zeller, D., & Pauly, D. (2017). Most fish destined for fishmeal production are food-grade fish. *Fish and Fisheries*, *18*(5), 837–844. https://doi.org/10.1111/FAF.12209

Cebrián, R., Baños, A., Valdivia, E., Pérez-Pulido, R., Martínez-Bueno, M., & Maqueda, M. (2012). Characterization of functional, safety, and probiotic properties of

Enterococcus faecalis UGRA10, a new AS-48-producer strain. *Food Microbiology*, *30*(1), 59–67. https://doi.org/10.1016/J.FM.2011.12.002

Cesari, V., Mangiagalli, M. G., Giardini, A., Galimberti, P., Carteri, S., Gallazzi, D., & Toschi, I. (2014). Egg quality and productive performance of laying hens fed different levels of skimmed milk powder added to a diet containing Lactobacillus acidophilus. *Poultry Science*, *93*(5), 1197–1201. https://doi.org/10.3382/PS.2013-03518

Chu, B., Zhu, Y., Su, J., Xia, B., Zou, Y., Nie, J., … Wang, J. (2020). Butyrate-mediated autophagy inhibition limits cytosolic Salmonella Infantis replication in the colon of pigs treated with a mixture of Lactobacillus and Bacillus. *Veterinary Research*, *51*(1), 1–16. https://doi.org/10.1186/S13567-020-00823-8

Egerton, S., Wan, A., Murphy, K., Collins, F., Ahern, G., Sugrue, I., … Stanton, C. (2020). Replacing fishmeal with plant protein in Atlantic salmon (Salmo salar) diets by supplementation with fish protein hydrolysate. *Scientific Reports*, *10*(1), 1–16. https://doi.org/10.1038/s41598-020-60325-7

Elokil, A. A., Magdy, M., Melak, S., Ishfaq, H., Bhuiyan, A., Cui, L., … Li, S. (2020). Faecal microbiome sequences in relation to the egg-laying performance of hens using amplicon-based metagenomic association analysis. *Animal*, *14*(4), 706–715. https://doi.org/10.1017/S1751731119002428

Estruch, G., Collado, M. C., Peñaranda, D. S., Vidal, A. T., Cerdá, M. J., Martínez, G. P., … Moreau, C. S. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (Sparus aurata) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. *PLoS ONE*, *10*(8), 1–22. https://doi.org/10.1371/journal.pone.0136389

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., & Rediers, H. (2011). Vibrio anguillarum as a fish pathogen: virulence factors, diagnosis and prevention. *Journal of Fish Diseases*, *34*(9), 643–661. https://doi.org/10.1111/j.1365-2761.2011.01279.x

Franz, C. M. A. P., Huch, M., Abriouel, H., Holzapfel, W., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology*, *151*(2), 125–140. https://doi.org/10.1016/J.IJFOODMICRO.2011.08.014

Froese, R. (2006). Cube law, condition factor and weight–length relationships: history, meta-analysis and recommendations. *Journal of Applied Ichthyology*, *22*(4), 241–253. https://doi.org/10.1111/J.1439-0426.2006.00805.X

Gabriel, N. N., Wilhelm, M. R., Habte-Tsion, H. M., Chimwamurombe, P., & Omoregie, E. (2019). Dietary garlic (Allium sativum) crude polysaccharides supplementation on growth, haematological parameters, whole body composition and survival at low water pH challenge in African catfish (Clarias gariepinus) juveniles. *Scientific African*, *5*, e00128. https://doi.org/10.1016/J.SCIAF.2019.E00128

Galafat, A., Sáez, M. I., Martínez, T. F., Macías-Vidal, J., Marqués, R., Rojas-Crespo, E., … Alarcón, F. J. (2021). Evaluación de un preparado funcional de microalgas y fitasa para la alimentación de lubina. In *Poster presentado en: X Foro Iberoamericano de los Recursos Marinos y la Acuicultura*.

