UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

Functional additives as a tool to improve robustness of European sea bass (*Dicentrarchus labrax*) juveniles

Thesis for the degree of Doctor of Philosophy
University of las Palmas de Gran Canaria

Antonio Serradell Pastor

Las Palmas de Gran Canaria
2023

Supervisors:
Prof. Daniel Montero Vítores
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PhD programme in Sustainable Aquaculture and Marine Ecosystems
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ESCUELA DE DOCTORADO

Programa de doctorado **Acuicultura Sostenible y Ecosistemas Marinos**

Título de la Tesis: **Functional additives as a tool to improve robustness of European sea bass (*Dicentrarchus labrax*) juveniles**

Tesis Doctoral presentada por D. Antonio Serradell Pastor

Dirigida por el Dr. D. Daniel Montero Vítores

Codirigida por el Dra. Dª. Silvia Torrecillas Burriel

Las Palmas de Gran Canaria, a de Diciembre de 2023

El Director, La Codirectora, El Doctorando,

FUNDING

This PhD thesis was funded by the University of las Palmas de Gran Canaria under the gran agreement PIFULPGC-2019-Ciencias1 (Convocatoria 2019 de contratos predoctorales (BOC nº32, 15/02/2019)

Unión Europea

This study was partially funded under the EU funded projects PROINMUNOIL PLUS (AGL2016-79725-P): "Functional additives and alternative oils to fish oils in Aquaculture: an effective tool to increase fish disease resistance" and AquaIMPACT Project (EU Horizon 2020 no. 818367): Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture

Agradecimientos/ Aknowledgments

Aquí estoy, al final…

Cansado y feliz al mismo tiempo, después de tantos años dedicados a una misión, aquí estoy. Esta tesis doctoral no solo es un manuscrito recopilando el estudio y trabajo que hemos llevado a cabo estos años, es un fragmento importantísimo de mi vida. Ocho años hace que llegué a esta isla, pero todos los pequeños detalles están grabados a fuego de forma que no marcharán nunca. Una isla que se convirtió en hogar y refugio, que a pesar de todas las mareas y vendavales que hayan podido llegar, me ha dado calor y calma. Os doy las gracias a todos los pilares que habéis sostenido esta isla en mitad del Atlántico.

En primer lugar, os quiero dar las gracias a vosotros Dani y Silvia, por el apoyo que habéis supuesto todos estos años a nivel profesional y por supuesto a nivel personal. Si en algún momento he tenido problemas, he podido hablar con vosotros y me he sentido comprendido y apoyado. Sé que tengo muchos defectos que han hecho de mi tesis un camino especialmente largo, pero lo habéis recorrido a mi lado con paciencia y dedicación. Habéis intentado que crezca como investigador y como persona, y creo que lo habéis logrado. Tras estos años con vosotros, conozco mis puntos débiles y mis puntos fuertes, me habéis enseñado como trabajar los débiles y me habéis impulsado a potenciar los fuertes…esto no lo hace un profesor, esto lo hace un mentor. De nuevo, gracias.

El segundo pilar sosteniendo esta isla lo componen mis amigos y amigas. No concibo ni un solo día en esta aventura sin haber tenido el placer de compartir vida con todos vosotros. Antonio y Sara, mis grandes amigos y compañeros de buceo, que siempre que he necesitado una cerveza (por buenas o por malas) han estado ahí para tomársela conmigo. Mi amigo Álvaro, que me vio empezar este camino siguiendo algunos de sus pasitos (con sonrisa picarona porque él ya sabía lo que se me venía), que siempre me ha brindado apoyo y conocimiento. Mi amigo Néstor, el pecado y la ecología hechas carne. Ha sido un placer conocer a alguien como tu (y tus padres), que siempre tenéis una palabra amable y la casa abierta con un plato de garbanzada en la mesa para cuando voy a Anaga. Tampoco debo dejar atrás a Gonzalo, que lleva ahí desde mi segundo año de carrera, un gran amigo y proveedor de buceo (de hecho me diste el primer buceo en esta isla, en Sardina, y me has dado el último antes de acabar este manuscrito, en Sardina). Finalmente, aunque no menos importante, Adri que empezó como mi profesor de escalada y ha acabado convertido en uno de mis grandes amigos.

Mi tercer apoyo, como siempre y para siempre, mi familia que es fundamental. Mi hermano Ángel, al que debo más de lo que podré devolverle. Mi padre, que siempre va a tener para mi un abrazo y toda la ayuda y apoyo que necesite (bueno, también lo de reservar en el Jauja para ir a echarnos un par de jarras y unos callos siempre que vuelvo, es importante). Mi madre, que siempre nos va a querer y va a estar dispuesta a ayudarnos y darlo todo por nosotros. Mis abuelos, Mon y Leonor, que han actuado siempre y siguen actuando como padres, formándome y aconsejándome de la mejor forma posible. Por supuesto el amor del resto de mi

familia, que siempre que llego tienen el corazón abierto para mi, por muy lejos que me vaya y por mucho tiempo que esté fuera. A todos vosotros, os quiero.

El cuarto pilar, pero al mismo tiempo piedra angular, mis hermanos y hermanas. Raquel Q., Ale y Raquel P. (con sus ratas respectivas, Lu y Gina) son las personas que llevan ahí desde el día 1, compañeros de nada mas y nada menos que ocho años de vida. Ya sabéis lo mucho que os quiero, aunque a veces no sepa expresarlo. Me habéis dado alegría, tristeza, risas, compañerismo, sinceridad, silencio… todo lo que he podido necesitar, de una forma u otra ha llegado por vuestra parte. Si me lo preguntáis, tengo clara la respuesta, no debo perderos. Aunque haya habido años turbulentos, la brasa que encendisteis está candente y dudo mucho que se llegue a apagar nunca. No puedo olvidarme tampoco del cuarto de los hermanos que me llevo de esta isla, David. Mi compañero de cordada y aventuras, la persona que sabe llevarme al limite de mi capacidad piscológica y física. A ti te debo una progresión enorme como montañero, pero más allá te debo la increíble amistad y apoyo que me has brindado siempre. Con pocas personas te ves a las 5 de la mañana en mitad de un barranco, después de escalar durante toda la noche, calentando arroz y charlando de los problemas que uno tiene. A vosotros cuatro, con la mano en el corazón, os doy las gracias por hacerme crecer.

Tampoco dejo de lado a todas las personas que de una forma u otra se han cruzado en mi camino. Todos mis compañeros del GIA, mis buenos compañeros calisténicos, toda la gente que he conocido gracias a la montaña y al buceo y toda esa gente que he conocido gracias a la ciencia, como al equipo del IRTA en Sant Carles de la Ràpita y todos los jóvenes investigadores e investigadoras que he podido conocer en congresos.

A todos, os atribuyo quien soy hoy.

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Abbreviations list

ABW	Average body weight
ACH50	Alternative complement activity 50
ACTH	Adrenocorticotrophic hormone
ANFs	Anti-nutritional factors
ANOVA	Analysis of variance
ARA	Arachidonic acid
ARG	Arginine
ATP	Adenosine triphosphate
BW	Body weight
CA	Catecholamines
casp-3	Caspase 3
casp-9	Caspase 9
CAT	Catalase
cfu	Colony forming units
CI treatment	Confinement + infection *V. aguillarum*
cox	Cytochrome c
CRH	Cortisol releasing factor
C treatment	Confinement stress challenge
Cu	Copper
cyp11B	P450 11β-hydroxylase enzyme
CYS	Cysteine
DGI	Daily growth index
diet_ash	Dietary ash content
diet_CL	Dietary crude lipid
diet_CP	Dietary crude protein
diet_CP/GE	Dietary Protein to Energy level
diet_GE	Dietary crude energy
diet_moisture	Diet moisture
diet_Others	Dietary “others” additives
diet_Prebiotics	Dietary prebiotics
diet_Probiotics	Dietary probiotics
dl	Deciliters
DHA	Cocosahexaenoic acid
DNA	Deoxyribonucleic acid
dph	Days post hatch
EBI ENA	European Nucleotide Archive
EF1	Elongation factor 1α
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
ETC	Electronic transport chain
FAs	Functional additives
FBW	Final body weight
FCR	Feed conversion ratio
Fe	Iron
FI	Feed intake
FI_norm	Normalized individual feed intake
FM	Fish meal
FO	Fish oil
FOS	Fructo oligosaccharides
FW	Forward primer sequence
g	Grams
GABA	Gamma-aminobutyric acid receptor complex
galt	Gut-associated lymphoid tissue
GC	Glucocorticoids
GIALT	Gill-associated lymphoid tissue
GMOS	Galactomannan oligosaccharides
GOS	Galacto oligosaccharides
GPX	Glutathione peroxidase
gr	Glucocorticoid receptor
GS	Genetically selected
HBSS	Hank’s Buffered Salt Solution
HG	High growth genotype
hif-1 α	Hypoxia inducible factor 1α
HIS	Histidine
HPI	Hypothalamus-Pitutiary-Interrenal axis
HSP70	Heat shot protein 70
HSP90	Heat shot protein 90
IBW	Initial body weight
Ig	Immunoglobulins
IGF-1	Insuline-like growth factor 1
il-1β	Interleukine 1β
ILE	Isoleucine
IMM	Inner mitochondrial membrane
IMS	Inner mitocondrial space
K	Potasium
kg	Kilograms
KPI	Key performance indicator
L	Liters
lab	Lactic acid-producing bacteria
LC-PUFA	Long chain polyunsaturated fatty acids
LEU	Leucine
LYS	Lysine
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinases

MET	Methionine
MJ	Megajoules
ml	Mililiters
MLR	Multiple linear regression
Mn	Manganese
MOS	Mannan oligosaccharides
MRCs	Mitochondria rich cell/ ionocyte
Na	Aodium
nd5	NADH dehydrogenase subunit 5)
NF-kB	Nuclear factor kappa beta
nfKβ2	Nuclear factor kappa beta gene
NKA α1a	Na+/K+ ATPase)
ns	Not significant
ocln	Occludin
OMM	Outer mitocondrial membrane
ORG	Organic acids
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular pattern
PCTM	Parque Científico-Tecnológico Marino de Taliarte
PFIs	Phytogenic feed ingredients
PHE	Phenylalanine
PHYTO	Phytogenic compound
PLS-DA	Partial least square-discriminant analysis
ppm	Parts per million
PROB	Probiotics
PUFA	Polyunsaturated fatty acids
PVCs	Pavements cells
RAS	Recirculating aquaculture system
RNA	Ribonucleic acid
RONS	Reactinve nitrogenspecies
ROS	Reactive oxygen species
rpl17	Ribosomal protein L17
RPS	Relative survival percentage
RT-PCR	Real-time PCR
RV	Reverse primer sequence
SC	Sympathetic Chromaffin axis
SCFAs	Short chain fatty acids
SGR	Specific growth rate
SOD	Superoxide dismutase
StAR	Steroidogenic acute regulatory protein
TBM	Tetraethylbenzidine
TCR-β	T-cell receptor β
Temp	Temperature
THR	Threonine
tnf-1α	Tumor necrosis factor α
TYR	Tyrosine
ucp1	Uncouping protein 1
uL	Microliters
ULPGC	University of las palmas de gran canaria
USD	United State dollars
VAL	Valine
WG	Weight gain
WT	Wild type genotype
XOS	Xylose oligosaccharides
Zn	Zinc
zo-1	Zonula occludens
α-tub	α-tubulin

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Table 7.4. Coefficients of linear regression obtained for the normalized feed intake (FI_norm) observational models. Temp (temperature ◦C); ABW (average body weight (g)); diet_CP (dietary protein content (%)); diet_GE (dietary energy content (MJ/kg)); diet_CP /diet_GE (Protein – Energy ratio (g /MJ)); diet_moisture (diet mositure content (%)); diet_Prebiotics (dietary prebiotics content (g/kg)); diet_Probiotics (dietary probiotics content (g/kg)); diet_Others (dietary Others content (g/kg)). a FI simple model (trimmed simple model). b FI simple model with prebiotics dietary inclusion as quantitative variable. c FI simple model with probiotics dietary inclusion as quantitative variable. d FI simple model with Others dietary inclusion as quantitative variable. (page 114)

Table 7.5. Coefficients of linear regression obtained for the normalized feed conversión ratio (FCR_norm) observational models. Temp (water temperature ◦C); Oxygen (dissolved oxygen (ppm)); ABW (average body weight (g)); diet_CP (dietary protein content (%)); diet_GE (dietary energy content (MJ/kg)); diet_moisture (diet moisture content (%)); diet_Prebiotics (dietary prebiotics content (g/kg)); diet_Probiotics (dietary probiotics content (g/kg)); diet_Others (dietary Others content (g/kg)). a FCR simple model (trimmed simple model). b FCR simple model with prebiotics dietary inclusion as quantitative variable. c FCR simple model with probiotics dietary inclusion as quantitative variable. d FCR simple model with others dietary inclusion as quantitative variable. (page 117)

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Chapter I
Introduction

1.1. The European sea bass (*Dicentrarchus labrax*): aquaculture production

Aquatic foods have become one of the main sources of nutrients for human consumption, supposing in the present, the 20% of world's total animal protein consumed. In the year 2020, the sector produced a total of 178 million tons fish and shellfish live weight, supposing a total outcome of USD 406 billion. The 51% of total production (90.3 million tons) account for capture fisheries, whereas 87.7 million tons were produced by aquaculture (48% total production) [SOFIA, 2022]. Despite annual production is similar for both industries, they present different production trends [Carvalho and Guillen, 2021]. Natural fish stocks overexploitation by extractive fishing has led to the stagnation of capture fisheries production, with values ranging between 86 and 90 million tons since the late 1980s. On the contrary, aquaculture industry production has increased its production in a 609% in the last 30 decades, from 14.4 millions tons in 1990s to the actual 87.7 million tons [FEAP, 2021; SOFIA, 2022].

The European sea bass is a carnivorous marine teleost fish species, distributed along the north-eastern Atlantic ocean and Mediterranean and Black seas. With an eurythermal (2-32°C) and euryhaline (2-40 ppt) ecology, the European sea bass can be found on coastal shallow waters over 100m depth and brackish water bodies such as, estuaries and coastal lagoons [Kousoulakis et al., 2015; Vandeputte et al., 2019]. The European sea bass is a clear example of aquaculture industry development and technification. Since 1980s with the development of reproductive and larval rearing protocols ensuring the continuous supply of individuals, the European sea bass intensive aquaculture production started its expansion [Le Boucher et al., 2010; Boudry et al., 2011; Carvalho and Guillen, 2021]. In 1992, the Mediterranean sea aquaculture has been producing over 96% of world's total consumed European sea bass, accounting 243.9 thousand tons of live weight in 2020. The main producers of European sea bass in the Mediterranean area are Egypt, Turkey, Greece and Italy accounting the 86% of the total production [Stavrakidis-Zachou et al., 2019; Carvalho and Guillen, 2021].

The production cycle of this fish species is divided in two phases, with a total duration of 24 months approximately [Vandeputte et al., 2009]. A hatchery-pre growing phase up to 20g (in three to eight months) and an on growing phase producing fish between 250 to 400 g (in twelve to twenty months) which are the main market product consumed [Vandeputte et al., 2019].

1.2. European sea bass nutrition: production challenges

European sea bass nutritional requirements are well stablished, ensuring fish maximum growth performance and health status under intensive culture conditions [Cerdá et al., 1994; Rizzo et al., 1996; Dias et al., 1998; Zambonino and Cahu, 1999; Lupatsch et al., 2001; Kaushik et al., 2002; Boujard et al., 2004; Oliva-Teles and Pimentel-Rodrigues, 2004; Enes et al., 2011]. As a carnivorous fish species, the European sea bass requires high dietary protein contents with an also highly specific amino acid profile [Kaushik et al., 1998; Tibaldi and Kaushik, 2005; Kousoulaki et al., 2015] presented in Table 1. On the other hand, carnivorous fish species have lost the capacity to elongate and/or desaturate polyunsaturated fatty acids (PUFA) to long chain polyunsaturated fatty acids (LC-PUFA). In the case of the European sea bass, the essential fatty acids (EFA) requirements is covered with the total inclusion of 1% n-3 LC PUFA in the diet [NRC, 2011; Oliva-Teles et al., 2015; Kousoulaki et al., 2015].

Table 1.1. Average proximal composition and essential amino acids requirements on commercial diets for European sea bass (*Dicentrarchus labrax*)

Fish weight (g)	Pellet size (mm)	Crude Protein (%)	Crude Lipid (%)	Crude Fibre (%)	Ash (%)	Total P (%)	Digestible Energy (MJ kg^{-1})
< 0.1	< 0.3	57	12	0.7-10	10	1.9	17.8-20
0.3-20	0.3-1.9	52-60	11-18	0.4-1.9	10-13	1.4-1.9	17.4-20.2
20-250	2.2-5	45-50	11.5-24	1-3.2	8.2-13	10-2	17.1-21.6
250-600	5-7	35-45	10.5-26	1.7-2	8.2-12	0.9-2	18-21.6
> 600	6-8	33.8-45	10.5-26	1.7-2	9-11	0.9-2	18-21.6

Essential amino acids requirements (% protein)					
ARG	3.9 - 4.6	LYS	4.4 - 4.8	MET + CYS	4 – 4.4
HIS	1.6*	THR	2.3 - 2.6	PHE + TYR	2.6*
ILE	2.6*	VAL	2.9*		
LEU	4.3*				

ARG (arginine); HIS (histidine); ILE (isoleucine); LEU (leucine); LYS (lysine); THR (threonine); VAL (valine); MET+CYS (methionine + cysteine); PHE+TYR (phenylalanine + tyrosine); n-3 LC-PUFA (omega-3 long chain polyunsaturated fatty acids). *Estimated levels for European sea bass, after Kaushik, 1998.

Aquaculture industry has employed fish meal (FM) and fish oil (FO) as the main sources of proteins and lipids for fish nutrition due to their excellent nutritional features with high quality micronutrients, amino acids and fatty acids profiles and an elevated digestibility and nutrient bioavailability. Additionally, this raw materials confer the aquafeeds high palatability and odor properties, stimulating fish feed consumption and thus favoring growth [Hardy, 2002; Turchini et al., 2009; Montero and Izquierdo, 2010; Oliva-Teles, 2015].

Nonetheless, FM and FO production relies directly on extractive fisheries which supposes an important handicap for their use on aquafeed production. In the year 2020, from the total 90.3 million tons produced by extractive fisheries, 16 million tons were destined to produce FM and FO for animal feed production [FAO, 2020]. Natural fish stocks depletion has translated into the shortage of FM and FO availability and leading to highly volatile prices. In 2012, FM and FO reached the total price of 1600 USD and 1800 USD per metric ton respectively [Hodar et al., 2020]. This economical sustainability issues, added to the higher demand in response to aquaculture industry growth has led to the necessity to look for alternative protein and lipid sources in order to develop a more economically and environmentally sustainable aquaculture production [Turchini et al., 2009; Hardy et al., 2010; Olsen and Hasan, 2012; Oliva-Teles, 2015; Turchini et al., 2019].

In the last decades an intensive research effort has been carried out in order to find more economic and environmental sustainable protein [Torstensen et al., 2008; Oliva-Teles et al., 2015; Salze and Davis, 2015; Hamre et al., 2016; Hemre et al., 2016; Lock et al., 2018; Benedito-Palos et al., 2016; Hua et al., 2019; Lazzarotto et al., 2018; Turchini et al., 2019; Carvalho et al., 2020; Parma et al., 2020; Carvalho et al., 2021; Gesto et a., 2021; Luthada et al., 2021] and lipid sources [Turchini et al., 2009; Turchini et al., 2010; Lenihan-Geels et al., 2013; Oliva-Teles et al., 2015; Gasco et al., 2018; Carvalho et al., 2020; Hodar et al., 2020; Carvalho et al., 2021]. Nevertheless, the use of these alternative raw materials may lead to nutritional inadequacies such as essential amino acid deficiencies or unbalances on n-3/n-6 ratios, negatively affecting fish growth and health performance [Turchini et al., 2009; Montero and Izquierdo, 2010; Oliva-Teles et al., 2015]. Besides, alternative raw materials such as those derived from plants may present anti-nutritional factors (ANFs) reducing feed digestibility and nutrient bio-availability as well as food palatability [Kousoulaki et al., 2015; Oliva-Teles, 2015; Daniel, 2018].

In 2017, within the framework of ARRAINA project (N288925: “Advanced Research Initiatives for Nutrition & Aquaculture” funded by EU7FP), Torrecillas and co-authors

analyzed the effects of combined graded levels of substitution of FM and FO by raw terrestrial materials in practical diets for European sea bass juveniles [Torrecillas et al., 2017 a,b,c]. The authors carried out a 90 days feeding experience in which European sea bass juveniles with an initial body weight of 9.78 ± 1.50 g were fed dietary treatments formulated to contain graded levels of FM%/FO%: 58/15, 20/6, 20/3, 10/6, 10/3, 5/6, 5/3, 0/0. The authors reported a feasible substitution of FM/FO down to 10%/3% respectively without affecting fish growth and feed intake in comparison to the dietary treatment with 58%FM/15%FO [Torrecillas et al., 2017 a,b]. Further reduction of FM down to 5% and 0% markedly reduced fish feed intake which resulted in reduced growth rates. Additionally, this levels of FM replacement led to alterations on fish gut microbiota profiles and an enlarged submucosa in the posterior intestine, with higher mucus production and an increased relative gene expression of the pro-inflammatory cytokines IL-1β and TNF-α [Torrecillas et al., 2017a,b]. Regarding FO replacement by VO down to 3% and 0% led to increased lipid deposition in the liver of fish, pointing to a reduction in dietary lipid utilization and reduced feed efficiencies. In the gut, high VO content resulted in the accumulation of lypoproteins in the anterior gut *lamina propria*, as a possible consequence of changes on the digested lipids reacylation and transport processes [Torrecillas et al., 2017 b,c]. Total reduction of FO from 6% down to 3% also resulted in changes in tissue composition and morphology, leading to higher bacterial translocation rates. All these physiolofical alterations synergized impairing fish ability to mount a proper immune response and increasing mortality rates against the pathogen a *Vibrio anguillarum* [Torrecillas et al., 2017c].

Thus, the successful replacement of FM and FO by alternative raw materials in practical diets for European sea bass must consider the potential nutritional unbalances as a source of *stress* or an aggravating factor on *stress processes*, negatively affecting fish health and welfare [Ashley, 2007; Montero and Izquierdo, 2010; Oliva-Teles, 2012].

1.3. European sea bass physiology: an stress susceptible species

1.3.1. Stress physiology

Fish, as well as other organisms, are characterized by the ability to maintain a dynamic and complex equilibrium called *homeostasis*. When this equilibrium is challenged by any external or internal agent, called *stressor*, the organism initiates a cascade of physiological and behavioral changes, known as *stress response*, in order to reestablish homeostasis and survive. The total energetic cost of facing and surviving an stressor is called *allostatic load* and will be directly proportional to the stressor severity [Aluru et al., 2009; Ellis et al., 2012; Tort, 2011; Schreck and Tort, 2016; Mateus et al., 2017].

The stress response is an energy demanding process, consisting in the allocation and relocation of organism's energetic resources in order to cope with the stressor. This process can be divided in three different stages; *(1)* the alarm stage, in which organism energetic resources are rapidly allocated in order to face the stressor (flight or fight response). Second, *(2)* a resistance stage in which organism energetic resources are relocated in order to reestablish the pre-stress homeostatic conditions. When a stressor acquires a severe character or is repeated in time, the organism may suffer an *allostatic overload*, leading to a third stage *(3)* of exhaustion. In this stage, organism general performance is affected, with decreased growth and reproductive capacity, increased disease susceptibility and even death (Figure 1) [Ellis et al., 2012; Nardocci et al., 2014; Schreck and Tort, 2016].

Immediately after the perception of a stressor the stress response is initiated and mediated by two neural-endocrine axes, the Sympathetic Chromaffin (SC) axis and the Hypothalamus-Pituitary-Interrenal (HPI) axis, triggering the synthesis an release of catecholamines (CA) and glucocorticoids (GC) respectively. Overall, both hormones main role is to ensure energy

availability but they exert their effects at different moments during the stress response process [Reid et al., 1998; Ellis et al., 2012; Schreck et al., 2016].

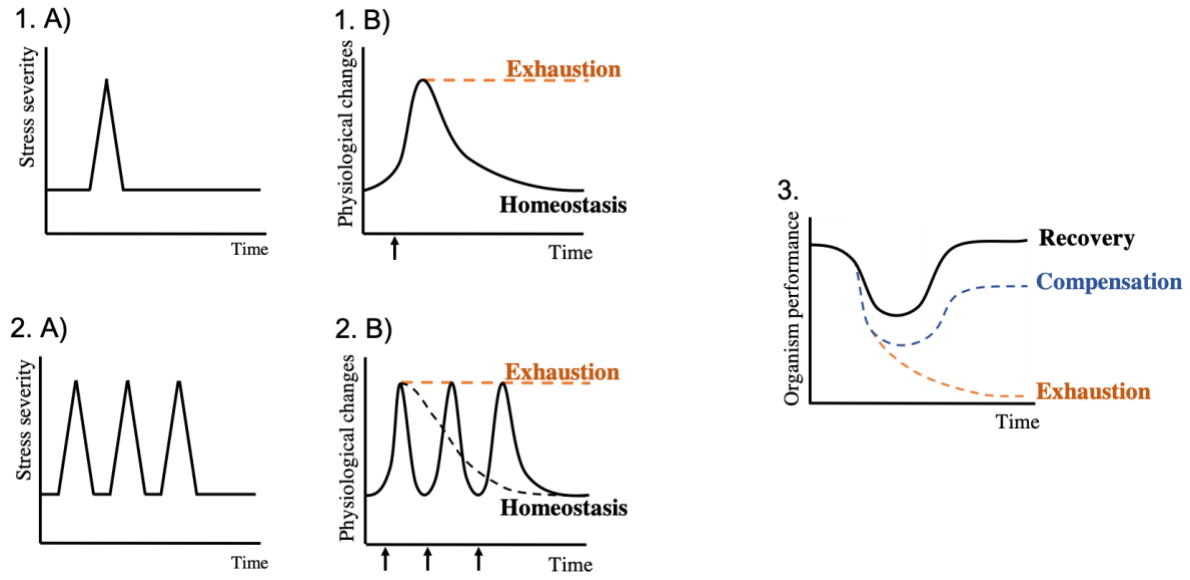


Figure 1.1. Temporal patterns of a stress process. (1.A) Single stressor; (1.B) Single occurrence of a stressprocess ↑: can be soft or mild resulting in homeostasis reestablishment or can be severe, resulting in the inability to recover homeostasis and leading to exhaustion; (2.A) Occurrence of different consecutive stress processes; (2.B) Chronic stress process indicated by ↑: A prolonged stressor or repetitive stress processes may lead to the exhaustion stage. (3) A stress process may present three different final outcomes depending on organism homeostasis reestablishment capacity: the full ability to face and overcome a stressor will lead to the total recovery of pre-stress conditions. The ability to face and survive a stressor but not being capable to reestablish totally the pre-stress conditions will lead to compensation. The incapacity to overcome a stressor or to recover the homeostatic balance after stressor occurrence will lead to the exhaustion. Adapted from Schreck and Tort, 2016.

Both CA hormones, adrenaline and noradrenaline, are synthesized in matter of seconds by direct stimulation of Blachsko pathway within chromaffin cells [Reid et al., 1998; Gorissen and Flik, 2016]. CA release into blood stream will result in an increased cardiovascular and respiratory rates and the mobilization of energy reserves supplying the increased metabolic demands [Schreck et al., 2016; Rodnick and Planas, 2016]. CA will trigger a rapid increase on circulating plasma glucose levels through the stimulation of hepatic glycogenolysis and anaerobic glycolysis. At cardiovascular level, CA stimulation will result in an increased cardiac rate and the stimulation of splenic contractions, leading to increased erythrocytes populations facilitating oxygen and glucose transport to the different tissues involved in the flight or fight response [Pottinger, 2008; Peter, 2011]. Regarding fish respiratory system, CA will increase gill water permeability, increasing oxygen uptake [Rodnick and Planas, 2016]. CA will also exert important changes at immune level, activating fish innate immune response through the production of acute phase proteins, peptides and proinflammatory cytokines. CA will stimulate head kidney macrophages and surveillance T cells production with increased kinetics and infiltration capacity, favoring inflammatory processes [Sarkar et al., 2011; Yada and Tort, 2016; Urbinati et al., 2020].

These series of changes associated to CA activity are accompanied by significant physiological unbalances that must be addressed in order to reestablish fish pre-stress conditions. A clear example of such unbalances is the phenomena called osmoregulatory compromise, consisting in the loose of osmotic and hydromineral balance as direct consequence of an increased gill diffusive capacity and an accelerated hear rate. Blood pH will also be disturbed by the release of lactate and protons into the bloodstream as by-product of an increased muscular activity in response to CA [Rodnick and Planas, 2016.] The up-regulation of fish immune activity in response to CA will also impose an elevated energetic cost, with

processes such as cell proliferation and protein synthesis [Aluru et al., 2009; Tort, 2011; Yada and Tort, 2016].

The processes involved on fish homeostatic balance reestablishment will be mediated by the GC, mainly, the cortisol [Schreck and Tort, 2016]. Cortisol synthesis is also triggered immediately after stressor perception, nevertheless this process is mediated by a cascade of chemical signals lasting minutes to hours [Schreck and Tort, 2016]. Upon activation, the hypothalamus triggers the secretion of cortisol releasing factor (CRF) that in turn will stimulate pituitary's production and release of the adrenocorticotrophic hormone (ACTH). Once in the bloodstream, the ACTH will target fish head kidney triggering the cortisol steroidogenesis [Ellis et al., 2012; Schreck and Tort, 2016; Mateus et al., 2017]. Cortisol will be synthesized from cholesterol in the mitochondria [Ellis et al., 2012; Nardocci et al., 2014; Schreck and Tort, 2016], in a series of isomerizations and hydroxilations with a final step catalyzed by the P450 11β-hydroxylase enzyme, a cytochrome encoded by the *cyp11β* gene expression [Vukelic et al., 2011]. This process will be rate-limited by cytosolic cholesterol transport into the mitochondria, by action of the Steroidogenic acute regulatory protein (StAR) [Stocco et al., 2005]. Upon arriving the target tissues, cortisol effects will be mediated by the glucocorticoid receptors (GR). This receptors can be find on the membrane surface, mediating the non-genomic actions exerted by cortisol, or in the cytoplasm complexed with the co-chaperone heat shock proteins 70 (hsp70) and 90 (hsp90) [Balasch and Tort, 2019]. After entering the cell, the cortisol will bind to the GR inducing the heat shock proteins detachment. The complex GG-cortisol then will translocate into the nucleus inducing the transcription of the cortisol responsive factors [Terova et al., 2005; Ellis et al., 2012; Nardocci et al., 2014] (Figure 2).

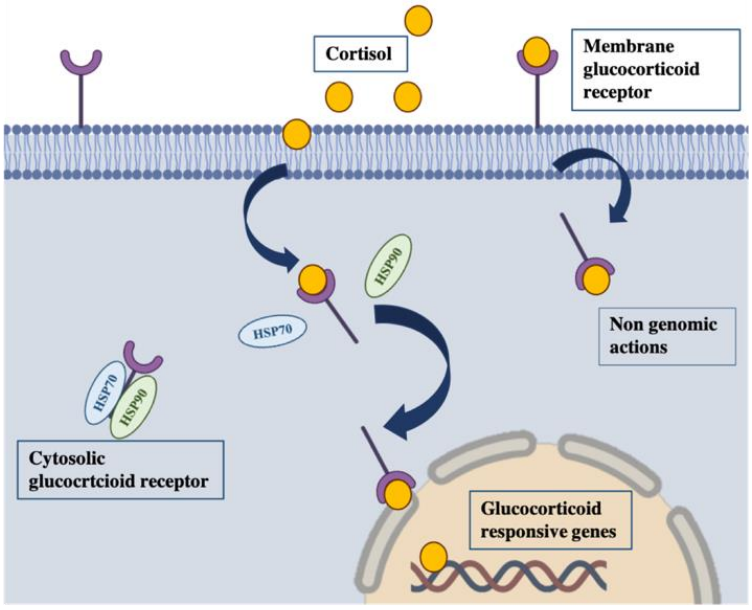


Figure 1.2. Cortisol effects mediation by glucocorticoid receptors. HSP70 (heat shock protein 70); HSP90 (heat shock protein 90).

Cortisol main role is to secure more proper and lasting sources of energy in order to supply fish metabolic demands. Cortisol stimulates fish aerobic metabolism, increasing cellular oxidative phosphorylation (OXPHOS) processes which is a highly efficient mean to produce ATP (aprox. 34 ATP per cycle) [Ballard and Towarnicki, 2020] compared to glycogenolysis

and glycolysis (with 3 and 2 ATP per glucose, respectively) [Rodnick and Planas, 2016]. Besides, cortisol regulates hepatic glucose and protein metabolism and will stimulate gluconeogenic activity [Kuo et al., 2015; Faught and Vijayan, 2016]. This energy availability will supply the Na^+K^+ ATPase pumps activity, leading to the osmotic and hydromineral balance reestablishment [Nardocci et al., 2014; Rodnick and Planas, 2016, Schreck and Tort, 2016]. Cortisol will also play a fundamental role on inflammatory processes resolution, inhibiting pro-inflammatory and stimulating anti-inflammatory cytokines release through nuclear factor kappa beta (NFkβ) modulation [Baker et al., 2011; Liu et al., 2017]. Cortisol activity will also result in a generalized reduction on circulating leukocytes and their concentration in affected tissues. Leucocyte clearance will be favored through inhibited mitosis and promoted apoptotic processes [Aluru et al., 2009; Tort, 2011; Yada and Tort, 2016; Mateus et al., 2017].

1.3.2. Chronic stress

In a perfect scenario, fish will be capable to face the stressor and recover the initial conditions. Nevertheless, when a stressor acquires an elevated severity or is prolonged or repeated in time, fish can suffer an allostatic overload leading to the exhaustion stage (Figure 3). Furthermore, stressors can be concomitant or little spaced in time, leading to an overlapped effect overcoming fish capability. The repeated activation of HPI axis may lead to several negative-side effects associated to cortisol on metabolism and immune responses [Ellis et al., 2012; Nardocci et al., 2014; Schreck et al., 2016]. One of the most characteristic behavioral changes exerted by cortisol is the loose of appetite, possibly due to neural signals of satiation induced by the increased levels of plasmatic glucose [Ellis et al., 2012; Rodnick and Planas, 2016]. With this condition, all the metabolic costs associated to stress response will rely on endogenous reserves, leading to a process of energy deprivation for secondary processes such as growth and reproduction capacity. [Rodnick and Planas, 2016; Gorissen and Flik, 2016] (Figure 3). Chronic stress processes will also result in an increased pathogen susceptibility due to the immunosuppressive effects of cortisol, with reduced populations of immune cells. In particular, cortisol will drastically decrease B cells populations negatively affecting antibody production and phagocytic activity [Tort, 2011; Schreck and Tort, 2016; Yada and Tort, 2016]. A prolonged exposure to cortisol may also induce an elevated reactive oxygen species (ROS) production as by-product of an accelerated aerobic metabolism, leading to oxidative stress processes [Spiers et al., 2015; Mittler, 2017].

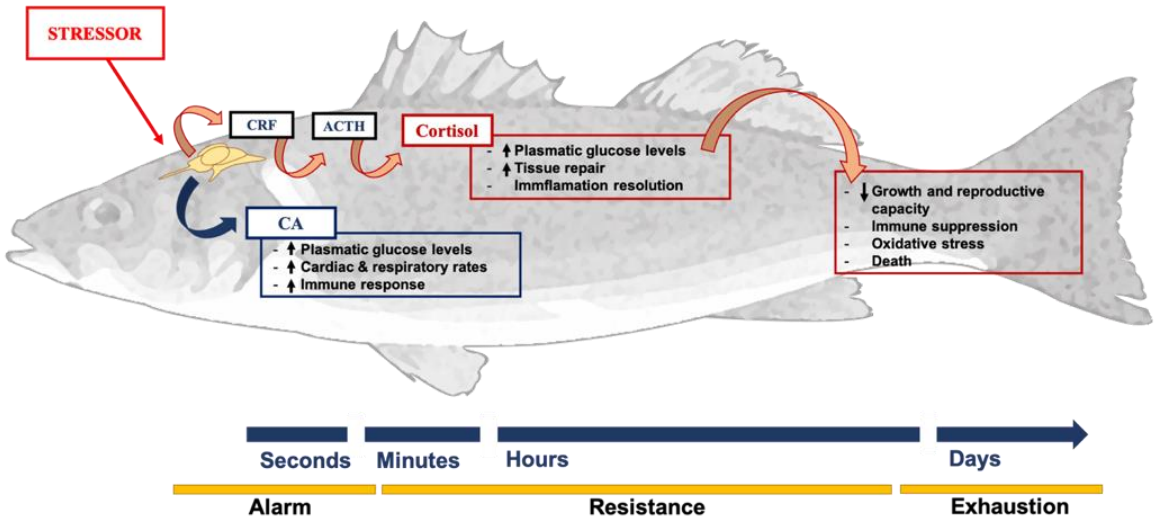


Figure 1.3. Describing the temporal development of a stress response and its physiological effects. Stressor (Perceived stressor); CRF (cortisol releasing factor); ACTH (adrenocorticotrophic hormone); CA (catecholamines); Cortisol (circulating plasma cortisol); Alarm (Alarm stage of the stress response); Resistance (Resistance stage of the stress response); Exhaustion (Exhaustion stage of the stress response).

1.3.3. Oxidative stress

Cortisol, among other effects, stimulates the mitochondrial membrane receptor protein BCL-2, triggering the activation of the electronic transport chain (ETC). The ETC is composed by a series of four intermembrane proteins (Complexes I, II, III and IV) that generate an electron flow that is coupled with the production of a proton gradient across mitochondrial inner membrane [Orrenius et al., 2007; Spiers et al., 2015; Zhao et al., 2019] (Figure 4). The energy accumulated in this proton gradient will supply the ATP synthesis by the ATP synthase (Complex V) [Cash et al., 2007; Spiers et al., 2015]. Nevertheless, this process is not totally efficient, since 1 to 3% of the total electrons may escape the ETC in a process known as "proton leak" [Brand et al., 1994; Brand et al., 2010; Spiers et al., 2015; Zhao et al., 2019]. This leaked electrons will react with oxygen molecules (O_2), both in the mitochondrial intermembrane space and in the mitochondrial cytosol, leading to superoxide radicals formation (O2-) [Brand et al., 2010; Zhao et al., 2019]. Generated superoxide radicals will be dismutated by the mitochondrial antioxidant enzyme copper-zinc superoxide dismutase (Cu, Zn-SOD) into hydrogen peroxide (H2O2) which will passively diffuse out of the mitochondria [Grivennikova and Vinogradov, 2006; Brand et al., 2010]. In turn, the H2O2 will be detoxified by two antioxidant enzymes, the catalase (CAT) and the glutathione peroxidase (GPX). The ROS produced as normal by-product of aerobic metabolism is detoxified by fish enzymatic and non-enzymatic defenses [Martínez-Álvarez et al., 2005; Zafir et al., 2009; Pamplona and Constantini, 2011]. An insufficient antioxidant capacity may lead to the formation of hydroxyl radicals (OH^-) which is the mots unstable ROS and it can not be detoxified [Abele and Puntarulo, 2004; Birben et al., 2012; Spiers et al., 2015]. This ROS will be formatted by H_2O_2 oxidation of the cytosolic transition metals Fe^{2+} and Cu^{2+} by Haber-Weiss and the Fenton reactions, respectively [Birben et al., 2012; Marciano and Vajro, 2017; Demirci-Cekic et al., 2022]. The oxidative damages induced by this radical will lead to the exponential formation of new ROS [Ballard and Towarnicki, 2020; Marciano and Vajro, 2017; Zhao et al., 2019].

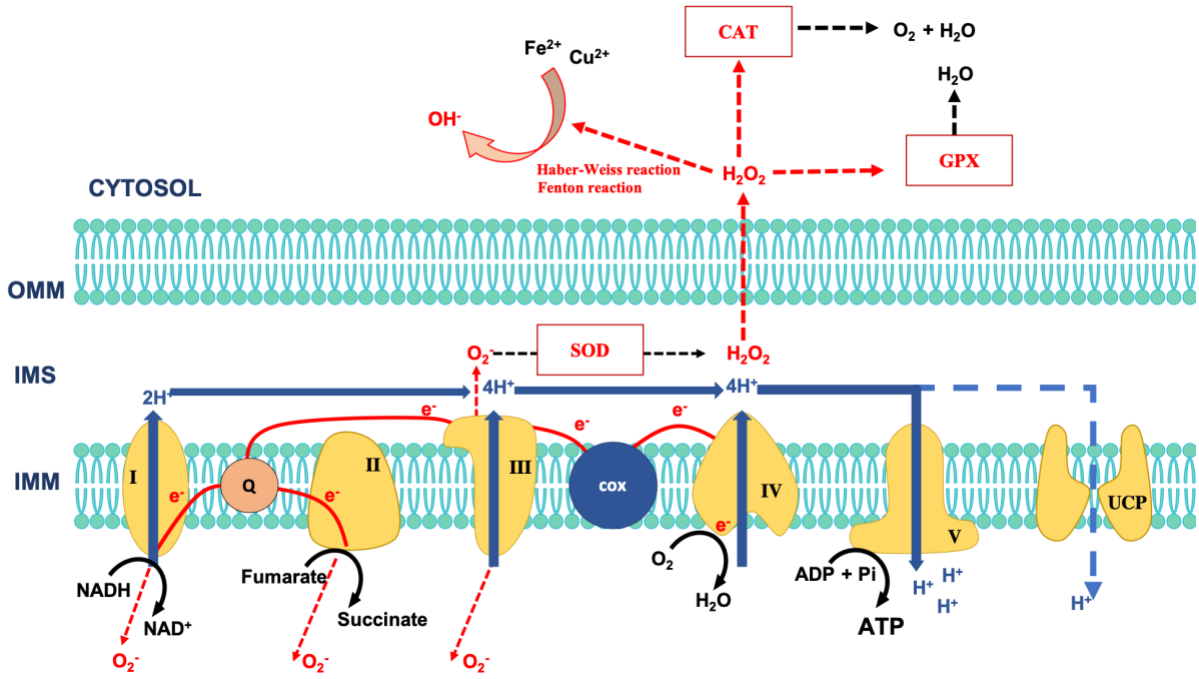


Figure 1.4. Schematic representation of the Electronic Transport Chain (ETC). The complexes I,II, III and IV pass through IMM the electrons derived from oxidizable substrates, driving the proton pumping into the IMS. The energy generated in the proton pumping will be consumed by the complex 5, the ATP synthase [Continuation] or the UCPs. The O^{2-} generated will be dismutated by SOD into H_2O_2 which will passively diffuse through OMM into the cytosol. H_2O_2 will be detoxified by CAT and GPX activity into O_2 and H_2O_2. H_2O_2 may react with Fe^{2+} or Cu^{2+} by Haber-Weiss and Fenton reaction, leading to the formation of OH^-. Blue lines indicate proton pump. Red lines indicate electron transfer. Red dashed lines indicate oxidation. Black dashed lines indicate detoxification. I, II, III, IV and V (intermembrane protein complexes); Q (Ubiquinone); cox (Cytochrome c); SOD (superoxide dismutase); CAT (catalase); GPX (glutathione peroxidase); H_2O (water); O_2^- (superoxide); H_2O_2 (hydrogen peroxide). OMM (outer mitochondrial membrane); IMS (intermebrane space); IMM (inner mitochondrial membrane). Adapted from Zhao et al., 2019.

When ROS formation overcomes fish antioxidant capacity oxidative stress processes begin, leading to severe damages to important biomolecules, including lipid and protein peroxidation and DNA damages [Burton et al., 2011; Preiser, 2012; Sies et al., 2017]. Altogether leading to the loss of functionality and even to cellular death. The mitochondria play an important role on apoptotic processes, hosting a wide variety of critical apoptotic activators, such as the cytochrome c (*cox*) [Moll and Zaika, 2001]. An oxidative stress process may induce to a mitochondrial dysfunction, with the collapse of the inner membrane gradient and leading to its permeabilization and the release of all these apoptotic factors [Orrenius et al., 2007; Ott et al., 2007; Sinha et al., 2016].

Mitochondria is the main organelle for endogenous ROS formation places, nevertheless other biological processes may suppose a source of ROS. Among them, lipid metabolism and immune system activity are also important sources of endogenous ROS and nitrogen reactive species (RONS). Thus, those organs and tissues with functions such as biosynthesis, ion transport, contractile or immune defense will be especially susceptible to suffer oxidative stress processes [Lushchak, 2014; Rodnick and Planas, 2016; Chowdhury and Saikia, 2020].

Fish gill interact directly with the external environment, acting as physical and biochemical semipermeable barrier with fundamental roles on gas exchange, hydro-mineral balance and immune response [Hwang et al., 2011; Li et al., 2019]. Gill epithelia is composed by two main cell types, the pavements cells (PVCs) and the mitochondria rich cells (MRCs). The MRCs are involved in the active ion transportation, being key players on fish osmoregulatory and hydromineral balance [Hwang et al., 2011; Hiroi et al., 2012]. This cells are characterized by a mitochondria-rich cytoplasm supplying the Na^+K^+ ATPase pumps activity [Hwang et al., 2011; Torrecillas et al., 2019]. Gill tissue also presents an important function on fish immune response, through the gill-associated lymphoid tissue (GIALT) activity. GIALT, as other

mucosal associated tissues, is composed mainly by B cells, T cells, macrophages, neutrophils, eosinophilic granulocytes, goblet cells and humoral immune related molecules such as lysozyme, bacteriocins and complement proteins [Salinas et al., 2011; Torrecillas et al., 2021]. Thus, as result of its important role as both regulatory and immune tissue, fish gills will be highly oxidative active tissues with an elevated risk of oxidative stress damages.

1.3.4. Stress and culture conditions

In aquaculture production, fish are subjected to a wide variety of stimuli which may suppose or can be interpreted as potential threats, leading to the activation of the stress response. For example, those derived from common aquaculture procedures such as husbandry protocols including handling, vaccination and sanitary biocides exposure, culture density and hierarchy relationships [Ashley, 2007; Gabriel and Akinrotimi, 2011; Faouraki et al., 2011; Samaras et al., 2017; Rehman et al., 2017] or nutritional unbalances [Ashley, 2007; Montero and Izquierdo, 2010; Oliva-Teles, 2012]. Additionally, it must be taken into account fish susceptibility to water physicho-chemical variations [Gabriel and Akinrotimi, 2011; Rehman et al., 2017; Abisha et al., 2022] and to potential pathogens such as bacteria, viruses, fungi and parasites [Paperna, 1991; Yanong, 2003; Johnson et al., 2004; Dos Santos and Howgate, 2011; Pridgeon and Klesius, 2012; Gozlan et al., 2014; Zhang and Gui, 2015; Kibenge, 2019].

European sea bass is a highly stress susceptible fish species [Rotllant et al., 2003; Di Marco et al., 2008; Fanouraki et al., 2011] and in special to handling stressors [Samaras et al., 2017]. In 2011, Fanouraki and collaborators analyzed the stress response of several cultured fish against a chasing and air exposure challenge test. Results obtained determined that European sea bass was the most stress sensitive fish species, with cortisol levels twenty-fold higher than the dusky grouper (*Epinephelus marginatus*), the meagre (*Argyrosomus regius*) and the common dentex (*Dentex dentex*), and between two to four-fold higher than the gilthead sea bream (*Sparus aurata*), the sharpsnout sea bream (*Diplodus puntazzo*) and the common Pandora (*Pagellus erythrinus*). Moreover, challenged European sea bass required extra time to reestablish the pre-stress conditions [Fanouraki et al., 2011].

Stress susceptibility has associated negative-side effects, as increased incidence of opportunistic pathogens on European sea bass aquaculture production. Among the variety of pathogens affecting this fish species [Pridgeon and Kresius; 2012; Zhang and Gui, 2015], the *Vibrio* sp. are known to induce loses of over 50% of the population in farms soon after outbreaks [Arab et al., 2020; Regev et al., 2020]. Opportunistic pathogens belonging to Vibrionaceae family, are one of the main causes of diseases outbreaks in marine and estuarine-type fish species and can be found freely in the marine water environment or associated to mucosal tissues and gastrointestinal tract of fish [Laganà et al., 2011; Regev et al., 2020]. Upon infection, vibriosis induces loss of appetite and discoloration. Advanced stages of infection are characterized by skin swollen sores accompanied by redness around mouth and gills. Finally when the infection becomes systemic it can produce exophthalmia and gut and rectum flood with blood and fluid [Regev et al., 2020]. This situation is worsened by the banning of several antibiotic treatments, by the law 1831/2003/EC from the European union The law was approved in 2006, as a result of the development of multidrug-resistant bacteria as result of this products leakage from aquaculture production facilities [Giannenas et al., 2012; Monzón-Atienza et al., 2023].

Considering the prevalence of stress processes under culture conditions and the potential negative side-effects of FM/FO on fish health status, the development of preventive strategies attenuating European sea bass stress susceptibility and reinforcing immune response and antioxidant status will acquire a vital role on culture productivity and profitability.

1.4. Functional additives: improving fish health and welfare

Concerning this necessity, the projects "PROINMUNOIL (AGL2012-39919) and PROINMUNOIL PLUS (AGL2016-79725-P): Functional additives and alternative oils to fish oils in Aquaculture: an effective tool to increase fish disease resistance" were developed in order to investigate the potential use of functional additives (FA) as European sea bass health and welfare promoters under a low FM/FO based dietary regime. FA are nutrients with a wide variety of beneficial properties capable of stimulate fish immune defense and tissue integrity, attenuate the stress response, reinforce their antioxidant status and increase feed digestibility and nutrient biodisponibility [Hoseinifar 2015; Encarnação, 2016; Dawood et al., 2016]. The PROINMUNOIL project series investigated; probiotics, prebiotics and phytogenic compounds. This PhD thesis is involved in the studies developed within the PROINMUNOIL PLUS (AGL2016-79725-P) project, with special focus on dietary prebiotic and phytogenic compounds dietary supplementation.

1.4.1. Prebiotic compounds

The prebiotic compounds are plant derived non digestible food ingredients that could benefit host by selectively stimulating the growth and/or activity of a limited number of bacterial species already present in host intestinal tract [Gibson, 2004; Hoseinifar et al., 2015; Guerreiro et al., 2017]. Prebiotic supplementation have shown to significantly increase fish growth, feed utilization and conversion efficiency [Ringø et al., 2010, 2014; Guerreiro et al., 2017; Torrecillas et al., 2015a,b; Torrecillas et al., 2018] through the modulation of gut bacterial population digestive enzymes secretion leading to an increased nutrient availability [Guerreiro et al., 2017]. Besides, prebiotic compounds have shown the ability to enhance gut epithelium integrity, leading to an increased absorptive area and functionality [Ringø et al., 2010; Guerreiro et al., 2015; Torrecillas et al., 2018; Butt et al., 2019; Dawood et al., 2021].