Galafat, Alba, Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Arizcun, M., Chaves-Pozo, E., & Alarcón, F. J. (2022). Assessment of dietary inclusion of crude or hydrolysed Arthrospira platensis biomass in starter diets for gilthead seabream (Sparus aurata).

Aquaculture, *548*, 737680. https://doi.org/10.1016/J.AQUACULTURE.2021.737680

Galafat, Alba, Vizcaíno, A. J., Sáez, M. I., Martínez, T. F., Jérez-Cepa, I., Mancera, J. M., & Alarcón, F. J. (2020). Evaluation of Arthrospira sp. enzyme hydrolysate as dietary additive in gilthead seabream (Sparus aurata) juveniles. *Journal of Applied Phycology*, *32*(5), 3089–3100. https://doi.org/10.1007/s10811-020-02141-0

Gasco, L., Gai, F., Maricchiolo, G., Genovese, L., Ragonese, S., Bottari, T., & Caruso, G. (2018). *Feeds for the Aquaculture Sector - Current Situation and Alternative Sources* (1st ed.). Springer International Publishing. https://doi.org/10.1007/978-3-319-77941-6

Gheisar, M. M., & Kim, I. H. (2018). Phytobiotics in poultry and swine nutrition–a review. *Italian Journal of Animal Science*, *17*(1), 92–99. https://doi.org/10.1080/1828051X.2017.1350120

Guillamón, E., Andreo-Martínez, P., Mut-Salud, N., Fonollá, J., & Baños, A. (2021). Beneficial Effects of Organosulfur Compounds from Allium cepa on Gut Health: A Systematic Review. *Foods*, *10*(8), 1680. https://doi.org/10.3390/FOODS10081680

Guo, J. R., Dong, X. F., Liu, S., & Tong, J. M. (2017). Effects of long-term Bacillus subtilis CGMCC 1.921 supplementation on performance, egg quality, and fecal and cecal microbiota of laying hens. *Poultry Science*, *96*(5), 1280–1289. https://doi.org/10.3382/PS/PEW389

Haldar, S., Maharajan, A., Chatterjee, S., Hunter, S. A., Chowdhury, N., Hinenoya, A., … Yamasaki, S. (2010). Identification of Vibrio harveyi as a causative bacterium for a tail rot disease of sea bream Sparus aurata from research hatchery in Malta. *Microbiological Research*, *165*(8), 639–648. https://doi.org/10.1016/j.micres.2009.12.001

Han, G. G., Kim, E. B., Lee, J., Lee, J.-Y., Jin, G., Park, J., … Kong, C. (2016). Relationship between the microbiota in different sections of the gastrointestinal tract, and the body weight of broiler chickens. *SpringerPlus*, *5*(1), 1–9. https://doi.org/10.1186/S40064-016-2604-8

Hashemi, S. R., & Davoodi, H. (2011). Herbal plants and their derivatives as growth and health promoters in animal nutrition. *Veterinary Research Communications*. https://doi.org/10.1007/s11259-010-9458-2

Hu, Y., Dun, Y., Li, S., Zhang, D., Peng, N., Zhao, S., & Liang, Y. (2015). Dietary Enterococcus faecalis LAB31 improves growth performance, reduces diarrhea, and increases fecal Lactobacillus number of weaned piglets. *PloS One*, *10*(1), e0116635. https://doi.org/10.1371/JOURNAL.PONE.0116635

Kajihara, Y., Yoshikawa, S., Cho, Y., Ito, T., Miyamoto, H., & Kodama, H. (2017). Preferential isolation of Megasphaera elsdenii from pig feces. *Anaerobe*, *48*, 160–164. https://doi.org/10.1016/J.ANAEROBE.2017.08.013

Kim, D. K., Lillehoj, H. S., Lee, S. H., Lillehoj, E. P., & Bravo, D. (2013). Improved resistance to Eimeria acervulina infection in chickens due to dietary supplementation with garlic metabolites. *British Journal of Nutrition*, *109*(01), 76–88. https://doi.org/10.1017/S0007114512000530

Kim, J. H., Pitargue, F. M., Jung, H., Han, G. P., Choi, H. S., & Kil, D. Y. (2017). Effect of superdosing phytase on productive performance and egg quality in laying hens. *Asian-Australasian Journal of Animal Sciences*, *30*(7), 994. https://doi.org/10.5713/AJAS.17.0149

Kraimi, N., Dawkins, M., Gebhardt-Henrich, S. G., Velge, P., Rychlik, I., Volf, J., … Leterrier, C. (2019). Influence of the microbiota-gut-brain axis on behavior and welfare in farm animals: A review. *Physiology & Behavior*, *210*, 112658. https://doi.org/10.1016/J.PHYSBEH.2019.112658

Kumar, V., Sinha, A. K., Makkar, H. P., Boeck, G. De, & Becker, K. (2012). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, *96*(3), 335–364. https://doi.org/10.1111/J.1439-0396.2011.01169.X

Lee, J.-Y., & Gao, Y. (2012). Review of the Application of Garlic, Allium sativum, in Aquaculture. *Journal of the World Aquaculture Society*, *43*(4), 447–458. https://doi.org/10.1111/j.1749-7345.2012.00581.x

Lemos, D., & Tacon, A. G. J. (2017). Use of phytases in fish and shrimp feeds: a review. *Reviews in Aquaculture*, *9*(3), 266–282. https://doi.org/10.1111/RAQ.12138

Li, X., Yan, Q., Ringø, E., Wu, X., He, Y., & Yang, D. (2016). The influence of weight and gender on intestinal bacterial community of wild largemouth bronze gudgeon (Coreius guichenoti, 1874). *BMC Microbiology*, *16*(1), 191. https://doi.org/10.1186/s12866-016-0809-1

Luo, L., Xu, Q., Xu, W., Li, J., Wang, C., Wang, L., & Zhao, Z. (2020). Effect of Bacillus megaterium-Coated Diets on the Growth, Digestive Enzyme Activity, and Intestinal Microbial Diversity of Songpu Mirror Carp Cyprinus specularis Songpu. *BioMed Research International*, *2020*. https://doi.org/10.1155/2020/8863737

Mahesh, M. S., Mohanta, R. K., Patra, A. K., Krishi, R. K. M., Kendra, V., & Patra, A. K. (2021). Probiotics in Livestock and Poultry Nutrition and Health. (G. Goel & A. Kumar, Eds.), *Advances in Probiotics for Sustainable Food and Medicine*. Springer, Singapore. https://doi.org/10.1007/978-981-15-6795-7_7

Mahmoud, K. Z., Gharaibeh, S. M., Zakaria, H. A., & Qatramiz, A. M. (2010). Garlic (Allium sativum) Supplementation: Influence on Egg Production, Quality, and Yolk Cholesterol Level in Layer Hens. *Asian-Australasian Journal of Animal Sciences*, *23*(11), 1503–1509. https://doi.org/10.5713/AJAS.2010.10124

Markowiak, P., & Ślizewska, K. (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathogens*, *10*(1), 1–20. https://doi.org/10.1186/S13099-018-0250-0

Mazanko, M. S., Gorlov, I. F., Prazdnova, E. V, Makarenko, M. S., Usatov, A. V, Bren, A. B., … Chikindas, M. L. (2018). Bacillus Probiotic Supplementations Improve Laying Performance, Egg Quality, Hatching of Laying Hens, and Sperm Quality of Roosters. *Probiotics and Antimicrobial Proteins*, *10*(2), 367–373. https://doi.org/10.1007/S12602-017-9369-4

Menni, C., Jackson, M. A., Pallister, T., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Gut microbiome diversity and high-fibre intake are related to lower long-term weight gain. *International Journal of Obesity*, *41*, 1099–1105. https://doi.org/10.1038/ijo.2017.66

Mikulski, D., Jankowski, J., Mikulska, M., & Demey, V. (2020). Effects of dietary probiotic (Pediococcus acidilactici) supplementation on productive performance, egg quality, and body composition in laying hens fed diets varying in energy density. *Poultry Science*, *99*(4), 2275–2285. https://doi.org/10.1016/J.PSJ.2019.11.046