Regarding fish immune response and pathogen resistance, dietary prebiotic have also showed an elevated potential as an immunomodulatory tool through a wide variety of mechanisms. Between them, the promotion of host beneficial microbiome species may reinforce fish health status through competitive exclusion and the production of chemical substances as organic acids, H_2O_2, antibiotics, bacteriocins and lysozyme [Buentello et al., 2010; Roberfroid et al., 2010; Zhou et al., 2010; Akrami et al. 2013; Guerreiro et al. 2016]. Dietary prebiotics may also interact with host microbe-associated molecular patterns (MAMPs) recognition systems, leading to the stimulation of fish immune response [Torrecillas et al., 2011a; Cerezuela et a., 2013; Song et al., 2014]. On the same way, the promotion of gut tissue integrity and mucosal secretions by dietary prebiotic supplementation may act as first line of defense hampering pathogen bacteria adherence and translocation capacity [Torrecillas et al., 2011b; Hoseinifar et al., 2015; Torrecillas et al., 2019].

Prebiotic supplementation have also shown potential as an antioxidant status reinforcement tool, decreasing oxidative stress damages [Guerreiro et al., 2017; Hoseinifar et al. 2017]. Prebiotic compounds present a phenolic nucleus with a powerful antioxidant capacity, which could directly quench ROS preventing oxidative damages [Carbone et al., 2016; Guerreiro et al., 2016; Guerreiro et al., 2017; Hoseinifar et al., 2017; Abasubong et al., 2022] or the production of short chain fatty acids (SCFAs), such as butyrate, propionate and acetate which may promote host's antioxidant enzymatic activity [Yuan et al., 2018; Cuciniello et al., 2023].

Table 1.2. Prebiotics effects on fish growth performance, feed efficiency and health status.

Prebiotic	Fish species/ Body weight	Dosage	Results	Reference
MOS (Bio-Mos®)	*S. aurata* BW ≈ 24 g	0.2 and 0.4% 9weeks	Increased gut microbiota richness and diversity	Dimitroglou et al., 2010
MOS (Bio-Mos®)	*Sciaenops ocellatus* BW ≈ 7 g	1% 8weeks	Increased lysozyme activity	Zhou et al., 2010
MOS (Bio-Mos®)	*S. aurata* BW ≈ 170 g	0.2 and 0.4 %, 12 weeks	Increased FBW, WG, protein and energy digestibility and optimized FCR	Gültepe et al. (2011)
MOS (Bio-Mos®)	*D. labrax* BW ≈ 116 g	0.4%, 8 weeks	Increased gut folds height and width, increased secretion of acid mucins, increased and increased density of eosinophilic granulocytes.	Torrecillas et al., 2011a
FOS	*Rutilus rutilus* BW ≈ 0.67 g	1,2 and 3% 7 weeks	Increased Ig production, increased lysozyme activity and ACH50.	Soleimani et al., 2012
XOS	*D. labrax* BW ≈ 4.8 g	0.5 and 1% 12 weeks	Increased FBW, fish length and optimized FCR	Abdemlaek et al., 2015
cMOS	*D. labrax* BW ≈ 20 g	0.16%, 8 weeks	Increased fish length, SGR and relative growth rate	Torrecillas et al., 2015a,bb
MOS (Bio-Mos®)	*S. ocellatus* BW ≈ 11 g	1% 6weeks	Increased lysozyme activity	Buentello et al., 2017
GOS (Quingdao FTZ United International Inc.)	*D. sargus* BW ≈ 53 g	1% 15 days and 12 weeks	Increased gut trypsin (15 days) and decreased gut amylase (12 weeks)	Guerreiro et al., 2017
scFOS (PROFEED®)	*D. sargus* BW ≈ 53 g	1% 15 days and 12 weeks	Increased gut total alkaline protease (15 days), decreased gut amylase (12 weeks)	Guerreiro et al., 2017
MOS (Bio-Mos®)	*D. labrax* BW ≈ 20 g	0.3 and 0.6 % 13 weeks	Increased FBW (all), increased gut inflammatory markers (0.6%) and increased resistance to a *V. anguillarum* (0.3% diet)	Torrecillas et al., 2018

SGR (specific growth rate); Ig (immunoglobulins); ACH50 (alternative complement activity 50); FBW (final body weight); WG (weigh gain); FCR (feed conversion ratio).

The correct supplementation of dietary prebiotic compounds will be determinant on their ability to enhance host health status and growth [Hoseinifar et al., 2015; Encarnação et a., 2016]. Prebiotic dosage will be highly dependent on factors such as fish species and size, the rearing conditions and prebiotic composition and properties [Guerreiro et al., 2017].

1.4.2. Phytogenic feed ingredients

The phytogenic feed ingredientes (PFIs) are a heterogeneous group of plant derived compounds originating from leaves, roots, tubers, herbs or fruits which can exert a wide variety of beneficial effects in fish growth, health and welfare [Chakraborty et al., 2011; Encarnação, 2016; Firmino et al., 2021; Reverter et al., 2021]. PFIs can be supplemented in fish dietary treatments as solid, dried, grounded and as extracts or essential oils [Encarnação, 2016]. *Lamiaceae* family and *Allium* spp., are both of the most spread PFIs employed in aquaculture dietary administration [Firmino et al., 2021] .

Phytogenic compounds are known to exert a antibacterial activity [Firmino et al., 2021; Kazempour, 2022], especially those belonging to the *Lamiaceae* family, which present an elevated cytotoxic activity due to their high contents in thymol and carvacrol [Memar et al., 2017; Kachur and Suntres, 2020]. PFIs dietary supplementation led to increased survival rates against pathogen outbreaks [Volpatti et al., 2013; Menanteau-Ledouble et al., 2015; Torrecillas et al., 2019; Caipang et al., 2021; Reverter et al., 2021]. In addition, PFIs containing this phenolic compounds have shown to interact with the NF-kB and MAPK pathways, modulating the expression of pro-inflammatory and anti-inflammatory cytokines [Zhou et al., 2014; Huang and Lee, 2018; Liu et al., 2019]. These compounds have shown antistress properties, attenuating fish stress response [Chakraborty et al., 2014; Choubey et al., 2015; Yilmaz and Ergün, 2015; Gbadamosi et al., 2016; Mohiseni et al., 2017; Pahor-Filho et al., 2017; Hoseini and Yousefi, 2019; Yonar et al., 2019; Firmino et al., 2021].

Due to their phenolic composition, PFIs present an elevated capacity to inhibit the formation or directly quenching ROS leading to an increased antioxidant status in fish [Alloui et al., 2014; Irkin et al., 2014; Firmino et al., 2021; Kazempour, 2022]. In addition, PFIs have shown the ability to selectively enhance and suppress fish antioxidant enzymes, directly modulating fish antioxidant and immune status [Giannenas et al., 2012; Chowdhury et al., 2020; Chowdhury et al., 2021; Firmino, 2021; Firmino et al., 2021; Ibrahim et al., 2021; Liu et al., 2022; Magouz et al., 2022; Poolsawat et al., 2022].

The PFIs have shown to improve fish growth by stimulating fish digestive enzymes secretion, leading to increased feed efficiency and feed conversion ratio [Peterson et al., 2014; Habiba et al., 2021; Mansour et al., 2021; Kazempour, 2022]. Furthermore, PFIs dietary administration may lead to improved fish fillet organoleptic properties [Peterson et al., 2014; Steiner and Syed, 2015; Firmino, 2021].

Table 1.3. Phytogenic feed additives effects on fish growth performance, feed efficiency and health status.

Phytogenic feed additive	Fish species/ Body weight	Dosage	Results	Reference
Allium sativum (extract)	*Oreochromis niloticus* BW ≈ 100 g	1,4 and 8 % 2 weeks	Increased resistance to *Trichodina* sp and *Aeromonas hydrophila*	El Deen and Razin, 2009
Allium sativum (extract)	*Lates calcarifer* BW ≈ 21 g	1, 2, 3 ,4 and 5 % 2 weeks	Increased FBW, optimized FCR and increased resistance to *Vibrio harveyii*	Talpur and Ikhwanuddin, 2012
Thymus vulgaris or *Rosmarinus officinalis* or *Trigonella foenum graecum* L. (powder)	*D.labrax* BW ≈ 21 g	1% 7 weeks	Increased protein efficiency ratio, fillet protein levels, protein and energy retentions	Yilmaz et al., 2012
Thymol (25 %) and carvacrol	*S. aurata*	0.01 % 9 weeks	Increased gut epithelial tissue health and functionality and	Pérez-Sánchez et al., 2015

(25 %) commercial additive			induction of anti-inflammatory and anti-proliferative gene expression	
Origanum vulgare (essential oil)	*Onchorhynchus keta*	0.01, 0.02, 0.05 and 0.1 % 8 weeks + 28 days immune challenge	Increased feed efficiency and increased survival against *Ichthyobodo salmonis* and *Trichodina truttae*.	Mizuno et al., 2018
Thymus vulgaris (aqueous extracted)	*Onchorhynchus mykiss*	0.5, 1 and 2 % 2 weeks	Increased intestine antioxidant enzyme activity and decreased levels of malondialdehyde	Hoseini and Yousefi, 2019
Allium sativum essential oils + carvacrol and thymol commercial additives	*S. aurata*	0.5 % 9 weeks + 39 days immune challenge	Modulation of pro-inflammatory gene expression, sustained antioxidant and anti-inflammatory response in gills and increased gill epithelium and mucus content in carboxylate glycoproteins containing sialic acid	Firmino et al., 2020
Origanum vulgare (ethanolic extracted)	*O. niloticus*	0.2 and 0.5 % 9 weeks + 7 days immune challenge	Increased FBW, weight gain, SGR, skin mucus total Ig, increased survival against *A. hydrophyla*	Mohammadi et al., 2020
Origanum vulgare (essential oil)	*Cyprinus carpio* BW ≈ 16 g	0.5, 1.5 and 4.5 % 8 weeks	Increased digestive enzyme activity, antioxidant capability and increased resistance to *A. hydrophyla*	Zhang et al., 2020
Origanum vulgare (powder)	*Danio rerio*	0.5, 1 and 2 % 8 weeks	Increased FBW, SGR and weigh gain, increased skin mucus lysozyme, protease and alkaline phosphatase activities and increased survival against *A. hydrophyla*	Rashidian et al., 2021

SGR (specific growth rate); FBW (final body weight); WG (weigh gain); FCR (feed conversion ratio).

Despite the wide variety of beneficial effects of PFIs on fish health and welfare, they also present important disadvantages. As plant origin dietary ingredients, they may induce nutritional imbalances associated to the presence of anti-nutritional factors and reducing feed palatability [Guerreiro et al., 2017]. In addition, these compounds are highly volatile leading to low availability during their digestion or changes on the dose administration [Firmino et al., 2021].

1.4.3. Probiotic compounds

Probiotics are live microbial organisms which can benefit a host animal by improving its intestinal balance or their environment [Lazado et al., 2014; Merrifield and Carnevalli, 2014; Merrifield and Ringo, 2014; Encarnação, 2016; Dawood et al., 2016]. Probiotic compounds can be delivered via injection, direct addition to the water column or via dietary

supplementation, being the last one the most common practice on aquaculture [Lazado et al., 2014; Monzón-Atienza et al., 2023].

Oral administration of probiotic compounds will target primarily host gut as health promoters, potentially increasing fish immune response and growth performance. Dietary probiotic supplementation have shown the ability to stimulate bacterial production of digestive enzymes facilitating feed digestibility and increasing nutrient availability leading to increased feed utilization and growth rates [Avella et al., 2011; Pérez-Sánchez et al., 2014; Eissa et al., 2022]. Probiotic supplementation also present a high potential as fish immune stimulators, through microbial populations modulation and systemic and local immune response [Balcázar et al., 2006; Lazado et al., 2014; Dawood et al., 2016]. This modulation of host gut microbial populations can be exerted through the competition for essential nutrients and adhesion sites and the inhibition of virulence gene expression or disruption of quorum sensing of pathogen bacterial species [Hasan et al., 2023; Monzón-Atienza et al., 2023; Moroni et al., 2023]. Probiotic strains can also lead to the production of inhibitory compounds against pathogens such as lysozyme and bacteriocins and the modulation of pro-inflammatory cytokines, enhancing fish immune response [Burbank et al., 2011; Dawood et al., 20017; Monzón-Atienza et al., 2023] (Table 4).

Between the wide variety of probiotic strains with potential beneficial effects as fish immunomodulators [Merrifield et al., 2010], the lactic acid bacteria (*Lactobacillus* spp., *Peridococcus* spp., *Enteroccoccus* spp.) have been one of the most accepted group [Lazado et al., 2014; Encarnação, 2016]. Lactic acid bacteria present an elevated adequacy to probiotic strains selection criteria, such as, the capacity to adhere and grow in the host intestinal cavity, being free of antibiotic resistance and not modifying host heritable traits, and the capacity to enhance the growth or/and development of the immune system against pathogens [Monzón-Atienza, 2023].

Table 1.4. Probiotic feed additives effects on fish growth performance, feed efficiency and health status.

Probiotic feed additive	Fish species/ Body weight	Dosage	Results	Reference
Lactobacillus delbrueckii delbrueckii	*D. labrax* Larvae	10^5 bacteria ml^{-1} 25 or 59 days	Increased growth performance (81% increase by long term administration and 28% by short administration), reduced cortisol levels, increased IGF-1 gene expression	Carnevali et al., 2006
L. lactis sbsp. lactis, *L. sakei* or *Leuconostoc mesenteroides*	*Salmo trutta* BW ≈ 70 g	10^6 cfu g^{-1} diet 2 weeks	Increased serum complement activity, lysozyme activity and immunoglobulin	Balcázar et al., 2007
L. delbrueckii delbrueckii or a multispecies probiotic (*L. fructivorans* + *L. plantarum*	*D.labrax* and *S.aurata* Larvae	10^5 bacteria mL^{-1} 63 days	Increased body weight, attenuated stress response and enhanced immune response. In *D.labrax* increased body TCR- β, increased acidophilic granulocytes and low pro-inflammatory gene expression. In *S.aurata* immunoglobulin (Ig+) cells and acidophilic granulocytes	Abelli et al., 2009
Enterococcus faecalis	*O. mykiss* BW ≈ 13 g	1% diet 12 weeks	Improved hematocrit value, phagocytic index and activity and mucus production, increased resistance against *V.anguillarum*	Rodríguez-Estrada et al., 2009

Debaryomyces hansenii	*D.labrax* Larvae (2 dph)	4.3% live yeast 48 days	Increased growth performance and enhanced antioxidant gene expression of SOD and GPX	Tovar-Ramírez et al., 2010
Pdp11	*S.aurta* BW ≈ 38 g	10^9 cfu g^{-1} diet 116 days	Increased growth performance, attenuated stress under high stocking density	Varela et al., 2010
Multispecies probiotic (*Bacillus sp., Pediococcus sp., Enterococcus sp., L. sp.*) or single *Pediococcus acidilactici*	*O. mykiss* BW ≈ 16 g	10^9 cfu g^{-1} dry powder 96 days	Improved growth performance, host gut microbiome modulation	Ramos et al., 2013
L. plantarum	*D.labrax* BW ≈ 75 g	10x10^9 CFU kg^{-1} diet 90 days	Increased survival and higher cholesterol and triglycerides levels	Piccolo et al., 2015
Multispecies probiotic (Aqua Star® Growout: *Bacillus* sp., *P.* sp., *Enterococcus* sp., *L.* sp)	*Solea senegalensis* BW ≈ 33 g	1.34 ×10^{10} cfu kg^{-1}diet 73 days	Enhanced antioxidant status with up-regulated CAT and GPX gene expression when fed low FM/FO based diets	Batista et al., 2016
Bacillus velezensis strain V4 CGMCC 10149 and *Rhodotorula mucilaginosa* strain CGMCC 1013	*S.salar* BW ≈ 180 g	*B.velezensis* V4 5×10^6 cfu g^{-1}, *R.mucilaginosa* 5×10^7 cfu g^{-1}; *B.velezensis* V4 1.5 × 10^7 cfu^{-1}, *R.mucilaginosa* 1.5×10^8 cfu g^{-1}; *B.velezensis* V4 2.5×10^7 cfu g^{-1}, *R.mucilaginosa* 2.5×10^8 cfu g^{-1} 62 days	Increased growth performance and food utilization, enhanced immune response, better antioxidant status and increased resistance against *Aeromonas salmonicida*	Wang et al., 2019
Debaryomyces hansenii strain BCS004	*S.aurata* BW ≈ 80 g		Increased phagocytosis capacity, peroxidase and respiratory burst activities in leucocytes, increased haemolytic complement activity and total IgM in serum and increased TNF TNFα, C3 and IgM gut gene expression	Reyes-Becerril et al., 2021
P. acidilactici	*D.labrax* BW ≈ 16 g	2, 2.5 or 3 g/ kg ~ 1 × 1010 CFU 100 days	Increased growth performance, increased gut villi length, reduced ammonia excretion and increased survival rates (n.s.s.)	Eissa et al. 2022

cfu (colony forming units); IGF-1 (Insuline-like growth factor 1); TCR-β (T-cell receptor β); SOD (superoxide dismutase); CAT (catalase); GPX (glutathione peroxidase).

The survival rates of the strains during the extrusion and palletization phase of feed production can be one of the main factors limiting probiotic viability and thus limiting this functional additives development in feed industry [Rokka et al., 2010; Encarnação, 2016; Dawood et al., 2016; Guerreiro et al., 2017]

1.5. Selective breeding

Selective breeding is also a potential strategy in order to provide robust fish that will cope with the nutritional unbalances with low FM/FO dietary levels. Genotype selection is based on the calculation of fish breeding values based on phenotype measurements and genotype information of the fish presenting the desired trait [Louro et al., 2014; Boudry et al., 2021].

Selective breeding can target a wide variety of phenotypic characteristics, including growth performance and feed utilization, disease resistance or stress tolerance [Gjedrem et al., 2012; Stear et al., 2012; Das et al., 2014; Hermesch et al., 2015]. This programs can also target simultaneously different phenotypic traits, leading to multi-trait selection programs [Sae-Lim et al., 2013; Thodesen et al., 2013; Boudry et al., 2021]. Besides, selective breeding may lead to the indirect coselection of other traits, as selection for optimized FCR when targeting fast growing traits [Knap and Kause, 2018; Besson et al., 2019; Vandeputte et al., 2019] or the induction of domestication processes, by which a captive population get adapted to the rearing environment due to its genotype modification along generations [Vandeputte et al., 2009; Vandeputte et al., 2019].

Currently, seven private companies distributed along Italy, Turkey, Greece (Niveuus) and France (Ecloserie Marine de Graveline, Ferme Marine du Douhet) are developing European sea bass breeding programs [Vandeputte et al., 2019]. The European sea bass show medium to high selection response for weight, with increases ranging from 23-42% of growth per generation [Vandeputte et al., 2009; Dupont-Nivet et al., 2010].

The AquaIMPACT Project (EU Horizon 2020 no. 818367): Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture, was developed in order to integrate the field of selective breeding and nutrition trying to increase European Aquaculture competitiveness. This PhD thesis was developed within the AquaIMPACT Project.

1.6. General Objectives

The main objective of the present thesis was to investigate the combined effect of selective breeding strategies and functional additives dietary supplementation in order to minimize the negative side effects derived of low FM/FO based diets in European sea bass health status and welfare.

- To evaluate the potential of functional additives dietary supplementation to reduce the negative side effects derived from low FM/FO based diets in European sea bass juveniles stress and health indicators .

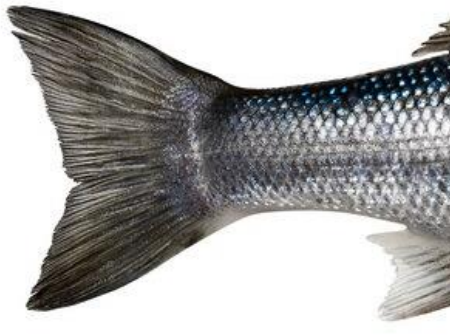
- To evaluate the effects of functional additives as gill antioxidant status boosters in European sea bass juveniles fed low FM/FO diets and subjected to physical and biological stressors.

- To evaluate the possible synergies between multi-trait selection strategies and functional additives dietary supplementation in order to address the physiological unbalances derived from low FM/FO utilization and enhance European sea bass juveniles gill antioxidant capacity when subjected to an H_2O_2 exposure stress challenge.

- To determine whether multi-trait selected European sea bass genotypes can benefit from functional additives dietary supplementation in order to address the limitations of FM/FO reduction in terms of improving fish growth performance, gut health and disease resistance.

- To develop a nutritional multiple-linear regression model to characterize the possible effects of different functional additives dietary inclusion on European sea bass growth and feed utilization key performance indicators.

Chapter II
Materials and Methods



2.1. Experimental diets

The details of each experimental diet used in the different feeding trials are detailed in its respective chapters. All the experimental diets used in the present thesis were isonitrogenous and isoenergetic and were formulated according to commercial standards with a low FM (10%) and FO(6%) basis content, covering European seabass nutritional requirements. Diets were formulated and produced by commercial producers using an extrusion process.

Table 2.1. Main ingredients and analyzed proximate composition of the diets used in trials belonging to chapters III, IV and V.

Ingredients	Control	PHYTO0.02	PHYTO0.1	GMOS
Fish meal[1]	9.6	9.6	9.6	9.6
Soya protein concentrate	18.2	18.2	18.2	18.2
Soya meal	11.6	11.6	11.6	11.6
Corn gluten meal	24.1	24.1	24.1	24.1
Wheat	8.5	8.5	8.5	8.5
Wheat gluten	1.9	1.9	1.9	1.9
Guar meal	7.7	7.7	7.7	7.7
Rapeseed extracted	3.0	3.0	3.0	3.0
Fish oil[2]	6.5	6.5	6.5	6.5
Rapeseed oil[3]	5.2	5.2	5.2	5.2
Vitamin and mineral premix[4]	3.6	3.6	3.6	3.6
Antioxidant[5]	0.06	0.06	0.06	0.06
GMOS[6]	-	-	-	0.5
PHYTO 1[7]	-	0.02	-	-
PHYTO 2[8]	-	-	0.1	-
Proximate composition (% dry mater)				
Crude fat	19.91	20.47	20.47	20.44
Crude protein	49.30	49.76	49.76	49.27
Moisture	5.10	5.06	5.06	5.01
Ash	7.02	6.49	6.49	6.41
Gross enery (MJ/kg, as is)[9]	22.07	22.17	22.17	22.11

[1]South-American, Superprime 68% (63-68% protein; 8-9.5% lipids). [2]South American fish oil. [3]DLG AS, Denmark. [5]BAROX BECP, BHT. [6]Delacon Biotechnik GmbH, Austria. [7]Delacon Biotechnik GmbH, Austria. [8]Delacon Biotechnik GmbH, Austria. [9]Determined using a calorimetric bomb (Eurofins Food & Feed testing, Norway, AS).

Table 2.2. Main ingredients and analyzed proximate composition of the diets used in trial belonging to chapter VI.

Ingredients		Low level dietary supplementation ingredient			High level dietary supplementation ingredient		
	Control	PROB	ORG	PHYTO	PROB	ORG	PHYTO
Corn gluten	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Hi Pro Soybean meal[1]	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Wheat gluten	10.2	10.2	10.2	10.2	10.2	10.2	10.2
Faba bean dehulled[2]	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Wheat	19.95	19.95	19.95	19.95	19.95	19.95	19.95

Soy protein concentrate[3]	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Fish oil[4]	-	-	-	-	-	-	-
Fish meal[5]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Rapseed oil	8.98	8.98	8.98	8.98	8.98	8.98	8.98
Phophate	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Vitamin & mineral mix[6]	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Poultry meal[7]	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Poultry oil[8]	1.37	1.37	1.37	1.37	1.37	1.37	1.37
DHA oil[9]	2.75	2.75	2.75	2.75	2.75	2.75	2.75
Lecithin	2.0	2.0	2.0	2.0	2.0	2.0	2.0
PROBIOTIC	-	0.2	-	-	1	-	-
ORGANIC ACID	-	-	0.3	-	-	0.75	-
PHYTO 3	-	-	-	0.5	-	-	0.75
Proximate composition (% dry mater)							
Crude fat	17.67	17.67	17.67	17.67	17.67	17.67	17.67
Crude protein	51.18	51.18	51.18	51.18	51.18	51.18	51.18
Moisture	6.25	6.25	6.25	6.25	6.25	6.25	6.25
Ash	4.57	4.57	4.57	4.57	4.57	4.57	4.57

Yttrium premix: 0.1% ; [1]Soya bean meal: CJ Selecta S.A (Brasil) ; [2]Faba beans: Cefetra BV (The Netherlands) ; [3]Soya protein concentrate: CJ Selecta S.A (Brasil); [4]Fish oil: Copeinca, S. A. (Perú); [5]Fish meal: Norsildmel AS (Norway) ;[6]Mineral and Vitamin premix: Trouw Nutrition (The Netherlands) ; [7]Poultry meal: Sonac (Belgium); [8]Poultry oil: Sonac (Belgium); [9]DHA: Veramaris (Evonik).

For the different experiments, the basal diet (Control) was supplemented with either three different functional additives produced by Delacon Biotechnik (Delacon, Austria)(Table 2).

Table 2.3. Functional additives employed in the different trials

Functional additive	Composition	Inclusion method
GMOS	Plant derived galactomannan-oligosaccharides	Powder
PHYTO 1	Blend of garlic and Labiatae plant oils (87.5 mg terpenes/kg diet	Oil-top-coating
PHYTO 2	Mixture of citrus flavonoids and *Asteraceae* and *Labiatae* plant essential oils (57 mg terpenes/kg diet)	Powder
PHYTO 3	Mixture consisting in 16% natural plant extracts of garlic combined with medium-chain fatty acid sources (Aquagarlic P Protec, Domca, Spain)	Oil-top-coating

PROB	Mixture in equal ratio of Bacillus species: *B. subtilis*, *B. licheniformis*, and *B. pumilus* at a total bacterial concentration of 2×10^{10} CFU (colony forming units)/g of product	Oil-top-coating
ORG (organic acids)	70% butyric acid sodium salt (GBM CMR, Sanluc, Belgium)	Oil-top-coating

In order to ensure product stability, GMOS (Delacon internal product code: SBPMH01) and PHYTO 2 (Delacon internal product code:X) were included in the dietary treatments as solid compounds during the pre-extrusion process. PHYTO 1 (Delacon internal product code: SBPMG02) functional additive was included as an oil in the dietary mix, by vacuum coating after the extrusion process. The stability of the functional additives was checked before and after diet production and before feeding trials. Chapters III and IV analyzed the dietary inclusion of GMOS and PHYTO 1. Chapter V analyzed the dietary inclusion of GMOS, PHYTO 1 and PHYTO 2. Chapter VI analyzed the dietary inclusion of ORG, PROB 1 and PHYTO 3.

2.2. Experimental conditions

The experiences conducted in this thesis were developed in the facilities of the Parque Científico-Tecnológico Marino de Taliarte (PCTM) of the University of Las Palmas de Gran Canaria (Canary Islands, Spain). Trials I and II were developed within the framework of PROINMUNOIL PLUS (AGL2016-79725-P) project: "Functional diets for marine raw materials replacement: boosting the fish disease resistance through epithelial barriers reinforcement and immunization tools", founded by the Spanish Ministry of Economy, Industry and Competitiveness ("Subprograma Estatal de Generación de Conocimiento, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013–2016"). Trial III, IV and V developed within the framework of AquaIMPACT: Nutrition and breeding (EU Horizon 2020 no. 818367) project, founded by the European Union's Horizon 2020 research and innovation program. All the procedures described in the present thesis comply the guidelines of the European Union council (2010/63/EU) on the protection of animals used for scientific purposes and approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (approval no. 007/2012C EBA ULPGC for Chapters III, IV; approval no OEBA_ULPGC_14/2020 for trial V and VI).

A detailed experimental design experimental design and protocols for each trial are described in the different chapters corresponding to each experiment. The experimental procedures developed in the different chapters of the present thesis are summarized in the following visual abstracts.

2.2.1. Chapter III graphical abstract

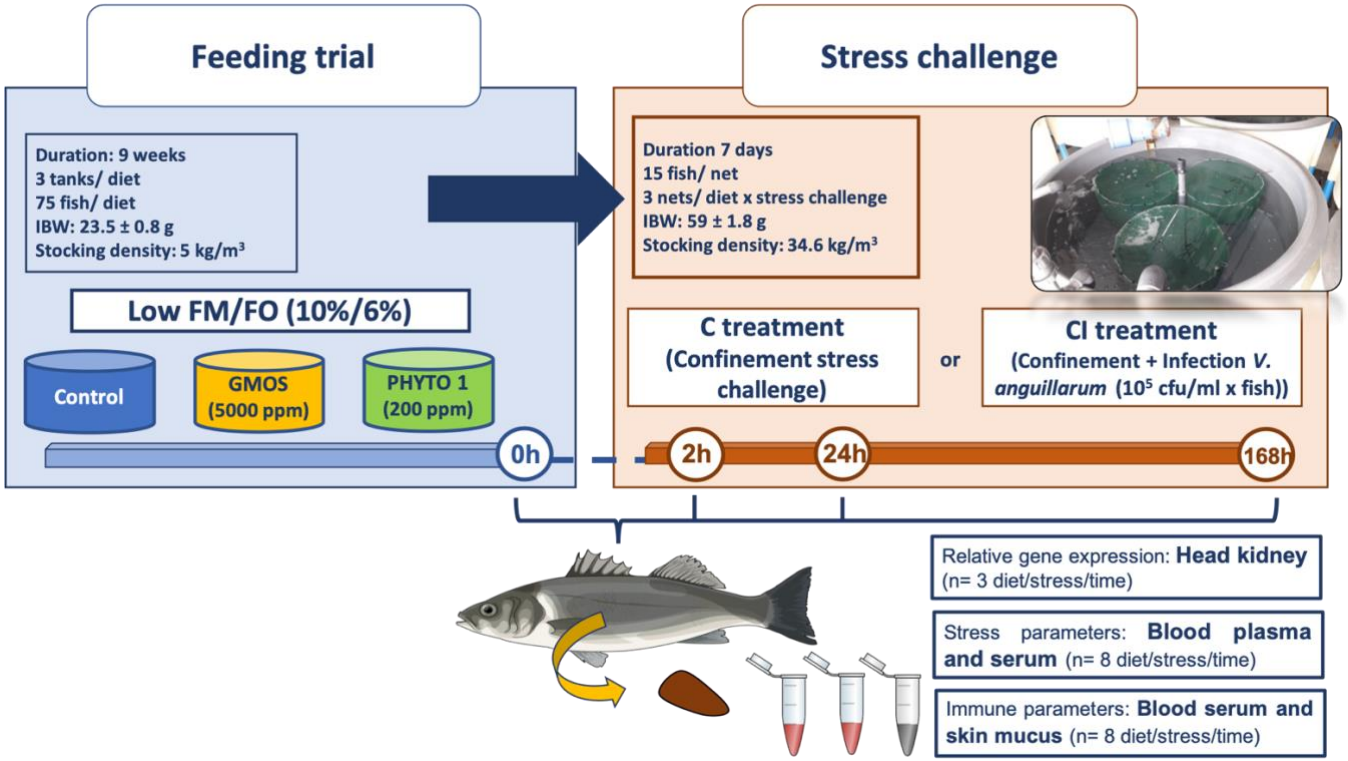


Figure 2.1. Chapter III graphical abstract. Nine weeks feeding trial (three experimental treatments fed in triplicate (Control (no supplementation); GMOS (GMOS supplemented, 5000 ppm); PHYTO 1 (PHYTO 1 supplemented, 200 ppm); 3 times/ day, 6 days/ week until apparent satiation; n= 75 fish/tank, initial body weight (IBW) = 23.5 ± 0.8 g). Seven days stress challenge with two experimental treatments: (C treatment (confinement stress challenge; 3 nets/ dietary treatment, 15 fish/ net, initial body weight (IBW) = 59 ± 1.8 g) or CI treatment (confinement stress challenge + intestinal infection with *V.anguillarum* (10^5 cfu/ml x fish); 3 nets/ dietary treatment, 15 fish/ net, initial body weight (IBW) = 59 ± 1.8 g). Sampling points after feeding trial (t=0h) and at t= 2h, 24h and 168h after stress challenge: Relative gene expression (head kidney, n= 3 diet/ stress treatment/ sampling point); Stress parameters (Blood plasma and serum, n= 8 diet/ stress treatment/ sampling point); Immune parameters (Blood serum and skin mucus, n= 8 diet/ stress treatment/ sampling point).

2.2.2. Chapter IV graphical abstract

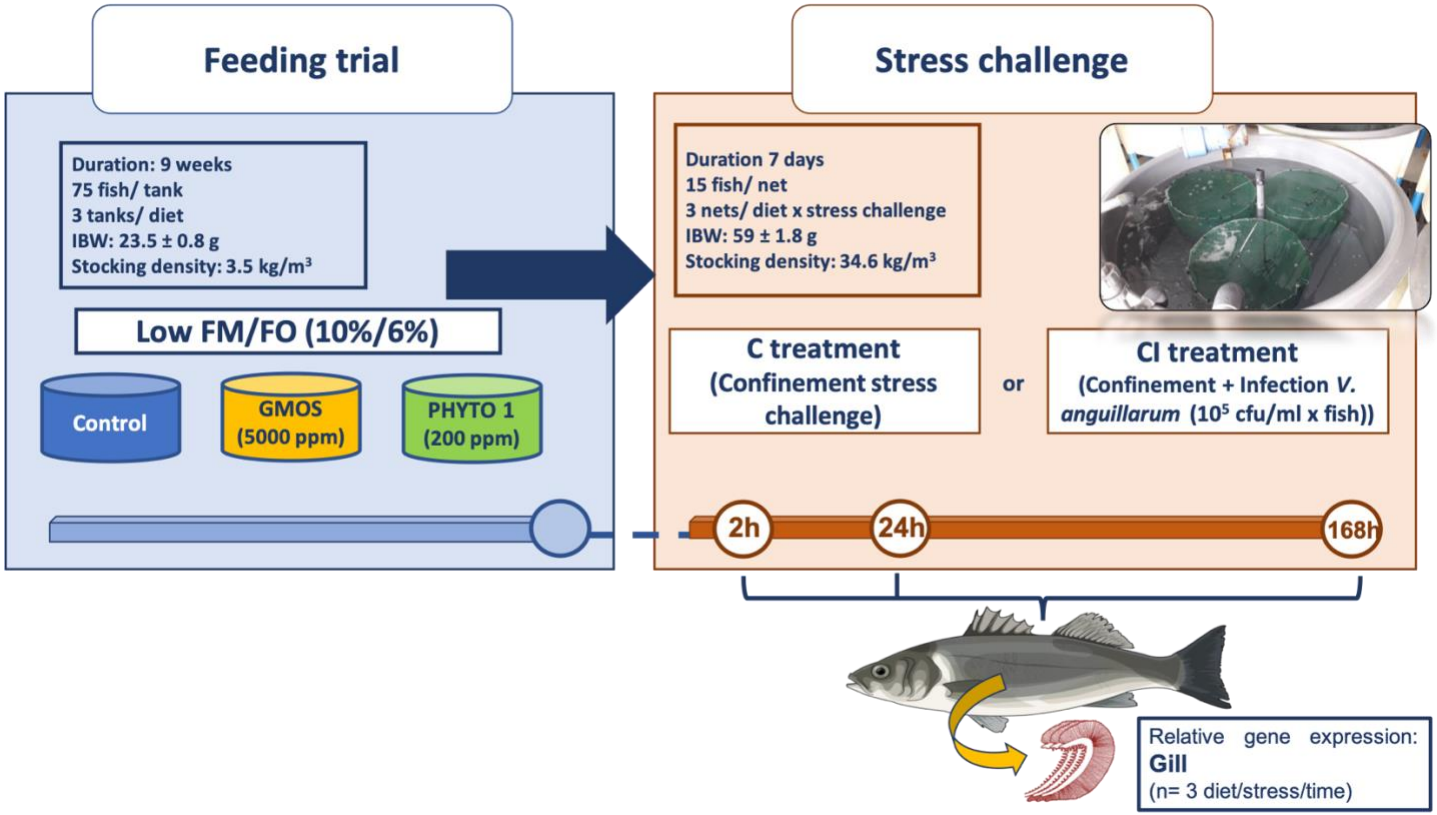


Figure 2.2. Chapter IV graphical abstract. Nine weeks feeding trial; three experimental treatments fed in triplicate (Control (no supplementation); GMOS (GMOS supplemented, 5000 ppm); PHYTO 1 (PHYTO 1 supplemented, 200 ppm); 3 times/ day, 6 days/ week until apparent satiation) (n= 75 fish/tank ; initial body weight (IBW) = 23.5 ± 0.8 g). Seven days stress challenge with two experimental treatments: C treatment (confinement stress challenge; 3 nets/ dietary treatment, 15 fish/ net, initial body weight (IBW) = 59 ± 1.8 g) or CI treatment (confinement stress challenge + intestinal infection with *V.anguillarum* (10^5 cfu/ml x fish); 3 nets/ dietary treatment, 15 fish/ net, initial body weight (IBW) = 59 ± 1.8 g). Sampling points at t= 2h, 24h and 168h after stress challenge: Relative gene expression (gill, n= 3 diet/ stress treatment/ sampling point).

2.2.3. Chapter V graphical abstract

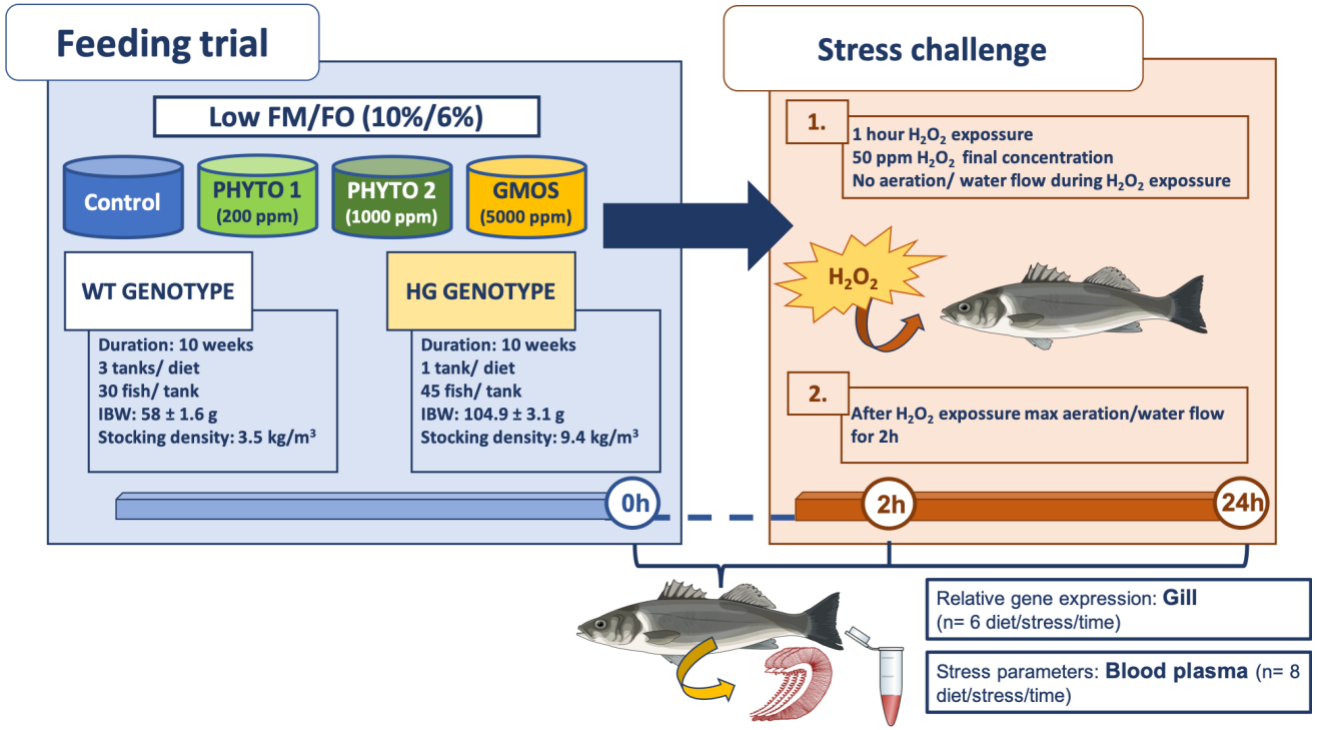


Figure 2.3. Chapter V graphical abstract. Ten weeks feeding trial; 2 genotypes: Wild genotype (WT) fed four experimental treatments fed in triplicate (Control (no supplementation); PHYTO0.02 (PHYTO 1 supplemented, 200 ppm); PHYTO0.1 (PHYTO 2 supplemented, 200 ppm); GMOS0.5 (GMOS supplemented, 5000 ppm); 3 times/ day, 6 days/ week until apparent satiation) (n= 30 fish/tank ; initial body weight (IBW) = 58 ± 1.6 g) and high growth genotype (HG) fed four experimental treatments (one tank per dietary treatment) (Control (no supplementation); PHYTO0 (PHYTO 1 supplemented, 200 ppm); PHYTO0 (PHYTO 2 supplemented, 200 ppm); GMOS0 (GMOS supplemented, 5000 ppm); 3 times/ day, 6 days/ week until apparent satiation) (n= 45 fish/tank ; initial body weight (IBW) = 104.9 ± 3.1 g). Oxidative stress challenge (all tanks) by H_2O_2 exposure (1hour exposure, 50 ppm, no aeration or water renovation) followed by 2h maximum aeration and water flow (chemicals removal). Sampling points after feeding trial (t=0h) and at t= 2h and 24h after stress challenge: Relative gene expression (gill, n= 6 diet/ stress treatment/ sampling point); Stress parameters (Blood plasma, n= 8 diet/ stress treatment/ sampling point.

2.2.4. Chapter VI graphical abstract

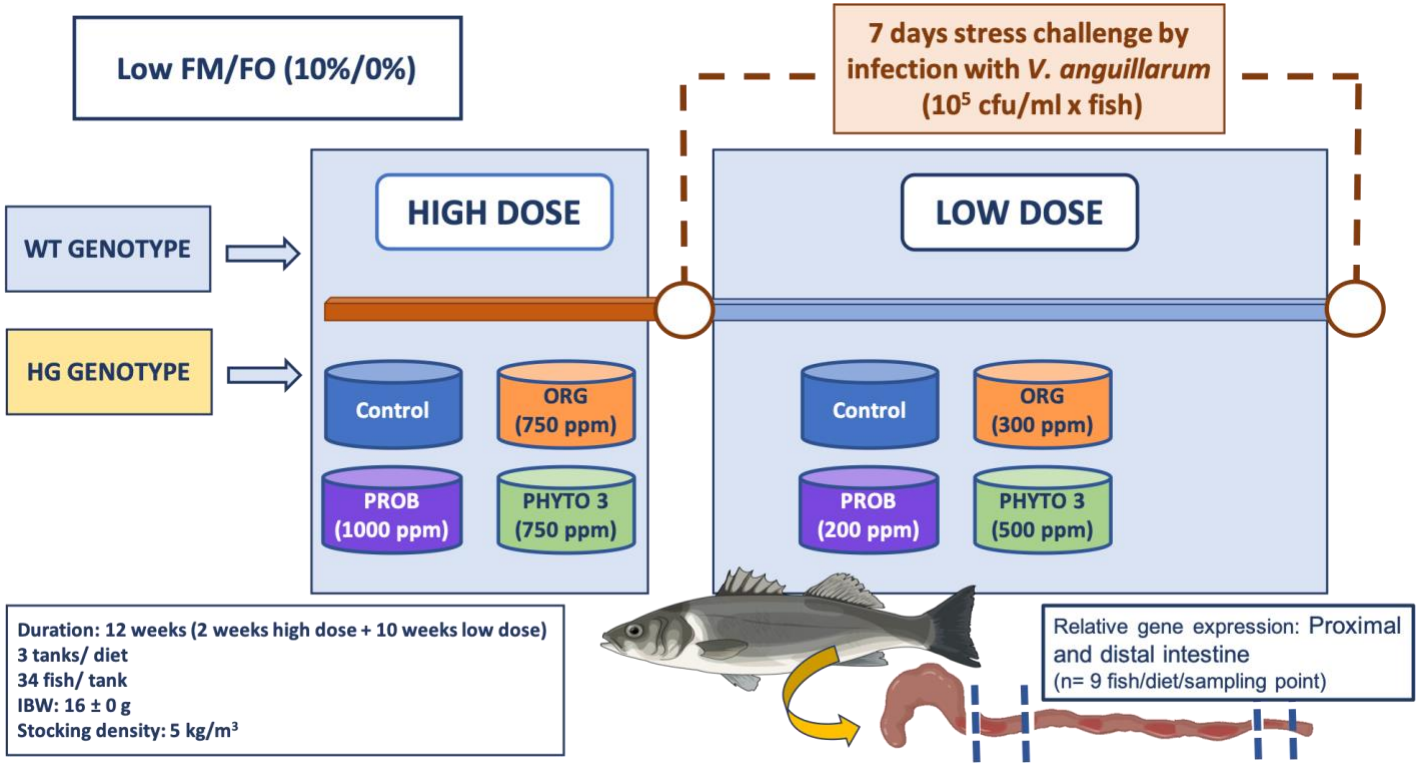


Figure 2.4. Chapter VI graphical abstract. Twelve weeks feeding trial (2 weeks high dose functional additive dietary supplementation + 10 weeks low dose functional additive dietary supplementation); 2 genotypes: Wild genotype (WT) and High growth genotype (HG) fed four experimental treatments in triplicate (Control (no supplementation); PROB (probiotic supplemented, 1000 ppm (high dose) or 200 ppm (low dose); ORG (organic acid supplemented, 750 ppm (high dose) or 300 ppm (low dose); PHYTO 3 (phytogenics supplemented, 750 ppm (high dose) or 500 ppm (low dose); 3 times/ day, 6 days/ week until apparent satiation) (n= 34 fish/tank ; initial body weight (IBW) = 16 ± 0.1 g).Seven days stress challenge by confinement (3 nets/ dietary treatment, 15 fish/ net) + intestinal infection with *V.anguillarum* (10^5 cfu/ml x fish) after each feeding period; Sampling points at t= 168h after stress challenge: Relative gene expression (proximal and distal intestine, n= 9 samples per dietary treatment (3 fish/ tank)).

2.3. Experimental fish and feeding trials

All European sea bass juveniles used in Chapters III and VI were procured by a local farm, Aquanaria S.L. (Castillo del Romeral, Gran Canaria, Canary Islands, Spain) whereas. After arrival to the PCTM facilities, fish were maintained in 1000 L fiberglass tanks at least two weeks until being adapted to the new environmental conditions. During the acclimation period fish were fed commercial diets three times a day, six days a week until apparent satiation. After acclimation period, fish were randomly pooled and distributed into the experimental tanks.

The European sea bass juveniles employed in Chapters V and VI were procured by the French Research Institute for the Exploitation of Marine resources (IFREMER) (Brest, France), and shipped to the PCTM-ULPGC facilities. Both HG and WT genotypes were produced following the same breeding scheme. A total of 7 growth selected dams were mated with 33 sires (GS) derived from the breeding nucleus of the EMG Ecloserie Marine de Gravelines (Gravelines, France) breeding company or 32 wild sires captured in the gulf of Lion (Wild type genotype, WT). Eggs were collected by stripping and pooled in equal representation between dams and transferred into 65 tubes, one tube per sire. The fertilized eggs were incubated separately at 14 °C until hatching. One day post hatching (dph), larvae were pooled by the equi-representation of each dam, placed into oxygen-saturated water transport bags that were kept in insulated boxes and shipped by airplane to the facilities of the University of Las Palmas de Gran Canaria (ULPGC, Las Palmas de Gran Canaria, Spain). The larvae were grown in separated tanks following the methodology previously standardized by the Research Group in Aquaculture (GIA) at the ULPGC facilities [Izquierdo et al., 2010; Atalah et al., 2011]. Both progenies were kept at similar on growing conditions from the larval stage to early juvenile growing stage.

All the trials developed in this thesis employed 500 L fiberglass experimental tanks supplied with filtered sea water (36-37ppm) in an open system under natural photoperiod (12L:12D). In all the experiences, water temperature and dissolved oxygen ranged between 18.2 - 20.2 °C and 6.1 - 6.6 ppm, respectively. To reduce stress in European sea bass, water micro-aeration systems were used in all the trials [Carvalho et al, 2004]. Fish were manually fed three times a day, six days a week until apparent satiation with the different experimental diets. In all the trials, feed intake was monitored daily, and growth performance and feed efficiency were calculated at the end of each feeding trial.

2.4. Stress challenges

2.4.1. Crowding stress and bacterial infection stress challenges

In trials I and II, the experimental fish were subjected to a confinement stress challenge (C treatment) or a confinement stress challenge combined with an infection with a pathogen (CI treatment). These experiences were carried out in the Marine Biosecurity (MBS) facilities of the PCTM-ULPGC (Canary Islands, Spain). C treatment consisted in a crowding stress challenge in which fish were confined in cylindrical 25.6 l (40 cm x 20 cm) submerged nets at a stocking density of 34.6 kg/ m^3. CI treatment, meanwhile, consisted in the same crowding stress combined with an *in vivo* gut inoculation with the pathogen *Vibrio anguillarum* (10^7 cfu/ml per fish; strain 507, isolated from a clinical outbreak in Canary Islands). During all the stress challenge, fish were fed the corresponding experimental diets (3 times/ day, 6 days/ week) until apparent satiation.

Fish mortality was registered daily along the stress challenge in order to calculate relative percent survival (RPS).

2.4.2. Oxidative stress challenge

In trial III, the experimental fish were subjected to an oxidative stress challenge by exposure to hydrogen peroxide (H_2O_2). Fish were subjected to a one hour bath at a final H_2O_2 concentration of 50 ppm in 500 L cylindroconical fiberglass tanks as described by before [Roque et al., 2010] without aeration to avoid any chemical removal. At the end of the oxidative stress challenge aeration and the waterflow were restarted again in a renovation rate of 1000 L per hour for one hour. Prior to oxidative stress challenge (0h) and at 2h and 24h after oxidative stress challenge, blood plasma samples for stress parameters analysis and for gill relative gene expression of stress-related genes.

2.5. General sampling protocols

2.5.1. Anesthesia and husbandry protocols

In all the experiences, prior manipulation, fish were anesthetized with clove oil 0.2 mL/L (Guinama S.L; Spain, Ref. Mg83168) diluted in alcohol 100% (1:2), to reduce stress processes following the recommendations of Tort et al., 2002. After handling, fish were introduced in a 100 L recovery tank in a continuous flow through of filtered sea water and high aeration until recovering normal motor activity and returned to the experimental tanks.