Motlagh, H. A., Paolucci, M., Bami, M. L., & Safari, O. (2020). Sexual parameters, digestive enzyme activities, and growth performance of guppy (Poecilia reticulata) fed garlic (Allium sativum) extract supplemented diets. *Journal of the World*

Aquaculture Society, *51*(5), 1087–1097. https://doi.org/10.1111/jwas.12729

Motlagh, H. A., Safari, O., Selahvarzi, Y., Baghalian, A., & Kia, E. (2020). Non-specific immunity promotion in response to garlic extract supplemented diets in female Guppy (Poecilia reticulata). *Fish & Shellfish Immunology*, *97*, 96–99. https://doi.org/10.1016/J.FSI.2019.12.007

Ogbuewu, I. P., Okoro, V. M., Mbajiorgu, E. F., & Mbajiorgu, C. A. (2019). Beneficial Effects of Garlic in Livestock and Poultry Nutrition: A Review. *Agricultural Research*, *8*(4), 411–426. https://doi.org/10.1007/S40003-018-0390-Y

Omer, H. A. A., Ahmed, S. M., Abdel-Magid, S. S., El-Mallah, G. M. H., Bakr, A. A., & Fattah, M. M. A. (2019). Nutritional impact of inclusion of garlic (Allium sativum) and/or onion (Allium cepa L.) powder in laying hens' diets on their performance, egg quality, and some blood constituents. *Bulletin of the National Research Centre*, *43*(1), 1–9. https://doi.org/10.1186/s42269-019-0061-6

Omonijo, F. A., Ni, L., Gong, J., Wang, Q., Lahaye, L., & Yang, C. (2018). Essential oils as alternatives to antibiotics in swine production. *Animal Nutrition*, *4*(2), 126–136. https://doi.org/10.1016/j.aninu.2017.09.001

Pan, D., & Yu, Z. (2013). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), 108–119. https://doi.org/10.4161/gmic.26945

Park, J. H., Song, T. H., & Kim, I. (2016). Egg production, egg quality, and cecal microbial populations of layers fed diets supplemented with fermented phytogenic feed additive. *Turkish Journal of Veterinary and Animal Sciences*, *40*, 660–666. https://doi.org/10.3906/vet-1512-55

Peinado, M. J., Ruiz, R., Echávarri, A., Aranda-Olmedo, I., & Rubio, L. A. (2013). Garlic derivative PTS-O modulates intestinal microbiota composition and improves digestibility in growing broiler chickens. *Animal Feed Science and Technology*, *181*(1–4), 87–92. https://doi.org/10.1016/j.anifeedsci.2013.03.001

Peinado, M. J., Ruiz, R., Echavarri, A., & Rubio, L. A. (2012). Garlic derivative propyl propane thiosulfonate is effective against broiler enteropathogens in vivo. *Poultry Science*, *91*(9), 2148–2157. https://doi.org/10.3382/ps.2012-02280

Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., & Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: Current status and future perspectives. *Aquaculture*. Elsevier. https://doi.org/10.1016/j.aquaculture.2014.05.048

Rhouma, M., Fairbrother, J. M., Beaudry, F., & Letellier, A. (2017). Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. *Acta Veterinaria Scandinavica*, *59*(1), 1–19. https://doi.org/10.1186/S13028-017-0299-7

Ribeiro, V., Albino, L. F. T., Rostagno, H. S., Barreto, S. L. T., Hannas, M. I., Harrington, D., … Ferreira, M. A. (2014). Effects of the dietary supplementation of Bacillus subtilis levels on performance, egg quality and excreta moisture of layers. *Animal Feed Science and Technology*, *195*, 142–146. https://doi.org/10.1016/J.ANIFEEDSCI.2014.06.001

Romano, N., & Kumar, V. (2018). Phytase in animal feed. *Enzymes in Human and Animal Nutrition: Principles and Perspectives*, 73–88. https://doi.org/10.1016/B978-0-12-805419-2.00004-6