2.5.2. Euthanasia protocols

In order to obtain tissue samples to perform the different analysis after the different trials, two different standardized euthanasia protocols were followed depending on the nature of the analysis. In the trials I and II, fish were euthanized by clove oil overdose of 0.5 mL/L (Guinama S.L; Spain, Ref. Mg83168) diluted in alcohol 100% (1:2). In trials III and IV fish were euthanized by a sharp head blow in order to reduce branchial tissue contamination with erythrocytes [Castro et al., 2018].

2.5.3. Blood collection and component separation

Blood samples were collected by caudal sinus puncture with 1 mL syringes. To obtain plasma samples, immediately after extraction the blood was stored in 1.5 mL Eppendorf tubes coated with heparin avoiding blood coagulation. Immediately, blood was centrifugated at 3000 g at 4ºC for 5 minutes. The obtained plasma samples were rapidly kept at -80ºC until analysis.

To obtain serum samples, blood was stored in non-coated 1.5 ml Eppendorf tubes and stored at 4ºC between 4 and 24h to ensure blood coagulation. Afterwards, blood was centrifugated at 5000 g at 4ºC for 3 minutes. The obtained serum samples were rapidly conserved at -80ºC until analysis

2.5.4. Skin mucus collection

Skin mucus samples obtained by gently scraping fish skin with a sterile microscope slide, avoiding samples contamination with blood or secretions of epithelial cells. The mucus samples were stored in 1.5 mL Eppendorf and diluted 1:2 volumes of a Tris-buffered saline solution (TBS, 50 mMTris-HCl, ph= 8. 0, 150 mM NaCl) [Palaksha et al., 2008]. Diluted mucus samples were purified by centrifugation at 18000 g at 4ºC for 40 minutes. After centrifugation, the supernatant was separated and rapidly stored at -80ºC until analysis.

2.5.5. Tissue samples for relative gene expression analysis

Tissue samples were collected employing sterilized dissection material and stored in 1.5 mL Eppendorf tubes with 1 mL RNA later (1L deonised water, 650 g ammonium sulphate ($NH_42(SO_4)$), 7.4 g sodium citrate di hydrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$), 7.4 g EDTA di sodium salt ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$)). Then the samples were stored at 4ºC for 24h-72h and then RNA later was removed and the fixed samples stored at -80ºC until analysis. Between each fish dissection, the dissection material was cleaned with propane AF and washed with miliQ water in order to avoid samples cross contamination.

2.6. Biological parameters evaluation

To evaluate the effects of the different functional additives on fish growth performance, individual fish body weight (g) and size (cm) was obtained for initial and final sampling points. In addition, daily feed intake was monitored in order to evaluate feed efficiency.

2.6.1. Relative growth

Relative growth was calculated as the relation between the increase on body weight (g) and the initial body weight (g) expressed as a percentage

$$\text{Relative growth} = \left[\frac{[\text{Final body weight} - \text{Initial body weight}]}{Initial\ body\ weight}\right] x\ 100$$

2.6.2. Specific growth rate

Specific weight gain was calculated as the percentage of body weight gain (g) in a time frame (days)

$$\text{Specific growth rate} = \left[\frac{(\text{Final body weight} - \text{Initial body weight})}{Number\ of\ days}\right] x\ 100$$

2.6.3. Feed conversion ratio

The feed conversion ratio was calculated as the relation between the increase on body weight ((g) and the total feed intake (g)

$$\text{Feed conversion ratio} = \left[\frac{\text{Total feed intake}}{(\text{Final body weight} - \text{Initial body weight})}\right]$$

2.6.4. Relative percent survival

Relative percent survival was calculated has the percentage of mortality of fish fed a functional diet compared to the mortality of fish fed the Control diet.

$$\text{Relative percent survival} = \left[1 - \frac{\text{Mortality of fish fed a supplemented diet}}{\text{Mortality of fish fed the control diet}}\right] x\ 100$$

2.7. Stress related parameters

2.7.1. Circulating plasma cortisol determination

The circulating plasma cortisol concentration was determined using the assay kit Access Cortisol ref 33600, ©2010 Beckman Coulter, Inc. by the external laboratory AnimaLab (Las Palmas de Gran Canaria, Gran Canaria, Canary Island, Spain). Briefly, the determination protocol consists on an immunofluorescence assay employing rabbit cortisol antibodies and goat rabbit-anti-antibodies using the chemo-lumminiscent substrate Lumi-Phos*530.

2.7.2. Circulating plasma glucose determination

The circulating plasma glucose concentration was determined using the hexokinase method for *in vitro* diagnosis. The assay was performed using the glucose reactive OSR6521 from Beckman Coulter AU, employable on AU2700 y AU5400 equipment. Briefly, this assay is based on the glucose phosphorylation by the hexokinase (HK) in the presence of ATP and magnesium ions, producing glucose 6-phosphate. Then, the glucose 6-phosphate is oxidized by the 6-phosphate glucose dehydrogenase (G6P-DH) into gluconate 6-phosphatse by reducing NAD^+ into NADH. The absorbance increase at 340nm will be directly proportional to sample's glucose concentration.

2.8. Immune related parameters

2.8.1. Serum and skin mucus protein content

Both serum and skin mucus protein content was determined by Bradford (1976) assay. Briefly, this assay consists on turbidimetric method employing a standard curve of known bovine serum albumin (BSA) protein concentration.

2.8.2. Serum and skin mucus lysozyme activity

The quantification of the serum and skin mucus lysozyme activity was carried out by Ellis method (1990). Lysozyme activity was determined by *Micrococcus lysodeikticus* (0.2 mg/ ml) in a sodium phosphate buffer (0.05M, pH= 6.3) and employing and egg whites in a phosphate buffered solution as standard.

2.8.3. Serum and skin mucus bactericidal activity

The bactericidal activity on both serum and skin mucus was determined employing the methodology described by Sunyer and Tort (1995). This is a turbidimetric method in which the effect of the problem sample (serum or mucus) on a bacterial growth curve is evaluated. The employed bacteria was the same as the one in the stress challenge of trials I and II; the *Vibrio anguillarum* (10^5 cfu/mL ; strain 507, isolated from a clinical outbreak in Canary Islands).

$$\text{Bactericidal activity (\%)} = \left[\frac{\text{Positive control absorbance} - \text{Sample absorbance}}{\text{Negative control absorbance}}\right] x\ 100$$

2.8.4. Serum and skin mucus peroxidase activity

Both serum and skin mucus peroxidase activity were determined employing an adaptation of the methodology described by Sitjà-Bobadilla and collaborators in 2005. Briefly, 15 µL of sample (serum or mucus) were diluted on 85 µL of HBSS (Hank's Buffered Salt Solution, ph= 7.2)and incubated for 2 minutes with 100 µLTBM (3,3',5,5'-Tetraethylbenzidine, Sigma, St. Louis, MO,USA) and 100 µL hydrogen peroxide (H_2O_2 1M, 33%). Afterwards, the reaction was stopped employing 50 µL sulfuric acid (1N H_2SO_4) and the absorbance of each sample was measured at 450 nm. One unit of peroxidase activity produced an absorbance of 1.

2.9. Relative gene expression analysis

The real-time PCR (RT-PCR) analysis were performed with the different target tissues total mRNA from the different target tissues extracted by using TRI reagent (Sigma-Aldrich, Sant Louis, MO, USA) and the from an extraction kit RNeasy® Minikit from Qiagen. The mRNA extraction quantity and quality were evaluated by nanodrop reading and electrophoresis analysis. When mRNA quality was checked an iScript™ cDNA synthesis Kit (Bio-Rad Hercules, California) was employed to perform the reverse transcriptions to obtain cDNA in a 20 µL reaction containing 1 µL of total mRNA.

All the RT-PCR analysis on the present thesis were performed employing a 1-cycler with optical module (Bio-Rad Hercules, CA, USA), nevertheless RT-PCR conditions were different depending on the target tissue. For head kidney relative gene expression analysis, the RT-PCR was carried out in a final volume of 15 µL containing 7.5 µL iQTM-

SYBER® Green Supermix (Bio-Rad Hercules, CA, USA), 5 µl of cDNA (1:10 dilution), 1 µL of each primer and 0.5 µL of RNAase free water. The real time conditions were: 95 °C for 2 min followed by 40 cycles of 94 °C for 15 s, annealing temperature of each primer sequence (Table 3) for 30 s and final step of 72 °C for 45 s for elongation. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [Livak & Schmittgen, 2001; Livak & Schmittgen, 2001; Schmittgen & Livak, 2008]. More detailed protocol on relative gene expression are given on each chapter corresponding to the different trials.

For gill relative gene expression analysis, the RT-PCR was carried out in a final volume of 20 µL containing 10 µL of iQTM-SYBER® Green Supermix (Bio-Rad Hercules,Ca,USA), 5 µL of free-nuclease water, 3 µL of cDNA (1:10 dilution) and 1 µL of forward and reverse primers. The real time running conditions were: 95 °C, 1 min followed by 40 cycles at 95 °C for 10 s and annealing temperature for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [Livak & Schmittgen, 2001; Schmittgen & Livak, 2008]. More detailed protocol on relative gene expression are given on each chapter corresponding to the different trials.

Table 2.3. Primer sequences of the different genes analyzed and their RT-PCR conditions.

Gene	Access Number	Primer	Nucleotide sequence 5' - 3'	Annealing Temperature (°C)
nfKβ2	KM225790	Fw	CTGGAGGAAACTGGCGGAGAAGC	60
		Rv	CAGGTACAGGTGAGTCAGCGTCAC	
hif-1 α	DQ171936	Fw	GACTTCAGCTGCCCTGATTC	60
		Rv	GGCTGGTTTATAGCGCTGAG	
il-1b	AJ53742	Fw	ATTACCCACCACCCACTGAC	60
		Rv	TCTCTTCCACTATGCTCTCCAG	
tnf-1α	DQ200910	Fw	GCCAAGCAAACAGCAGGAC	60
		Rv	ACAGCGGATATGGACGGTG	
casp-9	DQ345775	Fw	GGCAGGACTCGACGAGATAG	60
		Rv	CTCGCTCTGAGGAGCAAACT	
casp-3	DQ345774	Fw	ACGAAGCAGGTCAATCATCC	60
		Rv	GCAGTTTAAGGGTATCCAGAGC	
cyp11B	AF449173	Fw	GGCCCTCTTCTCCTCATCTC	60
		Rv	GGCTGAAGATGTGATCCC	
StAR	EF409994	Fw	AAGCCCTGTGCTGAAGACC	60
		Rv	AGAATCTTCTGCTTGTCGTCG	
gr	AY549305.1	Fw	GTGGGCCTACAAGACCAGAA	60
		Rv	CGGACGACTCTCCATACCTG	
nd5	KF857307	Fw	CCCGATTTCTGTGCCCTACTA	60
		Rv	AGGAAAGGAGTGCCTGTGA	
coxi	KF857308	Fw	ATACTTCACATCCGCAACCATAA	60
		Rv	AAGCCTCCGACTGTAAATAAGAA	
ucp1	MH138003	Fw	CGATTCCAAGCCCAGACGAACCT	60
		Rv	TGCCAGTGTAGCGACGAGCC	

sod	FJ860004.1	Fw	CATGTTGGAGACCTGGGAGA	60
		Rv	TGAGCATCTTGTCCGTGATGT	
cat	FJ860003.1	Fw	TGGGACTTCTGGAGCCTGAG	60
		Rv	GCAAACCTCGATCGCTGAAC	
gpx	FM013606.1	Fw	AGTTCGTGCAGTTAATCCGGA	60
		Rv	GCTTAGCTGTCAGGTCGTAAAAC	
zo-1	MH321323.1	Fw	CGGCCTGCAGATGTTCCTAA	60
		Rv	GCTGAGGGAATTGGCTTTGA	
ocln	MH321322.1	Fw	GGACGAAGACGACAACAACGA	60
		Rv	CCATGGGAGAAAGCCTCTGA	
hsp70	AY423555.2	Fw	GGACATCAGCCAGAACAAGAGA	60
		Rv	GCTGGAGGACAGGGTTCTC	
hsp90	AY395632	Fw	GCTTCGAGGTCCTGTACATG	62.7
		Rv	GCCTTATCCTCCTCCATC	
NKA α1a	KP400258	Fw	AACCTCAGATGGCAAGGAGAAG	60
		Rv	GAGACTGGTACATTCAGGCGG	
α-tub (hk)	AY326429.1	Fw	AGGCTCATTGGCCAGATTGT	60
		Rv	CAACATTCAGGGCTCCATCA	
EF1	AJ866727	Fw	GCTTCGAGGAAATCACCAAG	60
		Rv	CAACCTTCCATCCCTTGAAC	
rpl17	AF139590	Fw	GAGGACGTGGTGGTTCATCT	60
		Rv	CTGGCTTGCCTTTCTTGACT	

Fw: Forward primer sequence, Rv: Reverse primer sequence.

2.10. Statistical analysis

All the statistical analysis were performed with R Project for Statistical Computing. Mean and data standard deviation (SD) was calculated for each parameter measured. Prior to analysis, all data were tested for outlying values through linear regression adjustment, defining the outside cut-offs as 1.5 times the Inter-Quantile Range (IQR) below the first and above the third quantiles [Hoaglin and Iglewicz,1987; Feng et al., 2008].

Trials I, II and III results were analyzed by ANOVA statistical analysis. All data were tested for normality and homogeneity. Before the analysis, a Kolmogorov–Smirnov test was used to assess the quantile normality, and Levene's test was used to assess the homogeneity of the variance. Where there was significant variance heterogeneity, the data were transformed by the square root or log transformation. When transformations did not remove the heterogeneity, the analysis was performed with untransformed data with the F-test α-value set at 0.01 [Underwood et al., 1997]. When significant differences were obtained, a Tukey post-hoc test was performed for multiple-means comparison.

Trial IV consisted in the development of an observational multiple linear regression model describing the effects of functional additives supplementation on fish growth, feed intake and feed utilization.

2.10.1. Trial I: ANOVA design

Trial I statistical design contemplated a three-way ANOVA in which all factors were fixed and orthogonal between them.

$$X_{ijk}: \text{Diet}_i + \text{Stress}_j + \text{Time}_k + \text{Diet x Stress x Time} + \text{Diet x Stress} + \text{Diet x Time} + \text{Stress x Time} + \text{Residuals}_{n(ijk)}$$

In which Diet: Three levels a) Control, b) GMOS, c) PHYTO0.02; Stress: Two levels a) C treatment and b) CI treatment; Time: Three levels a)2 hours, b) 24 hours and c) 168 hours.

2.10.2. Trial II: ANOVA design

Trial II statistical design contemplated a three-way ANOVA in which all factors were fixed and orthogonal between them.

$$X_{ijk}: \text{Diet}_i + \text{Stress}_j + \text{Time}_k + \text{Diet x Stress x Time} + \text{Diet x Stress} + \text{Diet x Time} + \text{Stress x Time} + \text{Residuals}_{n(ijk)}$$

In which Diet: Three levels a) Control, b) GMOS, c) PHYTO0.02; Stress: Two levels a) C treatment and b) CI treatment; Time: Three levels a)2 hours, b) 24 hours and c) 168 hours.

2.10.3. Trial III: ANOVA design

Trial III statistical design contemplated a three-way ANOVA in which all factors were fixed and orthogonal between them.

$$X_{ijk}: \text{Diet}_i + \text{Genotype } j + \text{Time}_k + \text{Diet x Genotype x Time} + \text{Diet x Genotype} + \text{Diet x Time} + \text{Genotype x Time} + \text{Residuals}_{n(ijk)}$$

In which Diet: four levels a) Control, b) GMOS, c) PHYTO0.02 and d) PHYTO0.1; Genotype: Two levels a) GS and b) WT; Time: Three levels a) 0 hours, b) 2 hours and c) 24 hours.

2.10.4. Trial V: Multiple linear regression model (MLR)

The development of an observation MLR evaluating functional additives dietary supplementation of fish growth, feed consumption and feed efficiency may allow the detection of significant effects on diet supplementation through the exclusion of possible confounding variables. This statistical methodology will allow the normalization of fish growth and nutritional traits such as SGR, FCR and feed intake, removing the effect of important confounding variables (i.e., fish size and temperature). The normalization will be based on the formula:

$$normalized\ trait\ = \frac{measured\ raw\ trait}{maximum\ trait\ value}$$

Where *measured raw trait* consists on the direct calculation of each parameter as follow:

SGR (day^{-1}) = [ln (FBW) – ln (IBW)] / days;

FI (% body weight/day) = [(individual feed intake)/ (IBW + FBW)/2/days] x 100;

FCR (g feed intake/g weight gain) = individual feed intake / (FBW – IBW).

And the *maximum trait value* consists on the value obtained from three reference models for the predition of European sea bass maximum growth, feed intake and feed conversion. The models were developed and provided by Sparos Lda. (Olhão, Portugal). These models were obtained using quantile regression (to estimate quantiles 0.95 for SGR_max and FI_max, and quantile 0.50 for FCR_typical) of log transformed responses, based in an aggregated data base including information about European sea bass growth trials from 37 sources.

The models used are described by the following equations:

$\ln (SGR_{max}) = - 7.93079 + [0.50781 \times \ln(ABW)] – [0.00133 \times ABW] – [0.09766 \times (\ln (ABW)^2)] + [0.2524 \times Temp] – [0.0041 \times (Temp)^2]$,

$\ln (FI_{max}) = 5.11608 + [0.61529 \times \ln (ABW)] + [0.14896 \times Temp] - 0.00136 \times (Temp)^2]$,

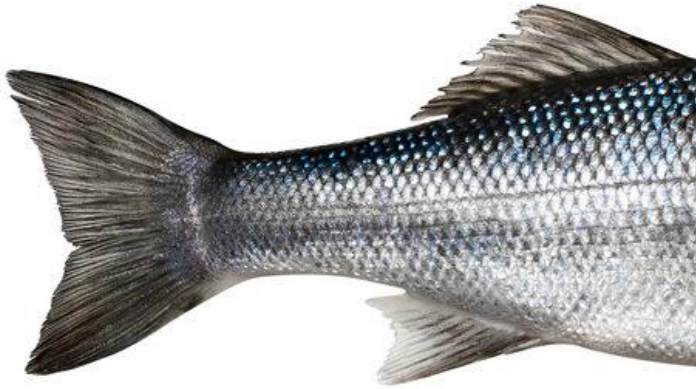
$FCR_{typical} = 0.9036676 \times (ABW)^{0.1082725}$.

*ABW (average body weight (g)) = $(IBW * FBW)^{0.5}$
*Temp (temperature (°C))

After model fitting a stepwise backward selective regression was performed by iteratively adding and removing coefficients in order to find the simplest and best performing model [Agostinelli et al., 2002; Wang et al., 2007]. Model quality was evaluated in reference to the model selection criteria and residuals analysis. [Sanquetta et al., 2018]. The simplification of the descriptive models will allow the detection of significant effects of functional additive variable on model final outcome.

Chapter III

Prebiotics and Phytogenics functional additives in low FM/FO based diets for European sea bass (*Dicentrarchus labrax*) : Effects on stress and immune repsonses



Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Prebiotics and phytogenics functional additives in low fish meal and fish oil based diets for European sea bass (*Dicentrarchus labrax*): Effects on stress and immune responses

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ARTICLE INFO

Keywords:
Functional feeds
Prebiotics
Phytogenics
Cortisol
European seabass
Low fish meal
Low fish oil

ABSTRACT

The use of terrestrial raw materials to replace fish meal (FM) and fish oil (FO) in marine fish diets may affect fish growth performance and health. In the last years functional additives have been profiled as good candidates to reduce the effects on health and disease resistance derived from this replacement, via reinforcement of the fish immune system. In the present study, three isoenergetic and isonitrogenous diets with low FM and FO (10% and 6% respectively) were tested based on supplementation either with 0.5% galactomannanoligosaccharides (GMOS diet) or 0.02% of a mixture of essential oils (PHYTO diet), a non-supplemented diet was defined as a control diet. Fish were fed the experimental diets in triplicate for 9 weeks and then they were subjected to a stress by confinement as a single challenge (C treatment) or combined with an experimental intestinal infection with *Vibrio anguillarum* (CI treatment). Along the challenge test, selected stress and immunological parameters were evaluated at 2, 24 and 168h after C or CI challenges. As stress indicators, circulating plasma cortisol and glucose concentrations were analyzed as well as the relative gene expression of cyp11b hydroxylase, hypoxia inducible factor, steroidogenic acute regulatory protein, heat shock protein 70 and heat shock protein 90 (*cyp11b, hif-1α, StAR, hsp70* and *hsp90*). As immune markers, serum and skin mucus lysozyme, bactericidal and peroxidase activities were measured, as well as gene expression of Caspase-3 (*casp-3*) and interleukin 1β (*il-1ß*). The use of functional additives induced a significant ($p < 0.05$) reduction of circulating plasma cortisol concentration when confinement was the unique challenge test applied. Supplementation of PHYTO induced a down-regulation of *cyp11b, hif-1α, casp-3* and *il*-1β gene expression 2h after stress test, whereas *StAR* expression was significantly ($p < 0.05$) up-regulated. However, when combination of confinement stress and infection was applied (CI treatment), the use of PHYTO significantly ($p < 0.05$) down-regulated *StAR* and *casp-3* gene expression 2h after challenge test, denoting that PHYTO diet reinforced fish capacity of stress response via protection of head kidney leucocytes from stress-related apoptotic processes, with lower caspase-3 gene expression and a higher *il-1β* gene expression when an infection occurs. Additionally, dietary supplementation with GMOS and PHYTO compounds increased fish serum lysozyme after infection. Both functional additives entailed a better capability of the animals to cope with infection in European sea bass when fed low FM and FO diets.

1. Introduction

Handling or alterations on water physicochemical parameters are known to cause stress in cultured fish especially when they are living under intensive farming conditions. Stress induces an allostatic physiological response that tends to reestablish fish homeostasis, through the activation of the Hypothalamus-Pituitary-Interrenal (HPI) axis and the release of cortisol into the bloodstream [1]. Cortisol effects are mediated by the intracellular glucocorticoid receptors (GR), which are members of the nuclear receptor superfamily and act as ligand-

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https://doi.org/10.1016/j.fsi.2020.03.016
Received 17 February 2020; Received in revised form 6 March 2020; Accepted 7 March 2020

dependent transcription factors to control and regulate gene expression within the allostatic process [2]. Cortisol is synthetized from cholesterol through a series of isomerizations and hydroxylations, including a final step catalyzed by the P450 11β-hydroxylase enzyme (11β). This enzyme belongs to the cytochrome P450 (CYP) family proteins, and is encoded by the CYP11β gene [3]. To trigger cortisol synthesis, the cytosolic cholesterol is transported across the mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) [4], and it is regulated by several factors such as the hypoxia inducible factor (HIF), through its role on cholesterol synthesis [5].

When a stressor acquires a chronic character, it can induce a persistent increase of plasma cortisol with negative effects on fish health and metabolism [1]. The continuous cortisol secretion to the bloodstream reduces fish feed intake [6] and alters protein and lipid metabolism [7], altogether reducing fish growth performance. Furthermore, an allostatic load produces immunosuppression in fish, increasing its susceptibility to pathogenic outbreaks [8].

Among the different stressors, nutritional imbalances have been described to alter fish stress response both *in vivo* [9,10] and *in vitro* [11–13]. Among them, high substitution of fish meal (FM) and fish oil (FO) in fish diets have been associated with several side-effects on fish health and stress tolerance [14–18], depending on the species susceptibility, the substitution levels and the origin of the raw material used [19].

To face these side effects, feed functional additives are good candidates as they enhance fish health and welfare. A functional additive is a nutrient with the ability to enhance the animal health by stimulating their immune system [20], improving tissues integrity [21], attenuating the stress response [22] and increasing feed digestibility and nutrients biodisponibility [23,24].

There is a wide range of active substances that could be used as functional additives to supplement fish commercial diets [25]. Immunostimulants from herbal origin, including galacto and galactomannan oligosaccharides (GOS and GMOS, respectively) are prebiotic compounds mainly composed of indigestible fibers that could benefit the host by selectively stimulating its growth and the activity of a limited number of intestinal bacteria species, enhancing thus host im- mune system [26]. Previous studies have demonstrated the effective- ness of the herbal immunostimulants on fish to attenuate stress re- sponse [27], enhance immune system, improve gut tissue integrity [21,28,29], and increase feed digestibility and fish growth performance [27,30].

Other active compounds used as functional ingredients are the phytogenic feed additives (PFA). These are commonly defined as plant-based feed additives or botanicals, representing a group of natural substances used in animal nutrition. Phytogenics are derived from herbs, spices, other plants and their extracts consisting of highly active substances [31]. They encompass much more than essential oils, including spicy or bitter substances, saponins, flavonoids, mucilages or tannins [31]. Among the wide variety of products and extracts available, garlic and labiate plants essential oils haven been studied for their antibacterial and antioxidant characteristics [32,33], as well as their ability to improve feed conversion ratio by increasing feed palatability [34,35], feed digestibility and nutrients transport [36]. These PFAs have been described as having the ability to enhance fish immune system [36,37] and increase fish survival to pathological outbreaks [38]. Several studies have demonstrated that PFA compounds can elicit anti-stress properties on pigs [39], broilers [40] and fish [41,42].

The European sea bass (*Dicentrarchus labrax*) is a marine fish species with a high susceptibility to suffer deleterious problems derived from stressful culture conditions and pathogen outbreaks [43]. In addition, the diet formulation for this species is in constant evolution, leading to a certain level of substitution of marine raw materials [44–46]. Thus, the aim of this study was to evaluate the potential of different func- tional additives, including prebiotics and PFAs, to ameliorate the de- leterious effects of high dietary substitution of FM/FO on the stress and health indicators of European sea bass juveniles.

Table 3.1
Main ingredients and analyzed proximate composition of the diets.

Ingredients	Diet (%)		
	C	GMOS	PHYTOO
Fish meal	10	10	10
Soya protein concentrate	18.9	18.9	18.9
Soya Meal	12.0	12.0	12.0
Corn gluten meal	25.0	25.0	25.0
Wheat	8.7	8.2	8.7
Wheat gluten	2.0	2.0	2.0
Guar Meal	8.0	8.0	8.0
Rapeseed extracted	3.0	3.0	3.0
Fish oil	6.7	6.7	6.7
Rapeseed oil	5.4	5.4	5.4
Vitamin and mineral premix	3.7	3.7	3.7
Antioxidant	0.06	0.06	0.06
Galactomannan oligosaccharides [6]	0	0.5	0
Phytogenic	0	0	0.02
Proximate composition (% of dry matter)			
Crude lipids	19.91	20.44	20.47
Crude protein	49.30	49.27	49.76
Moisture	5.10	5.01	5.06
Ash	7.02	6.41	6.49

2. Material and methods

2.1. Diets

Three isonitrogenous and isoenergetic diets were prepared by an extrusion process in the Biomar Tech-Centre (May 2017; Brande, Denmark) and were based on a 10% FM and a 6% FO levels as detailed previously in Ref. [47], covering the nutritional requirements for sea bass (Table 1). The experimental diets were supplemented either with 5000 ppm of galactomannan oligosaccharides from plant origin (Delacon, Austria) (GMOS diet) or 200 ppm of a mixture of garlic and labiatae essential oils (Delacon, Austria) (PHYTO diet). The specific levels of supplementation were chosen accordingly to producer's recommendations (Delacon; Austria). To ensure product stability GMOS was included in the mix pre-extrusion process. The additive of PHYTO diet was included post extrusion process by vacuum coating and homogenized with the dietary oil. The stability of the PFAs used was checked previously to diet production and at the beginning of the feeding trial.

2.2. Experimental

2.2.1. Experiment I: feeding trial

Each diet was manually fed in triplicate (3 tanks/diet) for 9 weeks, 6 days per week, 3 times a day until apparent satiation. Each tank contained 75 fish randomly distributed with an initial weight of 23.5 ± 0.8 g (mean ± SD). The cylindroconical 500L tanks were supplied with filtered sea water at a temperature of 18.2–20.2 °C in a flow-through system under natural photoperiod (12L:12D). Water dissolved oxygen levels ranged between 6.1 and 6.6 ppm. Feed intake was monitored daily, and growth parameters and feed efficiency were calculated at the end of the growth experimental period.

At the end of the feeding trial, three fish per tank were used to obtain plasma and serum samples (N = 9 fish/diet) and two extra fish were used to obtain skin mucus samples (N = 6 fish/diet). Blood samples were taken by caudal sinus puncture using 1 mL syringes. Plasma samples were obtained by centrifugation at 3000 g for 5 min at 4 °C and then stored at −80 °C. Serum samples were obtained after 24h coagulation at 4 °C, followed by centrifugation and storage at −80°C until analysis. Mucus samples were scraped from fish skin by using a glass slide and purified by centrifugation at 18,000 g during 40 min at

4ºC. After centrifugation the samples were diluted 1:3 in a 50 mM NaCl solution [48] and conserved at −20ºC until analysis. Three additional fish per tank were euthanized by an anesthetic overdose with clove oil [49] and head kidney excised, pooled and rapidly placed in RNA later and frozen at −80ºC until gene expression analyses.

2.2.2. Experiment II: stress challenge test

After the feeding trial, 90 disease-free fish per dietary treatment with an average weight of 59.0 ± 1.8 g (mean ± SD) were randomly selected and subjected to a challenge test consisting on either a confinement stress or a combination of stress and infection. This procedure was done in the Biosecurity Station of the Scientific Technological Park of the University of Las Palmas de Gran Canaria (ULPGC). The confinement stress challenge (C) consisted on confining fish using 25.6 L (40 cm × 20 cm) submerged cages at a stocking density of 34.6 kg m^{-3} (N_{tank} = 15 fish/cage, 3 cages per dietary treatment, N_{diet} = 45 fish/diet) for a week. The confinement plus infection challenge consisted on a similar confinement protocol plus an *in vivo* exposure to *Vibrio anguillarum* (CI) (10^5 CFU per fish) via anal inoculation [50] (N_{tank} = 15 fish/cage, 3 cages per dietary treatment, N_{diet} = 45 fish/diet). For each dietary treatment, the cages were distributed among 6 cylindroconical 500L tanks on a RAS system supplied with filtered water at a temperature of 22ºC, 3 tanks for C fish and 3 tanks for CI fish. Fish were manually fed their respective diets until apparent satiation 3 times per day during a week. At 2h, 24h and 7 days post confinement/infection, 15 fish per dietary treatment from the stress strategy were sampled individually for immune parameters and stress indicators in serum, plasma and mucus and analyses of the expression of stress-related gene in the head kidney. Seven fish were used to obtain serum and plasma samples and eight fish were used to obtain skin mucus and head kidney samples. Fish mortality was monitored along the challenge test and relative percent survival (RPS) was calculated at the end of challenge test as described in Ref. [51] following the equation: RPS = [1- Mortality of fish fed supplemented diet (%)/Mortality of fish fed control diet (%)] x 100.

2.3. RNA extraction and real-time quantitative PCR analyses

Total RNA of head kidney was extracted using TRI reagent (Sigma-Aldrich, Sant Louis, MO, USA) from an extraction kit RNeasy® MiniKit from Quiagen. The reverse transcription (RT) reactions were performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California) in 20 µl reaction containing 1 µl of total RNA. Real time PCRs were performed employing a l-cycler with optical module (Bio-Rad Hercules, Ca, USA) in a final volume of 15 µl containing 7.5 µl of iQTM-SYBER® Green Supermix (Bio-Rad Hercules, Ca, USA), 5 µl of cDNA (1:10 dilution) and variable volumes of the different primers detailed in Table 2. The real time reaction conditions were: 95 °C for 2 min followed by 40 cycles of 94 °C for 15 s, annealing temperature for 30 s and 72 °C for 45 s for elongation,. All reactions were performed in duplicate for each template cDNA and blank control reactions were performed with water replacing cDNA. Relative gene expression was estimated by the $2^{-\Delta\Delta CT}$ method [60], using RPL17 as housekeeping.

2.4. Analyses of immune

For both serum and skin mucus samples, lysozyme activity was determined by a turbidimetric assay described in Ref. [61]. Bactericidal activity was determined evaluating the effect of different samples on the growth pattern of *V. anguillarum* [62]. Peroxidase activity was analyzed using the methodology described in Ref. [63] with some modifications. For this assay, 15 µl of each sample were diluted in 85 µl of Hank's buffered salt solution (HBSS) and incubated for 2 min with TBM (3,3′,5,5′ ytetramethyl bencidine; Sigma, St. Louis, MO, USA) and H_2O_2. The reaction was stopped by acidification with H_2SO_4 and its absorbance was measured at 450 nm.

2.5. Statistical analyses

All the analyses were performed with R Project for Statistical Computing. Means and standard deviations (SD) were calculated for each parameter measured. All data presented were tested for normality and homogeneity of variance. A One-way ANOVA analysis was employed to analyze differences among fish fed the different dietary treatments on growth and biological parameters including SGR, FCR and K factor, as well as for immune and stress parameters and gene expression levels, at each sampling time point. Significant differences were considered when $p < 0.05$.

3. Results

3.1. Fish growth and feed utilization

Results obtained for growth performance and feed efficiency have been reported previously as detailed in Ref. [47]. Briefly, functional diets did not affect ($p > 0.05$) fish growth performance after nine weeks of supplementation and fish presented a 2.6x increase in body weight along the experimental period, representing a relative growth (%) of a 158.8 ± 16.3 [47]. The recorded mortality during the feeding trial was negligible (< 1%) and not associated to a specific diet [47].

Table 3.2.
Primer sequences of the different genes analyzed and their RT-PCR conditions..

Gene	Access. Number	Primer sequence 5' – 3'		Anneling T (ºC)	Ref.
HSP90	AY395632	F	GCTTCGAGGTCCTGTACATG	62.7	[52]
		R	GCCTTATCCTCCTCCATC		
HSP70	AY423555	F	CAACCTCCTGGGAAAGTTTG	56.3	[52]
		R	AGAATCTTCTGCTTGTCGTCG		
StAR	EF409994	F	AAGCCCTGTGCTGAAGACC	60.0	[53]
		R	AGAATCTTCTGCTTGTCGTCG		
HIF-1α	DQ171936	F	GACTTCAGCTGCCCTGATTC	60.0	[54]
		R	GGCTGGTTTATAGCGCTGAG		
CYP11 β hydroxylase	AF449173	F	GGCCCTCTTCTCCTCATCTC	63.5	[55]
		R	GGCTGAAGATGTGATCCC		
Casp-3	DQ345774	F	ACGAAGCAGGTCAATCATCC	60.0	[56]
		R	GCAGTTTAAGGGTATCCAGAGC		
Interleukine-1β	AJ53742	F	ATTACCCACCACCCACTGAC	60.0	[57]
		R	TCTCTTCCACTATGCTCTCCAG		
RPL17	AF139590	F	GAGGACGTGGTGGTTCATCT	60.0	[58]
		R	CTGGCTTGCCTTTCTTGACT		
EF1	AJ866727	F	GCTTCGAGGAAATCACCAAG	60.0	[59]
		R	CAACCTTCCATCCCTTGAAC		

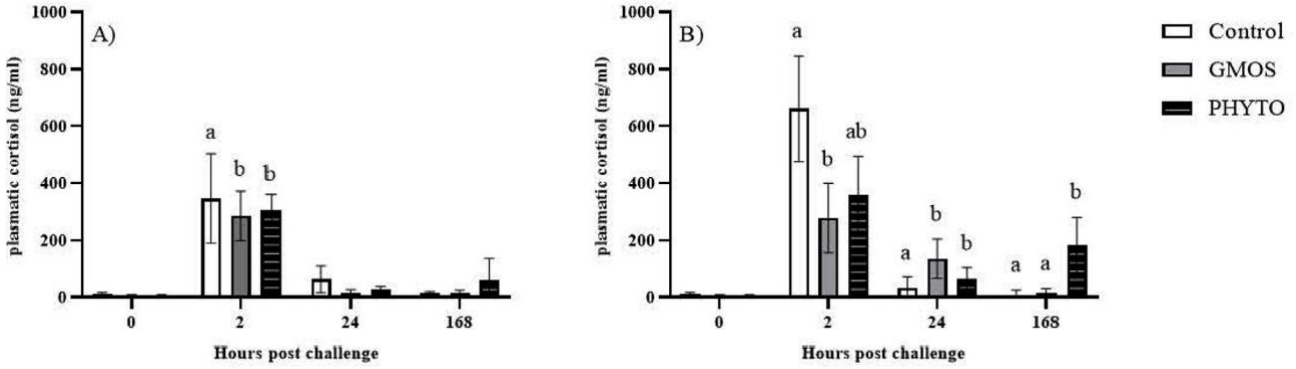

Fig. 3.1. Plasma cortisol levels in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. A) Fish subjected to confinement treatment and B) Fish subjected to confinement plus infection treatment. Different letters denote significant differences among dietary treatments at each sampling time ($p < 0.05$; one-way ANOVA followed by Tukey post-hoc test). Values expressed in mean ± SD, n = 9 fish/diet/sampling point. Control (control diet), GMOS (0.5% galactomannan oligosaccharides), PHYTO (0.02% phytogenic).

Functional additives did not induce significant differences ($p > 0.05$) on feed intake or feed efficiency, with FCR values ranging from 1.5 to 1.8.

3.2. Stress challenge test

After the stress challenge, fish subjected to the confinement treatment (C) did not present mortality, regardless of the diet fed. However, the combination of stress and infection (CI treatment) resulted in a relative percent survival (RPS) of a 47% and a 33% for animals fed with PHYTO and GMOS diets, respectively in comparison to fish fed the control diet. More details and discussion on these results have been previously published in Refs. [47].

In terms of stress resistance, functional diets did not induce any significant effect ($p > 0.05$) on basal (pre-challenge) circulating level of plasma cortisol. Indeed, during the feeding trial, values ranged from 4.0 to 5.8 ng/mL regardless of the dietary treatment (Fig. 1).

As expected, crowding stress treatment (C), resulted in a general increase ($p < 0.05$) of plasma cortisol at 2h post confinement (Fig. 1A), which was lower ($p < 0.05$) in fish fed GMOS and PHYTO diets compared with fish fed with the control diet. Fish of the control group did not show an increase in plasma cortisol at any other sampling point (Fig. 1A). Fish confined and infected (CI) presented a similar pattern of response in terms of circulating plasma cortisol after 2h, with fish fed GMOS diet presenting lower levels ($p < 0.05$) than fish fed control diet (Fig. 1B). Indeed, 2h post challenge, fish subjected to the CI treatment and fed the control diet presented almost a two fold higher circulating plasma cortisol concentration than fish subjected only to C treatment (Fig. 1). Contrary to the pattern observed at 2h post-chal- lenge, at 24h post challenge for CI treatment, fish fed control diet presented lower ($p < 0.05$) circulating cortisol concentration when compared to fish fed functional diets (Fig. 1). Finally, at 7 days post applying the CI treatment, those animals fed with PHYTO diet pre- sented higher ($p < 0.05$) cortisol levels than fish fed with GMOS and control diets (Fig. 1B).

No differences ($p > 0.05$) were detected in plasma glucose levels associated to the confinement treatment (Fig. 2A), regardless of the dietary treatment. For the CI treatment, control fish presented higher ($p < 0.05$) glucose levels after 2 h of infection than fish fed with PHYTO and GMOS diets, whereas after 7days of challenge, the opposite pattern was observed (Fig. 2B).

3.3. Combined effect of functional diets and stress in the expression of selected genes of head kidney

At the end of the feeding trial (t = 0, prechallenge), functional diets did not induce any significant ($p > 0.05$) effect on the relative expression levels of *StAR*, *hif-1α*, *cyp11B*, *casp-3* and *il-1β* (Fig. 3), neither of *hsp90* and *hsp70* genes (data not shown).

Two hours after confinement stress (C), fish fed with PHYTO diet presented a down-regulation ($p < 0.05$) of *cyp11β* (Fig. 3A), *hif-1α* (Fig. 3C), *casp-3* (Fig. 3G) and *il-1β* (Fig. 3I) gene expression levels in the head kidney in comparison to fish fed with control diet. However, these levels were not significantly lower than those presented by fish fed GMOS diet with the exception of *hif-1α* gene expression. For *StAR* gene expression fish fed PHYTO diet presented an up-regulation ($p < 0.05$) in comparison to fish fed control and GMOS diets (Fig. 3E). On the other hand, fish fed GMOS presented a down-regulation ($p < 0.05$) of *hif-1α* expression levels as compared to fish fed with control diet (Fig. 3C). In addition, when fish were subjected to CI treatment (2 h post challenge) fish fed PHYTO and GMOS diets pre- sented a down-regulation ($p < 0.05$) of StAR gene expression in the head kidney (Fig. 3F), in comparison to fish fed with control diet, whereas only fish fed PHYTO diet presented a down-regulation ($p < 0.05$) of *casp-3* gene expression (Fig. 3H) in comparison to fish fed with control diet.

Twenty-four hours of confinement induced an up-regulation ($p < 0.05$) of *StAR* gene expression in the head kidney of fish fed the PHYTO diet as compared to fish fed control and GMOS diets (Fig. 3E). However, when confinement was combined with the infection (CI), at 24 h post challenge, fish fed both functional diets presented an up-regulation ($p < 0.05$) of *Il-1b* gene expression compared to fish fed non-supplemented diet (Fig. 3J). Moreover, fish fed PHYTO diet pre- sented an up-regulation ($p < 0.05$) of *hif-1α* gene as compared to the rest of the dietary treatments (Fig. 3B).

At the end of the challenge test, fish fed with the PHYTO diet and subjected to CI treatment, presented an up-regulation ($p < 0.05$) of *StAR* gene expression in the head kidney in comparison to those fed with the GMOS diet (Fig. 3F). Besides, fish fed both functional diets presented a down-regulation ($p < 0.05$) of *cyp11β* (Fig. 3B) gene ex- pression when compared to fish fed with control diet, whereas fish fed PHYTO diet presented an up-regulation ($p < 0.05$) of *hif-1α* (Fig. 3D) in comparison to fish fed GMOS and control diets.

3.4. Lysozyme activity

At end of the feeding trial, no differences between fish fed the different dietary treatments were observed in serum and skin mucus lysozyme (t = 0h: Fig. 4). However, at the end of the stress challenge, serum lysozyme showed higher values on fish fed the control diet at the end of the stress challenge (Fig. 4A). After 2 and 24h of CI treatment, fish fed PHYTO diet presented a higher ($p < 0.05$) serum lysozyme activity than fish fed the rest of the dietary treatments (Fig. 4B). Similarly, fish fed both functional diets presented increased mucus lysozyme activity 2 h after CI compared to fish fed control diet (Fig. 4D).

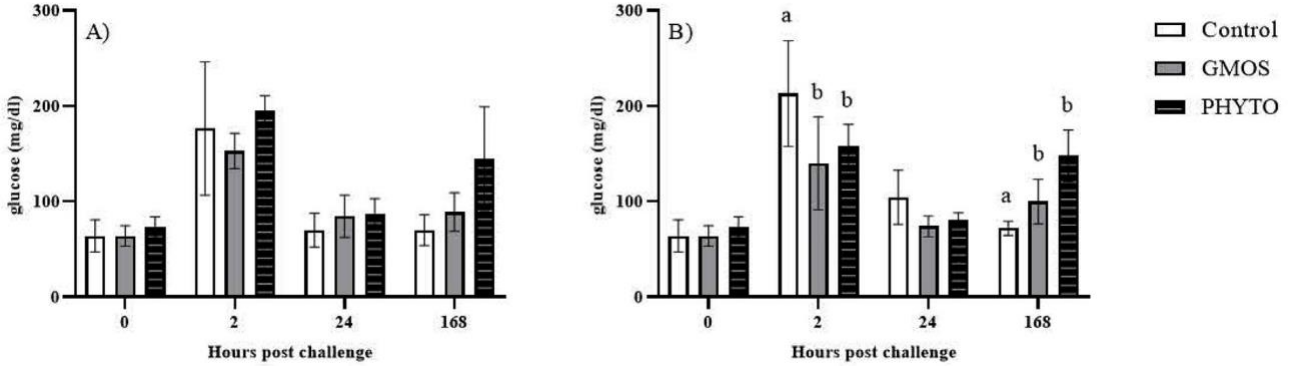

Fig. 3.2. Plasma glucose levels in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. A) Fish subjected to confinement treatment and B) Fish subjected to confinement plus infection treatment. Different letters denote significant differences among dietary treatments at each sampling time ($p < 0.05$; one-way ANOVA followed by Tukey post-hoc). Values expressed in mean ± SD, n = 9 fish/diet/sampling point. Control (control diet), GMOS (0.5% galactomannan oligosaccharides), PHYTO (0.02% phytogenic).

4. Discussion

Various extracts from herbs have been reported to improve animal performance by stimulating action on gut secretions or by having a direct effect on gut microbiome and inducing a higher protein synthesis [64,65]. Besides, addition of prebiotics such as GMOS has been also described to have growth promoting properties [19,29,66–68]. In the present study, dietary GMOS (galactomannan oligosaccharides from mucilage) and PHYTO (mixture of garlic oil and labiatae-plants oils) had no effects on European sea bass growth performance after nine weeks of feeding, being the fish growth obtained adequate and similar to that reported previously for the same fish species and diet composition (10%FM/6%FO [44]). Our findings are in agreement with previous results in Nile tilapia (*Oreochromis niloticus*) fed a dietary supplement containing another labiatae plants active compound, such as the monoterpenoid thymol-, at inclusion levels up to 0.05% for 63 days [68]. Similarly, there were no changes in growth parameters of Nile tilapia fed diets based on 15%FM/5.5%FO when supplemented with either cinnamaldehyde or thymol up to 2 mL/kg during 75 days [69]. Moreover, no effect was found on growth performance of Caspian white fish (*Rutilus friisi kutum*) fed with diets based on 40%FM/6%FO and supplemented with different levels of inclusion (1%, 2% and 3%) of herbal immunostimulants (GOS - Vivinal-GOS®, Zwolle, The Netherlands) for 8 weeks [70]. However, an improvement of European sea bass growth performance and nutrient utilization was found when fed a low FM/high FO (10%FM/15%FO) diet supplemented with a com- mercial blend of anise, citrus, and oregano essential oils (Digestarom PEP M.G.E 150; Biomin, Austria) over a 60-days feeding trial [46]. The utilization of garlic also has been described to be a growth promoter either used as powder at 0.15% inclusion rate for Caspian roach (*Rutilus*) [71], or used as ethanol-based extract at 0.5% inclusion rate for Sterlet Sturgeon (*Acipenser ruthenus*) [72]. The apparent discrepancy among the different studies, as occur with other functional ingredients, could be due to the formulation of the basal diet, the amount and/or type of functional ingredients used or the blend of ingredients used together with the ability of the different species to utilize low levels of FM and FO in the diet [19].

The utilization of low FM/FO diets for marine species has been shown to affect the stress response in different species (for review see Ref. [14]). Changes on the dietary lipid composition have been described to alter the capacity of larval fish to resist several stressors [73–75], to alter the time-course pattern of cortisol synthetized by in- terrenal cells of juvenile fish both *in vivo* [9,10,15,76–79] and *ex vivo* [11–13]. It has been suggested that the influence of dietary lipids, and in particular fatty acids, on the modulation and activation of the synthesis of cortisol and its release from interrenal cells during stress conditions is related to their electrophysiological effect on the ventromedial hypothalamus cells related to an inhibitory control on the activation of the HPI axis [80], similar to that described for mammals [81], or to a direct effect on the expression of steroidogenesis-related genes [13].

Complementary tools that will help to reduce the negative-side effects of the utilization of very low levels of FM/FO diets have been developed during the last decade. Functional additives such as prebiotics and phytogenics, among other compounds, have been proposed as potential candidates to reduce the deleterious effects of high reductions of FM/FO in fish diets [19,44,82–84]. Functional diets have also been shown to improve fish stress resistance capacity. For example, the use of a herbal mixture, including *Massa medicata fermentata*, *Crataegi fructus*, *Artemisia capillaries*, and *Cnidium officinale*, in the proportions 2:2:1:1, improved Japanese flounder (*Paralyhchtys olivaceous*) recovery after a 10-min air exposure test with five times repeat, and an anesthesia test for 2 min with 200 ppm 2-phenoxyethanol [85].

In the present study, the utilization of functional diets for 63 days did not affect the basal concentration of plasma cortisol. Values obtained were lower than the reference levels expected for this species [86], being European sea bass a species with a higher basal plasma cortisol level than other marine fish [8]. However, functional diets showed a regulatory effect on the time-course response pattern of plasma cortisol concentration after applying the C and CI stressful treatments. Fish fed with GMOS or PHYTO supplemented diets showed an attenuated stress-related increase of plasma cortisol when compared to those fed with the control diet. Those results are similar to the ones obtained for European sea bass supplemented with mannanoligosaccharides [22] or for greater amberjack (*Seriola dumerili*) supplemented with a similar phytogenic [87]. The modulatory effect of functional diets on metabolic indicators of stress response could be also observed 2 h after the induction of infection in combination with confinement, when plasma glucose concentration rose significantly on fish fed the non-supplemented diet. This increase corresponded to the higher plasma cortisol levels in the fish fed control diet, showing a more acute response to the stress factor. The amelioration of deleterious effects associated to a stressful situation obtained in the present study when dietary additives were used is also in agreement with those obtained for the common carp (*Cyprinus carpio*) exposed to a sub-lethal concentration of waterborne cadmium (1.5 mg L-1 free ion) for 15 days, using a diet supplemented with 1% of Shirazí thyme (*Zataria multiflora Boiss)* from the labiatae plant family) essential oil [42]. This reduction on the stress response was also comparable with a reduction in cortisol and lactate levels in silver catfish (*Rhamdia quelen*) fed with diet containing oil extracted from another similar herb, *Aloysia triphylla* (2.0 mL/kg) [88].

Besides the above-mentioned findings, little is known about the effect of functional diets and specifically phytogenics on specific stress

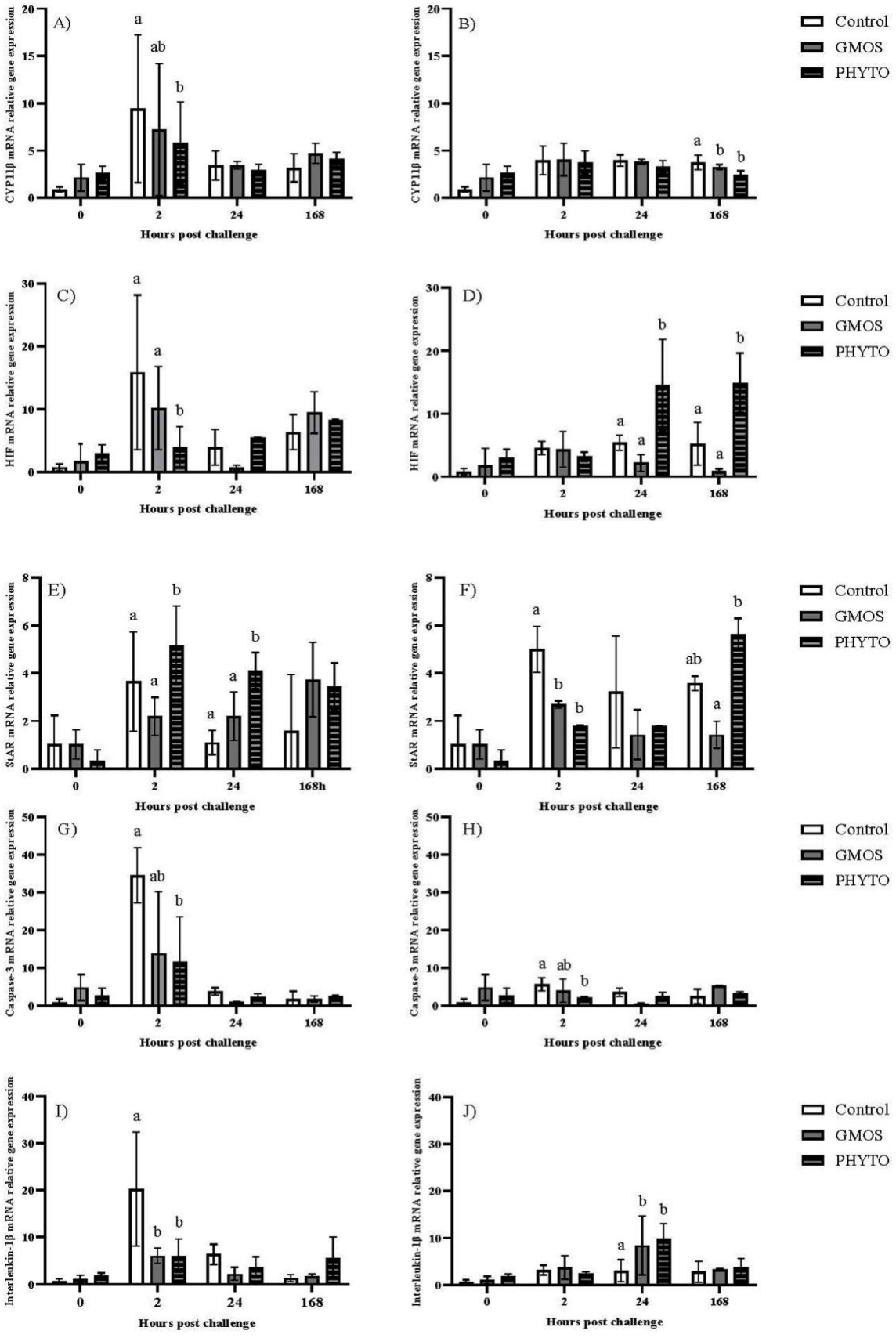

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Fig. 3.3. Head kidney relative gene expression in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. A) *cyp11β* expression of fish subjected to confinement treatment (C); B) *cyp11β* expression of fish subjected to confinement plus infection (CI) treatment; C) *hif-1α* expression of fish subjected to C treatment; D) *hif-1α* expression of fish subjected to CI treatment; E) *StAR* expression of fish subjected to C treatment; F) *StAR* expression of fish subjected to CI treatment; G) *casp-3* expression of fish subjected to C treatment; H) *casp-3* expression of fish subjected to CI treatment; I) *il-1β* expression of fish subjected to C treatment; J) *il-1β* expression of fish subjected to CI treatment. Different letters denote significant differences among dietary treatments at each sampling point ($p < 0.05$; one-way ANOVA; Tukey post-hoc). Values expressed in mean ± SD, n = 3 fish pooled/tank/sampling point; n = 3 pools/diet/sampling point. Control (control diet), GMOS (0.5% galactomannan oligosaccharides), PHYTO (0.02% phytogenic).

indicators of fish, although this effect has been described in other animals and in humans. As examples, the extracts of *Inula racemosa*, *Boerhaavia diffusa* and *Ocimum sanctum* decreased the concentration of both, cortisol and glucose in the serum of male mice [89] and essential oil from *Curcuma zanthorrhiza* has been shown to reduce the plasma corticosteroids after heat stress in broilers [40]. Dietary oregano essential oil supplementation alleviated transport stress by a reduction of circulating cortisol levels compared with the control diet, and reduced norepinephrine levels in pigs [90]. For fish, herbal extracts have been shown to ameliorate the plasma cortisol increase induced by a high stocking density in freshwater and marine species. This effect has been described for silver catfish fed a diet supplemented with 0.5 mL per kg of *L. alba* oil (linalool chemotype obtained from labiatae herbs) [91], or for gilthead sea bream (*Sparus aurata*) fed a diet supplemented with and extract of the labiatae herb *M. sylvatica* oil (2.0 mL per kg) for 90 days [92]. Garlic extract has been also shown to have beneficial effects on the circulating plasma corticosteroids in vertebrates. Lower plasma cortisol concentration was found on broilers fed a diet supplemented with fermented garlic powder (2 or 4 g per kg of diet) [66]. However, no effect was detected on plasma cortisol of pigs fed different essential oils from garlic and subjected afterwards to a fasting and dehydration stress challenge [40].

The attenuated response on plasmatic cortisol levels when the PHYTO diet was fed could be related to a decreased effect on cortisol synthesis-related enzymes activities [93]. The *cyp11β* gene encodes the expression of P450 11β-hydroxylase enzyme, which mediates the last step on cortisol synthesis in fish in a dose dependent manner [94]. An increased transcription of the 11β -hydroxylase gene has been proposed to be a longer term, chronic response to ACTH in rainbow trout [94]. As far as these authors know, there is no information about how GMOS and labiatae or garlic extracts can affect the expression of *cyp11β* gene, but it has been shown that some plants extracts such as the shrub (*Salsola tuberculatiformis*) acts as an stress alleviator by the inhibition of the enzymatic conversion of deoxycorticosterone to corticosterone by human 11β-hydroxylase [95]. Those authors suggested that Compound A, the precursor of aziridine found in the extract of the shrub, is the responsible of this inhibition and it is transported in the blood binding to steroid-binding globulins [95], being a competitive inhibitor of glucocorticoid binding to steroid-binding globulins [96] and also reducing the levels of circulating ACTH [97]. Aziridine has been isolated in several plants and marine organisms [98] including some labiatae plants such as *Lavandula stoechas* [99].

On the other hand, the expression of hypoxia inducible factor *(hif-1α)* was also decreased in fish fed PHYTO diet. The activation of this gene is related with the pro-inflammatory response [100,101] and plays a role on the accumulation of cholesterol in the cytoplasm [5], and hence influences directly the cortisol synthesis, as cholesterol is the substrate to produce cortisol [102]. Interestingly, when fish were stressed by confinement, without the associated effect of a pathogen, animals fed with the PHYTO diet showed lower gene expression values

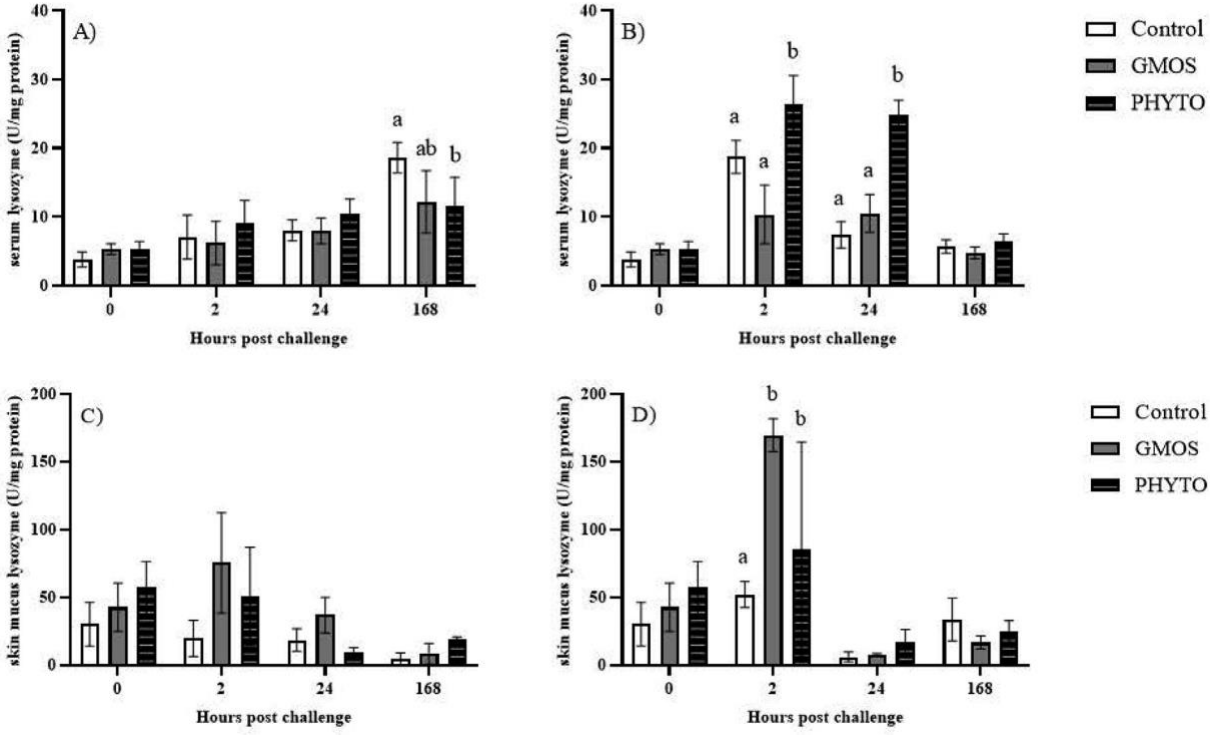


Fig. 3.4. Serum and mucus lysozyme activity in European sea bass (*Dicentrarchus labrax*) during the stress challenge test. A) Serum lysozyme activity of fish subjected to confinement treatment (C); B) Serum lysozyme activity of fish subjected to confinement plus infection (CI) treatment; C) Skin mucus lysozyme activity of fish subjected to C treatment; D) Skin mucus lysozyme activity of fish subjected to CI treatment. Different letters denote significant differences among dietary treatments at each sampling point ($p < 0.05$; one-way ANOVA; Tukey). Values expressed in mean ± SD, n = 2 fish/tank/sampling point; n = 8 fish/diet/sampling point. Control (control diet), GMOS (0.5% galactomannan oligosaccharides), PHYTO (0.02% phytogenic).

than fish fed the control and GMOS diet, suggesting an attenuation of the stress response. However, when the fish were subjected to the CI treatment, the opposite situation occurred. Twenty-four hours post infection, fish fed with phytogenics supplemented diets showed significantly higher *hif-1α* gene expression levels, suggesting a better immune system response against *Vibrio anguillarum* and supported by the lower infection rates found [47]. When an inflammatory response is triggered in response to an infection, a hypoxia condition can occur [103]. This fact directly implies an activation of the *hif-1α* transcription via nuclear factor-kappaB (NF-kB) in order to facilitate leucocytes survival in the inflammation-induced hypoxic medium generated [101], facilitating the organism fight against the invading pathogen and, which could explain the lower mortality found in fish fed PHYTO diet [47].

The expression of the *StAR* gene was higher on fish fed with the PHYTO diet. *StAR* is a transporter protein that mobilizes in tissue-specific manner cytoplasmic cholesterol into the mitochondria to synthetize cortisol by the interrenal cells of fish [94]. This process involves multiple signaling pathways, including protein kinase A (PKA) and protein kinase C (PKC) among others, that are related with ACTH activity, as an upstream regulator of cAMP production [104]. Due to this, in the present study it was expected that those fish fed with the PHYTO diet would have lower gene expression levels of this marker, which was corroborated by the more attenuated cortisol response in PHYTO-fed fish. However, it must be taken into account that head kidney is a leucocyte-rich tissue [105,106], which has high affinity receptors for cortisol and this partially mediates the impact of cortisol affecting also processes like apoptosis in these cells. At a physiological level, low-stress concentrations of cortisol are effective to induce increased apoptosis and to inhibit leucocyte proliferation [107]. In this sense, *StAR* plays a role on the immune cells' – in particular leucocytes - protection against apoptotic processes [8], via the mobilization of cholesterol into the mitochondria. The inhibition of intracellular cho- lesterol trafficking induces the apoptosis pathway in a caspase-depen- dent manner [108].

Caspase-3 catalyzes protein degradation during the apoptosis process [109] and the expression of this gene has been described to be a good indicator of apoptosis in fish leucocytes [110]. The results obtained in the present study showed that fish fed the diet supplemented with the phytogenic presented lower expression of *Caspase-3* gene. This correspond to the peak of higher expression of *StAR* gene, suggesting that phytogenics could have been inducing mechanisms associated to mitigation of apoptotic processes in response to stress, protecting leucocytes from deterioration. This preventive effect of garlic extract has been described in cardiomyocytes of rats in a dose-dependent manner, by a NO and H_2S dependent reduction of oxidative stress [111]. Dietary essential oils have been described as direct modulators of different oxidative stress indicators [64]. Catalase (CAT) and superoxide dismutase (SOD) were increased in Channel catfish fed with 0.5 mL per kg of *Origanum vulgare* oil [112] and Silver catfish fed 2.0 mL per kg of *A. triphylla essential oil* [85] or *L. alba* (around 0.5–2.0 mL per kg) [113]. Similarly, CAT was increased in Nile tilapia supplemented with *Cymbopogon citratus* (0.2 g per kg) and *Pelargonium graveolens* (0.4 g per kg) [114], or hepatic SOD was increased in rainbow trout fed 0.5–1.0 g per kg diet of *Salvia officinalis*, *Mentha spicata* and *Thymus vulgaris* essential oils [115], all of them belonging to the Labiatae plant family.

From this point of view, phytogenics seem to be inducing a higher effectiveness mitigating the stress response [116], hence boosting the immune response on a long-term basis. The expression of *interleukine-1β* (*il-1β*) gene was analyzed as one of the most important indicators of pro-inflammatory processes in fish [117]. Within the present study, the utilization of functional diets during 9 weeks did not induce any effect on the basal expression of *il-1β* in head kidney. However, it was observed that fish fed with GMOS or PHYTO diets showed reduced values of *il-1β* relative expression, concurrent with the lower infection and mortalities found after the confinement. In agreement with these results, a regulatory effect of the essential oil from Japanese criptomeria (*Cryptomeria japonica*) was described on the *il-1β* production [118]. These results suggest that animals fed a phytogenic-based diet presented a lower pro-inflammatory activity induced by the reduced increase of plasma cortisol after stress, since plasma cortisol and *il-1β* are positively correlated [8]. However, when the pathogen appeared in combination with the stressful situation, the animals fed with GMOS or PHYTO-supplemented diets showed higher expression of the gene encoding this cytokine, suggesting a better immune response by the enhancement of the specific pro-inflammatory response against the pathogen. Similar results have been observed in sea bass larvae in response to an infection with *V. anguillarum* [119]. This effect could be associated to a mitigation of the stress effects by the phytogenics in diet, as a lower cortisol production implies a lower inhibition of the pro- inflammatory response expression in response to the pathogenic agent [120], whereas those mechanisms seem to be inactivated in absence of the pathogen.

The supplementation of functional additives during 9 weeks did not induce any effect on serum or skin mucus lysozyme activity. A lack of effect on this parameter could be due to a time-dependent effect, as suggested for common carp fed with herbal extract supplemented diets, in which the lysozyme activity decreased after 45 days of treatment [121] or to a dose-dependent effect, as suggested for common carp supplemented in diet with Guava tree extract (*Psidium guajava*) [122]. Indeed, an increased lysozyme activity has been found in rainbow trout during the first 20 days of feeding a garlic extract in the diet, however this stimulatory effect disappeared after 28 days of feeding [36]. Similarly, no effect of confinement stress was observed in either serum or mucus lysozyme of Atlantic salmon subjected to short- or long-term handling stress [123]. However, serum lysozyme activity showed a significant increase in fish fed PHYTO diet in response to pathogen presence (2–24h post-inoculation), when comparing with fish fed either control or GMOS diets, suggesting a higher capacity to cope with the bacterial infection. In the case of skin mucus lysozyme, this positive effect was only found after 2h of bacterial inoculation, however lysozyme activity was increased after feeding both functional products, denoting the differences on the response pattern to the pathogen by the systemic and the mucosal immune systems after functional diets' supplementation.

In summary, the use of dietary GMOS (0.05%) or a mix of essential oils PHYTO (0.02%) attenuated the physiological response to stress in European sea bass, with lower plasmatic cortisol concentrations post-stress, a lower *hif-1α* expression and a higher *StAR* expression. The use of a dietary mixture of essential oils decreased the endocrine response after stress and had a protective role against apoptosis in head kidney cells in response to a stressful situation, with lower *casp-3* gene expression and a higher *il-1β* gene expression when an infection occurs. PHYTO and GMOS increased resistance to *Vibrio anguillarum* infection after stress. The ability of these functional additives to attenuate fish stress response entailed a better capability of the animals to cope with the infection, partially due to the protection of the head-kidney leu- cocyte population against apoptotic processes allowing a better re- sponse against the pathogen. This study confirms the protective effect of dietary functional additives supplemented during 9 weeks in diets for European sea bass juveniles.

CRediT authorship contribution

A. Serradell: Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. S. Torrecillas: Conceptualization, Software, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Methodology. A. Makol: Resources, Writing - review & editing. V. Valdenegro: Resources, Writing - review & editing. A. Fernández-Montero: Software, Validation, Formal analysis, Data curation. F. Acosta:

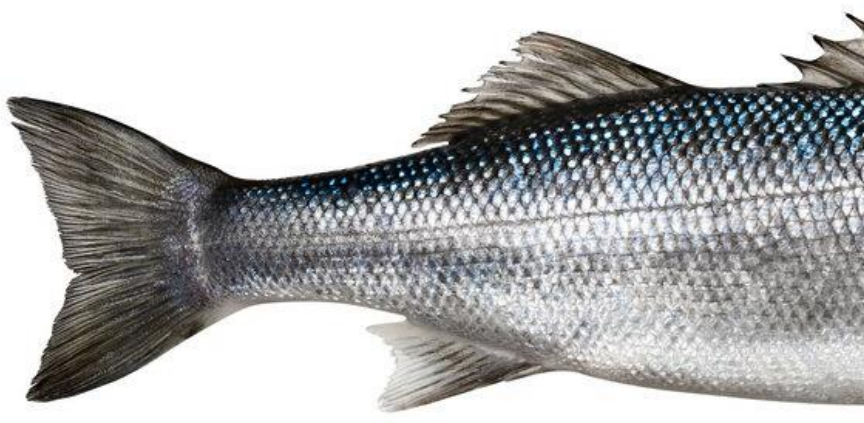
Conceptualization. M.S. Izquierdo: Methodology. D. Montero: Conceptualization, Validation, Investigation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition, Methodology, Visualization.

Aknowledgements and funding

This work was funded by the Spanish Ministry of Economy, Industry and Competitiveness ("Subprograma Estatal de Generación de Conocimiento, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013–2016") by the PROINMUNOIL PLUS (AGL2016-79725-P) project: "Functional diets for marine raw materials replacement: boosting the fish disease resistance through epithelial barriers reinforcement and immunization tools". The views expressed in this work are the sole responsibility of the authors and do not ne- cessary reflect the views of the Spanish Ministry of of Economy, Industry and Competitiveness. We also thank the Spanish Ministry of Economy, Industry and Competitiveness for the complementary funding for ST though the "Subprogama Juan de la Cierva- Incoproración, Convocatoria 2015, IJCI-2015-25748. Special thanks to Delacon Biotechnik GmbH and to BioMar A/S, which provided the functional products and diets.

Chapter IV

Gill Stress Oxidative Protection through the Use of Phytogenics and Galactomannanoligosaccharides as Functional Additives in Practical Diets for European sea bass (*Dicentrarchus labrax*) juveniles



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Gill Oxidative Stress Protection through the Use of Phytogenics and Galactomannan Oligosaccharides as Functional Additives in Practical Diets for European Sea Bass (*Dicentrarchus labrax*) Juveniles

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Simple Summary: Under intensive aquaculture conditions, fish are subjected to a wide variety of stressors, making fish prone to suffering chronic stress and impairing fish growth performance and immune response. Fishmeal (FM) and fish oil (FO) replacement by raw terrestrial materials may induce nutritional imbalances, leading to a chronic stress status and oxidative stress processes. The functional ingredients have been profiled as suitable candidates to face these negative side effects by reinforcing fish immune response, attenuating fish stress response and reducing fish oxidative stress. The present study evaluates the effects of two different functional ingredients, plant origin galactomannan-oligosaccharides (GMOS) and a mixture of garlic and labiate plant essential oils (PHYTO), as potential boosters of gill endogenous antioxidant capacity in European sea-bass (*Dicentrarchus labrax)* juveniles fed low-FM/FO-based diets. After a confinement stress challenge (C challenge) or confinement combined with an in vivo infection with the pathogen *Vibrio anguillarum* (CI challenge), the functional ingredients induced a controlled pro-inflammatory response against the stressor. The functional ingredients attenuated fish stress response, leading to a stable energy metabolism and an ameliorated antioxidant status, altogether indicating the potential of both functional additives to reduce the associated negative effects of stress in European sea bass fed a low-FM/FO diet.

Abstract: The aim of the present study is to evaluate the potential of two functional additives as gill endogenous antioxidant capacity boosters in European sea-bass juveniles fed low-FM/FO diets when challenged against physical and biological stressors. For that purpose, two isoenergetic and isonitrogenous diets with low FM (10%) and FO (6%) contents were supplemented with 5000 ppm plant-derived galactomannan–oligosaccharides (GMOS) or 200 ppm of a mixture of garlic and labiate plant essential oils (PHYTO). A control diet was void from supplementation. Fish were fed the experimental diet for nine weeks and subjected to a confinement stress challenge (C challenge) or a confinement stress challenge combined with an exposure to the pathogen *Vibrio anguillarum* (CI challenge). Both GMOS and PHYTO diets attenuated fish stress response, inducing lower circulating plasma cortisol and down-regulating *nfκβ2* and *gr* relative gene-expression levels in the gill. This attenuated stress response was associated with a minor energetic metabolism response in relation to the down-regulation of *nd5* and *coxi* gene expression.

Keywords: European sea bass; functional diets; galactomannan–oligosaccharides; gill relative gene expression; low-FM/FO diets; oxidative stress; phytogenics

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Citation: Serradell, A.; Montero, D.; Fernández-Montero, Á.; Terova, G.; Makol, A.; Valdenegro, V.; Acosta, F.; Izquierdo, M.S.; Torrecillas, S. Gill Oxidative Stress Protection through the Use of Phytogenics and Galactomannan Oligosaccharides as Functional Additives in Practical Diets for European Sea Bass (*Dicentrarchus labrax*) Juveniles. *Animals* **2022**, *12*, 3332. https://doi.org/10.3390/ani12233332

Academic Editor: Jorge S. Olmos-Soto

Received: 22 October 2022
Accepted: 23 November 2022
Published: 28 November 2022

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1. Introduction

Fish reared under intensive aquaculture conditions are subjected to a wide variety of stressors. Between them, the nutritional imbalances may induce a chronic stress status [1–3] compromising fish growth performance and impairing fish immune response and tissue integrity [4–8].

Fish gills have essential functions for fish physiological balance, gas exchange, hydro–mineral balance [9], and immune response [10]. As gills interact directly with the external environment, they are the first barrier of protection against external agents such as pathogens and chemicals, acquiring a significant importance in fish development and disease resistance [10,11]. One of the main cell types composing gill epithelia are mitochondria- rich cells (MRCs), which are involved in gas exchange, ion transport, and blood acid–base balance regulation [9].

As a direct consequence of a stressful process, cortisol will target gills, increasing oxidative phosphorylation to ensure the energy availability to conduct all the physiological changes required to cope with the stress processes and an up-regulating Na^+/K^+ ATPase pump activity to maintain tissue hydro–mineral balance and functioning [12]. Cortisol effects are mediated through glucocorticoid receptors (GR), which reside in the cytoplasm complexed with the co-chaperone heat-shock proteins heat-shock protein 70 (HSP70) and heat-shock protein 90 (HSP90) [13]. Cortisol binds to the GR, inducing the dissociation of the chaperon proteins; then, cortisol–GR complex translocates into the nucleus to regulate gene expression of different stress-responsive factors, such as the pro-inflammatory nuclear factor *κβ* (*nfκβ2*). The NF*κβ* protein is one of the most important mediators of inflammatory response, which can be activated by different extracellular stimuli, such as pro-inflammatory cytokines [14,15], reactive oxygen species (ROS) [15,16], pathogen-associated molecular patterns (PAMPs) [17,18], and acute stress events [19]. In parallel, the GR can bind to the BCl-2 receptor in the mitochondrial membrane, inducing an increase in the oxidative phosphorylation rate by the mitochondrial electron-transport chain (ETC), generating energy to supply the adenosine triphosphate (ATP) synthase to produce ATP [20]. However, not all the electrons in the ETC are transferred to the final acceptor, generating an electron leak, which leads to the formation of reactive oxygen species (ROS)—namely, superoxide-radical (O_2^-) formation. Superoxide radicals are transformed by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2), which diffuses to the cytoplasm to be detoxified by glutathione peroxidase (GPX) and catalase (CAT) [21,22]. Oxidative stress results from an imbalance between ROS production and its neutralization by the antioxidant-defense system. It leads to the oxidation of essential biomolecules such as proteins and lipids, DNA damage, and the impairment of mitochondrial activity, causing cell death [21]. Insufficient ATP production will also impair Na^+/K^+ ATPase activity, causing hydro–mineral imbalances [23,24].

Functional ingredients have been profiled as suitable tools to face these harmful side effects, boosting fish health and promoting fish welfare, reinforcing fish immune response [25–28], attenuating fish stress response [28–31], and reducing oxidative stress processes [32–34]. This is of remarkable interest in European sea bass, which is an especially susceptible fish species to stress processes [35–37]. Among the functional ingredients, phy- togenic feed additives (PFAs) are plant-derived bioactive compounds such as flavonoids, mucilages, and tannins with antioxidant properties [38–40]. In previous studies, dietary supplementation with a mixture of garlic and labiate plant essential oils attenuated European sea-bass juveniles' stress response, with the fish fed the supplemented diets presenting lower circulating cortisol levels in comparison to fish fed a control diet [30,41,42]. In ad- dition, PFA supplementation enhanced fish gut-mucosal health, reducing pre-ileorectal valve-segment goblet-cell size as compared to fish fed the control diet [41]. Another exam- ple of plant-derived functional ingredients are prebiotics, which are indigestible fibers with the ability to enhance host health by selectively stimulating the growth and activity of a limited number of intestinal bacterial species [28,43–46]. Between them, galactomannan–oligosaccharides (GMOS) have demonstrated in previously reported studies to increase

host antioxidant capacity, modulate gut microbiota, and promote gut health in this fish species [41,42,47].

A scarce number of studies have investigated the effects of functional ingredients to offset the negative effects derived from low-FM/FO diet formulation and especially in fish subjected to stress processes. Thus, the aim of this study is to evaluate the effects of functional additives (PFAs and GMOS) as potential boosters of the gill endogenous antioxidant capacity of European sea-bass juveniles fed low-fish meal (FM)/fish oil (FO)-based diets when challenged against physical and biological stressors.

2. Materials and Methods

2.1. Experimental Diets

Three isonitrogenous and isoenergetic low-fishmeal- and -fish oil-based diets (10% FM/6% FO) were formulated and produced by Biomar (Brande, Denmark), covering all the nutritional requirements for European sea bass. The control diet was void of functional ingredients (control diet), the GMOS diet was supplemented with 5000 ppm plant-derived galactomannan–oligosaccharides, and the PHYTO diet was supplemented with 200 ppm of a mixture of garlic and labiate plant essential oils. Functional additives were supplemented according to the producer's commercial recommendations (Delacon Biotechnik GmbH, Engerwitzdorf, Austria). To ensure product stability, GMOS was included in the mix pre-extrusion process and replaced standard carbohydrates. PHYTO additive was included in the post-extrusion process by vacuum coating and homogenized with dietary oil. The stability of the used PFAs was checked prior to diet production and at the beginning of the feeding trial. The ingredients used in the diets and their proximate composition are detailed in Table 1, below [43].

Table 4.1. Main ingredients and analyzed proximal composition of the experimental

Ingredients	Diet (%)		
	Control	GMOS	PHYTO
Fishmeal [1]	9.6	9.6	9.6
Soya protein concentrate	18.2	18.2	18.2
Soya meal	11.6	11.6	11.6
Corn gluten meal	24.1	24.1	24.1
Wheat	8.54	8.04	8.52
Wheat gluten	1.9	1.9	1.9
Guar meal	7.7	7.7	7.7
Rapeseed extracted	3.0	3.0	3.0
Fish oil [2]	6.5	6.5	6.5
Rapeseed oil [3]	5.2	5.2	5.2
Vitamin and mineral premix [4]	3.6	3.6	3.6
Antioxidant [5] Galactomannan–oligosaccharides (GMOS) [6]	0.06	0.06	0.06
Phytogenic [7]	0	0.5	0
Proximate composition (% of dry matter)	0	0	0.02
Crude lipids			
Crude protein	19.91	20.44	20.47
Moisture	49.30	49.27	49.76
Ash	5.10	5.01	5.06
	7.02	6.41	6.49

Dietary-ingredient composition and proximal composition expressed as % of dry weight. Control (Control diet), GMOS κGMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils); [1] South American, Superprime 68%; [2] South American fish oil; [3] DLG AS, Denmark; [4] Vilomix, Denmark; [5] BAROX BECP, Ethoxyquin; [6] Delacon Biotechnik GmbH, Austria; [7] Delacon Biotechnik GmbH, Austria.

2.2. Feeding Trial

This experiment is part of a series of experiments belonging to the project PROIN-MUNOIL PLUS, funded by the Spanish Ministry of Economy, Industry and Competi-

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tiveness. All the experiments were conducted in the facilities of the Parque Científico-Tecnológico Marino (PCTM) of the University of Las Palmas de Gran Canaria (ULPGC) (Canary Islands, Spain). A total of 675 European sea-bass juveniles from a local farm (Aqua- naria, Castillo del Romeral, Gran Canaria, Canary Islands, Spain) were transferred and acclimatized for 4 weeks to the PCTM facility's water conditions (6.1−6.6 ppm dissolved O_2, 18.2–20 ˚C, 36 ppm salinity) under a natural photoperiod (12L:12D). After acclimation, the fish were randomly distributed in 9 fiberglass tanks of 500 L (75 fish/tank), having an initial mean weight of 23.5 ± 0.8 g. Each experimental group was triplicated (3 tanks/diet) and the experimental fish were fed 6 days a week, 3 times a day until apparent satiation for 9 weeks. Feed intake was monitored daily, and growth and feed efficiency were calculated at the end of the experimental period. The Bioethical Committee of the University of Las Palmas de Gran Canaria approved all the protocols used in the present study (approval no. OEBA_ULPGC_14/2020).

At the end of the feeding trial (t = 0 h sampling point), three fish per tank (*n* = 9 fish per dietary treatment) were used to obtain blood samples in order to evaluate fish plasma circulating cortisol and glucose levels as stress indicators (Figure 1).

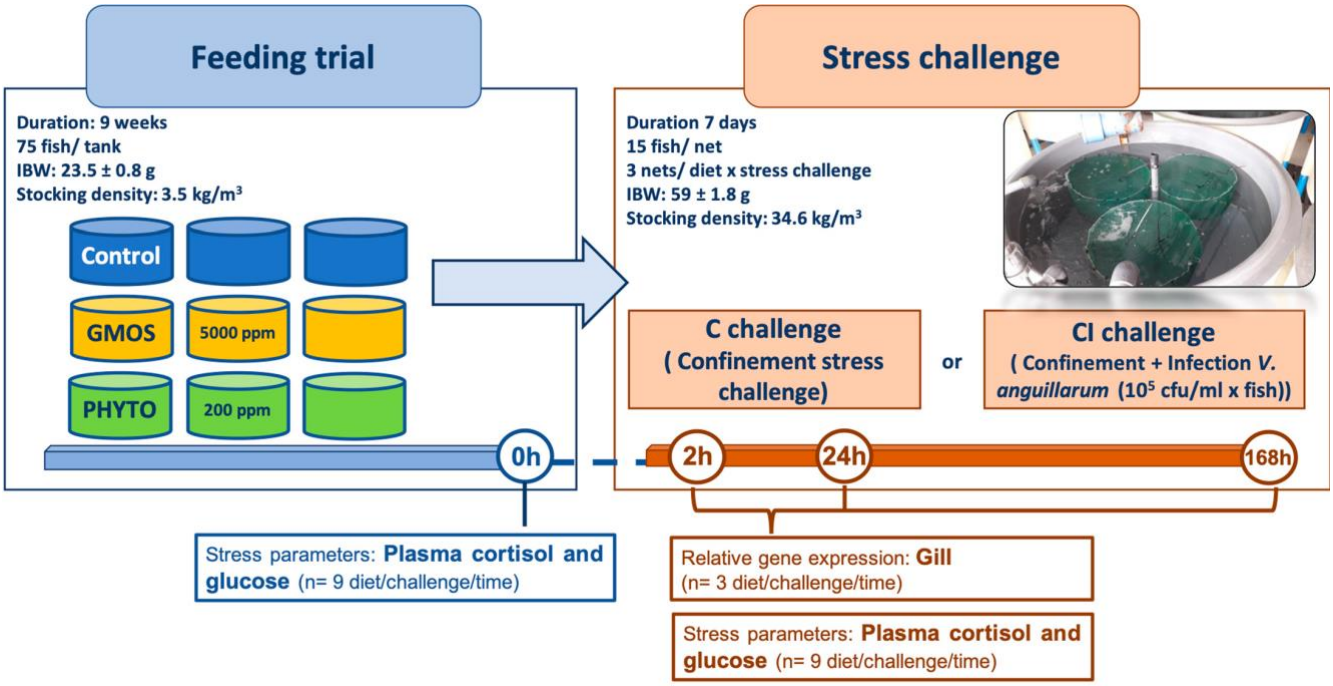

Figure 4.1. Experimental-design scheme. Nine-week feeding trial; three experimental treatments fed in triplicate (Control (no supplementation); GMOS (GMOS supplemented, 5000 ppm galactomannan-oligosaccharides); PHYTO (PHYTO supplemented, 200 ppm mixture of garlic and labiate plant essential oils); 3 times/day, 6 days/week until apparent satiation) (*n* = 75 fish/tank; initial body weight (IBW) = 23.5 ± 0.8 g). Seven-day stress challenge with two experimental treatments: C challenge (confinement stress challenge; 3 nets/dietary treatment, 15 fish/net, initial body weight (IBW) = 59 ± 1.8 g) or CI challenge (confinement stress challenge + intestinal infection with *V. anguillarum* (10^5 cfu/mL × fish); 3 nets/dietary treatment, 15 fish/net, initial body weight (IBW) = 59 ± 1.8 g). Sampling points at t = 0 h, 2 h, 24 h, and 168 h after stress challenge: Stress parameters (blood plasma cortisol and glucose concentration, *n* = 9 diet/stress challenge/sampling point); relative gene expression (gill, *n* = 3 diet/stress challenge/sampling point).

2.3. Stress Challenge

After 9 weeks of the feeding experiment, a total of 90 fish per dietary treatment were transferred to the Marine Biosecurity (MBS) facilities of the PCTM-ULPGC (Taliarte, Canary Islands, Spain) and exposed to a stress challenge. Forty-five fish per dietary

treatment were subjected to a confinement stress challenge (C challenge), consisting of a culture-density increase [48] (final stress challenge density = 35 kg/m^3) by confinement in submerged nets (15 fish/net, 3 nets/dietary treatment). The other 45 fish per dietary treatment were exposed to the same confinement stress challenge combined with an in vivo exposure to *Vibrio anguillarum* (CI challenge) (10^5 cfu/mL per fish, strain 7507, isolated from a clinical outbreak in Canary Islands) via intestinal inoculation as described before for *V. alginolyticus* [49]. The nets were placed in 6 fiberglass cylindroconical 500 L tanks on a RAS system supplied with filtered seawater at temperature of 22 °C, with 3 tanks for the C challenge and 3 tanks for the CI challenge. The fish were fed daily 3 times per day until apparent satiation during the entire stress challenge.

At 2 h, 24 h, and 168 h a whole net per dietary treatment and stress challenge was sampled to obtain blood for stress-indicator analysis (n = 9 fish per dietary treatment and stress challenge) and gill samples (n = 3 fish per dietary treatment and stress challenge) for stress and antioxidant response-related relative gene-expression analysis (Figure 1).

2.4. Sampling Methodology

In order to obtain blood samples, the fish were anesthetized with clove oil 0.02 mL/L (Guinama S.L; Pobla de Vallbona, Valencia, Spain; Ref. Mg83168) diluted in alcohol 100% (1:2). Afterwards, blood samples were obtained by caudal-sinus puncture with 1 mL syringes. Blood was stored in 1.5 mL Eppendorf tubes coated with heparin to avoid blood coagulation. Immediately, the blood was centrifuged at 3000× *g* at 4 °C for 5 minutes. The obtained plasma samples were rapidly kept at −80 °C until plasmatic-cortisol and glucose-concentration analysis. Plasmatic-cortisol concentration was determined using the assay kit Access Cortisol ref 33600, ©2010 Beckman Coulter, Inc., by the external laboratory AnimaLab (Las Palmas de Gran Canaria, Gran Canaria, Canary Island, Spain). Circulating plasma-glucose concentration was determined using the hexokinase method for in vitro diagnosis. The assay was performed using glucose-reactive OSR6521 from Beckman Coulter AU, employable on AU2700® and AU5400® chemistry analyzers (Beckman Coulter AU, PN B06960AA; March 2012).

Gill samples for relative gene expression were obtained after fish euthanasia by a clove-oil overdose of 0.5 mL/L (Guinama S.L; Spain, Ref. Mg83168) diluted in alcohol 100% (1:2). The second holobranch on the left side of the fish was excised with sterile dissection material and placed individually in 2 mL Eppendorf tubes filled with RNA later (for later 1.4 L RNA preparation: dilute in 1 L deionized water 650 g ammonium sulphate, 7.4 g sodium citrate dihydrate, 7.4 g EDTA disodium salt, and 200–500 µL concentrated sulfuric acid; final pH 5.2) for 24 h. Afterwards, the RNA later was removed and samples were frozen at −80 °C until gene-expression analysis.

2.5. RNA Extraction and Real-Time PCR Analysis

To perform the real-time PCR analysis, total gill mRNA (ng/µL) was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) from an RNeasy Minikit from Qiagen. An iScript™ cDNA synthesis kit (Bio-Rad Hercules, California) was employed to perform the reverse transcriptions to obtain cDNA in a 20 µL reaction containing 1 µL of total mRNA.

The real-time PCR analysis was performed with an iCycler with an optical module (Bio-Rad Hercules, CA, USA) in a final volume of 20 µL containing 10 µL of iQTM-SYBER Green Supermix (Bio-Rad Hercules, CA, USA), 5 µL of free-nuclease water, 3 µL of cDNA (1:10 dilution), and 1 µL of forward and reverse primers. The target genes were nuclear factor kappa beta (*nfκβ2*), hypoxia inducible factor 1 α (*hif-1α*), glucocorticoid receptors (*gr*), NADH dehydrogenase subunit 5 (*nd5*), cytochrome oxidase c subunit 1 (*coxi*), superoxide dismutase (*sod*), catalase (*cat*), glutathione peroxidase (*gpx*), tight cell-junction occludins (*ocln*), zonula occludens-1 (*zo-1*), heat-shock protein 70 (*hsp70*), heat-shock protein 90 (*hsp90*), and Na^+/K^+ ATPase subunit α1a (*NKA* α1a). Specific primer sequences and accession numbers for each target gene assayed are reported in Table 2. The real-time running conditions were 95 °C, 1 min followed by 40 cycles at 95 °C for 10 s and annealing

temperature for 30 s (Table 2). All reactions were performed in duplicate for each template cDNA and a blank control containing nuclease-free water instead of cDNA was included in each assay as a negative control. Three constitutive genes were tested: α-tubuline (*α-tub*), eukaryotic translation elongation factor 1 α1 (*eEF1α1*), and β-actin (*β-act*). After applying the CFX Maestro™ Software selection tool (CFX Maestro™ Software User Guide Version 1.1, Biorad), the *α-tub* was selected as the most stable and amplification-efficient reference gene. Relative gene-expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [50], using *α-tubulin* as housekeeping gene.

Table 4.2. Primer sequences of the different genes analyzed and their RT-PCR

Gene	Accession Number	Primer	Nucleotide sequence 5′–3′	Annealing T (°C)
nfKβ2	KM225790	Fw	CTGGAGGAAACTGGCGGAGAAGC	60
		Rv	CAGGTACAGGTGAGTCAGCGTCAC	
hif-1α	DQ171936	Fw	GACTTCAGCTGCCCTGATTC	60
		Rv	GGCTGGTTTATAGCGCTGAG	
gr	AY549305.1	Fw	GTGGGCCTACAAGACCAGAA	60
		Rv	CGGACGACTCTCCATACCTG	
nd5	KF857307	Fw	CCCGATTTCTGTGCCCTACTA	60
		Rv	AGGAAAGGAGTGCCTGTGA	
coxi	KF857308	Fw	ATACTTCACATCCGCAACCATAA	60
		Rv	AAGCCTCCGACTGTAAATAAGAA	
sod	FJ860004.1	Fw	CATGTTGGAGACCTGGGAGA	60
		Rv	TGAGCATCTTGTCCGTGATGT	
cat	FJ860003.1	Fw	TGGGACTTCTGGAGCCTGAG	60
		Rv	GCAAACCTCGATCGCTGAAC	
gpx	FM013606.1	Fw	AGTTCGTGCAGTTAATCCGGA	60
		Rv	GCTTAGCTGTCAGGTCGTAAAAC	
zo-1	MH321323.1	Fw	CGGCCTGCAGATGTTCCTAA	60
		Rv	GCTGAGGGAATTGGCTTTGA	
ocln	MH321322.1	Fw	GGACGAAGACGACAACAACGA	60
		Rv	CCATGGGAGAAAGCCTCTGA	
hsp70	AY423555.2	Fw	GGACATCAGCCAGAACAAGAGA	60
		Rv	GCTGGAGGACAGGGTTCTC	
hsp90	AY395632	Fw	GCTTCGAGGTCCTGTACATG	62.7
		Rv	GCCTTATCCTCCTCCATC	
NKA α1a	KP400258	Fw	AACCTCAGATGGCAAGGAGAAG	60
		Rv	GAGACTGGTACATTCAGGCGG	
α-tub (hk)	AY326429.1	Fw	AGGCTCATTGGCCAGATTGT	60
		Rv	CAACATTCAGGGCTCCATCA	
eEF1α1	XM_051391260.1	Fw	GTTGCTGCTGGTGTTGGTGAG	60
		Rv	GAAACACACTGCTGGAGGCTC	
β-act	AY148350.1	Fw	TCTTCCAGCCTTCCTTCCTC	60
		Rv	GATGTCAACGTCGCACTTCA	

Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^+/K^+ ATPase subunit α1a, *α-tub: α-tubulin* (housekeeping), *eEF1α1: Eukaryotic translation elongation factor 1 α1*, *β-act: β-actin*. Fw: forward, Rv: reverse.

The gene expression was calculated relative to the transcript levels of 2 h post confinement stress challenge (C challenge) of fish fed the control diet.

2.6. Statistical Analyses

All the analyses were performed with R Project for Statistical Computing. Means and standard deviations (SD) were calculated for each parameter measured. For each sampling point, a two-way ANOVA analysis was performed to evaluate the effects of each dietary treatment on fish circulating plasma cortisol and glucose concentrations and gill relative gene expression in response to the different stress challenges. All data analyzed were tested for normality and homogeneity. When data did not accomplish homogeneity, the alpha-value was reduced to 0.01 in the analyses. When significant differences were obtained, a Tukey post-hoc test was performed for multiple-means comparison.

3. Results

As reported in our previous studies [44], fish grew properly during the feeding trial with no significant effects on fish growth performance associated with the use of the functional diets. After the nine-week feeding trial, the fish presented a mean increase of 2.6× body weight, representing a relative growth (%) of 158.8 ± 16.3. During the feeding trial, no mortality was registered in any of the specific dietary treatments.

Functional-ingredient dietary inclusion did not induce any differences on fish basal (t = 0 h) stress parameters, with values ranging from 4.67 to 5.82 ng/mL for circulating plasma cortisol and from 67.43 to 67.71 mg/dL for circulating plasma glucose (Table 3). At 2 h after crowding stress a generalized increase ($p < 0.05$) in circulating plasma-cortisol concentration was observed, with significantly higher ($p < 0.05$) values in those fish fed the control diet. In the early hours after C challenge (2 h), all the dietary treatments presented an increase in circulating cortisol levels, especially those fish fed the control diet, with significantly higher ($p < 0.05$) levels than fish fed GMOS and PHYTO diets ($p < 0.05$). At 2 h after CI challenge, a similar trend was observed, with fish fed GMOS presenting lower ($p < 0.05$) cortisol levels than fish fed the control diet. On the contrary, at 24 h after CI challenge fish fed GMOS and PHYTO diets presented significantly higher ($p < 0.05$) circulating plasma-cortisol levels than those fed the control diet (Table 3). At the end of the CI challenge (t = 168 h), fish fed the PHYTO diet presented higher ($p < 0.05$) levels of circulating plasma cortisol than fish fed the control diet. Regarding circulating plasma- glucose concentrations, the use of functional additives did not induce significant differences in fish pattern of response against crowding stress (C challenge). Meanwhile, at 2 h and 168 h after the CI challenge, fish fed GMS and PHYTO diets presented significantly higher ($p < 0.05$) plasma-glucose levels than fish fed the control diet (Table 3).

Table 4.3. Concentration of circulating plasma cortisol (ng/mL) and glucose (mg/dL) in European sea-bass juveniles.

	Confinement (C Challenge)			**Confinement + Infection (CI Challenge)**		
Plasma Cortisol (ng/mL)	**Control**	**GMOS**	**PHYTO**	**Control**	**GMOS**	**PHYTO**
0 h (basal)	5.82 ± 3.45	5.33 ± 7.06	4.67 ± 8.04	5.82 ± 3.45	5.33 ± 7.06	4.67 ± 8.04
2 h	321.83 ^{a} ± 171.51	270.86 ^{b} ± 87.28	307.00 ^{b} ± 53.93	611.29 ^{a} ± 185.62	254.29 ^{b} ± 121.57	374.50 ^{ab} ± 133.29
24 h	71.00 ± 46.67	22.20 ± 10.43	29.43 ± 10.13	47.80 ^{a} ± 38.79	145.33 ^{b} ± 68.55	77.50 ^{b} ± 38.28
168 h	16.67 ± 4.73	15.60 ± 11.50	100.43 ± 76.97	14.17 ^{b} ± 18.17	16.40 ^{b} ± 15.16	217.43 ^{a} ± 96.14
Plasma glucose (mg/dL)						
0 h (basal)	67.63 ± 16.78	67.43 ± 10.66	67.71 ± 10.95	67.63 ± 16.78	67.43 ± 10.66	67.71 ± 10.95
2 h	143.33 ± 69.61	156.60 ± 18.61	194.50 ± 16.26	236.00 ^{a} ± 55.48	131.33 ^{b} ± 48.58	158.00 ^{b} ± 22.63
24 h	77.20 ± 17.54	96.50 ± 22.02	95.80 ± 16.08	102.33 ± 28.38	75.33 ± 10.98	80.50 ± 7.78
168 h	75.20 ± 16.08	93.00 ± 20.17	124.00 ± 54.21	74.25 ± 7.46	87.00 ± 23.39	148.00 ± 26.87

Different letters denote significant differences among dietary treatments at each stress challenge ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Values expressed in mean ± SD, $n = 9$ samples/diet/sampling point. Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils).

At the end of the stress challenge, fish subjected to the confinement (C challenge) did not present mortality regardless of dietary treatment. Nevertheless, the combination of both confinement and the pathogen gut inoculation (CI challenge) resulted in a relative percentage of survival (RPS = [1 – (%) surviving fish fed functional diet/(%) surviving fish fed control diet] × 100) of 47% and 33% in fish fed the PHYTO and GMOS diets, respectively (Figure 2), compared to fish fed the control diet [45].

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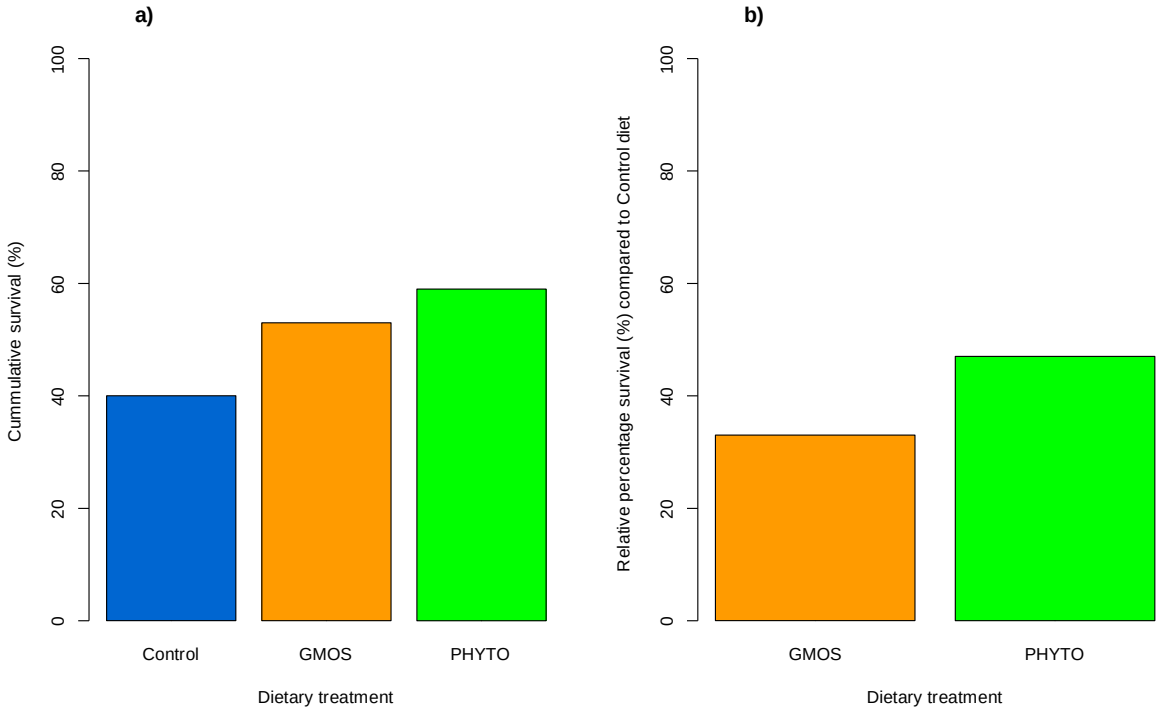

Figure 4.2. (**a**) Bar plot of cumulative survival (%) at the end of the CI challenge: control diet induced a 40% survival; GMOS diet (supplemented with 5000 ppm galactomannan–oligosaccharides) induced a 53% survival, PHYTO diet (supplemented with 200 ppm mixture of garlic and labiate plant essential oils) induced a 59% survival. (**b**) Bar plot of relative percentage of survival (RPS) (%) induced by GMOS and PHYTO diets in comparison to fish fed the control diet: GMOS diet (supplemented with 5000 ppm galactomannan–oligosaccharides) induced a 33% RPS; PHYTO diet (supplemented with 200 ppm mixture of garlic and labiate plant essential oils) induced a 47% RPS. Results previously reported in [41].