Sales, J. (2009). The effect of fish meal replacement by soyabean products on fish growth: a meta-analysis. *British Journal of Nutrition*, *102*(12), 1709–1722. https://doi.org/10.1017/S0007114509991279

Sánchez, C J, Martínez-Miró, S., Ariza, J. J., Madrid, J., Orengo, J., Aguinaga, M. A., … Hernández, F. (2020). Effect of Alliaceae Extract Supplementation on Performance and Intestinal Microbiota of Growing-Finishing Pig. *Animals*, *10*(9), 1–15. https://doi.org/10.3390/ANI10091557

Sánchez, Cristian Jesús, Martínez-Miró, S., Ariza, J. J., Madrid, J., Orengo, J., Aguinaga, M. A., … Hernández, F. (2020). Effect of alliaceae extract supplementation on performance and intestinal microbiota of growing-finishing pig. *Animals*, *10*(9), 1–15. https://doi.org/10.3390/ani10091557

Shah, M. R., Lutzu, G. A., Alam, A., Sarker, P., Chowdhury, M. A. K., Parsaeimehr, A., … Daroch, M. (2018). Microalgae in aquafeeds for a sustainable aquaculture industry. *Journal of Applied Phycology*, *30*(1), 197–213. https://doi.org/10.1007/s10811-017-1234-z

Shalaby, A. M., Khattab, Y. A., & Rahman, A. M. A. (2006). Effects of Garlic (Allium sativum) and chloramphenicol on growth performance, physiological parameters and survival of Nile tilapia (Oreochromis niloticus). *Journal of Venomous Animals and Toxins Including Tropical Diseases*, *12*(2), 172–201. https://doi.org/10.1590/S1678-91992006000200003

Shang, H., Zhao, J., Dong, X., Guo, Y., Zhang, H., Cheng, J., & Zhou, H. (2020). Inulin improves the egg production performance and affects the cecum microbiota of laying hens. *International Journal of Biological Macromolecules*, *155*, 1599–1609. https://doi.org/10.1016/J.IJBIOMAC.2019.11.137

Sharifuzzaman, S. M., Abbass, A., Tinsley, J. W., & Austin, B. (2011). Subcellular components of probiotics Kocuria SM1 and Rhodococcus SM2 induce protective immunity in rainbow trout (Oncorhynchus mykiss, Walbaum) against Vibrio anguillarum. *Fish & Shellfish Immunology*, *30*(1), 347–353. https://doi.org/10.1016/J.FSI.2010.11.005

Shepherd, C. J., & Jackson, A. J. (2013). Global fishmeal and fish-oil supply: inputs, outputs and marketsa. *Journal of Fish Biology*, *83*(4), 1046–1066. https://doi.org/10.1111/JFB.12224

Song, D., Wang, Y. W., Hou, Y. J., Dong, Z. L., Wang, W. W., & Li, A. K. (2016). The effects of dietary supplementation of microencapsulated Enterococcus faecalis and the extract of Camellia oleifera seed on growth performance, immune functions, and serum biochemical parameters in broiler chickens. *Journal of Animal Science*, *94*(8), 3271–3277. https://doi.org/10.2527/jas.2016-0286

Song, D., Wang, Y. W., Lu, Z. X., Wang, W. W., Miao, H. J., Zhou, H., … Li, A. K. (2019). Effects of dietary supplementation of microencapsulated Enterococcus fecalis and the extract of Camellia oleifera seed on laying performance, egg quality, serum biochemical parameters, and cecal microflora diversity in laying hens. *Poultry Science*, *98*(7), 2880–2887. https://doi.org/10.3382/PS/PEZ033

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Ariza-Romero, J. J., Baños-Arjona, A., Exposito-Ruiz, M., & Gutierrez-Fernandez, J. (2018). In vitro antibacterial activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate derived from allium spp. Against gram-negative and gram-positive multidrug-resistant bacteria isolated from human samples. *BioMed Research International*, *2018*, 7861207. https://doi.org/10.1155/2018/7861207

Sorlozano-Puerto, A., Albertuz-Crespo, M., Lopez-Machado, I., Gil-Martinez, L., Ariza-Romero, J. J., Maroto-Tello, A., … Gutierrez-Fernandez, J. (2021). Antibacterial

and antifungal activity of propyl-propane-thiosulfinate and propyl-propane-thiosulfonate, two organosulfur compounds from allium cepa: In vitro antimicrobial effect via the gas phase. *Pharmaceuticals*, *14*(1), 1–17. https://doi.org/10.3390/ph14010021

Srichanun, M., Tantikitti, C., Kortner, T. M., Krogdahl, Å., & Chotikachinda, R. (2014). Effects of different protein hydrolysate products and levels on growth, survival rate and digestive capacity in Asian seabass (Lates calcarifer Bloch) larvae. *Aquaculture*, *428–429*, 195–202. https://doi.org/10.1016/j.aquaculture.2014.03.004

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. M. (2015). The composition of the zebrafish intestinal microbial community varies across development. *The ISME Journal*, *10*(3), 644–654. https://doi.org/10.1038/ismej.2015.140

Tang, S. G. H., Sieo, C. C., Ramasamy, K., Saad, W. Z., Wong, H. K., & Ho, Y. W. (2017). Performance, biochemical and haematological responses, and relative organ weights of laying hens fed diets supplemented with prebiotic, probiotic and synbiotic. *BMC Veterinary Research*, *13*(1), 1–12. https://doi.org/10.1186/S12917-017-1160-Y

Thacker, P. A. (2013). Alternatives to antibiotics as growth promoters for use in swine production: A review. *Journal of Animal Science and Biotechnology*, *4*(1), 1–12. https://doi.org/10.1186/2049-1891-4-35

Torrecillas, S., Caballero, M. J., Mompel, D., Montero, D., Zamorano, M. J., Robaina, L., … Izquierdo, M. (2017). Disease resistance and response against Vibrio anguillarum intestinal infection in European seabass (Dicentrarchus labrax) fed low fish meal and fish oil diets. *Fish & Shellfish Immunology*, *67*, 302–311. https://doi.org/10.1016/J.FSI.2017.06.022

Tzeng, T.-R. J., Liu, T.-Y., Lin, C.-W., Chang, P.-E., Liao, P.-X., Yang, W.-Y., … Gonzalez, M. (2021). Effects of Dietary Inclusion of Dry Hydrastis canadensis on Laying Performance, Egg Quality, Serum Biochemical Parameters and Cecal Microbiota in Laying Hens. *Animals*, *11*(5), 1381. https://doi.org/10.3390/ANI11051381

Valente, L. M. P., Custódio, M., Batista, S., Fernandes, H., & Kiron, V. (2019). Defatted microalgae (Nannochloropsis sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets. *Fish Physiology and Biochemistry*, *45*(3), 1067–1081. https://doi.org/10.1007/s10695-019-00621-w

Valenzuela-Gutiérrez, R., Lago-Lestón, A., Vargas-Albores, F., Cicala, F., & Martínez-Porchas, M. (2021). Exploring the garlic (Allium sativum) properties for fish aquaculture. *Fish Physiology and Biochemistry*, *47*(4), 1179–1198. https://doi.org/10.1007/S10695-021-00952-7

Valeriano, V. D. V, Balolong, M. P., & Kang, D.-K. (2017). Probiotic roles of Lactobacillus sp. in swine: insights from gut microbiota. *Journal of Applied Microbiology*, *122*(3), 554–567. https://doi.org/10.1111/jam.13364

Vezza, T., Garrido-Mesa, J., Diez-Echave, P., Hidalgo-García, L., Ruiz-Malagón, A. J., García, F., … Rodríguez-Cabezas, M. E. (2021). Allium-Derived Compound Propyl Propane Thiosulfonate (PTSO) Attenuates Metabolic Alterations in Mice Fed a High-Fat Diet through Its Anti-Inflammatory and Prebiotic Properties. *Nutrients*, *13*(8), 2595. https://doi.org/10.3390/nu13082595