When fish were subjected to the stress challenge, two hours after confinement (C challenge) those fed the control diet presented significantly higher ($p < 0.05$) gill-transcript levels of *nfκβ2*, *hif-1α*, *gr*, *nd5*, *coxi*, *sod*, *cat*, *hsp70*, *hsp90*, and *NKA α1a* genes than those fed GMOS and PHYTO diets (Table 4, Figure 3). No differences were found among fish fed the different dietary treatments and subjected to C challenge for *gpx*, *ocln*, and *zo-1* relative gene expression. Similarly, two hours after the CI challenge, fish fed the control diet presented a significant ($p < 0.05$) up-regulation of *nfκβ2*, *hif-1α*, *gr*, *sod*, *gpx*, *NKA α1a*, and *hsp90* gill gene expression compared to gills of fish fed GMOS and PHYTO diets. At this sampling point, fish fed the control diet and subjected to the CI challenge presented significantly lower ($p < 0.05$) *nd5* and *coxi* gill transcript levels than those fed the same diet but subjected to the C challenge.

At 24 h after confinement and in relation to the gene-expression levels observed after 2 h of confinement, fish fed the control diet presented a significant down-regulation ($p < 0.05$) of *nfκβ2*, *gr*, *nd5*, *coxi*, and *hsp70* and an up-regulation ($p < 0.05$) of *zo-1* gill relative gene expression compared to the initial transcript levels at 2 h after confinement (Figure 4, Table 5). On the contrary, fish fed the GMOS diet presented an up-regulation ($p < 0.05$) of *nfκβ2*, *hif-1α*, *gr*, *sod*, *cat*, *hsp70*, and *hsp90* gill relative gene expression and fish fed the PHYTO diet presented an up-regulation of *hif-1α* compared to the previous sampling point at 2 h.

Table 4.4. *Dicentrarchus labrax* gill relative gene-expression values at 2 h after confinement stress challenge (C challenge) or confinement combined with infection with the pathogen *Vibrio anguillarum* (CI challenge).

	Confinement (C Challenge)			Confinement + Infection (CI Challenge)		
	Control	**GMOS**	**PHYTO**	**Control**	**GMOS**	**PHYTO**
nfKβ2	1.02 ^{a} ± 0.26	0.2 ^{b} ± 0.22	0.29 ^{b} ± 0.22	0.99 ^{a} ± 0.20	0.2 ^{b} ± 0.03	0.23 ^{b} ± 0.10
hif-1α	1 ^{a} ± 0.01	0.3 ^{b} ± 0.1	0.4 ^{b} ± 0.2	1 ^{a} ± 0.2	0.4 ^{b} ± 0.01	0.6 ^{b} ± 0.1
gr	1.01 ^{a} ± 0.17	0.21 ^{b} ± 0.2	0.18 ^{b} ± 0.13	0.94 ^{a} ± 0.25	0.21 ^{b} ± 0.02	0.24 ^{b} ± 0.03
nd5	1.06 ^{a*} ± 0.50	0.19 ^{b} ± 0.07	0.30 ^{b} ± 0.19	0.37 ** ± 0.07	0.2 ± 0.02	0.46 ± 0.24
coxi	1 ^{a*} ± 0.14	0.7 ^{b} ± 0.06	0.1 ^{b} ± 0.1	0.22 ** ± 0.09	0.1 ± 0.03	0.1 ± 0.07
sod	1.08 ^{a} ± 0.58	0.2 ^{b} ± 0.26	0.22 ^{b} ± 0.21	0.85 ^{a} ± 0.48	0.3 ^{b} ± 0.13	0.17 ^{b} ± 0.08
cat	1 ^{a} ± 0.09	0.19 ^{b} ± 0.16	0.26 ^{b} ± 0.2	0.73 ± 0.31	0.26 ± 0.17	0.35 ± 0.17
gpx	1 ± 0.13	0.94 ± 0.89	0.42 ± 0.18	3.57 ^{a} ± 2.36	0.42 ^{b} ± 0.26	0.62 ^{b} ± 0.26
ocln	1.01 ± 0.18	0.82 ± 0.74	0.8 ± 0.29	1.48 ± 0.75	0.78 ± 0.01	1.2 ± 0.38
zo-1	1.08 ± 0.56	2.18 ± 0.62	3.02 ± 0.88	2.64 ± 1.7	3.59 ± 1.32	2.94 ± 1.33
hsp70	1 ^{a} ± 0.09	0.11 ^{b} ± 0.08	0.19 ^{b} ± 0.13	0.56 ± 0.04	0.28 ± 0.2	0.16 ± 0.05
hsp90	1 ^{a} ± 0.01	0.2 ^{b} ± 0.2	0.2 ^{b} ± 0.2	1.3 ^{a} ± 0.8	0.2 ^{b} ± 0.01	0.2 ^{b} ± 0.2
NKA α1a	1 ^{a} ± 0.11	0.28 ^{b} ± 0.32	0.26 ^{b} ± 0.13	1.48 ^{a} ± 0.18	0.17 ^{b} ± 0.01	0.44 ^{b} ± 0.26

Different letters denote significant differences among dietary treatments at each stress challenge ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Different numbers of asterisks (*) denote significant differences among C and CI challenge for each dietary treatment ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Values expressed in mean ± SD, $n = 3$ samples/diet/sampling point. Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils). Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^+/K^+ ATPase subunit α1a, *α-tubulin* (housekeeping).

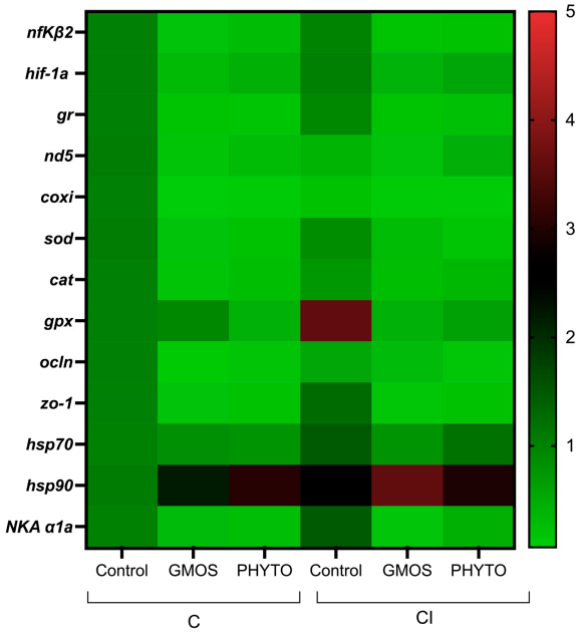

Figure 4.3. Heatmap of *Dicentrarchus labrax* gill relative gene expression at 2 h post stress challenge. Confinement stress challenge (C challenge). Confinement combined with infection with *Vibrio anguillarum* stress challenge (CI challenge). Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils). $n = 3$ samples/diet/challenge. Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^+/K^+ ATPase subunit α1a, *α-tubulin* (housekeeping).

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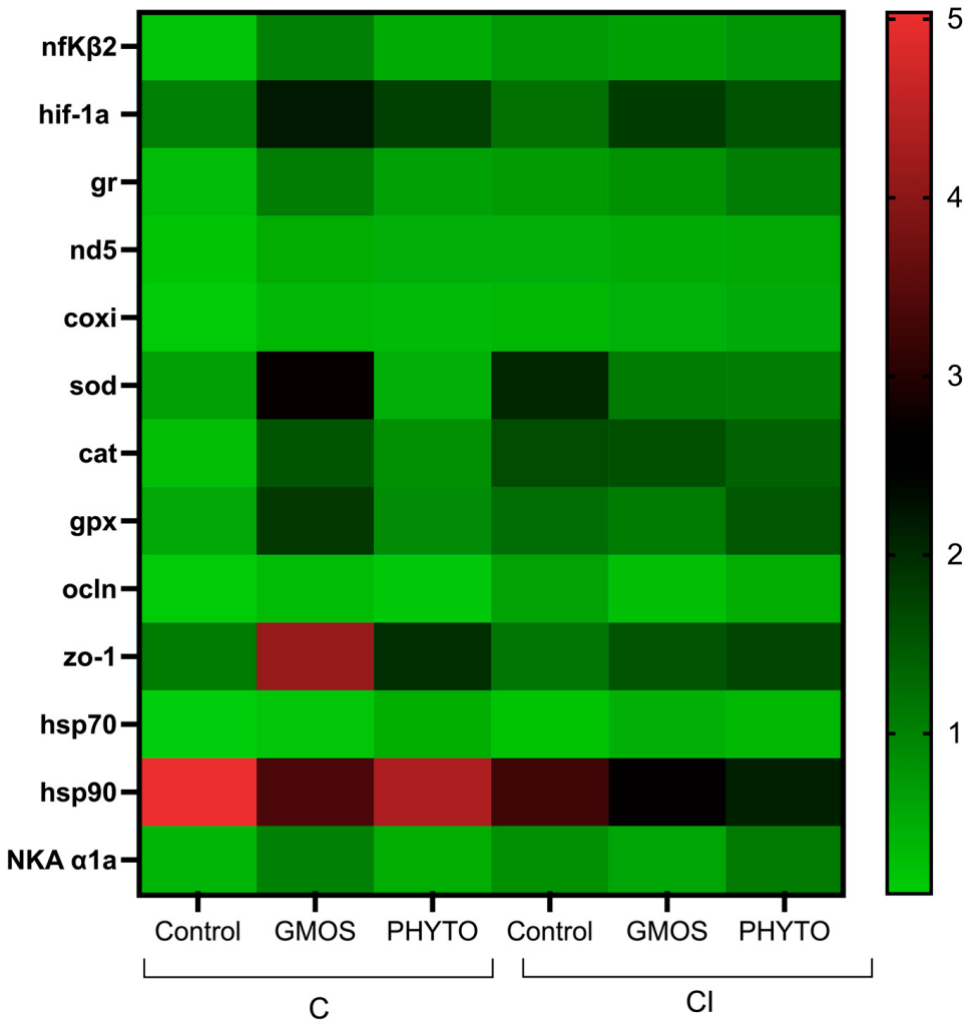


Figure 4.4. Heatmap of *Dicentrarchus labrax* gill relative gene expression at 24 h post stress challenge. Confinement stress challenge (C challenge). Confinement combined with infection with *Vibrio anguillarum* stress challenge (CI challenge). Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils). n = 3 samples/diet/challenge. Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^+/K^+ ATPase subunit α1a, *α-tubulin* (housekeeping).

For fish confined and infected after 24 h post challenge the pattern of response for all the dietary treatments resulted in a down-regulation ($p < 0.05$) of *ocln* gill relative gene expression compared to the previous sampling point, whereas *coxi* and *cat* transcript levels presented a significant up-regulation ($p < 0.05$) compared to the initial transcript levels. Besides, fish fed the control and PHYTO diets presented an up-regulation ($p < 0.05$) of *sod* gill relative gene expression compared to 2 h post CI challenge. Fish fed the GMOS diet, and in relation to 2 h post CI challenge, presented a significant up-regulation ($p < 0.05$) of *hif-1α* gill transcript levels, whereas fish fed the PHYTO diet presented an up-regulation ($p < 0.05$) of *nfκβ2*, *gr*, and *NKA α1a* gill gene expression compared to 2 h post CI challenge.

Table 4.5. *Dicentrarchus labrax* gill relative gene-expression values at 24 h after confinement stress challenge (C challenge) or confinement combined with infection with the pathogen *Vibrio anguillarum* (CI challenge).

	Confinement (C Challenge)			Confinement + Infection (CI Challenge)		
	Control	**GMOS**	**PHYTO**	**Control**	**GMOS**	**PHYTO**
nfKβ2	0.22 ^{b} ± 0.05	1.01^{a} ± 0.04	0.51^{ab} ± 0.31	0.73 ± 0.15	0.66 ± 0.24	0.78 ± 0.11
hif-1a	1 ± 0.3	2.22 ± 0.9	1.82 ± 0.9	1.2 ± 0.2	1.8 ± 1	1.6 ± 0.2
gr	0.30 ± 0.08	1.08 ± 0.35	0.64 ± 0.37	0.71 ± 0.19	0.82 ± 0.52	1.07 ± 0.34
nd5	0.21 ± 0.07	0.5 ± 0.19	0.48 ± 0.39	0.48 ± 0.2	0.52 ± 0.19	0.57 ± 0.29
coxi	0.12 ± 0.06	0.36 ± 0.14	0.33 ± 0.29	0.38 ± 0.10	0.45 ± 0.34	0.54 ± 0.4
sod	0.63 ^{b}* ± 0.23	2.72 ^{a}* ± 0.24	0.48 ^{b} ± 0.15	2.4 ** ± 0.03	1.1 ** ± 0.17	1.06 ± 0.17
cat	0.28 ± 0.17	1.53 ± 0.23	0.84 ± 0.63	1.64 ± 1.34	1.59 ± 0.99	1.38 ± 1.03
gpx	0.55 ^{b} ± 0.26	1.85 ^{a} ± 0.6	0.88 ^{ab} ± 0.57	1.24 ± 0.09	1.1 ± 0.49	1.51 ± 0.29
ocln	0.1 ^{b} ± 0.03	0.18 ^{ab} ± 0.11	0.49 ^{a} ± 0.3	0.24 ± 0.1	0.47 ± 0.07	0.38 ± 0.15
zo-1	5.04 * ± 1.69	3.35 * ± 0.93	4.33 * ± 1.04	3.24 ** ± 0.6	2.68 ** ± 1.21	2.14 ** ± 1.08
hsp70	0.12 * ± 0.04	0.32 ± 0.01	0.16 ± 0.04	0.63 ** ± 0.2	0.28 ± 0.13	0.51 ± 0.23
hsp90	1.1 ^{b} ± 0.5	4.12 ^{a} ± 1.3	2 ^{ab} ± 1.9	1.2 ± 0.4	1.5 ± 1.7	1.7 ± 0.4
NKA α1a	0.39 ± 0.24	1.03 ± 0.45	0.51 ± 0.08	0.85 ± 0.42	0.6 ± 0.22	1.1 ± 0.09

Different letters denote significant differences among dietary treatments at each stress challenge ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Different numbers of asterisks (*) denote significant differences among C and CI challenge for each dietary treatment ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Values expressed in mean ± SD, $n = 3$ samples/diet/sampling point. Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils). Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^{+}/K^{+} ATPase subunit α1a, *α-tubulin* (housekeeping).

Within the 24 h sampling point for the C challenge, fish fed the GMOS diet presented higher ($p < 0.05$) gill gene-transcript levels of *nfκβ2*, *sod*, *gpx*, and *hsp90* than those fed the control diet, as well as higher ($p < 0.05$) *sod* gill relative gene-expression values than those fed the PHTYO diet. Similarly, fish fed the PHYTO diet presented higher ($p < 0.05$) gill relative gene expression of *ocln* than those fed the control diet. On the other hand, in fish subjected to the CI challenge after 24 h of stress challenge, the diet did not induce significant differences in the gill relative gene expression of the target genes, despite both stress challenges differing between them in the gill relative gene-expression patterns presented. At 24 h post CI challenge, those fish fed the control diet presented higher ($p < 0.05$) *sod* and *hsp70* gene-expression levels than those fed the same dietary treatment but subjected to the C challenge. On the contrary, the CI challenge induced a significant down-regulation ($p < 0.05$) of *sod* gill transcript levels in fish fed the GMOS diet and subjected to the C challenge. Regardless of the dietary treatment, the CI challenge induced a significant down-regulation ($p < 0.05$) of *zo-1* relative gene expression compared to C challenge after 24 h post challenge.

At the end of the confinement stress challenge (168 h after C challenge), fish fed the GMOS diet presented a significant down-regulation ($p < 0.05$) of *sod* and *cat* gill transcript levels (Table 6, Figure 5) down to the levels observed at 2 h post challenge, as presented by fish fed the PHYTO diet for *zo-1* gill relative gene expression. Fish fed the control diet and confined presented a significant down-regulation ($p < 0.05$) of *NKA α1a* gill relative gene expression compared to the previous sampling points at 2 h and 24 h post C challenge. In the case of the CI challenge, fish fed GMOS presented a significant down-regulation ($p < 0.05$) of sod gill gene-expression levels down to the initial levels.

Table 4.6. *Dicentrarchus labrax* gill relative gene-expression values at 168 h after confinement stress challenge (C challenge) or confinement combined with infection with the pathogen *Vibrio anguillarum* (CI challenge).

	Confinement (C Challenge)			Confinement + Infection (CI Challenge)		
	Control	**GMOS**	**PHYTO**	**Control**	**GMOS**	**PHYTO**
nfKβ2	0.27 ± 0.17	0.47 ± 0.18	0.40 ± 0.08	0.55 ± 0.08	0.34 ± 0.11	0.43 ± 0.10
hif-1a	1 ± 0.3	1.3 ± 0.3	1 ± 0.3	1.1 ± 0.2	0.7 ± 0.3	0.7 ± 0.2
gr	0.24 ± 0.18	0.46 ± 0.09	0.6 ± 0.13	0.53 ^{a} ± 0.04	0.27 ^{b} ± 0.11	0.65 ^{a} ± 0.17
nd5	0.25 ± 0.16	0.26 ± 0.11	0.39 ± 0.11	0.35 ± 0.03	0.19 ± 0.06	0.46 ± 0.21
coxi	0.17 ± 0.16	0.17 ± 0.13	0.21 ± 0.07	0.28 ± 0.02	0.11 ± 0.06	0.29 ± 0.1
sod	0.31 * ± 0.2	0.47 ± 0.19	0.38 ± 0.19	1.6 ^{a}** ± 0.56	0.31 ^{b} ± 0.17	0.54 ^{b} ± 0.13
cat	0.37 ± 0.33	0.54 ± 0.33	0.59 ± 0.12	1.11 ± 0.62	0.51 ± 0.10	0.68 ± 0.25
gpx	0.74 ± 0.22	1.17 ± 0.19	1.02 ± 0.11	0.96 ^{b} ± 0.25	0.46 ^{b} ± 0.03	1.39 ^{a} ± 0.38
ocln	0.68 ^{a}* ± 0.09	0.51 ^{ab}*± 0.19	0.20 ^{b} ± 0.02	0.17 ^{b}** ± 0.02	0.89 ^{a}** ± 0.14	0.43 ^{ab} ± 0.01
zo-1	2.47 ± 0.15	1.44 * ± 0.64	1.57 ± 0.66	2.62 ^{ab} ± 1.31	3.57 ^{a}** ± 0.62	0.86 ^{b} ± 0.11
hsp70	0.12 * ± 0.05	0.17 ± 0.06	0.15 ± 0.06	0.47 ** ± 0.01	0.19 ± 0.05	0.26 ± 0.08
hsp90	2 ± 2.1	4.5 * ± 1.4	3.3 ± 0.7	1.1 ± 0.3	0.7 ** ± 0.4	1.2 ± 0.1
NKA α1a	0.2 ^{b}* ± 0.1	0.51 ^{a} ± 0.04	0.37 ^{ab} ± 0.08	0.61 ** ± 0.11	0.64 ± 0.08	0.49 ± 0.1

Different letters denote significant differences among dietary treatments at each stress challenge ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Different numbers of asterisks (*) denote significant differences among C and CI challenge for each dietary treatment ($p < 0.05$, two-way ANOVA: stress challenge x dietary treatment, Tukey post-hoc test). Values expressed in mean ± SD, $n = 3$ samples/diet/sampling point. Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils). Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^+/K^+ ATPase subunit α1a, *α-tubulin* (housekeeping).

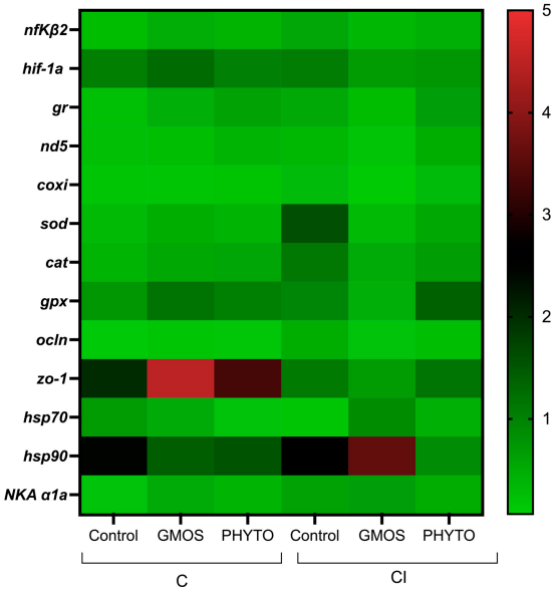


Figure 4.5. Heatmap of *Dicentrarchus labrax* gill relative gene expression at 168 h post stress challenge. Confinement stress challenge (C challenge). Confinement combined with infection with *Vibrio anguillarum* stress challenge (CI challenge). Control (control diet), GMOS (GMOS diet, 5000 ppm galactomannan–oligosaccharides), PHYTO (PHYTO diet, 200 ppm mixture of garlic and labiate plant essential oils). $n = 3$ samples/diet/challenge. Target genes: *nfκβ2*: nuclear factor kappa beta-2, *hif-1α:* hypoxia-inducible factor 1 alpha, *gr*: glucocorticoid receptor, *nd5*: NADH dehydrogenase subunit 5, *coxi*: cytochrome c oxidase subunit 1, *sod*: superoxide dismutase, *cat*: catalase, *gpx*: glutathione peroxidase, *zo-1*: zonula occludens-1, *ocln*: occludin, *hsp70*: heat-shock protein 70, *hsp90*: heat-shock protein 90, *NKA α1a*: Na^+/K^+ ATPase subunit α1a, *α-tubulin* (housekeeping).

Within this last sampling point (168h), fish from the C challenge and fed GMOS diet presented higher ($p < 0.05$) *NKA α1a* gill relative gene expression than those fed the control diet, whereas fish fed the PHYTO diet presented lower ($p < 0.05$) gill gene expression *ocln* than those fed the control diet. Regarding the CI challenge, fish fed the control diet presented higher ($p < 0.05$) gill *sod* transcript levels than fish fed the GMOS and PHYTO diets, whereas fish fed the control and PHYTO diet presented higher ($p < 0.05$) *gr* gill relative gene-expression values than those presented by fish fed the GMOS diet. On the contrary, fish fed the GMOS diet presented higher ($p < 0.05$) *ocln* and *zo-1* gill gene-expression levels than fish fed the control and PHYTO diets, respectively.

At 168 h after CI challenge, the infection itself induced significantly higher ($p < 0.05$) *sod*, *NKA α1a*, and *hsp70* gill gene-transcript levels, whereas it down-regulated ($p < 0.05$) *ocln* gill gene expression. In the case of the fish fed the GMOS diet, the CI challenge significantly increased ($p < 0.05$) *ocln* and *zo-1* gill relative gene expression and reduced ($p < 0.05$) *hsp90* in comparison to fish subjected to the C challenge. No effects were detected for fish fed the PHYTO diet when comparing the C and CI challenges at the end of the stress trial.

4. Discussion

Stress induces a physiological response to reestablish fish homeostasis, which is orchestrated by the release of cortisol into the bloodstream. As expected, in the present study an increased concentration of fish circulating plasma concentration was observed in the few hours after the initiation of both stress challenges and regardless of the dietary treatment. However, both GMOS and PHYTO diets attenuated fish stress response, with supplemented fish presenting lower circulating-cortisol levels than fish fed the control diet. In response to the stress process, the organism undergoes an alarm status in which the energetic resources are rearranged in order to cope with the surplus of activity. In this sense, gills play a fundamental role in energy supply with the activation of ATP synthesis [20,51]. In the present study, and in agreement with the attenuated stress response observed in cortisol patterns for supplemented fish, at 2 h after both the C and CI challenge, fish fed the GMOS and PHYTO diets presented lower *gr*, *nd5*, and *coxi* gill relative gene expression than fish fed the control diet. This may suggest a lower responsiveness of fish fed functional diets against the stressor, with a lower activity of the ETC and thus lower energetic requirements to cope with the stress process [51]. Nevertheless, fish fed the GMOS and PHYTO diets presented an up-regulation of *gr* relative gene expression at 24 h after the stress challenges. This delayed increase in *gr* gene expression in fish fed the supplemented diets could be understood as a mechanism to restore the GR protein content after exposure to glucocorticoids. Vijayan t al. (2013) reported on an in vitro experiment using hepatocytes, in which down-regulated *gr* gene expression was found in treatments presenting the higher GR protein contents [52].

Considering that the ETC is one of the major sources of endogenous ROS [20,34,53], an increased aerobic-metabolism rate in response to a stress process may induce elevated ROS production, leading to oxidative stress [21]. In the present study, at 2 h after the C challenge, fish fed the functional diets presented lower *sod* and *cat* gill gene-expression levels. A lower activation of the endogenous antioxidant defenses could suggest an attenuated stress response, leading to a lower production of mitochondrial ROS and thus an attenuated response against the stress processes. Although both functional diets reduced fish-stress and oxidative-stress response, each functional diet induced a different antioxidant response against the confinement stress challenge. In fact, along with the stress challenge, fish fed PHYTO did not presented an activation of endogenous antioxidant machinery gene expression. This lack of response in the endogenous antioxidant defenses could be present and associated with the antioxidant properties of the phytogenic compounds, making them capable of inhibiting ROS formation and quenching them once they are formed [54–56]. Indeed, our previous studies in this mucosal tissue indicated that both functional additives can reduce gill oxidative-stress status in basal conditions after dietary supplementation [42]. Between the different plant-origin biomolecules, the flavonoids are

polyphenolic compounds with high antioxidant properties and can be found in a wide spectrum of plant extracts as garlic oil and labiate plant extracts, such as origanum [57] and thyme [58]. On the other hand, fish fed the GMOS diet presented an increase in *sod* and *cat* gill gene expression at 24 h after the C challenge. In addition, fish fed the GMOS diet presented higher *gpx* gill transcript levels than fish fed the other dietary treatments. Interestingly, our previous studies indicated a similar delayed pattern of response against the stressor in other mucosal tissues, inducing GMOS supplementation as a controlled and prolonged intestinal-mucus secretion in response to the CI challenge, reducing gut bacterial-translocation rates and thus increasing pathogen resistance and survival rates [41]. Similarly, in the present study both functional diets also attenuated fish antioxidant-related gill gene response against the CI challenge, with fish fed the functional diets presenting lower values of *sod* and *gpx* gene expression than fish fed the control diet in the early hours after bacterial gut inoculation. Nevertheless, 24 h after the CI challenge the functional-diet supplementation induced an up-regulation of *sod*, *cat*, and *gpx* gill gene expression. This increase in the expression of antioxidant-related genes might suggest a response against the stress associated with the pathogen gut inoculation and with an organism arrangement to cope with future infection. In particular, dietary PHYTO increased fish *gpx* gene expression and kept it up-regulated throughout the entire stress challenge. Similarly, Mansour et al. (2020) observed a higher antioxidant capacity in gilthead sea-bream (*Sparus aurata*) gills and skin, with an increased gene expression of *sod* and *cat* after feeding diets supplemented with *Moringa oleifera* leaf extracts [59].

The gills, skin, and intestine are the first barrier interacting with the external environment, playing a fundamental role in maintaining tissue structure and integrity, regulating solute trafficking across the gill epithelium and thus facilitating or limiting paracellular-ion movement [60]. For this reason, cell-junction complexes play a fundamental role in maintaining gill-epithelium integrity and functioning. Damage to fish-gill structural integrity like that originated by oxidative-stress processes may lead to degenerative processes such as gas-exchange disturbances [61] and impairments to immune functions [62]. In our previous studies, Torrecillas et al. (2021) observed the ability of GMOS and PHYTO diets to induce higher gill gene expression of *zo-1* and *ocln*, respectively, in basal conditions [42]. In the present study, the use of different dietary treatments did not affect fish gill *ocln* and *zo-1* relative gene expression in the early hours after both stress challenges. However, 24 h after confinement, fish fed all the dietary treatments presented an increase in *zo-1* gill gene expression despite it only being significant in fish fed the control diet, with fish fed PHYTO diet at 24 h post confinement presenting the highest expression levels. In concordance with these results, Zhao et al. (2020) described an up-regulated gene expression of *ocln* and *zo-1* genes in the gills of *Ctenopharyngodon idella*-fed diets supplemented with *Allium monoglicum* Regel flavonoids (AMRF), alleviating the oxidative stress and toxicity derived from chromium exposure [56]. In the same way, Trujillo et al. (2015), described the ability of curcumin to prevent cisplatin-induced fibrosis and decreased tight-junction proteins in rat kidney [63]. These protective effects of phytogenic feed additives could be related to the ability of those plant-origin compounds to interact with MAPK receptors, preventing H_2O_2-induced tight-junction disruptions [64]. On the contrary, at 24 h after challenge when the fish were subjected to the CI challenge and at 24 h after stress all the dietary treatments presented a down-regulation of *ocln* gill gene expression. Moreover, the CI challenge induced a down-regulation of *zo-1* compared to the C challenge. Acute inflammatory processes are characterized by the hyper-permeabilization of tissues, allowing inflammatory mediators and immune cells to infiltrate the damaged tissues [65]. In this sense, a down-regulation of genes related to tight-junction structure maintenance could be related to a preparation process to facilitate the response against a future infection. The inflammatory response acquires a critical importance in the gills, considering the high number of permanent-resident lymphocytes and immune cells associated with the gill-associated lymphoid tissue (GIALT) [66]. In the present study, both the C and CI challenge induced an acute response of *nfκβ2* gill gene expression after stress challenge in fish fed the control diet,

whereas fish fed the GMOS and PHYTO diets presented an attenuated pro-inflammatory response against the stressors, with the highest *nfκβ2* gill gene-expression levels at 24 h after being subjected to the C and CI challenge. Previous studies have reported on the ability of phytogenic compounds derived from oregano, curcumin, and thymol to modulate pro-inflammatory response in fish [67–69]. These compounds have been shown to be able to directly regulate the NFKB- and mitogen-activated protein kinase (MAPK)-signaling pathways, attenuating the inflammatory response [27]. In the case of GMOS, the mechanism that can modulate European sea-bass immune and stress response differs from the phytogenic compounds, as the animal does not directly harness the prebiotics. The by-products from prebiotic fermentation generated by the host microbiome may produce short-chain fatty acids (SCFAs), which can modulate fish innate immune response and inflammatory cells [70] by interacting with immune-cell pattern-recognition receptors [71]. Inflammatory processes are characterized by an increased leukocyte infiltration [5], which may lead to hypoxia conditions due to the high amount of O_2 consumed by the increased phagocytic activity [72]. In response to the hypoxia, the *nfκβ2* triggers the activation of the *hif-1α*, inducing a metabolic swift into a glycolytic strategy, facilitating leukocyte survival in a hypoxic medium [73]. In the present study, fish fed the functional diets presented lower *hif-1α* gill gene-expression levels than those fed the control diet, supporting the idea of an attenuated pro-inflammatory response in the early hours after the stress. Nevertheless, at 24 h after stress challenge, and in parallel with an increased transcription of the *nfκβ2*, both functional diets induced an up-regulation of *hif-1α* gill gene expression. This could suggest a better protection of the immune-cell populations, leading to a better ability to cope with the deleterious effects derived from a prolonged inflammatory response against a stressor, which may also be related with the lower infection rates and higher survival observed in CI fish fed the functional diets [41]. Indeed, the fish fed the control diet presented no significant changes in *hif-1α* gill gene-expression levels regardless of the variations in the *nfκβ2* gene transcripts during the different stress challenges. A similar response was observed in previous reports, in which the same functional diets protected head-kidney leukocyte populations against apoptotic processes by attenuating head-kidney pro-inflammatory response and increasing *hif-1α* head-kidney relative gene expression after CI challenge [30]. Another mechanism promoting tissue integrity in response to a stressor is the activation of the heat-shock proteins, which are overexpressed to act as molecular chaperones associated with the GR avoiding protein denaturation, refolding denatured proteins and promoting misfolded-protein degradation [74–76]. In response to an acute stress process, the *hsp70* and *hsp90* gene expression is increased, activating the necessary mechanisms to respond to the stressor [77]. In the present study, in fish fed the control diet both C and CI challenges induced an overexpression of gill *hsp70* in the first hours after the challenge, followed by a strong down-regulation until the end of the stress challenges. Meanwhile, the fish fed the GMOS diet presented a delayed *hsp70* and *hsp90* gene-expression pattern, with the highest expression levels at 24 h after stress, indicating an attenuated response to the stress. In concordance, in previous studies dietary supplementation with fructo-oligosaccharides in blunt-snout bream (*Megalobrama amblycephala*) induced an increase in *hsp70* and *hsp90* at 24 hours after confinement stress [78].

Na^+/K^+ ATPase is an ATP-dependent transmembrane enzyme that plays a fundamental role in maintaining cell ionic homeostasis. This protein is highly represented in the gills and confers an important osmoregulatory role to the tissue [79]. In the present study, at 2 h after C and CI challenges, the fish fed the control diet presented a strong up-regulation of *NKA α1a* gill gene expression, indicating an acute response to the stressor. At the end of the confinement stress challenge, these fish presented a down-regulated gene expression of the *NKA α1a*. Alterations to cellular ionic balance may lead to the entrance of sodium (Na^+) [23,80] and thus disturb the osmotic balance, leading to membrane ruptures [43]. When fish were subjected to the CI challenge, the control group presented the same pattern of response but the *NKA α1a* gene expression remained unchanged throughout the stress challenge, being highest at the end of the CI challenge. Meanwhile, and regardless of the

stress challenge, fish fed the functional diets presented the higher values of relative gene expression of *NKA α1a* at 24 h after stress, indicating an attenuated response to the stressor. The fish fed the functional dies did not show a down-regulation of *NKA α1a*, which could suggest a more prolonged activity of the Na^+/K^+ ATPase and thus a better capacity to cope with the imbalances originated during the stress process, which in turn may also be related to the lower infection rates observed in supplemented fish.

5. Conclusions

In conclusion, both GMOS (5000 ppm) and PHYTO (200ppm) functional additives in 10% FM/6% FO diets induced a down-regulation of the *nf-κβ* relative gene expression in the gill during the stress challenge, leading to a controlled inflammatory response against the stressor. The functional diets attenuated fish stress response, leading to a stable energy metabolism and an ameliorated antioxidant status. Altogether, this indicates the potential of both functional additives to reduce the associated negative effects of stress in European sea bass fed a low-FM/FO diet. Owing to the diverse methods of action of the different functional additives analyzed in the present study, more experiments must be carried out to fully understand the potential effects on fish health and stress response.

Author Contributions: Conceptualization, S.T., M.S.I., A.M. and D.M.; formal analysis, G.T.; data curation, A.S.; software, A.S.; validation, S.T., Á.F.-M. and D.M.; writing—original draft preparation, A.S.; writing—review and editing, S.T., D.M, G.T., Á.F.-M. and V.V.; project administration, D.M.; funding acquisition, S.T., M.S.I., F.A. and D.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Spanish Ministry of Economy, Industry and Competitiveness ("Subprograma Estatal de Generación de Conocimiento, en el marco del Plan Estatal de Investigación Científica y Técnica y de Innovación 2013–2016") by the PROINMUNOIL PLUS (AGL2016-79725-P) project "Functional diets for marine raw materials replacement: boosting the fish disease resistance through epithelial barriers reinforcement and immunization tools".

Institutional Review Board Statement: The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the University of Las Palmas de Gran Canaria (approval no. OEBA_ULPGC_14/2020) for studies involving animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank the Spanish Ministry of Economy, Industry and Competitiveness for the complementary funding for ST though the "Subprogama Juan de la Cierva- Incoproración, Convocatoria 2015", IJCI-2015-25748. Special thanks to Delacon Biotechnik GmbH and to BioMar A/S, which provided the functional products and diets.

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

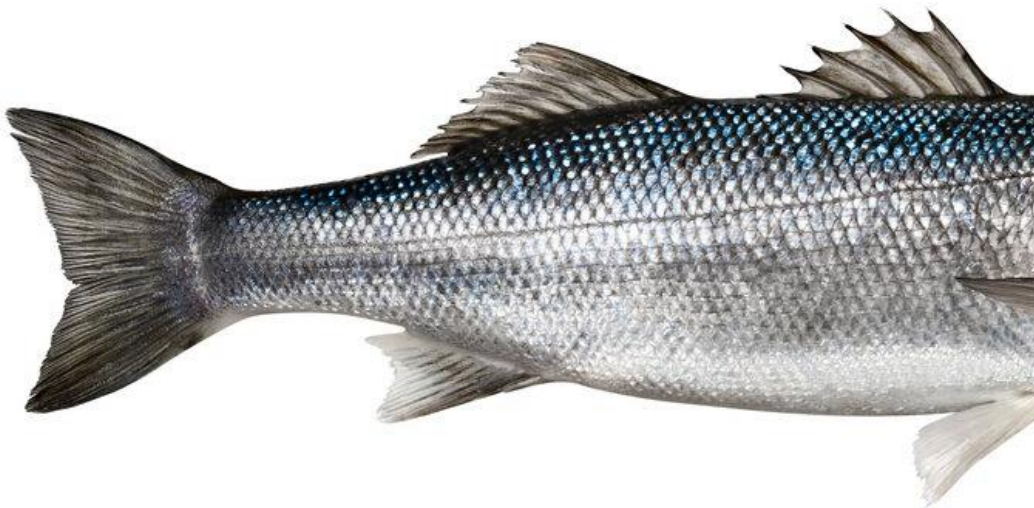
Abbreviations

ATP	(adenosine triphosphate)
C	challenge (confinement stress challenge)
cat	(catalase)
CI challenge	(confinement combined with infection stress challenge)
coxi	(cytochrome oxidase c)
ETC	(electron-transport chain)
FM	(fishmeal)
FO	(fish oil)
Fw	(forward primer sequence)
GIALT	(gill-associated lymphoid tissue)
GMOS	(galactomannan–oligosaccharides)
gpx	(glutathione peroxidase)

gr	(glucocorticoid receptor)
hif-1α	(hypoxia inducible factor 1 α)
hsp70	(heat-shock protein 70)
hsp90	(heat-shock protein 90)
MAPKs	(mitogen-activated protein kinases)
MRCs	(mitochondria-rich cells)
nd5	(NADH dehydrogenase subunit 5)
nfkb2	(nuclear factor kappa beta)
NKA α 1a	(Na^+/K^+ ATPase)
ocln	(occludin)
PAMPs	(pathogen-associated molecular pattern)
PFAs	(phyoteginc feed additives)
PHYTO	(phytogenic)
ROS	(reactive oxygen species)
Rv	(reverse primer sequence)
sod	(superoxide dismutase)
zo-1	(zonula occludens)

Chapter V

Functional Additives in a Selected European Sea Bass (*Dicentrarchus labrax*) Genotype: Effects on the Stress Response and Gill Antioxidant Status Response to Hydrogen Peroxide (H_2O_2) esposure



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Functional Additives in a Selected European Sea Bass (*Dicentrarchus labrax*) Genotype: Effects on the Stress Response and Gill Antioxidant Response to Hydrogen Peroxide (H_2O_2) Treatment

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Simple Summary: Husbandry practices in aquaculture production may lead to stress processes and oxidative stress damages on fish tissues. Functional ingredients have profiled as suitable candidates for reinforcing the fish antioxidant response and stress tolerance. In addition, selective breeding strategies have also demonstrated a correlation between fish growth and stress reactiveness, which may be a key component in species domestication. The present study evaluates the potential of three different functional additives for gill endogenous antioxidant capacity and stress relief in a growth selected genotype of European sea bass (*Dicentrarchus labrax*) juveniles fed low-FM/FO diets. For this purpose, after 72 days of a feeding trial, all fish were subjected to an oxidative stress challenge consisting of a 1 h bath exposure to hydrogen peroxide (H_2O_2) at a total concentration of 50 ppm. The functional additives induced a better recovery from the stress process, with a higher reduction in fish circulating plasma cortisol 24 h after oxidative stress. In addition, the functional additives induced higher catalase gill gene expression in response to the oxidative stress insult.

Abstract: Functional ingredients have profiled as suitable candidates for reinforcing the fish antioxidant response and stress tolerance. In addition, selective breeding strategies have also demonstrated a correlation between fish growth performance and susceptibility to stressful culture conditions as a key component in species domestication processes. The aim of the present study is to evaluate the ability of a selected high-growth genotype of 300 days post-hatch European sea bass (*Dicentrarchus labrax*) juveniles to use different functional additives as endogenous antioxidant capacity and stress re- sistance boosters when supplemented in low fish meal (FM) and fish oil (FO) diets. Three isoenergetic and isonitrogenous diets (10% FM/6% FO) were supplemented with 200 ppm of a blend of garlic and *Labiatae* plant oils (PHYTO0.02), 1000 ppm of a mixture of citrus flavonoids and *Asteraceae* and *Labiatae* plant essential oils (PHYTO0.1) or 5000 ppm of galactomannan-oligosaccharides (GMOS0.5). A reference diet was void of supplementation. The fish were fed the experimental diets for 72 days and subjected to a H_2O_2 exposure oxidative stress challenge. The fish stress response was evaluated through measuring the circulating plasma cortisol levels and the fish gill antioxidant response by the relative gene expression analysis of *nfKβ2*, *il-1b*, *hif-1a*, *nd5*, *cyb*, *cox*, *sod*, *cat*, *gpx*, *tnf-1α* and *caspase 9*. After the oxidative stress challenge, the genotype origin determined the capacity of the recovery of basal cortisol levels after an acute stress response, presenting GS fish with a better pattern of recovery. All functional diets induced a significant upregulation of *cat* gill gene expression levels compared to fish fed the control diet, regardless of the fish genotype. Altogether, suggesting an increased capacity

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Citation: Serradell, A.; Montero, D.; Terova, G.; Rimoldi, S.; Makol, A.; Acosta, F.; Bajek, A.; Haffray, P.; Allal, F.; Torrecillas, S. Functional Additives in a Selected European Sea Bass (*Dicentrarchus labrax*) Genotype: Effects on the Stress Response and Gill Antioxidant Response to Hydrogen Peroxide (H_2O_2) Treatment. *Animals* **2023**, *13*, 2265. https://doi.org/10.3390/ani13142265

Academic Editors: Sofia Engrola, Cláudia Aragão, Rita Teodósio and Maria João Xavier

Received: 26 April 2023
Revised: 6 July 2023
Accepted: 9 July 2023
Published: 11 July 2023

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of the growth selected European sea bass genotype to cope with the potential negative side-effects associated to an H_2O_2 bath exposure.

Keywords: phytogenics; galactomannan-oligosaccharides; selective breeding; European sea bass (*Dicentrarchus labrax*); oxidative stress; stress response

1. Introduction

The use of biocide compounds is an extended practice in aquaculture production in order to eliminate microorganisms and other pathogenic agents in aquaculture facilities [1,2]. Among them, hydrogen peroxide (H_2O_2) is a powerful oxidizer compound used against fish external parasites and bacteria [3–5] with proven effectiveness in treating diseases in European sea bass (*Dicentrarchus labrax*) [6,7]. However, this compound is an important source of reactive oxygen species (ROS), which may induce severe tissue damages, especially on those directly exposed to the surrounding environment [8,9].

Fish gills act as a physical and biochemical semipermeable barrier with an important role in fish respiratory processes, hydromineral balance and immune responses [10,11]. In response to a stress process, such as those derived from biocides or other pollutants exposure, cortisol will target gill tissue, triggering mitochondrial rich cells (MRCs) oxidative phosphorylation (OXPHOS) in order to supply ATP to the Na^+/K^+ ATPase pumps involved in fish hydromineral and osmotic balance reestablishment [12,13]. During OXPHOS, some electrons may leak the electronic transport chain (ETC) prior to being reduced by the cytochrome c oxidase, reacting in the mitochondrial intermembrane with oxygen (O_2) to form superoxide anions (O^{2-}) [14,15]. Then, superoxide$^-$ will be transformed by the superoxide dismutase enzyme (SOD) into H_2O_2, which will be finally detoxified by catalase (CAT) and glutathione peroxidase (GPX) into water and O_2 [16]. Nevertheless, in a high-stress-susceptible fish species such as the European sea bass [17,18], the cumulative effects of both internal and external ROS increased concentrations may overwhelm fish antioxidant defense, leading to oxidative stress processes including cellular membrane lipid peroxidation and protein and DNA destruction [14,19].

Supplementing fish diets with phytogenic feed additives (PFAs) has shown potential in reinforcing the fish antioxidant status [20,21]. PFAs are plant-derived bioactive compounds with elevated contents of flavonoids, tannins and mucilages with high antioxidant properties [22–24]. Additionally, supplementing fish diets with PFAS has been reported to be capable of attenuating different fish species' stress responses [25–29]. Plant-derived prebiotic compounds are another variety of functional additives with a potential reinforcing fish antioxidant defense [22,29]. Prebiotics have the ability to benefit the host health by selectively modulating the fish microbiome composition [30]. In previous studies, the galactomannan-oligosaccharides (GMOS) protected European sea bass juveniles' gills against the damages derived from oxidative stress [26,31,32]. Even though a wide variety of studies report the benefits of functional additives supplementation, the mechanisms by which these compounds may favor the fish health status and welfare are still not clearly defined. Several factors can define the functional additives' effects on fish performance, such as the different bioactive compounds' properties, dietary inclusion levels, dietary production methodologies and the fish's capacity to harness these products [29,31,33–35]. In this scenario, selective breeding has been recognized as a permanent and cumulative solution for improving fish feed efficiency and feed utilization [36–38], resulting in increased growth performance and better health and welfare [39]. Fish genotype selection may also contribute to increased growth performance, even coping with the nutritional variations associated with low fish meal (FM)- and fish oil (FO)-based diets [40]. Furthermore, one of the main effects of selective breeding is the fish species domestication processes by which the captive species becomes adapted to the rearing conditions [36], reducing the negative side effects associated with cultured conditions' stress processes [41].

Accordingly, the aim of the present study was to determine the gill antioxidant capacity and stress tolerance against an H_2O_2 exposure oxidative stress challenge in a growth selected European sea bass genotype fed low FM/FO-based diets supplemented with three different plant-derived functional additives, PHYTO0.02, PHYTO0.01 or GMOS0.5.

2. Materials and Methods

2.1. Experimental Diets

Four low FM/FO (10%/6%) diets with isoenergetic and isonitrogenous formulations were produced by Biomar (Brande, Denmark), meeting the nutritional requirements for European sea bass juveniles [42,43]. A reference diet void of supplementation (Control), a diet supplemented with 200 ppm of a blend of garlic and *Labiatae* plant oils (87.5 mg terpenes/kg diet; PHYTO0.02), a diet supplemented with 1000 ppm of a mixture of cit- rus flavonoids and *Asteraceae* and *Labiatae* plant essential oils (57 mg terpenes/kg diet; PHYTO0.1) and a diet supplemented with 5000 ppm of galactomannan-oligosaccharides (GMOS0.5). The functional ingredients were supplemented according to the producer's recommendations (Delacon, Engerwitzdorf, Austria). The PHYTO0.1 and GMOS0.05 additives were included in the mix during the pre-extrusion process in order to ensure product stability. The PHYTO0.02 additive was homogenized with the dietary oils and included by vacuum coating during the post-extrusion process (Table 1).

Table 5.1. Main ingredients and analyzed proximal composition of the experimental diets.

Ingredients	Diet (%)			
	Control	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**
Fish meal [1]	9.6	9.6	9.6	9.6
Soya protein concentrate	18.2	18.2	18.2	18.2
Soya meal	11.6	11.6	11.6	11.6
Corn gluten meal	24.1	24.1	24.1	24.1
Wheat	8.585	8.565	8.485	8.085
Wheat gluten	1.9	1.9	1.9	1.9
Guar meal	7.7	7.7	7.7	7.7
Rapeseed extracted	3.0	3.0	3.0	3.0
Fish oil [2]	6.5	6.5	6.5	6.5
Rapeseed oil [3]	5.2	5.2	5.2	5.2
Vitamin and mineral premix [4]	3.6	3.6	3.6	3.6
Antioxidant [5]	0.015	0.015	0.015	0.015
Phytogenic (garlic and *Labiatae* plant essential oils) [6]	0	0.02	0	0
Phytogenic (citrus fruits and *Asteraceae* and *Labiatae* plant essential oils) [7]	0	0	0.1	0
Galactomannan-oligosaccharides (GMOS) [8]	0	0	0	0.5
Proximate composition (% of dry matter)				
Crude lipids	19.91	20.44	20.44	20.47
Crude protein	49.30	49.27	49.27	49.76
Moisture	5.10	5.01	5.01	5.06
Ash	7.02	6.41	6.41	6.49

Dietary ingredient composition and proximal composition expressed as % of dry weight. Control (Control diet), PHYTO0.02 (PHYTO diet, 200 ppm mixture of garlic and *Labiatae* plant essential oils), PHYTO0.1 (PHYTO diet, 1000 ppm mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils), GMOS (GMOS diet, 5000 ppm galactomannan-oligosaccharides). [1] South American, Superprime 68%. [2] South American fish oil. [3] DLG AS, Denmark. [4] Vilomix, Denmark. [5] BAROX BECP, BHT. [6] Delacon Biotechnik GmbH, Austria. [7] Delacon Biotechnik GmbH, Austria. [8] Delacon Biotechnik GmbH, Austria.

2.2. Population Design and Fish Production

The experimental design contemplated two European sea bass genotypes, a high growth selected genotype (GS) and a wild type genotype (WT), with the same scheme of selection and details previously described in [40,44].

Briefly, both genotypes were produced in the facilities of Palavas-les-flot (France) by mating 7 dams selected for growth from the MARBEC-IFREMER broodstock with 33 sires

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(genetically selected, GS) derived from the breeding nucleus of the EMG Ecloserie Marine de Gravelines (Gravelines, France) breeding company or 32 wild sires captured in the gulf of Lion (Wild type genotype, WT). Dams' eggs were collected by stripping and pooled in equal representation between dams, and they were transferred into 65 tubes (one per sire). The two resulting genotypes were incubated separately at 14 °C until hatching. One- day-old hatched larvae were pooled by the equi-representation of each dam and shipped to the University of Las Palmas de Gran Canaria (ULPGC, Las Palmas de Gran Canaria, Spain) by airplane into oxygen-saturated water transport bags that were kept in insulated boxes. The larvae were grown in separated tanks following the standardized methodology of the Research Group in Aquaculture [45,46] at the ULPGC facilities. Progenies from both genotypes were kept at similar conditions during the preweaning, weaning and early juvenile growing phases.

2.3. Experimental Conditions

At 300 days post-hatching (dph), the fish genotype induced significant differences in fish growth. A total of 180 GS fish with a mean weight of 104.9 ± 3.1 g were randomly pooled and distributed in four 500 L tanks (30 fish/tank, 1 tank per dietary treatment). On the other hand, 360 WT fish with a mean weight of 58 ± 1.6 g were randomly pooled and distributed in twelve 500 L tanks (45 fish/tank, 3 tanks per dietary treatment). The experimental tanks presented similar initial culture densities. The tanks were supplied with filtered sea water (18.8–20 °C and 6.1–6.6 ppm dissolved oxygen) in a flow-through system under a natural photoperiod (12L:12D). The experimental diets were fed 3 times a day, 6 days a week until apparent satiation from 12 March to 29 May 2020 (72 days). The feed intake was monitored daily, and the growth performance and feed utilization were calculated at the end of the feeding experience.

At the end of the feeding experience, six fish per dietary treatment and genotype level (two fish/WT tank and six fish/GS tank) were used to obtain blood plasma samples for circulating plasma cortisol analysis and gill samples for relative gene expression analysis. This sampling point was considered as the basal point, t = 0 h (pre-stress challenge), in the statistical analysis.

2.4. Oxidative Stress Challenge

After 72 days of a feeding trial, experimental fish were subjected to an oxidative stress challenge consisting of a 1 h bath exposure to hydrogen peroxide (H_2O_2), following the procedure previously described by Roque and co-authors in 2010 [8]. Briefly, H_2O_2 treatment was applied by stopping experimental tanks' water flow and aeration and adding H_2O_2 at a nominal concentration of 50 ppm. After 1 h of exposure, the tanks' water flow and aeration were restored and kept at maximum renovation rate for 2 h in order to remove all the remaining H_2O_2.

At 2 h and 24 h after the oxidative stress challenge, six fish per dietary treatment and genotype level (two fish/WT tank and six fish/GS tank) were used to obtain blood plasma samples for circulating plasma cortisol analysis and gill samples for relative gene expression analysis.

2.5. Sampling Methodology

Prior to manipulation, the fish were anesthetized using diluted clove oil (diluted in ethanol 100% (1:2)) (Guinama S.L; La Pobla de Vall Bona, Valencia (46185), Spain, Ref. Mg83168) at a concentration of 0.02 mL/L.

Blood samples were obtained by a caudal sinus puncture with 1 mL syringes, stored on an heparin-coated Eppendorf and immediately centrifuged at 3000 g for 5 min at 4 °C in order to obtain plasma samples. Plasma samples were stored at −80 °C until plasmatic cortisol analysis. The plasmatic cortisol concentration was determined using the assay kit (Access Cortisol ref 33600, ©2010 Beckman Coulter, Inc.; Alcobendas, Madrid (28108),

Spain) by an external laboratory Animal Lab (Las Palmas de Gran Canaria, Gran Canaria, Canary Island, Spain).

Gill samples for relative gene expression were obtained after fish euthanasia by head blow. The second and third holobranch from the fish's left side were excised, placed in 1.5 mL Eppendorf with RNAlater and kept at 4 °C for 24 h. Afterwards, RNAlater was removed, and the samples were frozen at −80 °C until the relative gene expression analysis. RNAlater was prepared by dilution in 1 L deionized water of 650 g ammonium sulfate, 7.4 g sodium citrate dihydrate, 7.4 g EDTA di sodium salt and 200–500 µL concentrated sulfuric acid, with a final pH of 5.2, obtaining 1.4 L of RNAlater.

2.6. RNA Extraction and Real-Time PCR Analysis

The gill (approx. 50 mg/sample) total mRNA (ng/µL) was extracted by employing TRI-reagent (Sigma-Aldrich, Sant Louis, MO, USA) from the extraction kit RNeasy Minikit from Qiagen. An iScript^{TM} cDNA synthesis Kit (Bio-Rad, Hercules, CA, USA) was em- ployed to perform the reverse transcriptions to obtain cDNA in a 20 µL reaction containing 1 µL of the total mRNA at a concentration of 0.5 µg/µL.

The real-time PCR analysis was performed with an iCycler with the optical module in a final volume of a 20 µL reaction, containing 10 µL iQTM-SYBER® Green Supermix (Bio-Rad, Hercules, CA, USA), 5 µL of free-nuclease water, 3 µL of cDNA (1:10 dilution) and 1 µL of forward and reverse primer. The target genes were the nuclear factor kappa beta-2 (*nfκβ2*), interleukin 1β (*il1β*); hypoxia inducible factor 1α (*hif-1α*), NADH dehydrogenase subunit 5 (*nd5*), cytochrome b (*cyb*), cytochrome oxidase subunit 1 (*cox*), mitochondrial respiratory uncoupling protein 1 (*ucp1*), superoxide dismutase (*sod*), catalase (*cat*), glutathione peroxidase (*gpx*), tumor necrosis factor 1α (*tnf-1α*) and caspase 9 (*casp-9*). The specific primer sequences, annealing temperatures and accession numbers are presented in Table 2. The real-time running conditions were: 95 °C for 1 min, followed by 40 cycles at 95 °C for 10 s and an annealing temperature for 30 s (Table 2). All reactions were performed in duplicate for each sample, and a blank control containing nuclease-free water instead of cDNA in the final volume mix was included in each assay. Two constitutive genes were tested: α-tubulin *(α-tub*) and the ribosomal protein L17 (*rpl17*). Applying the CFX Maestro^{TM} Software selection tool (CFX Maestro™ Software User Guide Version 1.1, Biorad), the *α-tub* was selected as the most stable and amplification-efficient reference gene. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [47,48], using *α-tubulin* as the housekeeping gene. The gene expression was calculated relative to the transcript levels of WT fish fed the control diet at t = 0 h (pre-stress challenge).

2.7. Statistical Analyses

All the analyses were performed with R Project for Statistical Computing. Means and standard deviations (SD) were calculated for each parameter measured.

To assess differences in fish growth and feed utilization among the genotypes, differences in the mean specific growth rate (SGR), feed conversion ratio (FCR) and individual feed intake among the selected genotypes were tested by one-way analysis of variance (ANOVA) and a Tukey test. Similarly, to assess differences in fish growth and feed utilization among the experimental diets, differences in the mean SGR, FCR and individual feed intake among the selected experimental diets were tested by one-way analysis of variance (ANOVA) and a Tukey test. A three-way analysis of variance (ANOVA) and a Tukey test were performed to assess differences in the fish stress response and gill relative gene expression between the genotypes and experimental dietary treatments along the different sampling points.

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Table 5.2. Primer sequences of the different genes analyzed and their RT-PCR

Gene	Access. Number	Primer	Nucleotide Sequence 5^t-3^t	Annealing T (°C)	Reference
nfKβ2	KM225790	Fw Rv	CTGGAGGAAACTGGCGGAGAAGC CAGGTACAGGTGAGTCAGCGTCATC	60	[49]
il-1b	AJ53742	Fw Rv	ATTACCCACCACCCACTGAC TCTCTTCCACTATGCTCTCCAG	60	[50]
hif-1a	DQ171936	Fw Rv	GACTTCAGCTGCCCTGATTC GGCTGGTTTATAGCGCTGAG	60	[51]
nd5	KF857307	Fw Rv	CCCGATTTCTGTGCCCTACTA AGGAAAGGAGTGCCTGTGA	60	[52]
cyb	EF427553	Fw Rv	TGCCTACGCTTCCTTCGCTCGATCC TAACGCCAACACCCCGCCCAAT	60	[53]
cox	KF857308	Fw Rv	ATACTTCACATCCGCAACCATAA AAGCCTCCGACTGTAAATAAGAAA	60	[53]
ucp1	MH138003	Fw Rv	CGATTCCAAGCCCAGACGAACCT TGCCAGTGTAGCGACGAGCC	60	[53]
sod	FJ860004.1	Fw Rv	CATGTTGGAGACCTGGGAGA TGAGCATCTTGTCCGTGATGT	60	[54]
cat	FJ860003.1	Fw Rv	TGGGACTTCTGGAGCCTGAG GCAAACCTCGATCGCTGAAC	60	[54]
gpx	FM013606.1	Fw Rv	AGTTCGTGCAGTTAATCCGGA GCTTAGCTGTCAGGTCGTAAAAC	60	[54]
tnf-1α	DQ200910.1	Fw Rv	GCCAAGCAAACAGCAGGAC ACAGCGGATATGGACGGTG	60	[52]
casp-9	DQ345775	Fw Rv	GGCAGGACTCGACGAGATAG CTCGCTCTGAGGAGCAAACT	62.7	[55]
α-tub (hk)	AY326429.1	Fw Rv	AGGCTCATTGGCCAGATTGT CAACATTCAGGGCTCCATCA	60	[31]
rpl17	AF139590	Fw Rv	GAGGACGTGGTGGTTCATCT CTGGCTTGCCTTTCTTGACT	60	[56]

Fw: Forward primer sequence, Rv: Reverse primer sequence.

Prior to analysis, all data were tested for outlying values through linear regression adjustment, defining the outside cut-offs as 1.5 times the Inter-Quantile Range (IQR) below the first and above the third quantiles [57,58]. Before the analysis, a Kolmogorov–Smirnov test was used to assess the quantile normality, and Levene's test was used to assess the homogeneity of the variance. Where there was significant variance heterogeneity, the data were transformed by the square root or log transformation. When transformations did not remove the heterogeneity, the analysis was performed with untransformed data with the F-test α-value set at 0.01 [59].

3. Results

3.1. Feeding Experience

All fish grew properly along the feeding trial (72 days), presenting GS fish with significantly ($p < 0.05$) higher final body weights than those of WT fish ($p < 0.05$). GS fish presented significantly improved FCR values and lower individual feed intake values than WT fish (Table 3). Within each genotype, dietary functional additives did not affect the fish final body weight and length. No significant differences in the fish specific growth rate were found (Table 3).

Table 5.3. Growth parameters and feed utilization of European sea bass (*Dicentrarchus labrax*) juveniles (at age 372 dph) after 72 days of the feeding experience.

	WT Genotype				GS Genotype					
Diet	**Control**	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**	**Control**	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**		
									One-way ANOVA	
									Diet (Inside Each Genotype)	
IBW (g) (300 dph)	58 ± 9.2	57.8 ± 10.2	58.6 ± 10	57.5 ± 9.4	108.7 ± 15.4	106.2 ± 17.1	102.2 ± 17	102.4 ± 15.6	ns	
IL (300 dph)	17.6 ± 1	17.6 ± 0.9	17.7 ± 1	17.6 ± 0.87	20.8 ± 1.1	20.9 ± 1.1	20.5 ± 1.2	20.6 ± 1.3	ns	
FBW (g) (372 dps)	99.4 ± 18.3	95.3 ± 18.2	103 ± 19	99.8 ± 18.1	192.8 ± 31.7	189.7 ± 34	176.1 ± 33.2	180.4 ± 30.3	ns	
FL (cm) (372 dph)	21 ± 2.1	20.8 ± 1.2	21 ± 2.1	21.1 ± 1.2	25.6 ± 1.1	25.5 ± 1.3	25.1 ± 1.3	24.8 ± 1.4	ns	
									One-way ANOVA	
									Diet	Genotype
[1] SGR (%/day)	0.75 ± 0.03	0.70 ± 0.06	0.78 ± 0.04	0.76 ± 0.01	0.80 ± 0.01	0.83 ± 0.01	0.81 ± 0.01	0.80 ± 0.01	ns	ns
[2] FCR	1.84 [a] ± 0.16	1.99 [a] ± 0.29	1.78 [a] ± 0.17	1.70 [a] ± 0.12	1.48 [b] ± 0.01	1.46 [b] ± 0.01	1.58 [b] ± 0.01	1.58 [b] ± 0.01	ns	F = 8.335, *p*-val = 0.0119
[3] FI (g feed/ 100 g BW/day)	0.48 [a] ± 0.02	0.48 [a] ± 0.02	0.47 [a] ± 0.02	0.46 [a] ± 0.01	0.27 [b] ± 0.00	0.28 [b] ± 0.00	0.27 [b] ± 0.00	0.29 [b] ± 0.00	ns	F = 364.1, *p*-val = 2.03×10^{-11}

Different lowercase letters denote significant differences ($p < 0.05$) between genotypes in each sampling point (three-way ANOVA: Genotype × Diet × Time; Tukey post hoc test). ns = not significant. Values expressed in the mean ± SD. Control (Control diet); PHYTO0.02 (PHYTO0.02 diet, supplemented with a 200 ppm blend of phytogenic feed additives consisting of a mixture of garlic and *Labiatae* plant essential oils with 87.5 mg terpens/kg diet); PHYTO0.1 (PHYTO0.1 diet, supplemented with a 1000 ppm blend of phytogenic feed additives, consisting of a mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils with 57 mg terpens/kg diet); GMOS0.5 (GMOS0.5 diet; supplemented with 5000 ppm galactomannan-oligosaccharides); GS (high-growth selected genotype); WT (wild type genotype); IBW (initial body weight (g)); FBW (final body weight (g) 72 days after feeding experience); FL (final length (g) 72 days after feeding experience); SGR (specific growth rate 72 days after feeding experience). [1] SGR = [(ln average final body weight − ln average initial body weight/no days] × 100. [2] FCR = Feed consumption (g)/weight gain (g). [3] FI = Individual feed intake (g).

3.2. Stress Response

At the end of the feeding experience (pre-oxidative stress challenge, t = 0 h), the GS fish presented significantly lower ($p < 0.05$) levels of mean basal circulating plasma cortisol than the WT fish. The GS fish presented a mean basal concentration of 1.7 ± 0.51 ng/mL per fish g; meanwhile, the WT fish presented a mean basal concentration of 3.67 ± 0.15 ng/ mL per fish g (Table 4).

In response to the oxidative stress challenge, at 2 h after H_2O_2 exposure, all the experimental groups presented a significant increase ($p < 0.05$) in circulating cortisol levels compared to the basal levels, regardless of the genotype or the dietary treatment fed. The H_2O_2 exposure increased the GS fish cortisol up to levels ×2.4 fold higher compared to the basal levels. Meanwhile, the WT fish presented an increase in cortisol levels of ×1.7 fold compared to their basal levels.

After 24 h of the oxidative stress challenge, all experimental groups presented a significant reduction ($p < 0.05$) in plasmatic cortisol levels down to the basal levels observed at t = 0 h pre-oxidative stress challenge, regardless of the genotype or the dietary treatment fed. The GS fish presented a decrease in cortisol levels of ×2.2 fold compared to those levels observed at 2 h post-oxidative stress challenge, whereas the WT fish cortisol levels presented a decrease of ×1.7 fold compared to those levels observed at 2 h after the oxidative stress challenge.

Table 5.4. Circulating plasma cortisol level expressed in ng/mL per fish g of European sea bass (*Dicentrarchus labrax*) juveniles at t = 0 h pre-oxidative stress challenge and at t = 2 h and 24 h after the oxidative stress challenge.

	WT Genotype				GS Genotype			
	Control	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**	**Control**	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**
Time								
0 h	3.49 ^{a1} ± 1.10	3.77 ^{a1} ± 0.62	3.61 ^{a1} ± 0.92	3.83 ^{a1} ± 1.15	0.92 ^{b1} ± 0.23	1.96 ^{b1} ± 0.89	1.95 ^{b1} ± 0.21	1.97 ^{b1} ± 0.85
2 h	7.22 ^{a2} ± 2.95	5.80 ^{a2} ± 1.62	5.51 ^{a2} ± 1.29	6.20 ^{a2} ± 1.13	3.43 ^{b2} ± 0.53	3.43 ^{b2} ± 0.53	4.23 ^{b2} ± 1.43	3.81 ^{b2} ± 0.24
24 h	3.26 ^{a1} ± 0.31	3.54 ^{a1} ± 1.35	3.57 ^{a1} ± 1.73	4.10 ^{a1} ± 1.32	2.14 ^{b1} ± 0.41	1.61 ^{b1} ± 0.47	1.58 ^{b1} ± 0.13	1.79 ^{b1} ± 0.68
Three-way ANOVA								
	Diet	Genotype	Time	D × G	D × T	G × T	D × G × T	
Plasmatic cortisol	ns	F = 0.41; p-val = 2 × 10 $^{-16}$	F = 55.023; p-val = 3.16 × 10^{-16}	ns	ns	ns	ns	

Different lowercase letters denote significant differences ($p < 0.05$) between genotypes in each sampling point (three-way ANOVA: Diet × Genotype × Time; Tukey post hoc test). Different numbers denote significant differences ($p < 0.05$) between experimental sampling points (three-way ANOVA: Genotype × Diet × Time; Tukey post hoc test). ns = not significant. Values expressed in mean ± SD. Control (Control diet); PHYTO0.02 (PHYTO0.02 diet, supplemented with a 200 ppm blend of phytogenic feed additives consisting of a mixture of garlic and *Labiatae* plant essential oils with 87.5 mg terpens/kg diet); PHYTO0.1 (PHYTO0.1 diet, supplemented with a 1000 ppm blend of phytogenic feed additives, consisting of a mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils with 57 mg terpens/kg diet); GMOS0.5 (GMOS0.5 diet; supplemented with 5000 ppm galactomannan-oligosaccharides); GS (high-growth selected genotype); WT (wild type genotype).

3.3. Gill Relative Gene Expression

Prior to the oxidative stress challenge (t = 0 h), the fish gill antioxidant defense-related gene expression presented significant differences ($p < 0.05$) associated with the interaction between the genotype and the dietary treatment fed (Figure 1). The GS fish fed the control and PHYTO0.1 diets showed a higher ($p < 0.05$) cat basal gill expression compared to the WT fish fed the same dietary treatments. The GS fish fed the control diet also presented upregulated ($p < 0.05$) *sod* gene expression levels compared to the WT fish fed the same diet. On the contrary, the WT fish fed the GMOS0.5 diet presented a higher ($p < 0.05$) *sod* basal gene expression than the GS fish fed the same dietary treatment.

Within the WT fish genotype, the diet fed directly affected the fish gill basal antioxidant gene expression. The fish fed the GMOS0.5 diet presented the highest ($p < 0.05$) *sod* expression levels, followed by PHYTO0.02 and PHYTO0.1, respectively. Similarly, those fish fed with PHYTO0.1 and GMOS0.5 diets presented the highest ($p < 0.05$) gpx gill expression levels. Those fish fed the GMOS0.5 diet presented significantly higher ($p < 0.05$) *hif-1α* relative expression levels than those fish fed the control and PHYTO0.1 diets (Appendix A Table A1).

Two hours post-oxidative stress challenge, a generalized upregulation of antioxidant defense-related gene expression was observed. All fish presented a significant increase ($p < 0.05$) in *cat* and *gpx* gill expression levels (Appendix A Table A1), whereas the gill *sod* gene expression presented significant differences associated with the fish genotype and the dietary treatment fed. The GS fish fed the control diet presented an upregulation ($p < 0.05$) of gill *sod* relative expression levels compared to the WT fish fed the same dietary treatment. In fact, within the GS genotype, the fish fed the control diet induced the highest ($p < 0.05$) *sod* expression levels. Accordingly, as a response to the H_2O_2 exposure, an up-regulation of the mitochondrial ETC-related gene expression was observed. All the experimental fish groups presented a general increase ($p < 0.05$) in *cox* transcript levels, independently of the fish origin or diet fed. However, this response was more acute for GS fish fed the PHYTO0.1 diet, which presented a higher ($p < 0.05$) expression than the WT fish fed the same diet. The GS fish fed the control diet were the only experimental group presenting an increase ($p < 0.05$) in gill *ucp1* relative gene expression in relation to their basal levels (Figure 2).

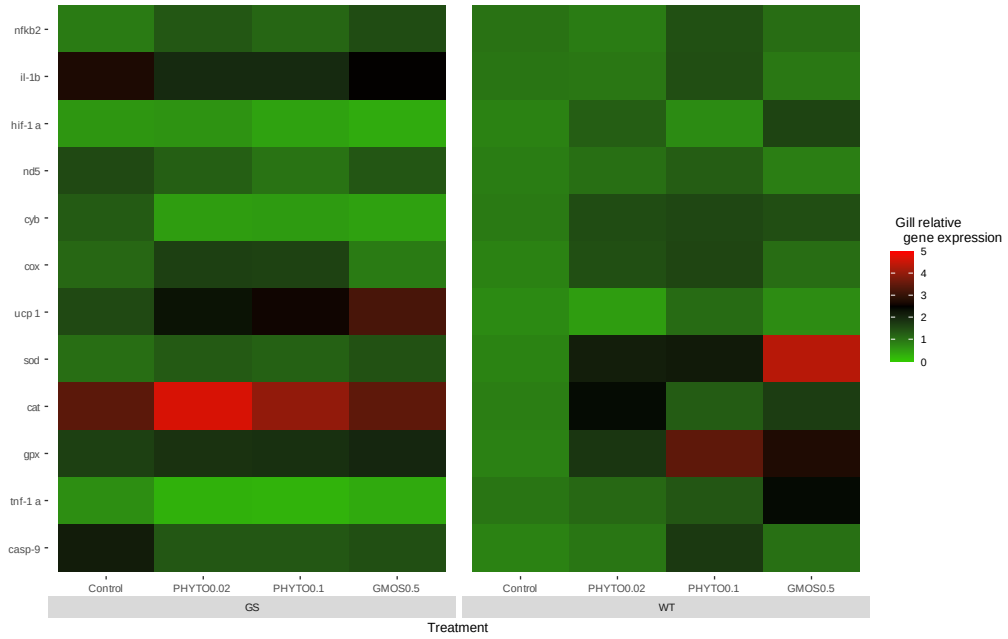

Figure 5.1. European sea bass gill relative gene expression heat map at 0 h pre-oxidative stress challenge for high-growth selected genotype (GS) and wild type genotype (WT) European sea bass. Control (Control diet); PHYTO0.02 (PHYTO0.02 diet, supplemented with a 200 ppm blend of phytogenic feed additives consisting of a mixture of garlic and *Labiatae* plant essential oils with 87.5 mg terpens/kg diet); PHYTO0.1 (PHYTO0.1 diet, supplemented with a 1000 ppm blend of phytogenic feed additives, consisting of a mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils with 57 mg terpens/kg diet); GMOS0.5 (GMOS0.5 diet; supplemented with 5000 ppm galactomannan-oligosaccharides).

In regard to the results observed on genes related with a proinflammatory response, 2 h after the oxidative stress challenge, all the experimental groups presented a significantly increased ($p < 0.05$) *nfKβ2* gill gene expression. Only the WT fish fed the PHYTO0.2 and GMOS0.5 diets presented significantly increased ($p < 0.05$) il-1*β* gill transcription levels in relation to basal levels. At this sampling point, feeding a GMOS0.5 diet to WT fish resulted in an increase ($p < 0.05$) in the *casp-9* gill relative gene expression, whereas the GS fish fed the GMOS0.5 diet presented an increased ($p < 0.05$) *hif-1α* gill relative gene expression.

Twenty-four hours after the oxidative stress challenge, the GS fish fed the control diet and the WT fish fed the GMOS0.5 diet presented a downregulation ($p < 0.05$) of *sod* and *gpx* gill relative gene expression, respectively. Despite the changes mentioned above, no significant differences were found among the different experimental groups in terms of the antioxidant defense gene response (Figure 3).

At this sampling point, the GS fish fed the PHYTO0.1 diet presented a significant downregulation ($p < 0.05$) of gill *cox* relative gene expression. No differences among the groups were observed in the fish cox gill gene expression. On the contrary, the WT fish fed the PHYTO0.1 diet presented higher ($p < 0.05$) *ucp1* gill relative gene expressions than the WT fish fed the GMOS0.5 diet.

At the end of the oxidative stress challenge, no significant change in the fish gill pro-inflammatory gene response was observed, with an exception for the il-1*β* gill relative gene expression, which was increased ($p < 0.05$) in the WT fish fed the control diet in relation to the previous sampling point (Appendix A Table A1).

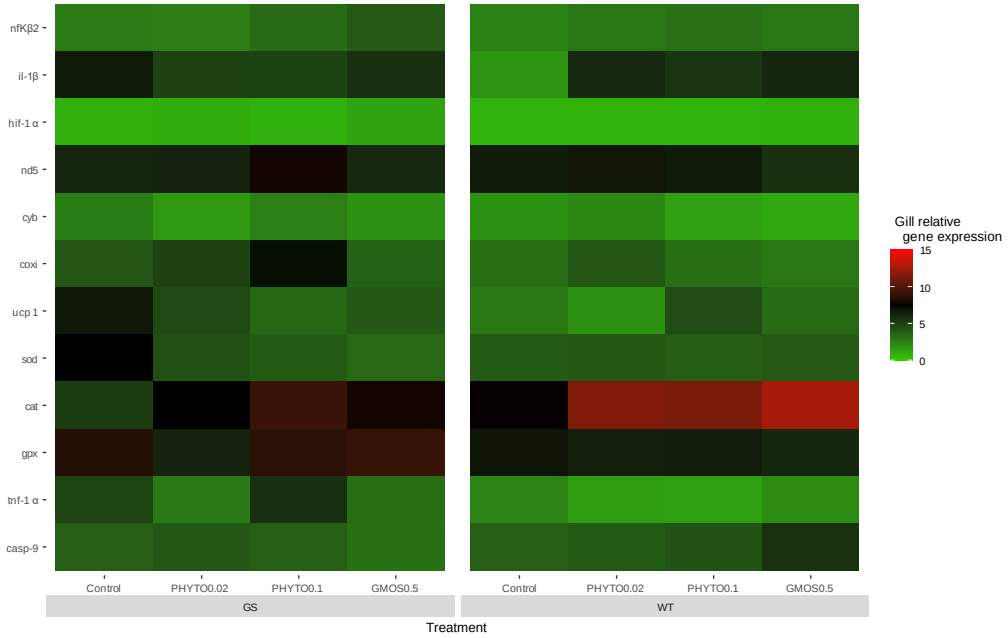

Figure 5.2. European sea bass gill relative gene expression heat map at 2 h after oxidative stress challenge for high-growth selected genotype (GS) and wild type genotype (WT) European sea bass. Control (Control diet); PHYTO0.02 (PHYTO0.02 diet, supplemented with a 200 ppm blend of phytogenic feed additives consisting of a mixture of garlic and *Labiatae* plant essential oils with 87.5 mg terpens/kg diet); PHYTO0.1 (PHYTO0.1 diet, supplemented with a 1000 ppm blend of phytogenic feed additives, consisting of a mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils with 57 mg terpens/kg diet); GMOS0.5 (GMOS0.5 diet; supplemented with 5000 ppm galactomannan-oligosaccharides).

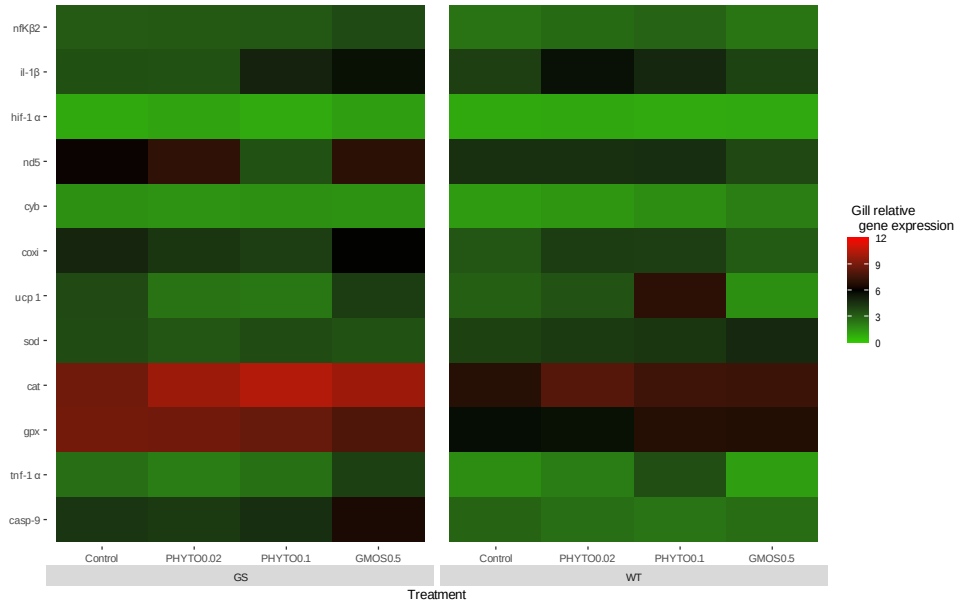

Figure 5.3. European sea bass gill relative gene expression heat map at 24 h after oxidative stress challenge for high-growth selected genotype (GS) and wild type genotype (WT) European sea bass. Control (Control diet); PHYTO0.02 (PHYTO0.02 diet, supplemented with a 200 ppm blend of phytogenic feed additives consisting of a mixture of garlic and *Labiatae* plant essential oils with 87.5 mg terpens/kg diet); PHYTO0.1 (PHYTO0.1 diet, supplemented with a 1000 ppm blend of phytogenic feed additives, consisting of a mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils with 57 mg terpens/kg diet); GMOS0.5 (GMOS0.5 diet; supplemented with 5000 ppm galactomannan-oligosaccharides).

4. Discussion

The results of the present study highlight the strong effect exerted by breeding selection, leading to a two-fold higher body weight for GS fish compared to WT at the same age, 300 days post-hatching. At the end of the feeding trial, both genotypes presented proper growth, almost doubling the initial body weight regardless of the dietary treatment fed. Despite the fish from both genotypes presenting similar specific growth rates, the GS fish presented improved feed conversion ratios and lower individual feed intakes than the WT fish, indicating a better capacity to harness feed even when dealing with low FM/FO-based diets. In agreement with these results, in the study carried out by Montero and co-authors in 2023 [40], GS fish belonging to the same breeding program presented a higher growth performance and a better plasticity to cope with the possible nutritional imbalances derived from low FM/FO-based diets. At the end of the feeding experience, GS fish presented a higher body weight, decreased fish perivisceral fat deposition and increased flesh DHA and ARA contents compared to WT fish.

The use of selective breeding strategies as a tool to increase fish growth performance may lead to favoring the selection of secondary functional phenotypes, such as stress tolerance and behavioral traits [38], which are keystones in domestication processes [60]. In 2016, Vandeputte and co-authors [61] studied the stress response of three different genotypes of European sea bass (wild, domesticated and selected for growth) subjected to acute confinement followed by a swimming stress challenge. The authors reported a negative correlation between the fish body weight and the circulating plasma cortisol levels after the stress challenge, concluding that selective breeding may favor fish's low stress responsiveness. Accordingly, in the present study, the GS fish presented significantly lower basal cortisol levels than the WT fish, pointing to a possible effect of growth selective breeding on fish stress indicators. Furthermore, and despite presenting higher cortisol levels than the WT fish in the first hours after the oxidative stress challenge, the GS fish presented a better recovery back to the basal cortisol levels at 24 h post-H_2O_2 exposure. A better competence for recovering a homeostatic status might be advantageous under aquaculture conditions in which fish are constantly exposed to stressful conditions [17,18]. An effective and controlled physiological stress response will avoid the negative side- effects associated with a chronic cortisol exposure [39,62]. An example of stress tolerance benefits for aquaculture production was reported by Øverli and co-authors in 2006 [63]. The authors studied the effects of a transport stress challenge on the feed utilization of two different genotypes of rainbow trout (*Oncorhynchus mykiss*) selected for low or high stress responsiveness. The low stress responsive genotype presented a significantly higher feed efficiency and a lower food waste production after the stress challenge.

In the present study, the genetic selection also induced differences in fish antioxidant defense gene expression. At the basal level, at t = 0 h pre-stress challenge, the GS fish presented higher *cat* gene expression levels than the WT fish. Similarly, other studies have reported higher antioxidant defenses in the selected genotypes of other fish species. For example, Solberg and co-authors, in 2012 [64], described higher glutathione reductase, Cu/Zn *sod* and *gpx* relative gene expression levels in response to environmental stress processes for a domesticated strain of Atlantic salmon (*Salmo salar*) in comparison to a wild strain. In 2010, Sauvage and co-authors [65] observed a higher gene expression of three genes associated with protective properties against oxidative stress processes (precursor of hemopexin, heme-binding protein 2, precursor of fibrinogen γ chain and precursor of the inter-α trypsin inhibitor heavy chain H2) on a selected strain of Brook charr (*Salvelinus fontinalis*) (F4 generation) compared to a reference population obtained from randomly mixed breeders (F1 generation) kept at the same environmental conditions. Palinska-Zarska and co-authors, in 2021 [66], compared the antioxidant enzymatic activity of two genotypes, domesticated and wild, of perch (*Perca fluviatilis*) larvae presenting higher *sod* and *cat* activities than the domesticated strain. These authors suggested that a higher antioxidant enzyme activity in the selected strain resulted in a better adaptation to the formulated feed, leading to better survival rates and performance during the larval weaning period. In the

present study, the functional additives also presented an effect on fish antioxidant defense. The WT fish fed functional diets presented higher *sod*, *cat* and *gpx* basal gene expression levels than the WT fish fed the control diet. In the same way, at two hours after H_2O_2 exposure, those fish fed the functional additives presented the highest *cat* gene expression levels compared to the fish fed the control diet, regardless of the genotype. This could suggest an enhanced antioxidant capacity associated with functional additives supplementation, as catalase is the main enzyme contributing to H_2O_2 removal when found in high concentrations in the intercellular space [66]. Dietary supplementation with plant origin compounds may reinforce the fish antioxidant status through the interaction with several signaling transcription factors modulating fish antioxidant-related gene expression [33]. Li and co-authors, in 2018 [67], evaluated the effects of pinostrobin, a potent flavonoid extracted from pines, on zebra fish's (*Danio rerio*) neural antioxidant status. This phytogenic compound increased fish GSH-PX, GSH/GSSG, SOD and CAT enzymes, reducing fish neural oxidative stress damages and apoptotic processes. Mansour and co-authors, in 2020 [68], analyzed the antioxidant capacity of sea bream (*Sparus aurata*) fed diets supplemented with Moringa (*Moringa oleifera*) against an H_2O_2 exposure at a concentration of 50 ppm. The authors reported an enhanced response of the fish fed the supplemented diets, with an increased gill *cat* gene expression compared to that of those fish fed diets void of supplementation. In addition, these compounds are rich in terpenes and flavonoids, which present high antioxidant properties preventing the formation or directly quenching the oxygen and nitrogen reactive species derived from aerobic metabolism [22,69].

An increased aerobic metabolism rate, in order to respond against a stress process, may also suppose an important source of oxidative stress processes. During oxidative phosphorylation, between 1 and 3% of all electrons may "leak" from the electron transport chain [14] being released into the mitochondrial intermembrane space, where they will react with O_2 generating ROS. In the present study, the stress challenge resulted in a generalized overexpression of the ETC-related genes *nd5*, *cyb* and *cox*, regardless of the fish genotype or the dietary treatment fed. However, an interesting response was observed for GS fish fed the control diet, which, unless presenting similar levels of expression as the other experimental groups, presented an increased *ucp1* gene expression after H_2O_2 exposure. In the absence of an external surplus of antioxidant defenses such as the antioxidant properties of functional additives, this may suggest a feedback mechanism limiting mitochondrial ROS formation, in a process called "uncoupling to survive" [15], and protecting gill tissue from oxidative stress processes.

ROS are important metabolic agents involved in fish inflammatory responses through the interaction with the nuclear factor kappa beta (NFKβ) [70,71] and leading to the activation of the pro-inflammatory cytokines IL-1β and TNF-α [72]. In the present study, all experimental treatments presented similar expressions of pro-inflammatory genes in gills after H_2O_2 exposure. Nevertheless, the GS fish fed the GMOS0.5 diet presented upregulated *hif-1α* gill expression levels compared to the fish fed the rest of the dietary treatments. Under hypoxic conditions associated with inflammatory processes [73], the *hif-1α* mediates the activation of the O_2-independent glycolytic pathway, ensuring ATP production to cope with the bio-energetic requirements [74,75]. On the other hand, the WT fish fed GMOS0.5, which did not present an increased expression of *hif-1α*, presented an increased expression of *caspase 9*, which is the activator of caspase-dependent apoptotic processes [76], suggesting a lower ability to cope with the side-effects associated with the inflammatory process.

5. Conclusions

In conclusion, H_2O_2 exposure induced the triggering of both the fish stress response and oxidative stress defense. The GS genotype fish presented a better capacity to recover the basal cortisol levels, suggesting a higher tolerance to potential stressful scenarios associated with fish rearing conditions. In addition, the use of functional additives enhanced the fish antioxidant response via upregulating the expression of *cat* in gill expression levels in

response to the oxidative insult. The GMOS0.5 diet induced the activation of hif-1α gene expression in the gills of GS fish, modulating the triggering of pro-inflammatory-associated processes. Nevertheless, in the view of the complexity of interactions between fish genetic traits and the diversity of functional ingredients, more experiences must be carried out to address the best nutritional and genetic selection strategies in order to promote fish health and welfare under rearing conditions.

Author Contributions: Conceptualization, S.T., A.M. and D.M.; formal analysis, A.S.; data curation, A.S.; software, A.S.; validation, S.T., P.H. and D.M.; writing—original draft preparation, A.S.; writing—review and editing, S.T., D.M., G.T., S.R., A.B., P.H. and F.A. (François Allal); project administration, D.M.; funding acquisition, S.T., F.A. (Félix Acosta) and D.M. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the European Union Horizon 2020 research and innovation program under grant agreement no. 818367; AquaIMPACT—Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture.

Institutional Review Board Statement: The described experiment complies with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals. The experimental protocol was approved by the Institutional Review Board (or Ethics Committee) of the University of Las Palmas de Gran Canaria (approval no. OEBA_ULPGC_14/2020) for studies involving animals.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Authors are thankful to the European Union for the funding of this study through the Horizon 2020 agreement no. 818367 concession (AquaIMPACT: Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture). Special thanks go to Delacon Biotechnik GmBH, which provided the functional additive products.

Conflicts of Interest: The authors declare no conflict of interest. The company had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Table 5.A1. Gill relative gene expression of European sea bass (*Dicentrarchus labrax*) juveniles at t = 0 h pre-oxidative stress challenge and at t = 2 h and 24 h after the oxidative stress challenge.

		High-Growth Selected Genotype (GS)				Wild Type Genotype (WT)			
		Control	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**	**Control**	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**
Sampling Point	**Target Gene**								
t = 0 h (pre-H_2O_2 exposure)	*nfKβ2*	0.89 ^{1} ± 0.26	1.36 ^{1} ± 0.19	1.17 ^{1} ± 0.13	1.49 ^{1} ± 0.35	1.02 ^{1} ± 0.17	0.90 ^{1} ± 0.14	1.41 ^{1} ± 0.48	0.98 ^{1} ± 0.58
	il-1β	2.58 ± 1.04	1.94 ± 0.43	1.94 ± 0.45	2.48 ± 0.48	0.95 ^{1} ± 0.33	0.93 ^{1} ± 0.33	1.42 ^{1} ± 0.49	0.93 ^{1} ± 0.23
	hif-1α	0.60 ± 0.16	0.64 ± 0.07	0.45 ± 0.19	0.33 ^{a1} ± 0.20	0.78 ^{A} ± 0.41	1.22 ^{AB} ± 0.44	0.73 ^{A} ± 0.13	1.61 ^{bB} ± 0.16
	nd5	1.42 ± 0.76	1.24 ± 0.31	1.01 ^{1} ± 0.14	1.34 ± 0.41	0.89 ^{1} ± 0.13	1.05 ^{1} ± 0.16	1.28 ^{1} ± 0.15	0.85 ± 0.31
	cyb	1.32 ± 0.07	0.53 ± 0.11	0.54 ^{1} ±0.18	0.46 ± 0.18	0.92 ± 0.20	1.20 ± 1.12	1.50 ± 0.55	1.39 ± 0.63
	cox	1.16 ^{1} ± 0.11	1.63 ^{1} ± 0.42	1.59 ^{1} ± 0.46	0.91 ^{1} ± 0.21	0.84 ^{1} ± 0.11	1.45 ^{1} ± 0.32	1.56 ^{1} ± 0.47	1.01 ^{1} ± 0.50
	ucp1	1.46 ^{1} ± 0.65	2.07 ± 1.27	2.39 ± 1.24	2.98 ± 1.52	0.75 ± 0.09	0.53 ± 0.07	1.10 ^{1} ± 0.20	0.68 ± 0.30
	sod	1.06 ^{a1}± 0.23	1.32 ^{1} ± 0.20	1.24 ^{1} ± 0.27	1.44 ^{a1}± 0.17	0.81 ^{bA1} ± 0.23	2.14 ^{B1} ± 0.19	2.00 ^{AB1} ± 1.13	4.28 ^{bC} ± 0.88
	cat	3.40 ^{a1}± 0.62	4.59 ^{1} ± 0.86	3.97 ^{a1} ± 0.34	3.44 ^{1} ± 0.50	0.85 ^{b1} ± 0.26	2.16 ^{1} ± 1.28	1.25 ^{b1} ± 0.45	1.60 ^{1} ± 0.74
	gpx	1.62 ^{1} ± 0.46	1.85 ^{1} ± 0.29	1.88 ^{1} ± 0.26	2.00 ^{1} ± 0.36	0.85 ^{A1} ± 0.18	1.80 ^{A1} ± 0.06	3.21 ^{C1} ± 1.59	2.55 ^{B1} ± 1.27
	tnf-1α	0.55 ^{1} ± 0.55	0.29 ± 0.11	0.28 ^{1} ± 0.07	0.30 ^{1} ± 0.28	0.96 ± 0.28	1.03 ± 0.62	1.15 ± 0.92	2.32 ± 0.80
	casp-9	1.69 ± 1.68	1.36 ± 0.24	1.33 ± 0.34	1.46 ± 0.21	0.82 ± 0.26	0.98 ± 0.17	1.70 ± 0.58	1.01 ^{1} ± 0.34
t = 2 h (post-H_2O_2 exposure)	*nfKβ2*	2.75 ^{2} ± 0.39	2.66 ^{2} ± 0.33	3.34 ^{2} ± 0.32	3.90 ^{2} ± 0.96	2.55 ^{2} ± 0.25	2.92 ^{2} ± 0.40	3.08 ^{2} ± 0.58	2.95 ^{2} ± 0.10
	il-1β	5.75 ± 4.14	4.80 ± 1.42	4.78 ± 1.28	5.65 ± 0.62	5.47 ^{12} ± 0.19	5.66 ^{2} ± 2.25	5.38 ± 1.04	6.06 ^{2} ± 0.79
	hif-1α	0.96 ± 0.12	1.02 ± 0.14	0.94 ± 0.11	1.29 ^{2} ± 0.41	0.84 ± 0.13	0.88 ± 0.06	0.89 ± 018	0.93 ± 0.06
	nd5	6.03 ± 1.46	5.99 ± 2.15	6.69 ^{2} ± 5.13	5.95 ± 1.08	6.56 ^{2} ± 0.34	6.70 ^{2} ± 1.50	6.62 ^{2} ± 0.52	5.55 ± 1.35
	cyb	2.59 ± 0.86	1.72 ± 0.48	2.59 ^{2} ± 0.23	1.90 ± 0.82	1.89 ± 0.87	2.21 ± 0.87	1.47 ± 0.25	1.15 ± 0.30
	cox	4.13 ^{AB2} ± 0.41	4.77 ^{AB2} ± 1.56	7.12 ^{aB2} ± 0.45	3.69 ^{A2} ± 0.46	3.21 ^{2} ± 0.49	4.10 ^{2} ± 0.94	3.20 ^{b12} ± 0.33	2.84 ^{12} ± 0.80
	ucp1	6.65 ^{2} ± 1.29	4.38 ± 1.93	2.94 ± 2.33	3.72 ± 1.94	2.56 ± 1.54	1.84 ± 0.90	3.72 ^{12} ± 3.14	3.30 ± 0.72
	sod	7.45 ^{aA2} ± 0.84	4.38 ^{B2} ± 0.27	3.90 ^{B2} ± 0.82	3.41 ^{B2} ± 0.66	3.88 ^{b2} ± 0.83	4.01 ^{2} ± 0.41	3.85 ^{2} ± 0.30	3.98 ± 0.37
	cat	4.97 ^{A2} ± 1.62	7.51 ^{AB12} ± 0.49	9.18 ^{B2} ± 0.81	7.86 ^{AB2} ± 0.42	7.39 ^{A2} ± 2.05	11.29 ^{AB2} ± 2.06	11.23 ^{AB2} ± 1.23	12.58 ^{B2} ± 0.11
	gpx	8.34 ^{2}± 1.41	6.24 ^{2} ± 0.18	8.62 ^{2} ± 1.21	8.99 ^{2} ± 0.79	6.83 ^{2} ± 0.84	6.47 ^{2} ± 0.80	6.44 ^{2} ± 1.29	6.00 ^{2} ± 0.70
	tnf-1α	4.70 ^{2} ± 1.34	2.47 ± 1.68	4.49 ^{2} ± 4.24	2.45 ^{12} ± 2.51	2.27 ± 1.23	1.55 ± 0.51	1.42 ± 0.39	2.02 ± 0.93
	casp-9	3.32 ± 2.36	3.64 ± 2.30	3.50 ± 1.73	2.98 ± 1.33	3.72 ± 1.00	3.93 ± 0.59	4.24 ± 0.51	5.34 ^{2} ± 2.14

Table A1. *Cont.*

		High-Growth Selected Genotype (GS)				Wild Type Genotype (WT)			
		Control	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**	**Control**	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**
Sampling Point	**Target Gene**								
t = 24 h (post-H_2O_2 exposure)	*nfKβ2*	3.20 ^{2} ± 0.17	3.20 ^{2} ± 0.24	3.21 ^{2} ± 0.55	3.69 ^{a2} ± 0.17	2.42 ^{2} ± 0.45	2.64 ^{2} ± 0.77	2.91 ^{2} ± 0.39	2.37 ^{b2} ± 0.15
	il-1β	3.50 ± 0.24	3.42 ± 0.22	4.88 ± 1.30	5.43 ± 1.79	3.81 ^{2} ± 1.60	5.27 ^{2} ± 2.50	4.72 ± 1.20	3.73 ^{2} ± 1.43
	hif-1α	0.91 ± 0.30	1.09 ± 0.13	0.90 ± 0.15	1.24 ^{2} ± 0.09	0.94 ± 0.15	1.00 ± 0.23	0.92 ± 0.14	0.93 ± 0.37
	nd5	6.05 ± 1.40	6.99 ± 1.43	3.22 ^{12} ± 1.41	6.28 ± 3.56	4.45 ^{12} ± 1.31	4.45 ^{12} ± 1.11	4.44 ^{12} ± 1.44	3.70 ± 0.72
	cyb	1.61 ± 0.28	1.53 ± 0.19	1.63 ^{12} ± 0.21	1.55 ± 0.41	1.27 ± 0.43	1.41 ± 0.43	1.69 ± 0.36	2.00 ± 0.86
	cox	4.88 ^{2} ± 0.71	4.33 ^{2} ± 0.47	4.05 ^{3} ± 0.33	5.47 ^{2} ± 2.84	3.30 ^{2} ± 0.71	4.00 ^{2} ± 1.03	3.92 ^{2} ± 1.36	3.14 ^{2} ± 0.22
	ucp1	3.42 ^{12} ± 1.82	2.31 ± 1.01	2.29 ± 0.54	3.90 ± 1.55	2.99 ^{AB} ± 0.58	3.22 ^{AB} ± 1.43	6.96 ^{A2} ± 1.23	1.54 ^{B} ± 0.74
	sod	3.60 ^{3} ± 0.66	3.35 ^{2} ± 0.30	3.64 ^{2} ± 0.43	3.41 ^{2} ± 0.28	3.93 ^{2} ± 0.57	4.16 ^{2} ± 0.68	4.32 ^{2} ± 0.38	4.77 ± 0.68
	cat	8.69 ^{2} ± 1.45	9.61 ^{2} ± 2.34	10.30 ^{2} ± 1.05	9.77 ^{2} ± 1.17	6.80 ^{2} ± 0.72	7.87 ^{2} ± 2.22	7.42 ^{2} ± 1.50	7.31 ^{3} ± 1.47
	gpx	8.82 ^{2} ± 0.94	8.74 ^{2} ± 1.12	8.49 ^{2} ± 0.45	7.89 ^{2} ± 0.86	5.69 ^{2} ± 0.84	5.5 ^{2} ± 1.10	6.76 ^{2} ± 0.73	6.68 ^{2} ± 0.79
	tnf-1α	2.44 ^{12} ± 1.08	2.04 ± 0.81	2.41 ^{12} ± 0.94	3.82 ^{2} ± 1.53	1.68 ± 0.40	2.04 ± 0.89	3.28 ± 1.65	1.23 ± 0.34
	casp-9	3.96 ± 2.26	4.06 ± 1.28	4.47 ± 1.32	5.83 ± 3.45	2.88 ± 0.38	2.54 ± 0.62	2.39 ± 0.33	2.63 ^{12} ± 0.30
		Three-way ANOVA							
		Diet	Genotype	Time	D × G	D × T	G × T	D × G × T	
	nfKβ2	F = 4.306 *p*-val = 0.009	F = 16.398 *p*-val = 0.0018	F = 158.176 *p*-val = < 2 × 10^{-16}	F = 3.481 *p*-val = 0.0229	ns	F = 3.335 *p*-val = 0.0474	ns	
	il-1β	ns	F = 8.798 *p*-val = 0.0047	F = 65.499 *p*-val = 1.91 × 10^{-14}	ns	ns	ns	ns	
	hif-1α	F = 5.231 *p*-val = 0.0033	F = 4.422 *p*-val = 0.041	F = 5.714 *p*-val = 0.006	ns	ns	F = 21.397 *p*-val = 2.27 × 10^{-7}	F = 4.260 *p*-val = 0.00162	
	nd5	ns	ns	F = 63.876 *p*-val = 2.97 × 10^{-14}	ns	ns	ns	ns	
	cyb	ns	ns	F = 19.325 *p*-val = 6.97 × 10^{-7}	ns	ns	F = 5.819 *p*-val = 0.005	ns	
	cox	F = 4.108 *p*-val = 0.0113	F = 21.469 *p*-val = 2.77 × 10^{-5}	F = 127.517 *p*-val = < 2 × 10^{-16}	ns	ns	F = 4.051 *p*-val = 0.0237	F = 2.616 *p*-val = 0.028	

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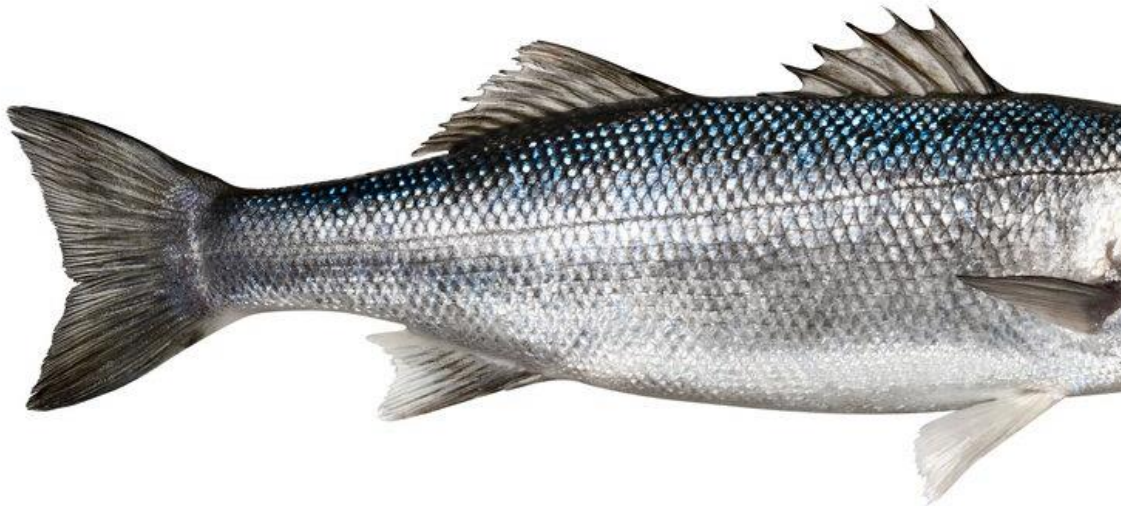
Table A1. *Cont.*

		High-Growth Selected Genotype (GS)				Wild Type Genotype (WT)			
		Control	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**	**Control**	**PHYTO0.02**	**PHYTO0.1**	**GMOS0.5**
Sampling Point	**Target Gene**								
		Three-way ANOVA							
		Diet	Genotype	Time	D × G	D × T	G × T	D × G × T	
	ucp1	ns	F = 7.93 p-val = 0.007	F = 19.895 p-val = 5.09×10^{-7}	F = 4.471 p-val = 0.007	ns	F = 5.287 p-val = 0.008	F = 2.699 p-val = 0.024	
	sod	ns	F = 10.086 p-val = 0.002	F = 181.372 p-val = $< 2 \times 10^{-16}$	F = 17.654 p-val = 7.36×10^{-8}	F = 13.457 p-val = 6.98×10^{-9}	F = 19.065 p-val = 8.06×10^{-7}	F = 2.748 p-val = 0.022	
	cat	F = 9.911 p-val = 3.38×10^{-5}	F = 10.017 p-val = 0.002	F = 242.091 p-val = $< 2 \times 10^{-16}$	ns	F = 2.914 p-val = 0.017	F = 44.054 p-val = 1.37×10^{-11}	ns	
	gpx	F = 2.859 p-val = 0.046	F = 35.012 p-val = 3.36×10^{7}	F = 294.593 p-val = $< 2 \times 10^{-16}$	ns	ns	F = 13.893 p-val = 1.74×10^{-5}	F = 3.079 p-val = 0.013	
	tnf-1α	ns	ns	F = 25.759 p-val = 2.51×10^{-8}	ns	ns	F = 10.79 p-val = 0.0001	ns	
	casp-9	ns	ns	F = 39.156 p-val = 8.23×10^{-11}	ns	ns	F = 5.946 p-val = 0.005	ns	

Different uppercase letters denote significant differences ($p < 0.05$) between dietary treatments inside each fish genotype at each sampling point (three-way ANOVA: Diet × Genotype × Time; Tukey post hoc test). Different lowercase letters denote significant differences ($p < 0.05$) between genotypes at each sampling point (three-way ANOVA: Diet × Genotype × Time; Tukey post hoc test). Different numbers denote significant differences ($p < 0.05$) between experimental sampling points (three-way ANOVA: Diet × Genotype × Time; Tukey post hoc test). ns = not significant. Values expressed in mean ± SD. Control (Control diet); PHYTO0.02 (PHYTO0.02 diet, supplemented with a 200 ppm blend of phytogenic feed additives consisting of a mixture of garlic and *Labiatae* plant essential oils with 87.5 mg terpens/kg diet); PHYTO0.1 (PHYTO0.1 diet, supplemented with a 1000 ppm blend of phytogenic feed additives, consisting of a mixture of citrus fruits and *Asteraceae* and *Labiatae* plant essential oils with 57 mg terpens/kg diet); GMOS0.5 (GMOS0.5 diet; supplemented with 5000 ppm galactomannan-oligosaccharides); GS (high-growth selected genotype); WT (wild type genotype).