Videnska, P., Sedlar, K., Lukac, M., Faldynova, M., Gerzova, L., Cejkova, D., …

Rychlik, I. (2014). Succession and Replacement of Bacterial Populations in the Caecum of Egg Laying Hens over Their Whole Life. *PLOS ONE*, *9*(12), e115142. https://doi.org/10.1371/JOURNAL.PONE.0115142

Vondruskova, H., Slamova, R., Trckova, M., Zraly, Z., & Pavlik, I. (2010). Alternatives to antibiotic growth promoters in prevention of diarrhoea in weaned piglets: A review. *Veterinarni Medicina*, *55*(5), 199–224. https://doi.org/10.17221/2998-VETMED

Wang, K., Cao, G., Zhang, H., Li, Q., & Yang, C. (2019). Effects of Clostridium butyricum and Enterococcus faecalis on growth performance, immune function, intestinal morphology, volatile fatty acids, and intestinal flora in a piglet model. *Food & Function*, *10*(12), 7844–7854. https://doi.org/10.1039/C9FO01650C

Wei, Q. T. (2014). Effect of dietary Enterococcus faecalis replacing of antibiotic on growth performance, diarrhea rate, humoral immunity and intestinal microflora of nursery pigs. *Journal of Nanjung Agricultural University*, *37*, 143–148.

Wiklund, T. (2016). Pseudomonas anguilliseptica infection as a threat to wild and farmed fish in the Baltic Sea. *Microbiology Australia*, *37*(3), 135. https://doi.org/10.1071/MA16046

Windisch, W., Schedle, K., Plitzner, C., & Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *Journal of Animal Science*, *86*(14), E140–E148. https://doi.org/10.2527/jas.2007-0459

Yan, W., & Reigh, R. C. (2002). Effects of Fungal Phytase on Utilization of Dietary Protein and Minerals, and Dephosphorylation of Phytic Acid in the Alimentary Tract of Channel Catfish Zctulurus punctutus Fed an All-Plant-Protein Diet. *Journal of the World Aquaculture Society*, *33*(1).

Yang, H., Xiao, Y., Wang, J., Xiang, Y., Gong, Y., Wen, X., & Li, D. (2018). Core gut microbiota in Jinhua pigs and its correlation with strain, farm and weaning age. *Journal of Microbiology 2018 56:5*, *56*(5), 346–355. https://doi.org/10.1007/S12275-018-7486-8

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B. A., & Arockiaraj, J. (2020). Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. *Reviews in Aquaculture*, *12*(3), 1903–1927. https://doi.org/10.1111/RAQ.12416

Żakowska-Biemans, S., & Tekień, A. (2017). Free Range, Organic? Polish Consumers Preferences Regarding Information on Farming System and Nutritional Enhancement of Eggs: A Discrete Choice Based Experiment. *Sustainability*, *9*(11), 1999. https://doi.org/10.3390/SU9111999

Zhang, G., Wang, H., Zhang, J., Tang, X., Raheem, A., Wang, M., … Qin, T. (2021). Modulatory Effects of Bacillus subtilis on the Performance, Morphology, Cecal Microbiota and Gut Barrier Function of Laying Hens. *Animals*, *11*(6), 1523. https://doi.org/10.3390/ANI11061523

Zhang, Y., Ma, W., Zhang, Z., Liu, F., Wang, J., Yin, Y., & Wang, Z. (2019). Effects of Enterococcus faecalis on egg production, egg quality and caecal microbiota of hens during the late laying period. *Archives of Animal Nutrition*, *73*(3), 208–221. https://doi.org/10.1080/1745039X.2019.1591128

Zhu, C., Xu, W., Tao, Z., Song, W., Liu, H., Zhang, S., & Li, H. (2020). Effects of Rearing Conditions and Sex on Cecal Microbiota in Ducks. *Frontiers in Microbiology*, *11*, 2424. https://doi.org/10.3389/FMICB.2020.565367