Chapter VI

Genetically superior European Sea Bass (*Dicentrarchus labrax*) and nutritional innovations: Effects of functional feeds on fish immune response, disease resistance and gut microbiota



Aquaculture Reports 33 (2023) 101747

Contents lists available at ScienceDirect

Aquaculture Reports

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

Genetically superior European sea bass (*Dicentrarchus labrax*) and nutritional innovations: Effects of functional feeds on fish immune response, disease resistance, and gut microbiota

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ARTICLE INFO

Keywords:
Bacilli
DNA barcoding
Firmicutes
Metagenomics
Proteobacteria

ABSTRACT

The objective of this study was to determine if selected fish genotypes could benefit from the use of functional additives in novel aqua feed formulations to improve growth performance, gut microbiota, immune response, and disease resistance in fish. Two batches of juvenile European sea bass selected for high growth (HG; selected sires x selected dams), and wild types (WT; wild sires x selected females) were fed a "future diet" coated with three different functional additives for 12 weeks as follows: (i) 2 weeks with a high dose, followed by (ii) 10 weeks with a low dose. The functional additives tested were a mixture of probiotics (PROB), organic acids (ORG), and phytogens (PHYTO). A pathogen challenge test (*Vibrio anguillarum*) and a stress condition (overcrowding) were performed after each dose. At the end of the feeding experiment, fish from the HG group performed better than fish from the WT group in terms of body weight, relative growth, SGR, and DGI. The results of the two challenge tests performed after two weeks of high dose and ten weeks of low dose showed a significant effect of diet on fish survival. GALT-associated gene expression analysis revealed an interaction between the genotype and diet for *il-1β* in the distal gut. Finally, regarding the gut microbiota, discriminant analysis showed no clear separation between fish fed the future diet and those fed the same diet with experimental additives. Nevertheless, the relative abundance of certain taxa varied between experimental groups. For example, fish fed the ORG diet had higher relative abundance of *Streptococcus* in both genotypes, whereas fish fed the PHYTO diet had higher abundance of Lactobacillales. In contrast, fish fed PROB had lower abundance of *Pseudomonas* and *Acinetobacter*.

1. Introduction

The development of the aquafeed industry is the first step in expanding aquaculture production. New strategies are needed to deal with the limited resources of wild fish traditionally used to produce raw materials such as fish meal (FM) and fish oil (FO). One of the biggest challenges in aquaculture is to find alternative and more sustainable feed ingredients that can replace FM and FO without compromising fish health and growth performance. To date, a wide range of raw materials have been successfully used in fish feeds without demonstrated negative effects on growth performance. These include plants (Hardy, 2010; Huyben et al., 2020; Torrecillas et al., 2017), animal by-products (Fontinha et al., 2021; Rimoldi et al., 2018b), insects (Rimoldi et al., 2021; Terova et al., 2021), unicellular microbes (Rimoldi et al., 2020a), and microalgae (Sarker et al., 2020). However, it is also true that optimal feeding strategies in intensive aquaculture systems should be

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https://doi.org/10.1016/j.aqrep.2023.101747
Received 3 August 2023; Received in revised form 4 September 2023; Accepted 19 September 2023
Available online 21 September 2023
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evaluated in conjunction with fish gut health. In particular, replacing FM and FO in aqua feeds with plant products may affect the health of carnivorous farmed fish by altering gut morphology and microbiota and modifying local gut and systemic fish immunity (Huyben et al., 2020; Martin and Kro´l, 2017; Simo´-Mirabet et al., 2018; Torrecillas et al., 2017). Indeed, insufficient nutritional value of the diet is the main factor affecting the gut microbiota, which provides essential health benefits to the host, especially by regulating immune homeostasis (Wu and Wu, 2012). Although gut immunity of fish is less developed than that of higher vertebrates, the gut immune components of teleosts contain both effector and inducer sites (Bruce et al., 2017). Unlike mammals, the gut immune system of fish lacks lymphoid tissue aggregates such as Peyer's patches, but instead consists of diffuse gut-associated lymphoid tissue (GALT) in which the induction and effector sites of the lamina propria are indistinguishable (Rombout Jan et al., 2011; Salinas et al., 2011). Similar to higher vertebrates, GALT contains mucosal immune cells such as lymphocytes, plasma cells, granulocytes, and macrophages that make this mucosal tissue an active immune organ. Intestinal microbiota plays a central role in the development of GALT (Dawood, 2021; Wu and Wu, 2012). Experiments in germ-free animal models have demonstrated the critical role of the commensal gut microbiota in priming neutrophils, enhancing disease resistance to pathogens, and in the initiation and progression of intestinal inflammation (Galindo-Villegas et al., 2012; Montalban-Arques et al., 2015; Tlaskalova-Hogenova et al., 2014). In fact, germ-free animals show a deficient development of the immune system and have an immature GALT (Ha et al., 2014).

In addition, beneficial bacteria break down and ferment indigestible dietary fiber, resulting in the production of large amounts of short-chain fatty acids (SCFAs). Among SCFAs, butyrate has received particular attention due to its multiple beneficial effects on the health of the intestinal tract and peripheral tissues of vertebrates, including fish. Butyrate has anti-inflammatory effects on the colon and stimulates the immune system of fish (Piazzon et al., 2017; Rimoldi et al., 2016; Terova et al., 2016). For instance, in grass carp (*Ctenopharyngodon idella*), administration of sodium butyrate leads to upregulation immune-related gene expression and downregulation of inflammatory and proinflammatory genes at the intestinal level (Tian et al., 2017). It is therefore undeniable that the content and diversity of the intestinal microbiota are strictly related to the general health of the host and its ability to resist infections and environmental stressors.

One strategy for improving intestinal immunity and ameliorating the composition of the commensal microbiota is the use of functional additives. The most important of these are probiotics, prebiotics, phytogenics, and organic acids. Several studies have shown that functional diets containing different bioactive compounds can improve growth (Rimoldi et al., 2018a), gut health (Estensoro et al., 2016; Nimalan et al., 2022; Rimoldi et al., 2020a, 2020b; Torrecillas et al., 2018, 2019), and immunity of fish (Ferna´ndez-Montero et al., 2021; Moroni et al., 2021; Piazzon et al., 2017; Serradell et al., 2020; Terova et al., 2016) and shrimp (Kesselring et al., 2021); thereby mitigating the negative side effects caused by the replacement of marine ingredients in the diet.

Most probiotics used in aquaculture belong to the Firmicutes phylum, particularly lactic acid-producing bacteria (LAB) and *Bacillus* spp. The modes of action of probiotics include exclusion of pathogens, improvement of feed digestion, absorption of macro- and micronutrients, improvement of immune response, and production of antimicrobial and functional compounds (El-Saadony et al., 2021; Simo´n et al., 2021). Recently, we showed that dietary administration of the probiotic strain *Lactococcus lactis* subsp. *lactis* SL242 stimulates the expression of interleukins *il-10* and *il-12*, and modulates the sea bream (*Sparus aurata*) gut microbiota even without the colonization of the probiotic in the host's intestinal mucosa (Moroni et al., 2021).

In recent years, our research has contributed significantly to the knowledge of the efficacy of additives in aquafeeds for the two most important species for aquaculture in the Mediterranean Sea: European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*). In particular, we found a positive effect of prebiotic and phytogenic additives on both immune response and gut microbiota in sea bass fed low FM and FO diets (Rimoldi et al., 2020b; Torrecillas et al., 2021, 2019). Our findings suggest that dietary intake of galactomannan oligosaccharides and phytogenics induces changes in the gut microbiota, modulates the expression of oxidative enzyme-related genes, and attenuates the stress response of fish. However, proper development of the aquaculture sector involves not only effective replacement of marine raw materials with novel substitutes, but also a successful breeding program to improve growth and feed utilization. Several strains of fish that can tolerate novel feeds with low (Belghit et al., 2019; Boudry et al., 2021; Gjedrem et al., 2012) or even zero amounts (Abernathy et al., 2017; Brezas and Hardy, 2020; Callet et al., 2017) of marine raw materials have already been developed.

Accordingly, the present study aimed to verify whether selected fish genotypes can benefit from the use of functional additives in aquafeeds by addressing the limitations of FM/FO availability in terms of improving fish growth performance, gut health, and disease resistance. To this end, three different types of additives (probiotics, organic acids, and phytogenics) were separately added to the feed and tested on two batches of juvenile European sea bass: selected high growth fish (HG) and wild types (WT). Fish growth performance, immune-related gene expression, gut microbiota composition, and disease resistance were evaluated for these two fish strains.

2. Materials and methods

2.1. Ethical

The animal experiments complied with the European Union Council Directives (2010/63/EU) for the use of experimental animals. All protocols used in the present study were approved by the Bioethics Committee of the University of Las Palmas de Gran Canaria (OEBA ULPGC 11/2020).

2.2. Fish and diets

Two batches of juvenile European sea bass, selected high growth population (HG; 32 selected sires x 7 selected dams), and "wildtype" population (WT; 33 wild sires x 7 selected females) produced at MARBEC-IFREMER, were reared in the facilities of the Parque Científico-Tecnolo´gico Marino (PCTM) at the University of Las Palmas de Gran Canaria (Telde, Canary Islands, Spain). The mating scheme of the genotypes HG and WT was described in detail by Montero et al. (2023). Briefly, seven dams were derived from experimental broodstocks selected for growth for three generations. Eggs were collected by stripping and pooled equally between dams. The batch HG was obtained by *in vitro* fertilization with thawed semen from 33 selected sires (breeding company EMG Ecloserie Marine de Gravelines) for multi-trait selection (growth, morphology, and low muscle fat content) over seven generations (>35 years). The genotype WT was bred using sperm from 32 wild European sea bass from the western Mediterranean caught in the Gulf of Lion. One-day-old hatched larvae from each batch of fish (HG or WT) were pooled in equal numbers from each dam and sent to the University of Las Palmas de Gran Canaria (ULPGC; Las Palmas de Gran Canaria, Spain). Selected (HG) or reference fish (WT) were maintained under similar conditions during the pre-weaning, weaning, and early juvenile phases. At 294 days post-hatching (dph), after the juveniles had reached a mean body mass of 10 g, they were nutritionally challenged. The fish were fed a "future diet" that represented our control diet, in which the total amount of FO was replaced by a combination of poultry oil (PO) and DHA-algae oil and 50% of FM by poultry meal (PM) (Skretting ARC, Norway), up to an initial experimental size of 16 g. The formulation and proximate composition of the "future" or control diet are shown in Table 1. Proprietary functional additives were produced by INVE Aquaculture (Belgium) and consisted of a probiotic mixture (PROB), a

Table 6.1.
Ingredients and proximal composition of the reference "Future" diet (CTRL).

Ingredients (%)	
Corn gluten	5.0
Hi Pro Soybean meal[a]	6.0
Wheat gluten	10.2
Faba bean dehulled[b]	8.0
Wheat	19.95
Soy protein concentrate [c]	15.0
Fish oil[d]	0.0
Fish meal[e]	10.0
Rapeseed oil	8.98
Phosphate	0.35
Vitamin & mineral mix[f]	0.3
Poultry meal[g]	10.0
Poultry oil[h]	1.37
DHA oil[i]	2.75
Lecithin	2.0
Proximal composition (% dry matter)	
Moisture	6.25
Crude protein	51.18
Crude fat	17.67
Ash	4.57

Yttrium premix: 0.1%
[a] Soya bean meal: CJ Selecta S.A (Brasil)
[b] Faba beans: Cefetra BV (The Netherlands)
[c] Soya protein concentrate: CJ Selecta S.A (Brasil)
[d] Fish oil: Copeinca, S. A. (Perú)
[e] Fish meal: Norsildmel AS (Norway)
[f] Mineral and Vitamin premix: Trouw Nutrition (The Netherlands)
[g] Poultry meal: Sonac (Belgium)
[h] Poultry oil: Sonac (Belgium)
[i] DHA: Veramaris (Evonik)

mixture of organic acids (ORG), and natural plant extracts (PHYTO). Specifically, the probiotic mixture (INVE, Belgium) contained three *Bacillus* species: *B. subtilis*, *B. licheniformis*, and *B. pumilus* at a total bacterial concentration of 2 × 10^{10} CFU (colony forming units)/g of product and with each of the bacterial species in an equal ratio. The organic acid booster was formulated based on the knowledge and known benefits of butyric acid and consisted of 70% butyric acid sodium salt of (GBM CMR, Sanluc, Belgium), while the phytogenic booster consisted of a robustness-enhancing additive based on 16% natural plant extracts of garlic combined with medium-chain fatty acid sources (Aquagarlic P Protec, Domca, Spain). After the acclimation period, fish were randomly distributed to 24 tanks of 500 l (34 fish/tank; 12 tanks per genotype; 19.0 ± 0.4 g) and fed until visual satiation with the future diet (CTRL) or with the future diet containing three different functional additives (INVE Aquaculture, Belgium): (i) 2 weeks with high dose, followed by (ii) 10 weeks with low dose (Fig. 1). The experimental feed additives were hand-oil-top-coated at two doses: low and high. For the PROB feed, the high and low doses were set at 10 g/kg and 2 g/kg, respectively. For the ORG feed, 7.5 g/kg corresponded to the high dose and 3 g/kg to the low dose, while natural plant extracts (PHYTO) were coated at 7.5 g/kg and 5 g/kg.

Growth performance parameters were calculated for both feeding periods (high and low doses). Before each sampling, fish were fasted for 24 h, anaesthetized with clove oil (4 ml clove oil / 100 l water) and measured individually. Growth parameters were calculated as follows:

Daily growth index (DGI) = [(final weight1/3- initial weight1/3)/number of days x 100];
Specific growth rate (SGR) = [(Ln (final weight-Ln (initial weight)) / number of days x 1 00]·

At each sampling point, 3 fish per tank (three tanks/feeding treatment) were euthanized with an overdose of anaesthetic (clove oil) and samples were collected at the end of the feeding experiment (12 weeks) for gut microbiota characterization (autochthonous) and gene expression analysis. After each dose feeding, a pathogen (*Vibrio anguillarum*) challenge test was performed in conjunction with stress conditions (overcrowding), as previously described (Serradell et al., 2020), to investigate the potential of additives to improve the resistance of fish exposed to both infection and stress conditions. Briefly, this stress test consisted of keeping fish submerged in 25.6-litre cages (40 cm × 20 cm) for one week (three tanks/feeding treatment) for one week and exposing them to *V. anguillarum* (CI) (10^5 CFU per fish) by anal inoculation. For each feeding treatment, the cages were distributed to 6 cylindrical conical 500-liter tanks in a recirculating aquaculture system (RAS) supplied with filtered water at a temperature of 22 °C. The water renewal rate of RAS was one per hour (500 l/hour), while aeration consisted of pumping air and, if necessary, automatically adding oxygen. Fish mortality was monitored during the experiment and relative sur- vival (RPS) was calculated at the end of the experiment using the following equation: RPS = [1- mortality of fish fed the diet with addi- tives (%)/mortality of fish fed the control diet (%)] x 100. Fish were fed the respective diets during the challenge test and fish survival was recorded daily.

2.3. Metabarcoding analysis of gut microbial communities

2.3.1. Sampling and bacterial DNA extraction

At the end of the feeding experiment (12 weeks), intestinal samples were collected from six fish in each feed/batch group were collected (48 samples in total). Feces were removed from each intestine by careful squeezing, and mucosa-associated microbiota (autochthonous microbiota) was obtained by scraping the intestinal mucosa (without pyloric ceca) with a sterile cotton swab. The tip of the swab was immediately

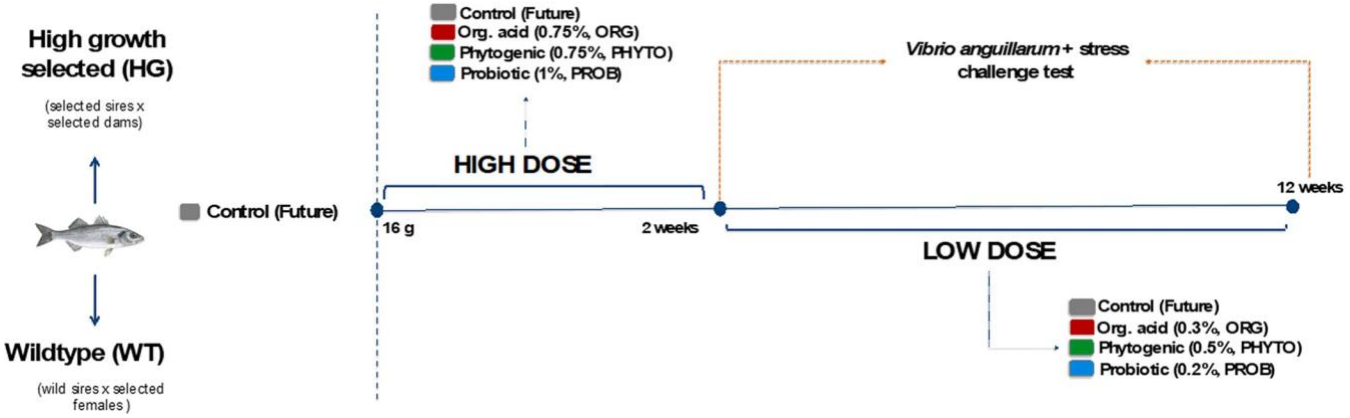


Fig. 6.1. Graphical schematic of the experimental

immersed in 300 μl Xpedition Lysis/Stabilization Solution (Zymo Research, Italy) and vortexed to facilitate bacterial release (Rimoldi et al., 2019). The tips of the swab and the solution were stored at 4 °C until DNA extraction.

DNA was extracted from 250 μl of gut bacterial suspension and from 200 mg of each feed (three aliquots/feed) using a DNeasy PowerSoil® Pro kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. The concentration and purity of DNA were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Milan, Italy). Bacterial DNA was stored at − 20 °C until amplicon library preparation.

2.3.2. 16S library preparation and MiSeq amplicon sequencing

Library preparation and sequencing on the Illumina MiSeq platform (Illumina, Italy) were performed by GalSeq SRL (Milan, Italy). The details of the methodology used for 16 S rRNA gene library preparation and sequencing have been reported previously (Terova et al., 2021). To identify the bacterial taxa in the gut, the hypervariable region V4 of the bacterial 16 S rRNA gene was amplified using aliquots of the isolated DNA from each sample and the oligonucleotides 515 F:5′-GTGY CAGCMGCCGCGGTAA-3' and 806 R:5′-GGACTACNVGGTWTCTAAT-3′. The expected size of the PCR products on the Agilent 2100 Bioanalyzer lane was ~ 400 bp. Amplicon libraries were quantified by qPCR, pooled at equimolar concentrations, diluted at 6 pM, multiplexed, and sequenced on an Illumina MiSeq instrument using a pair-ended sequencing starategy (2 × 250). All sequences were submitted to the European Nucleotide Archive (EBI ENA).

2.3.3. Bioinformatic analysis of raw sequencing data

The obtained reads were quality filtered (Q > 30), merged, and processed with the QIIME 2™ (v. 2018.4) pipeline using default settings (Bolyen et al., 2019). The remaining high-quality reads were dereplicated, and singletons and chimeric sequences were removed using the QIIME DADA2 denoise-paired command. The output of the DADA2 pipeline was a feature table of amplicon sequence variants (ASV table) in which the number of times each ASV occurred in each sample was recorded.

Taxonomic assignment was based on the Silva database (http://www.arb-silva.de) at the genus level. All ASVs assigned to chloroplasts and mitochondria were removed from the analysis because they were of eukaryotic origin. The alpha (within a single sample) and beta (between samples) diversity of bacterial communities were calculated. In particular, alpha diversity indices (Chao 1, Faith PD, observed ASVs, Shannon, and Simpson) were evaluated at the same level of rarefaction. Beta diversity was calculated using the weighted (presence/absence/abundance) and unweighted (presence/absence) UniFrac distance matrices (Lozupone and Knight, 2005; Lozupone et al., 2007).

To visualize the core microbiota (ASVs present in at least four of the six samples per diet/batch group), a Venn diagram was created using the Venny 2.1 tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.3.4. Predictive functional analysis of gut bacterial communities

The functional profile of the gut microbiome was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt1) (Langille et al., 2013). The 16 S rRNA gene data were referenced according to the Greengenes 13.8 database, and the resulting data were used for prediction analysis with PICRUSt via the Kyoto Encyclopedia of Genes and Genomes (KEGG). The identified KEGG orthology pathways were sorted into functional categories based on the KEGG pathway subsystem at hierarchical level 3. The PICRUSt1 output files (profile and metadata) were uploaded to the Statistical Analysis of Metagenomic Profiles software package (STAMP) (Parks et al., 2014) to generate extended error plots for each pairwise com- parison. Welch's two-tailed t-test was used to determine differences between the two groups with 95% confidence.

2.4. Gene expression analysis

For quantitative gene expression analysis, 0.5 cm samples of the proximal and distal intestinal regions (n = 6 fish /diet) were collected at the end of the feeding experiment and stored in RNAlater™ stabilization solution (ThermoFisher, Milan Italy) until they were delivered to the laboratory of the Department of Biotechnology and Life Sciences (Varese, Italy), where they were stored at − 80 °C until molecular analysis. Total RNA was automatically extracted from intestinal samples using the Maxwell® 16 LEV simplyRNA kit (Promega, Milan, Italy) in combination with the Maxwell® 16 instrument (Promega, Milan, Italy). The amount and purity of extracted RNA were determined spectrophotometrically using a NanoDrop™ 2000c spectrophotometer (Thermo Scientific, Milan, Italy). After extraction, 100 ng of RNA was subjected to qPCR using an iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad, Milan, Italy). This kit uses a combination of iScript™ RNase H + reverse transcriptase (to generate complementary DNA) and antibody hot-start iTAQ DNA polymerase to perform SYBR® Green real-time reactions in one step. PCR efficiency is improved over a wide dynamic range and under different conditions. All primer sequences used for quantification of target genes are listed in Supplementary Table 1. The qPCR reactions were run in triplicate on a Bio-Rad® CFX96™ system under the following conditions:10 min at 50 °C, 1 min at 95 °C, followed by 40 cycles consisting of 10 s at 95 °C and 30 s at 60 °C, followed by a melting curve (65–95 °C). Ct values were analyzed with Bio-Rad CFX Maestro software (Bio-Rad, Milan, Italy) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). β-actin was chosen as the housekeeping gene, and Ct values were transformed to a relative amount, using the lowest Ct value as the calibrator.

2.5. Statistics

All data were tested for normality and homogeneity of variance. Differences were considered statistically significant at $p \leq 0.05$. For quantitative data, individual effects of diet and genomic batch were analysed using two-way ANOVA. To ensure the assumptions required for the parametric tests, the relative abundance values (%) of the bacterial taxa were angle transformed. Analysis of the microbial dataset was performed only for those taxa whose overall abundance exceeded 1% up to the order level and 0.5% at the family and genus levels. The PERMANOVA test for beta diversity (999 permutations) was applied to compare the dissimilarity of microbial communities between groups using UniFrac distance matrices. The dissimilarities between experimental groups and the corresponding VIP values were calculated with Partial Least Squares Discriminant Analysis (PLS-DA) using R software. The remaining analyses were performed with Statistical Software Sys- tem v21.0 (SPSS, Chicago, IL, USA) and PAST3 software (Hammer et al., 2001).

3. Results

3.1. Fish growth performance

From the two-week feeding period (period of high-dose supplementation) to the end of the feeding experiment (period of high-dose + low-dose supplementation), fish from HG performed better ($p < 0.05$) than fish from WT in terms of body weight, relative growth, SGR, and DGI. Relative weight gain and SGR ranged from 365% to 400% and 1.3–1.4% in the HG groups to 290–310% and 1–1.1% in the WT fish group, respectively (Table 2). Functional feeding treatments only affected the performance of the HG fish at the end of the feeding trial, with fish fed the ORG diet having lower ($p < 0.05$) final body weight than fish fed the control diet, but similar to fish fed the PROB and PHYTO diets (Table 2).

The resistance of fish to *V. anguillarum* after feeding the experimental functional additives during the feeding experiment is shown in Fig. 2. At

Table 6.2.
Growth performance and feed utilization of wild (WT) and selected for growth (HG) European sea bass fed the experimental diets. W: mean body weight, WG: relative weight gain; SGR: specific growth rate; DGI: daily growth index.

		HG				WT				*Significance*		
	weeks	**CTRL**	**PROB**	**ORG**	**PHYTO**	**CTRL**	**PROB**	**ORG**	**PHYTO**	**Diet**	**Genotype**	**D*G**
W (g)	0	19.2 ± 0.3	19 ± 0.2	19.5 ± 0.1	18.5 ± 0.7	18.8 ± 0.3	18.9 ± 0.2	19.1 ± 0.4	19 ± 0.5	ns	ns	ns
	2	34.2 ± 3.1	37. 8 ± 4.4	36.7 ± 0.5	36.1 ± 2.1	32 ± 3.5	32.7 ± 3.3	29.7 ± 1.7	30.8 ± 3	ns	p < 0.05	ns
	12	79.7^{a} ± 11.3	74.2^{ab} ± 4.8	71.2^{b} ± 4.4	74.7^{ab} ± 2.6	55.9 ± 6.4	59.2 ± 2.3	57.2 ± 3.6	59.4 ± 2	ns	p < 0.05	p < 0.05
WG (%)	12	400.4 ± 53.7	388.1 ± 24.9	364.3 ± 2.8	401.6 ± 27.8	289.8 ± 37.1	307.9 ± 10	302.1 ± 16	311.6 ± 15.9	ns	p < 0.05	ns
SGR	12	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.01	1.4 ± 0.1	1 ± 0.1	1.1 ± 0.01	1.1 ± 0.1	1.1 ± 0.01	ns	p < 0.05	ns
DGI	12	18.8 ± 3.6	17.9 ± 1.6	16.9 ± 0.1	18.2 ± 1.1	11.6 ± 2.1	13 ± 0.7	12.5 ± 1.1	13.1 ± 0.7	ns	p < 0.05	ns

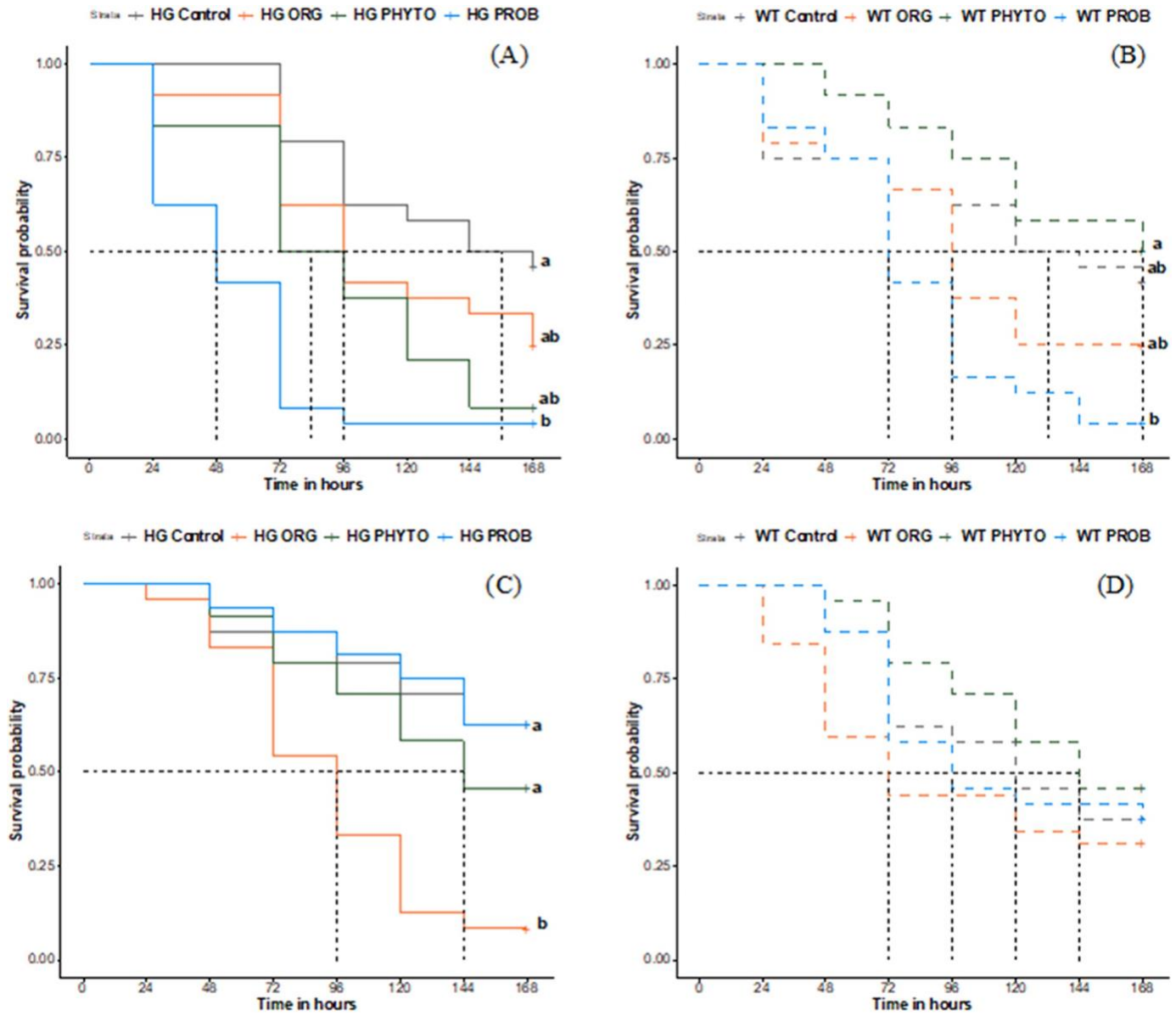


Fig. 2. Cumulative survival (%) of European sea bass (*Dicentrarchus labrax*) in the challenge test against *V. anguillarum* combined with confinement stress after 2 weeks of high dose for genotypes HG (A) and WT (B) and 12 weeks of feeding (2 weeks of high dose + 10 weeks of low dose) for genotypes HG (C) and WT (D). Different letters indicate statistical differences ($p < 0.05$; Kaplan-Meier survival). HG, genetically selected genotype; WT, wild-type genotype of European sea bass. Diets: probiotic mixture (PROB), organic acid mixture (ORG), phytogenic (PHYTO).

the end of the high-dose supplementation period (two weeks; Figs. 2A, 2B), the Kaplan-Meier curve showed a significant diet effect (F=3.469, p = 0.0411), indicating that fish fed a high dose PROB had a lower ($p < 0.05$) probability of survival than fish fed the CTRL diet, regardless of the batch of fish. After long-term supplementation (2 weeks high dose +10 weeks low dose), the results differed between fish families (Figs. 2C, 2D). HG fish fed the ORG diet showed the lowest survival rate ($p < 0.05$) compared to the other treatments (Fig. 2C), while no significant differ- ences were found between the experimental groups in WT fish, indi- cating an effect of genotype on the use of the tested additives (Fig. 2D).

3.2. Gut expression level of inflammation- and immune- related genes

In the proximal intestine, administration of any of the tested additives (PROB, PHYTO, and ORG) did not affect the expression of target genes compared to control fish. However, there was a significant effect of genotype batch ($p < 0.05$); expression of *cd4*, *cox-2*, and *il-10* was downregulated in HG compared to WT fish, regardless of diet (Fig. 3). In contrast, neither diet nor genotype affected transcript levels of the *mhc-II*, *il-1β*, and *tnf-α* genes in the proximal intestinal tract (data not shown). In contrast, there was an interaction effect between genotype and diet on *il-1β* gene expression in the distal intestine (Fig. 4). Selected fish fed a probiotic-supplemented diet showed upregulation of *il-1β* compared with the same batch fed CTRL or OA. No differences were found between experimental feeding groups in the expression levels of *cd4*, *tnf-α*, and *il-10* (data not shown), while the genes *mhc-II* and *cox-2* showed a batch effect and were significantly ($p < 0.05$) higher expressed in HG and WT fish, respectively (Fig. 4). However, there is no evidence of a correlation between gut microbiome profiles and host GALT gene expression.

3.3. Metabarcoding analysis outputs

All Illumina sequence files (FASTQ format) were deposited in the public database of the European Nucleotide Archive (EBI ENA) under accession code **PRJEB61519**.

A total of 840,441 reads were correctly classified using the database SILVA database. The Good's coverage value for all samples was ≥ 0.99, indicating that sequencing coverage was achieved and the ASVs found were representative of the microbial communities in feed and gut. To calculate the alpha diversity metrics, the feed and gut mucosal samples were normalized at a sequencing depth of 10,000 reads. The alpha diversity metrics are shown in Table 3. Statistical analysis using a two-way ANOVA showed no significant differences in species richness and diversity between the groups.

Beta diversity analysis revealed no overall effect of genetic batch and/or diet on microbial community profiles (Fig. 5A, B). Permutational multivariate analysis using Permanova's test with 999 permutations on weighted (F = 0.02; p = 0.926) and unweighted (F = 0.284; p = 0.639) UniFrac distance data fully confirmed the PCoA results.

Also, PLS-DA showed no clear separation between CTRL and fish fed additives, regardless of their genetic background (Fig. 6A, B). However, when we considered only the effect of genetic background, less inter-individual variability was observed at HG (Fig. 6A).

3.4. The feed-associate microbiota

The microbiota profile of the feed is outlined at the phylum, class, order, family, and genus levels. All samples included six different phyla, nine classes, 22 orders, 32 families, and 38 genera. However, when considering only the most abundant bacterial taxa, there were two phyla, three classes, 10 orders, 18 families, and 19 genera. The composition of the feed-associated microbiota was reported at the phylum and genus levels (Fig. 7). The relative abundances (%) of the most abundant taxa found in the feed samples and their statistical sig- nificance are shown in Supplementary Table 2. At the phylum level, the feeds showed a similar profile, with a higher proportion of Proteobac- teria (63–74%) followed by Firmicutes (24–35%) (Fig. 7A). As expected, the highest abundance of the genus *Bacillus* was found in the probiotic-

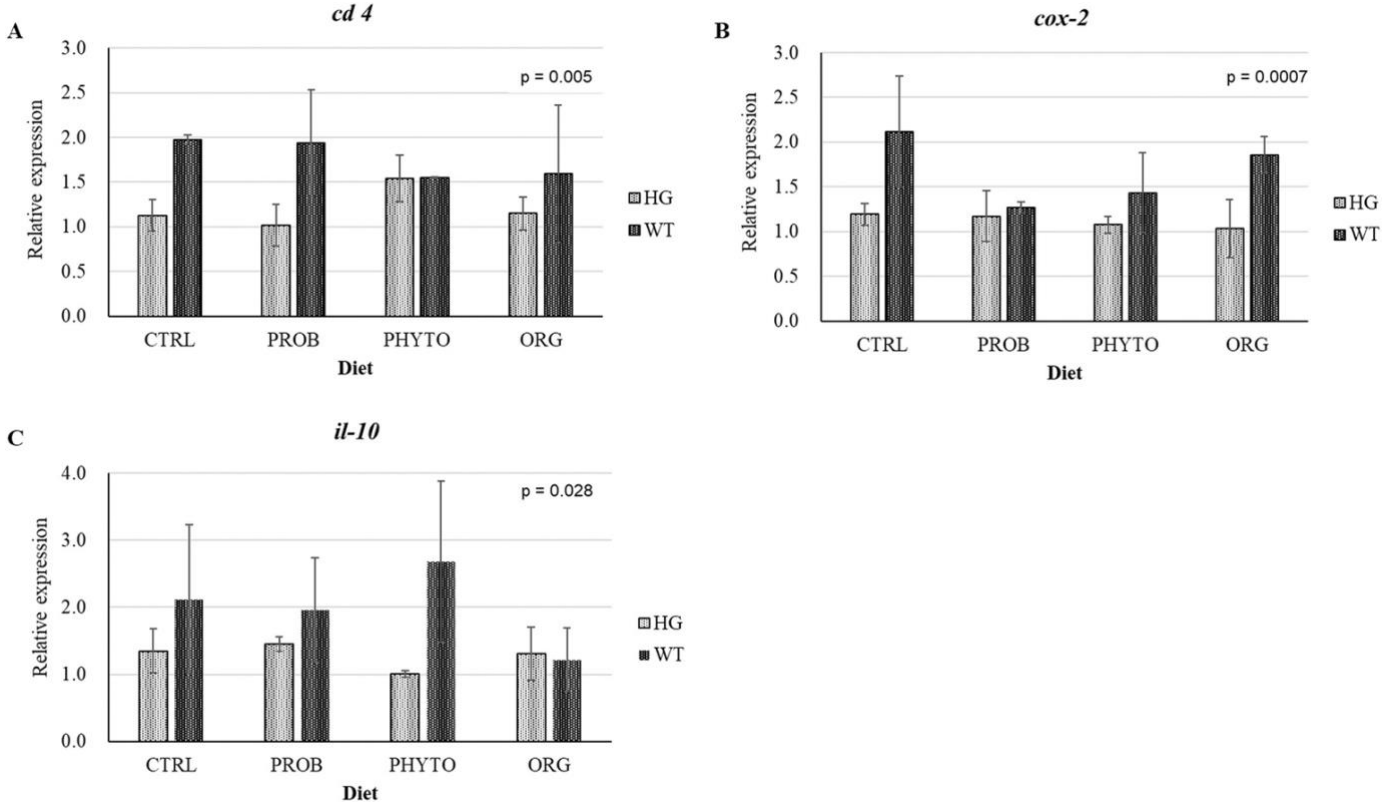


Fig. 6.3. Gene expression data in proximal gut. Data are mean ± SD.

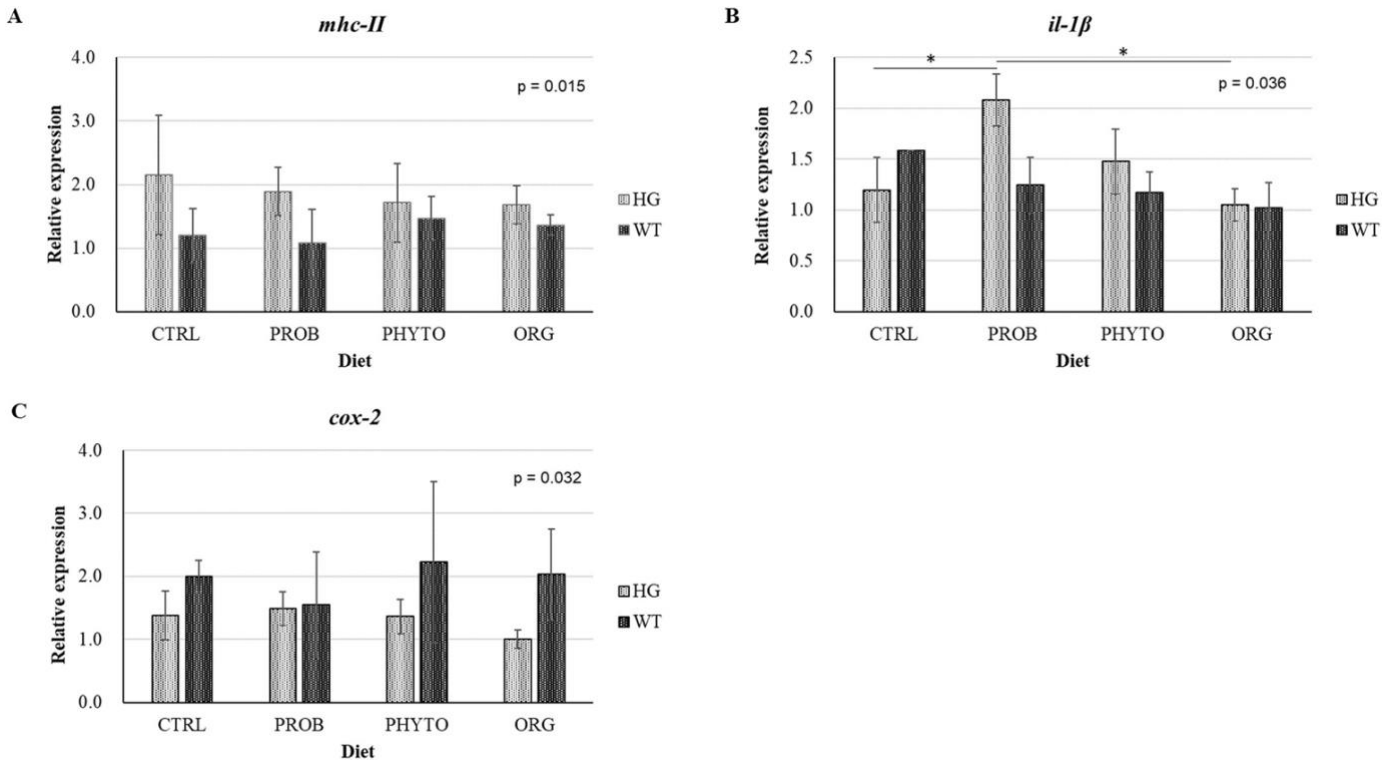


Fig. 6.4. Gene expression data in the distal intestine. (*) indicates significant difference between means ($p < 0.05$). Data are mean ±

Table 6.3.
Alpha diversity metrics of mucosa-associated microbial communities. The values are reported as mean values (n = 6) ± SD. The means were compared by two-way ANOVA test ($p < 0.05$).

		Chao 1	Faith PD	Observed OTUs	Shannon	Simpson
WT	**CTRL**	658 ± 281	9.1 ± 2.7	560 ± 238	6.2 ± 1.4	0.93 ± 0.04
	ORG	602 ± 249	10.0 ± 4.2	500 ± 215	5.8 ± 1.2	0.92 ± 0.04
	PROB	429 ± 178	7.3 ± 1.8	372 ± 169	5.3 ± 1.5	0.90 ± 0.05
	PHYTO	730 ± 219	11.0 ± 3.7	631 ± 209	6.7 ± 1.4	0.95 ± 0.04
HG	**CTRL**	537 ± 293	8.3 ± 4.1	469 ± 253	6.0 ± 1.4	0.93 ± 0.05
	ORG	665 ± 190	9.6 ± 0.9	558 ± 160	6.2 ± 1.4	0.93 ± 0.05
	PROB	465 ± 272	7.4 ± 2.5	384 ± 238	5.1 ± 1.2	0.89 ± 0.04
	PHYTO	463 ± 85	7.9 ± 2.1	406 ± 82	5.6 ± 0.7	0.92 ± 0.03
Significance		Diet: 0.231	Diet: 0.211	Diet: 0.241	Diet: 0.231	Diet: 0.156
		Genotype: 0.254	Genotype: 0.219	Genotype: 0.270	Genotype: 0.412	Genotype: 0.617
		D*G: 0.293	D*G: 0.610	D*G: 0.355	D*G: 0.540	D*G: 0.896

containing feed. Overall, the microbial community profile of the probiotic-containing feed differed most from that of the control.

3.5. Dietary gut microbiota modulation

The microbiota of the 48 intestinal mucosal samples was mainly composed of six phyla, 11 classes, 34 orders, 53 families, and 101 genera. Considering only the most representative taxa, it consisted of four phyla, six classes, 15 orders, 28 families, and 25 genera. The gut microbial community profiles for each feeding group are shown in Fig. 8 at the phylum, family, and genus levels. As expected, Firmicutes and Proteobacteria were the most abundant phyla in the sea bass gut (Fig. 8A) and accounted for more than 90% of the sequencing reads in all experimental groups (Supplementary Table 3). Regardless of the diet administered, the core microbiota was identified in both HG and WT samples. In both batches, the majority of genera were represented in all dietary groups (Fig. 9A). Even when only genotype was considered, the core microbiota was similar between the two fish strains, which shared 19 genera (Fig. 9B). Only six and four genera were exclusive to WT and HG, respectively.

The results of the two-way ANOVA showed an influence of diet and genotype on the relative abundance of certain taxa. However, the interaction effect between the two main factors (diet and genotype) was significant only in rare cases (Supplementary Table 3). When the main effects of diet and genotype were considered, the former was clearly more significant. At the phylum and class levels, statistical analysis by two-way ANOVA revealed no significant differences in relative taxa abundance. Dietary additive intake affected gut microbiota profiles at the family (Sphingomonadaceae, Moraxellaceae, Pseudomonadaceae) and genus (*Streptococcus*, *Pseudomonas*, *Sphingobium*, and *Novosphingobium*) levels. A significant interaction between diet and genotype was found at the order level for Lactobacillales, resulting in higher abundance in fish fed the PHYTO diet (Supplementary Table 3). Significant genotype-diet interactions were also observed for the family Weeksellaceae (enriched in WT-PHYTO) and for the genera *Enterovibrio* (enriched in HG-PHYTO) and *Acinetobacter* (lower in HG-PROB and WT-PROB). In general, the relative abundance of *Acinetobacter* and *Pseudomonas* was reduced in the guts of fish fed probiotics compared to controls for both strains (Supplementary Table 3, Figs. 8B, 8C). Probiotics and phytogenics negatively affected the abundance of *Novosphingobium* and

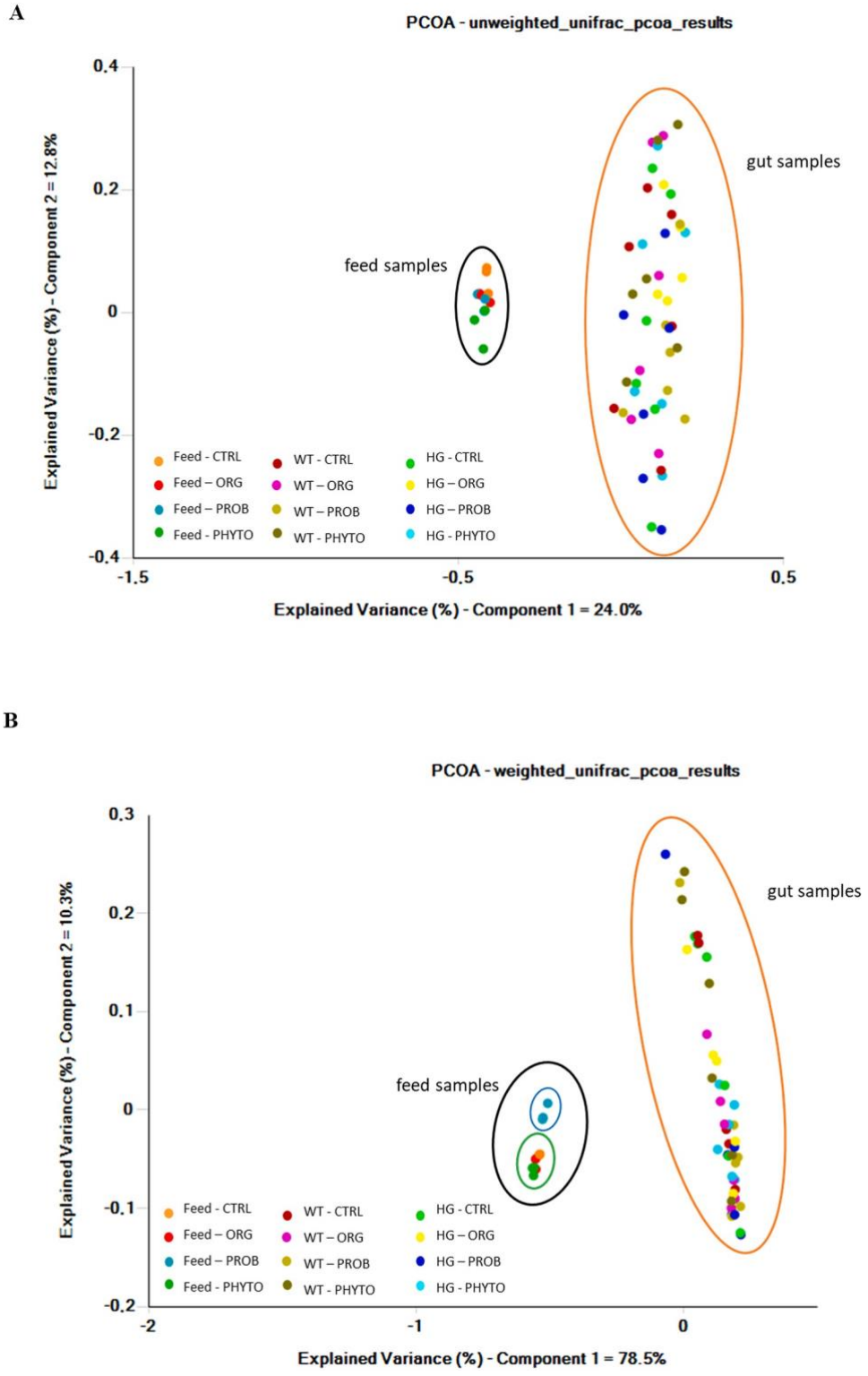


Fig. 6.5. Analysis of beta diversity between groups. (A) Unweighted and (B) weighted UniFrac PCoA plots of individual feed and gut samples from each group. In- dividual sample was represented as spot.

Sphingobium (Supplementary Table 3, Fig. 8C). Dietary supplementation with organic acids increased the relative abundance of Streptococcus, regardless of genotype, while bacteria of the genus *Photobacterium* were preferentially associated with WT sea bass (Supplementary Table 3, Fig. 8C).

3.6. Predictive functional profile of gut microbial communities

A functional profile of the gut microbiota was predicted using the PICRUSt tool. Functional analysis of the samples from WT samples showed higher abundance of carbohydrate metabolism when supple- mented with organic acids compared to controls (Fig. 10A). In response to the PHYTO diet, nucleotide metabolism improved (Fig. 10B), whereas

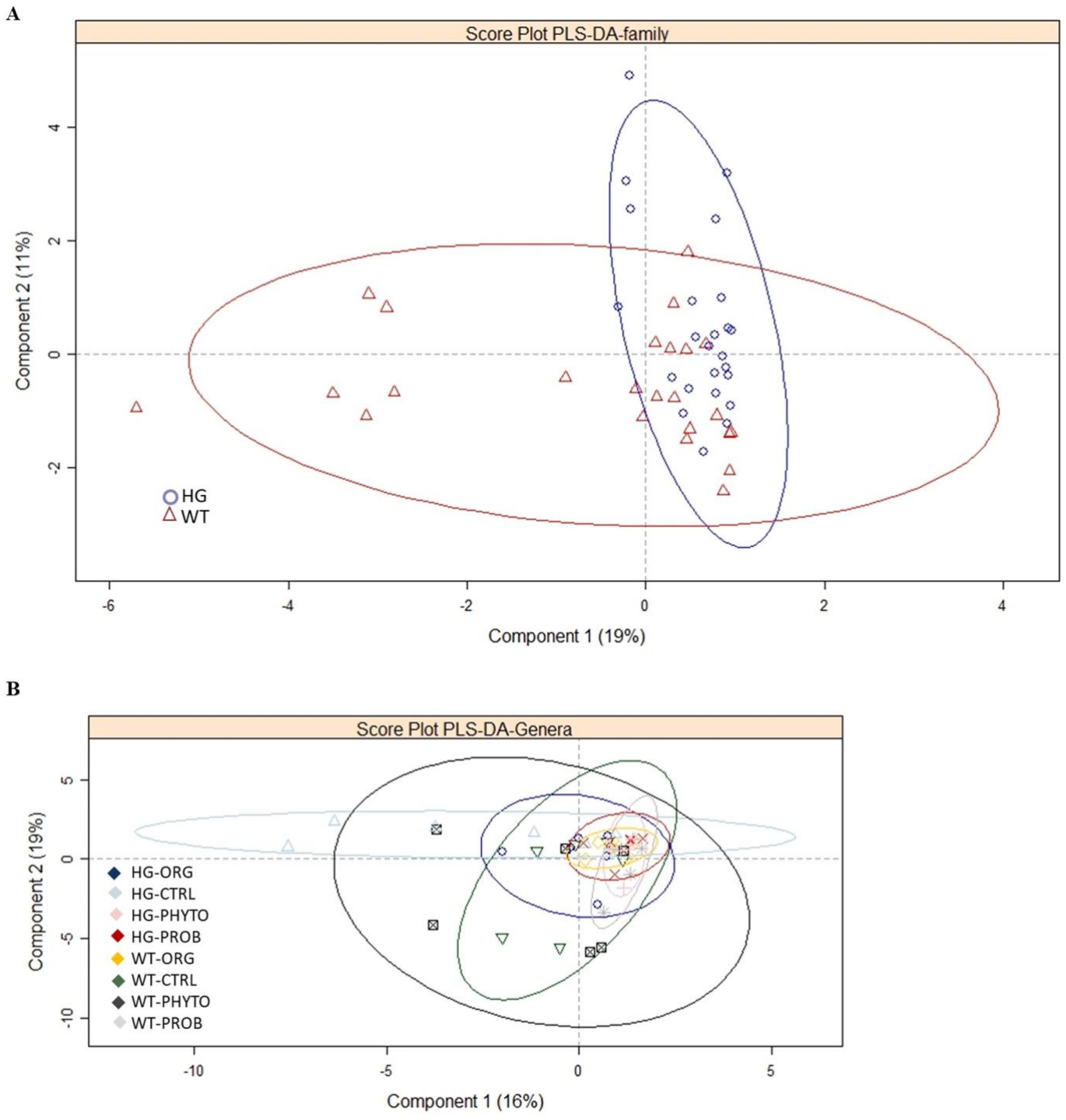


Fig. 6.6. Partial least square-discriminant analysis (PLS-DA) based on relative abundances of bacterial genera in the gut microbiota of the final samples. GS, genetically selected genotype; WT, wild-type genotype of European sea bass.

probiotic supplementation resulted in significant enrichment of DNA replication, ribosome biogenesis, and translation factor pathways (Fig. 10C).

In HG fish, metabolic pathways related to carbohydrate metabolism were more prominent in fish fed additives than in control fish. In particular, galactose metabolism and transport pathways were enriched in the gut microbiota of the HG-PHYTO (Fig. 11A) and HG-PRO (Fig. 11B) groups. In addition, the HG-PHYTO group had a higher abundance of metabolic pathways related to energy metabolism. Finally, a diet supplemented with organic acids resulted in enrichment of bacterial carbohydrate metabolism in genetically selected sea bass (Fig. 11C).

4. Discussion

The challenge of FM/FO replacement should be addressed by the aquatic feed industry with a strategy that includes a combination of technology, "complementary" raw materials, and innovation in selective breeding, rather than identifying individual substitutes (Turchini et al., 2019). In this context, the AquaIMPACT project (Horizon 2020), which

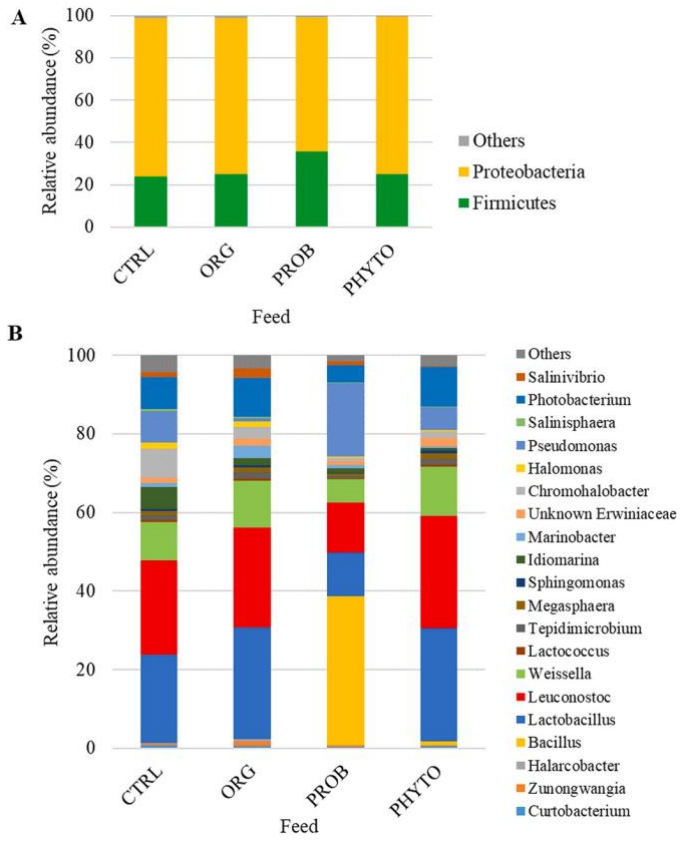


Fig. 6.7. Mean relative abundance (%) of bacteria most abundant in feed at phylum (A) and genus (B) taxonomic levels (N = 3). Only bacteria with a total abundance of 0.5% were reported. Bacteria with lower abundance were grouped together and reported as "other".

funded the present study, aims to integrate fish breeding and nutrition strategies to improve the competitiveness of European aquaculture and ensure a high-quality end product with limited environmental impact.

The aim of this study was to investigate the effects of a future diet supplemented with various functional additives on two different genotypes of sea bass. Effects on growth, host transcriptome, and resistance to vibriosis, and gut microbiota composition, were evaluated. The composition of the gut microbiota is strictly diet-dependent and has a major impact on host health. Therefore, evaluating the effects of novel formulations and functional additives on gut microbial communities of fish is critical for validating current feeding strategies in aquaculture. To date, high-throughput sequencing is the best strategy to characterize the profile of the gut microbiota of European sea bass in response to novel feeds (Busti et al., 2020; P´erez-Pascual et al., 2020; Rimoldi et al., 2020b; Serra et al., 2021).

In agreement with our previous study conducted with the same batches of fish, the genotype HG gave the best results in terms of growth and feed intake after 10 weeks at a low dose of feed additives (Montero et al., 2023). The present experiment confirmed that selected sea bass had a higher utilization capacity for a future diet poor in marine components than WT fish. Interestingly, in the HG group, only the fish fed PROB had a lower final weight than the fish in the control group. This is contrary to the widely held notion that the use of probiotics in aquaculture can improve fish survival, growth and health. In sea bream, administration of *Lactococcus lactis* did not improve feed conversion or specific growth rates, but had a positive effect on the final body weight of fish fed higher doses of probiotics compared to the control group (Moroni et al., 2021). Similarly, growth performance of turbot (*Psetta maxima*) was significantly improved when fish were fed different *Bacillus* species (*B. subtilis*, *B. licheniformis*, and *B. siamensis*), and the best results were obtained with *B. licheniformis* supplementation (Ma et al., 2022). However, contrary to all expectations, survival of fish exposed to pathogenic *V. anguillarum* in combination with overcrowding stress was lower in fish fed high-dose probiotic supplementation than in controls, regardless of genotype. In contrast to our results, a recent study on sea bass reported that *Bacillus velezensis* in the diet improved fish survival to *V. anguillarum* infection (Monzo´n-Atienza et al., 2022). The negative effect of the PROB diet on fish survival was reversed in the final stress test conducted at the end of 12 weeks of feeding after the phase of low-dose administration (2 weeks high-dose + 10 weeks low-dose). It is difficult to explain this unexpected reaction. Indeed, most probiotic modes of action have been observed in *in vitro* experiments, and the efficiency of a probiotic selected *in vitro* may change significantly when administered *in vivo*. In the host, probiotic organisms are influenced by complex factors, such as gut microbial interactions and/or the nutritional environment. Furthermore, probiotics may be more or less effective depending on the dose used (Moroni et al., 2021); however, the use of probiotics with multiple strains, as in the present study, should provide more benefit to the host than probiotics with only one strain (Puvanasundram et al., 2022; Shadrack et al., 2021).

After 12 weeks of feeding, fish fed the diet ORG had the lowest survival rates regardless of genotype, while survival rates were better for selected fish than for WT with all other additives.

Organic acids have long been used as feed additives in terrestrial livestock production, especially in swine and poultry nutrition (Khan et al., 2022; Nguyen et al., 2020). Their application in aquafeed, on the other hand, is new and little researched. Organic acids have been used as feed preservatives for centuries due to their antimicrobial activities (Ng and Koh, 2017). Organic acids are widely known as antibacterial, immune enhancing, and growth promoting agents in terrestrial animals. Fabay et al. (2022) recently studied the effects and importance of dietary supplementation with organic acids in farmed fish and concluded that their beneficial effects depend on the type and dose of organic acids as well as the species of fish used. Similar to the present study, sea bream fed a specific combination of short and medium fatty acids monoglycerides showed no effect on growth performance, but economic FCR improved compared to controls (Rimoldi et al., 2018a). Similarly, organic acids combined with natural identical compounds (thymol and vanillin) at the inclusion dose tested did not promote growth, feed conversion, or feed intake in juvenile sea bass reared under normal and suboptimal environmental conditions (Busti et al., 2020). In contrast, coconut oil, which is particularly rich in lauric acid (C12), improved growth and feed intake of sea bream (Simo´-Mirabet et al., 2017). In addition to the growth performance of the fish, no antibacterial effects were observed in fish fed the diet ORG, and they showed lower survival to *V. anguillarum* infection at the end of the feeding trial. Also, in a parallel experiment with sea bream, testing the same diet and functional additives, no significant effect of the diet on the growth and survival rate of the fish was observed. Even in this case, only an effect of genotype was observed; in particular, the selected fish performed better than the reference group (data not published).

Regarding the expression of immune-related genes, we found upregulation of *cd4*, *cox-2*, and *il-10* genes in the proximal intestine in a genotype-dependent manner, resulting in higher expression in HG fish, regardless of diet type. In contrast, an interaction effect between diet and genotype was observed in the distal part of the intestine, leading to an increase in *il-1β* expression in selected fish fed a probiotic diet.

Accordingly, significant intestinal upregulation of the proinflammatory cytokine *il-8* and anti-inflammatory cytokines *il-10* and *tgf-β* was observed in sea bass at increasing levels of dietary additives, particularly citric and sorbic acids and nature-identical compounds (Busti et al., 2020). The highest expression levels of *il-10* and *il-12* were observed in sea bream fed a high-dose probiotic diet (Moroni et al., 2021). Cytokines play an important role in the innate immune response of fish and are considered the best reference genes for studying their immune response (Sakai et al., 2021). *Il-1β* is an important cytokine

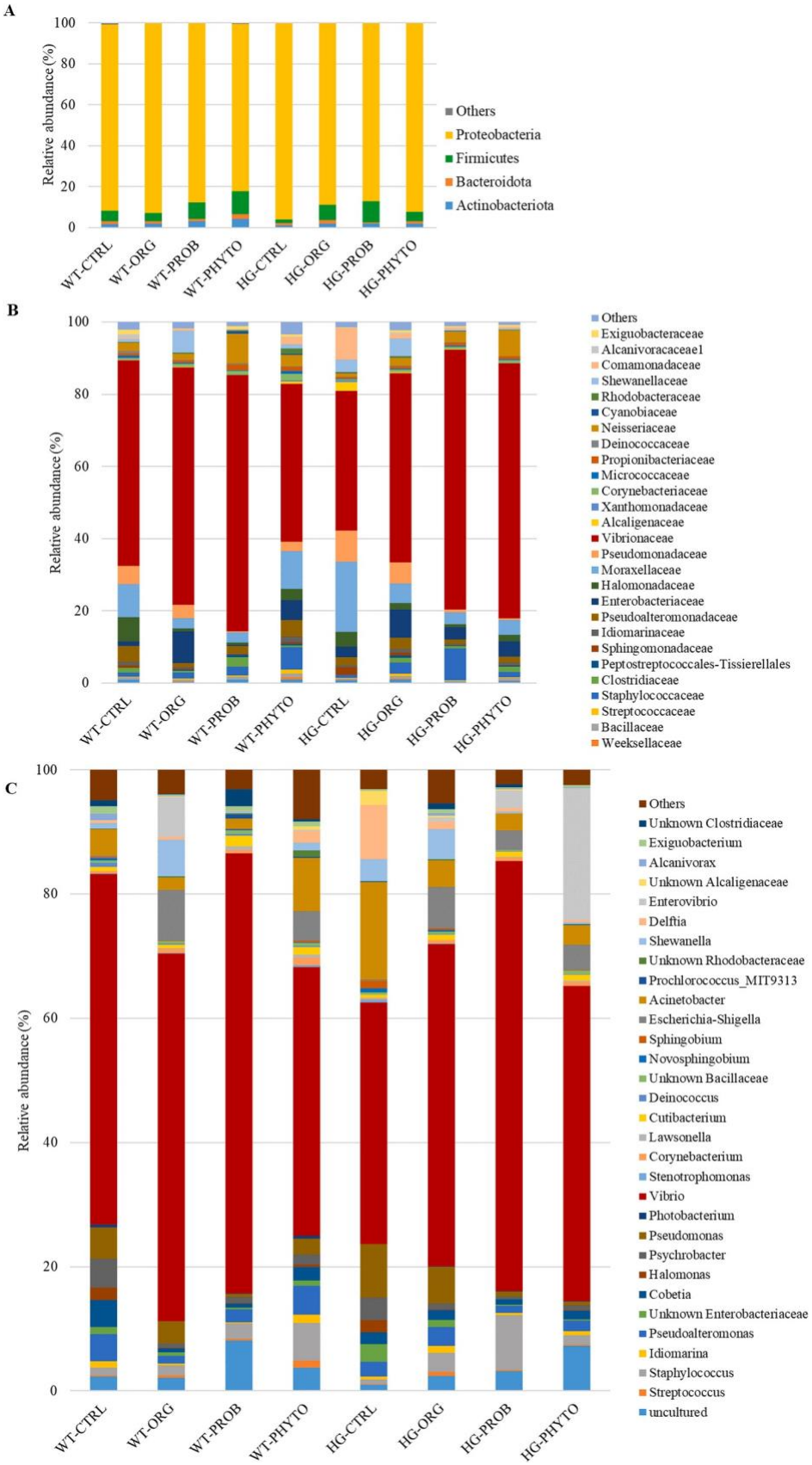


Fig. 6.8. Mean relative abundance (%) of the most abundant bacteria in the intestinal mucosa of sea bass at the end of feeding experiment at the phylum (A), family (B), and genus (C) taxonomic levels (N = 6). Only bacteria with a total abundance of 0.5% were reported. Bacteria with lower abundance were grouped together and reported as "other". GS, genetically selected genotype; WT, wild-type genotype of European sea bass.

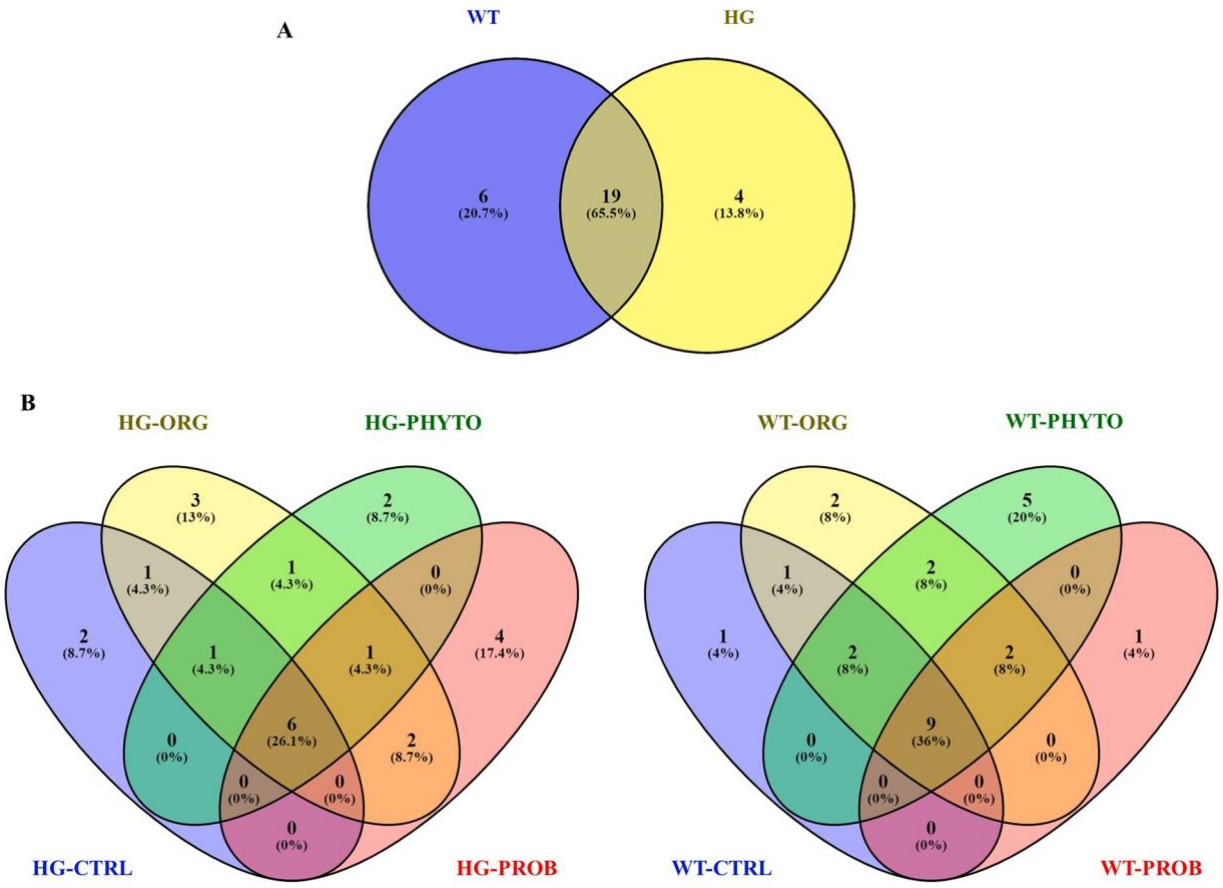


Fig. 6.9. Venn diagrams showing OTUs common between the two genotypes (A) or between feeding groups

involved in the activation of lymphocytes, phagocytic cells, and proinflammatory signaling pathways in peripheral tissues and brain. Interestingly, in the rainbow trout intestinal epithelial cell line RTgutGC and in intestinal explants, transcript levels of *il-1β* and *tnf-α* genes were significantly increased by exposure to the two *Bacillus* strains, whereas intraperitoneal injection of *B. subtilis* in trout upregulated the anti-inflammatory interleukins *il-4* and *il-13* (Docando et al., 2022). However, *in vivo* evaluation of probiotics is usually performed by feeding fish for an extended period of time. Administration of *Bacillus* probiotics for prolonged periods has been shown to upregulate transcription of cytokines in several fish species (Abarike et al., 2018; Cerezuela et al., 2013; Monzo´n-Atienza et al., 2022; Telli et al., 2014).

The greater influence of genotype on immune-related gene expression is easier to explain. This could be a consequence of multi-trait selection to improve fish growth parameters. As previously reported by Montero and colleagues (Montero et al., 2023), the GS fish used in this study were also indirectly co-selected for other traits, such as better adaptation to the rearing process and breeding manipulations, but also resistance to disease.

Despite the lack of significant growth enhancement in fish fed the tested additives, a modulatory effect of both diet and genotype on the resident gut microbial communities was observed. The overall composition of the gut bacterial communities did not differ between diets or genetic backgrounds, as confirmed by the high proportion of taxa forming the core microbiota. Also, no differences were observed in species richness, biodiversity, or beta diversity between experimental groups. However, when partial least-squares analysis was performed, the HG group showed lower individual variability in terms of gut microbiota composition than the WT group. This result confirms the improved ability of the selected fish to cope with changes in diet composition previously observed in the same sea bass batches (Torrecillas et al., 2023) and in sea bream (Naya-Catal`a et al., 2022; Piazzon et al., 2020).

Piazzon et al. (Piazzon et al., 2020) suggested that genetic selection for growth may indirectly lead to higher plasticity of the microbiota in response to changes in diet. In turn, the responses mediated by the microbiota could similarly influence host evolution. Unfortunately, the influence of the microbiota on host evolution remains poorly understood. What is certain is that the dynamics involved are complex and include feedback loops as well as non-genomic factors. In this context, Kolodny and Schulenburg (2020) have recently described and proposed several microbiome-mediated responses and/or the evolutionary consequences of plasticity. Among them, the microbiome-mediated Baldwin effect has piqued our interest, which refers to evolution via heritable traits in the host that provide for an enriched presence of beneficial microbes, leading to improved fitness.

In agreement with previous studies, 90% of the total gut resident bacteria were represented by the phyla *Firmicutes* and *Proteobacteria* (Montero et al., 2022; Rimoldi et al., 2020b). Although discriminant analysis did not clearly separate groups, two-way ANOVA revealed some effects of diet and/or genotype on the relative abundance of certain bacterial taxa. Specifically, ORG diet increased the relative abundance of *Streptococcus* regardless of genetic background. This genus belongs to the lactic acid bacteria family (LAB), which are considered the most promising probiotics in aquaculture. Similarly, dietary organic acids in combination with nature-identical compounds appear to exert prebiotic properties, leading to moderate increases in the genera *Lactobacillus* and *Leuconostoc* in sea bass (Busti et al., 2020). In agreement with these results, we previously found a positive effect of an organic acid mixture on *Leuconostocaceae* and *Lactobacillaceae* in the gut of sea bream (Rimoldi et al., 2018a). The genus *Photobacterium* appeared to be preferentially associated with the WT background. Some species of this

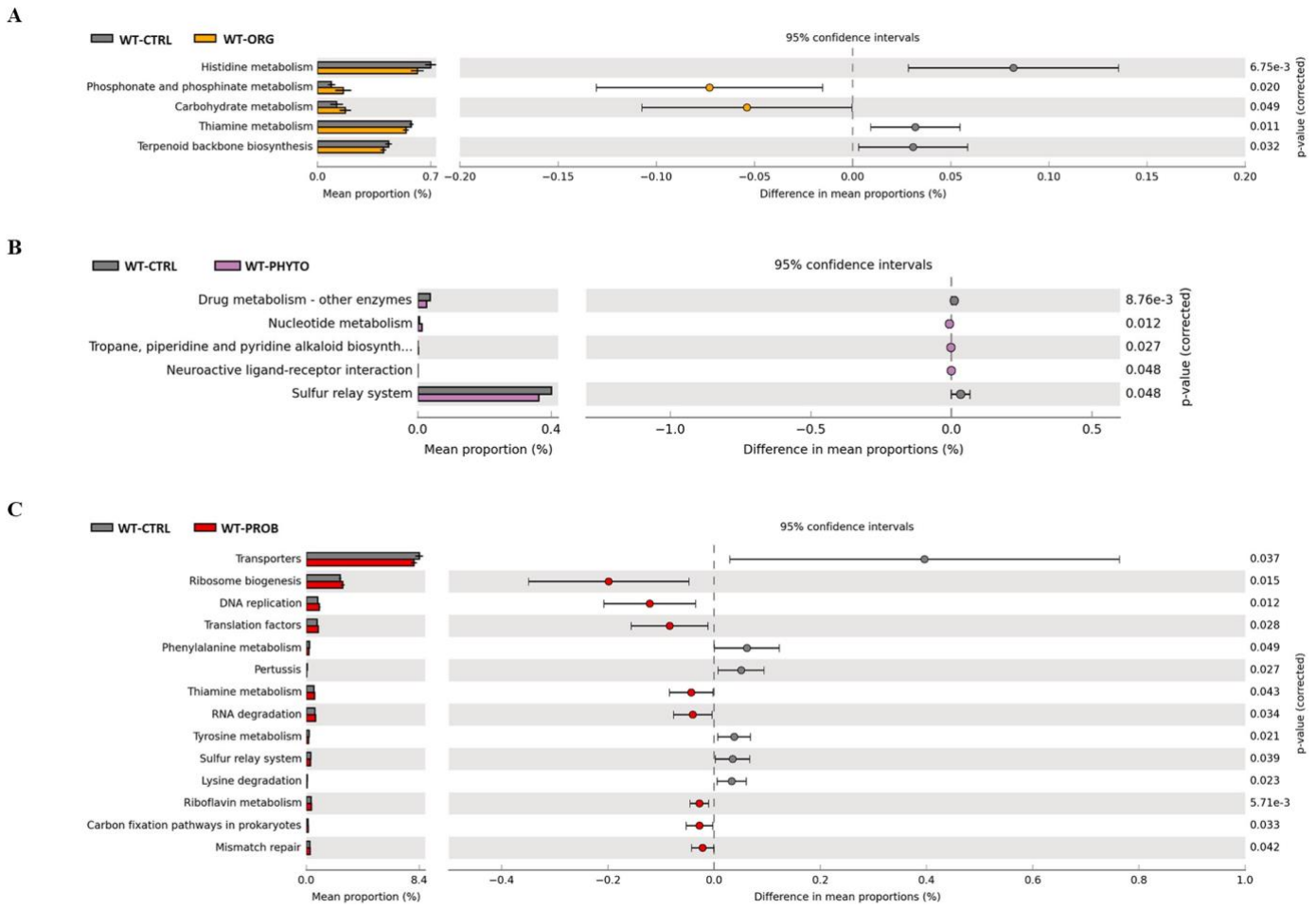


Fig. 6.10. PICRUSt analysis results of predicted functional pathways of gut mucosal microbiota of wild-type European sea bass

genus function as mutualistic bacteria in the gut of marine fish and support chitin digestion by secreting chitinase, while others, such as *P. damselae*, are common pathogens in aquatic animals (Huang et al., 2020). In our previous experiment using the same fish genotypes but fed a control diet or a non-supplemented "future" diet, the influence of genetic background on gut microbiota composition was more evident. Several bacterial genera, such as *Psychrobacter, Micrococcus, Enhydrobacter, Corynebacterium, Cutibacterium, Paracoccus,* and *Stenotrophomonas*, were associated with WT fish, regardless of diet (Torrecillas et al., 2023). Similarly, the strong influence of host genetics on gut microbiota composition was highlighted in sea bream. This influence was particularly pronounced in fish fed "future" diets with some genera, such as *Rubellimicrobium*, *Reyranella*, *Lactobacillus* and *Bifidobacterium*, emerging as HG-related taxa (Naya-Catal`a et al., 2022).

The effects of genetic and dietary interactions on gut microbiota composition were specific for each additive. Supplementation with phytogenics increased the number of members of Lactobacillales and Weeksellaceae in the gut of fish from WT. This is consistent with previous studies examining the effects of dietary supplementation with galactomannan oligosaccharides and phytogenics on the gut microbiota of European sea bass fed a FM/FO deficient diet, which found a reduction in potentially pathogenic taxa with an increase in Lactobacillales (Rimoldi et al., 2020b). In selected sea bass, both PHYTO and PROB diets reduced the abundance of opportunistic pathogenic genera *Novosphingobium* and *Sphingobium*. In addition, dietary supplementation with PROB showed bactericidal activity against the genera *Pseudomonas* and *Acinetobacter*, regardless of genotype. Our tested probiotic mixture contained three *Bacillus* species: *B. subtilis*, *B. licheniformis* and *B. pumilus*. The antimicrobial properties of several *Bacillus* species have been described for various aquatic pathogenic bacteria (Olmos et al., 2020; Soltani et al., 2019; Van Doan et al., 2019). For example, *B. amyloliquefaciens* shows good antagonistic activity *in vitro* against *Aeromonas hydrophila*, *Acinetobacter sp.* and *Acinetobacter tandoii* (Kavi- tha et al., 2018). However, although the highest abundance of *Bacillus* was found in the PROB diet, the results did not show significant change in the relative abundance of this genus at the gut level. The finding that Bacillus-based probiotics are able to regulate the microbiota of European sea bass, despite not colonizing of the host intestinal mucosa is not new. This result is further evidence that probiotics regulate the gut microbiota of European sea bass and thus provide health benefits even though they do not colonize the host intestinal mucosa (Moroni et al., 2021).

An increase in *Streptococcus* was observed in fish fed organic acids, regardless of genotype. Accordingly, increased levels of Lactobacillales, mainly represented by the genera *Lactobacillus* and *Leuconostoc*, were found in sea bream fed a specific combination of short- and medium- chain monoglycerides and in sea bass fed a combination of organic acids and natural compounds in their diet (Busti et al., 2020; Rimoldi et al., 2018a). LAB includes many bacterial genera, including *Lactoba- cillus, Lactococcus, Enterococcus, Streptococcus, Leuconostoc,* and *Pediococcus*. These genera differ in their pathogenic potential, and it is difficult to draw a clear dividing line between beneficial and virulent species. In general, *Lactobacilli* and *Lactococci* are considered harmless, while streptococcal infections are a major cause of disease in fish. However, all LABs have developed high acid tolerance and are stable during their fermentation processes, which produce organic acids as end

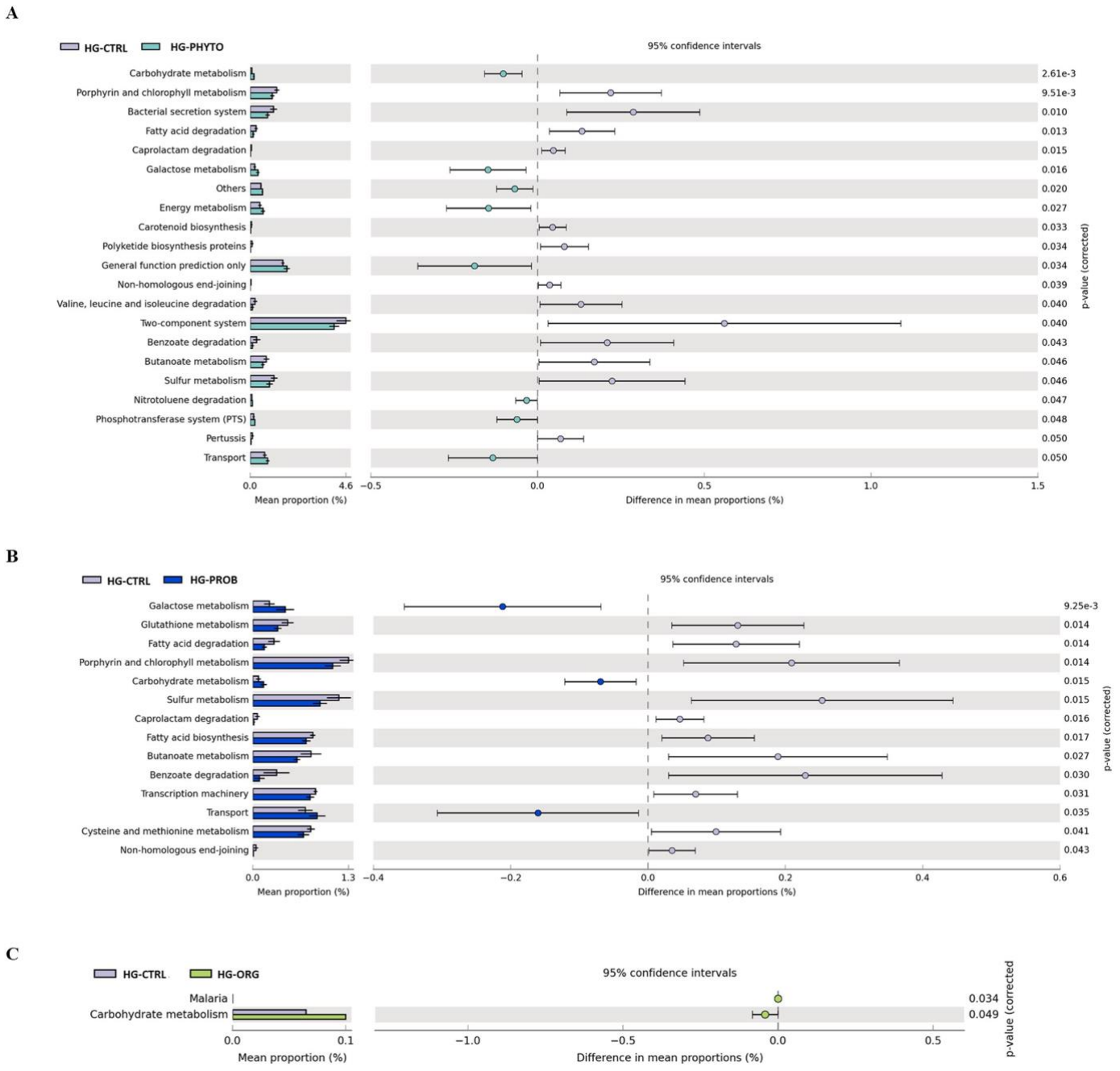


Fig. 6.11. PICRUSt analysis results of predicted functional pathways of gut mucosal microbiota of genetically selected European sea bass

products. Therefore, it can be assumed that acidifiers in the diet can improve their numbers.

Finally, PICRUSt analysis showed that fish from HG had a significant increase in carbohydrate and energy metabolism when fed with PROB, ORG, and PHYTO supplemented diets, while the WT fish had an improvement in carbohydrate metabolism only when fed with ORG supplemented diet. An increase in these metabolic pathways indicates a better ability of the selected fish to utilize carbohydrates. Upregulation of bacterial metabolic pathways involved in energy supply has been previously found in other fish species, such as rainbow trout selected for growth (Biasato et al., 2022). In both cases, the selected fish responded better to dietary changes the than WT fish by modulating their gut microbiota activity in response to changes in the diet.

5. Conclusions

In conclusion, the composition of the gut microbiota was strongly influenced by the genetic background. Genetically selected fish shared low individual variability but coped better with diet composition than WT. However, the effect of functional additives on the gut microbiota profile was both additive- and genotype-dependent. Low-dose *Bacillus*-based probiotics are most effective in modulating the resident gut microbiota and activating the gut immune system of European sea bass, even in the absence of gut mucosal colonization. In contrast, no direct beneficial effects on survival following bacterial challenge can be associated with dietary additives.

Funding

The current study was supported by the EU Horizon 2020 Aqua-IMPACT (Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture), number: 818367.

CRediT authorship contribution statement

S. Rimoldi: Methodology, Data collection, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **D. Montero**: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **S. Torrecillas**: Conceptualization, Methodology, Data collection, curation, Formal analysis, Writing – original draft, Writing – review & editing. **A. Serradell**: Methodology, Data collection, Data curation, Formal analysis. **F. Acosta**: Methodology, Data collection, curation, Formal analysis. **P. Haffray**: Writing – review & editing. **B. Hostins**: Additive producer. **R. Fontanillas**: Feed producer. **F. Allal**: Methodology, Data curation **A. Bajek**: Animal production. **G. Terova**: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

Data Availability

All the sequences were submitted to the public European Nucleotide Archive (EBI ENA).

Acknowledgements

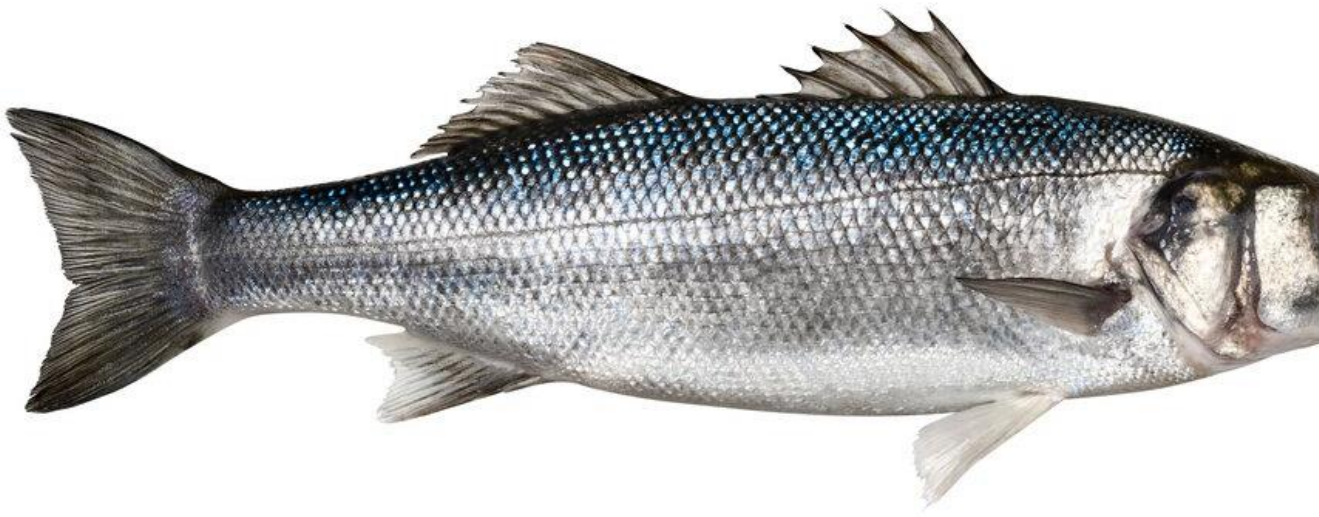
Authors are thankful to Prof. Marco Saroglia for helpful discussions and expert guidance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101747.

Chapter VII

Modelling the effect of prebiotics, probiotics and other functional additives on the growth , feed intake and feed conversión of European Sea Bass (*Dicentrarchus labrax*) juveniles



Aquaculture Reports 32 (2023) 101729

Contents lists available at ScienceDirect

Aquaculture Reports

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Modelling the effect of prebiotics, probiotics and other functional additives on the growth, feed intake and feed conversion of European sea bass (*Dicentrarchus labrax*) juveniles

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ARTICLE INFO

Keywords:
European sea bass (*Dicentrarchus labrax*)
Functional additives
Multiple lineal-regression
Specific growth rate
Feed utilization

ABSTRACT

Dietary supplementation of aquafeeds with functional additives is a commonly employed strategy in order to reduce the potential negative effects associated to fishmeal (FM) and fish oil (FO) replacement by alternative protein and oil sources. Nevertheless, the wide variety of functional ingredients with different bioactive properties hinders the selection of appropriate dietary supplementation strategies on feed formulation. The present study aimed to develop an observational multiple-linear regression (MLR) model to identify the effects of a variety of functional ingredients supplementation on European sea bass juveniles (*Dicentrarchus labrax*) growth performance and feed utilization. A literature survey was conducted gathering a total of 61 dietary treatments. The functional ingredients were classified in three main groups, namely, “probiotics”, “prebiotics” or “others” (including plant derived compounds such as essential oils, extracts and powders). Three different MLR were obtained and validated, allowing to describe the effects of functional ingredients supplementation on fish specific growth rate (SGR) (with a final R-squared (R2) = 0.96, adjusted R-squared (adj R2) = 0.92 and a p-value= 7.21E-08)), fish feed intake (FI) (R2 = 0.97, adj R2 = 0.95 and a p-value= 5.42E-12)) and fish feed conversion ratio (FCR) (R2 = 0.90, adj R2 = 0.80 and a p-value= 2.02E-05)). MLR model trimming, allowed the detection of a significant positive correlation (cor) between dietary prebiotics supplementation and SGR (cor= 0.32, p-value= 8.52E-04). On the contrary, prebiotic supplementation presented a negative correlation with fish FI (cor= −0.44, p-value= 6.27E-05) and FCR (cor= −0.41, p-value= 8.96E-05).

1. Introduction

The aquaculture industry has been facing new challenges in recent years to achieve a more economically and environmentally sustainable production. In this sense, a great effort has been made in the development of dietary strategies based on alternative protein sources that aim to reduce the dependence on marine raw materials (Fiorella et al.,2021). Nevertheless, feed formulation with terrestrial raw materials or other alternative protein sources such as terrestrial by-products and insects (Luthada-Raswiswi et al., 2021), may induce nutritional imbalances negatively affecting feed utilization and thus fish growth and health (Montero and Izquierdo, 2010; Schreck and Tort, 2016). Altogether, can lead to increased production costs, lower growth yields and higher amounts of feed requirements in a production cycle.

The deleterious effects associated to the reduction of fishmeal (FM) and fish oil (FO) in fish diets are of relevance in carnivorous fish species (Naylor et al., 2021), such as the European sea bass (*Dicentrarchus labrax*). Previous studies have reported a suitable reduction of up to 7.5 % FM on European sea bass diets without impairing growth performance compared to a control diet with 31.5 % FM (Campos et al., 2017). Nevertheless, fish health and welfare may also be compromised by FM and FO replacement, reducing pathogen resistance (Torrecillas et al., 2017a) and fish stress tolerance (Torrecillas et al., 2017b).

Dietary supplementation with functional additives is a well-known strategy to offset those negative effects (Kader et al., 2010; Estensoro et al., 2016; Torrecillas et al., 2018, 2019). Functional additives are compounds with the ability to enhance fish growth performance, health, and welfare by increasing, for example, nutrient digestibility and

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https://doi.org/10.1016/j.aqrep.2023.101729
Received 3 March 2023; Received in revised form 14 June 2023; Accepted 8 September 2023
Available online 14 September 2023
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Table 7.1.
Explanatory quantitative variables employed for full linear regression models fitting.

Abbreviation	Quantitative variable	Units
Temp	Temperature	ºC
Oxygen	Dissolved oxygen	gpm
ABW	Average body weight	g
FI_norm	Normalized individual feed intake	% body weight/day
diet_CP	Dietary crude protein	g/kg dry weight
diet_GE	Dietary crude energy	MJ/kg
diet_CP/GE	Dietary Protein to Energy level	g/MJ
diet_CL	Dietary crude lipid	g/kg dry weight
diet_moisture	Diet moisture	%
diet_ash	Dietary ash content	g/kg dry weight
diet_Prebiotics	Dietary prebiotics	g/kg dry weight
diet_Probiotics	Dietary probiotics	g/kg dry weight
diet_Others	Dietary "others" additives	g/kg dry weight

bioavailability, stimulating fish immune response and tissue integrity, and increasing fish stress resistance (Hoseinifar et al., 2021). Among the wide range of active substances, which can be used as functional additives, probiotics are live microbes with the ability to promote fish health by enhancing the internal microbiome balance. Several studies have investigated the effects of dietary probiotics supplementation reporting positive effects on fish growth (Geng et al., 2011; Ramos et al., 2017) and nutrient utilization (Sa´enz de Rodriga´n˜ez et al., 2009; Liu et al., 2012; Bunnoy et al., 2019; Hooshyar et al., 2020). However, there is a high variability on the reported effects regarding fish growth performance depending on the probiotic supplemented, dose, intake duration as well as the target fish species (Aly et al., 2008; He et al., 2013; Liu et al., 2013; Adeoye et al., 2016).

Other functional additives used in fish dietary supplementation are the prebiotics. Prebiotics are plant derived indigestible fibers that could selectively enhance a limited number of intestinal bacteria species (Hoseinifar et al., 2015), directly benefiting host health and feed utilization (Mazurkiewicz et al., 2008; Ebrahimi et al., 2012; Gültepe et al., 2011; Wu et al., 2013). Prebiotic supplementation has also been reported to promote growth of a wide variety of fish species (Torrecillas et al., 2007; Zhou et al., 2009; S¸ara et al., 2010; Soleimani et al., 2012; Guerreiro et al., 2018; Torrecillas et al., 2018). Meanwhile, other studies did not detected positive effects on fish growth performance (Grisdale-Helland et al., 2008; Řehulka et al., 2011; Serradell et al., 2020), again depending mainly on the prebiotic, dose, period of supplementation and fish species studied. Among the plant derived bioactive compounds a third group of functional ingredients can be found, the phytogenics, which include essential oils, extracts and powders. Those functional additives contain high concentrations of secondary metabolites with beneficial effects, enhancing fish health and improving growth in different fish species (Bello et al., 2012; Abdel-Latif et al., 2020; Rashidian et al., 2021; Yousefi et al., 2021). On the other hand, as occurs with pro- and prebiotics, there is a wide range of results observed depending on the phytogenic studies, fish species, dose and feeding strategy tested in terms of growth performance, including neutral or non-beneficial ones (Motlagh et al., 2020; Tasa et al., 2020; Fern´andez-Montero et al., 2021).

The wide variety of functional ingredients, the different effects associated with the level of inclusion and their still unclear mechanisms hinder the selection of appropriate strategies for dietary supplementation. Additionally, the experimental conditions in the different studies that analyze the effects of functional ingredients on fish health and growth are highly variable, preventing the direct comparison of the results obtained. In this sense, mathematical modeling has been identified as a powerful tool to analyze fish growth and feed utilization in response to different variations in diet composition and culture conditions (Van Dam, 1990; Galkanda-Arachchige et al., 2020; Luthada-Raswiswi et al., 2021). For example, regression models can be used to deduct the effects of factors that vary between studies (confounding variables) and therefore obtain normalized estimates of fish responses to different inclusion levels of functional ingredients. Thus, the objective of the present study was to develop an observational multiple-linear regression model to robustly isolate the effects of dietary functional ingredients may have on growth and feed utilization parameters of European sea bass juveniles by simultaneously considering the results of several growth trials performed under different contexts.

2. Materials and methods

2.1. Literature survey and selection

A literature survey was conducted employing the bibliographic databases Web of Science (-, 2022) and Scopus (-, 2022). The search for relevant bibliography was carried out following the title, abstract and keywords search strings: ("*Dicentrarchus labrax*" OR "*European sea bass*" OR "*European seabass*") AND ("*juveniles*" OR "*fry*") AND ("*functional feeds*" OR "*functional ingredients*" OR "*functional diets*" OR "*diet supple- mentation*" OR "*probiotics*" OR "*prebiotics*" OR "*phytogenics*" OR "*essential oils*" OR "*plant derived compounds*" OR "*phytogenic feed additives*" OR "*synbiotics*").

To be selected for compilation, studies had to meet the following criteria: (I) experiments carried out with European sea bass juveniles; (II) studies focused on dietary supplementation with probiotics, prebiotics and/or plant derived compounds; (III) at least one of the following growth and feed utilization parameters had to be reported in the study: specific growth rate (SGR), feed conversion ratio (FCR) and/or feed intake (FI); (IV) fish growth information, including initial (IBW) (g) and final body weight (FBW) (g), and a measure of dispersion in relation to the mean value (e.g., standard deviation [SD]); (V) feeding trial duration; (VI) dietary treatments composition or at least proximal composition, including dietary protein (diet_CP) and energy (diet_GE) concentrations; (VII) culture conditions information (at least mean water temperature and dissolved oxygen).

2.2. Data conditioning and analysis

Prior to data analysis, all the information gathered from the different studies was converted to standard units, expressing the different variables as: fish body weight (g); fish body length (cm); average body weight (ABW) (g); individual feed intake (g per fish day^{-1}); water temperature (ºC); water dissolved oxygen (ppm); tank volume (L); duration (days); dietary ingredients (% diet); dietary chemical composition (% diet); diet gross energy (MJ/kg). To facilitate data analysis, the different functional additives were classified in three global groups, namely, "*probiotics"*, "*prebiotics"* or "*others"* (including plant derived compounds or symbiotic compounds).

Since fish are poikilothermic animals (Bell et al., 1986), important traits as feed intake and growth rate are conditioned by water temperature. Thus, in order to evaluate the effect of the inclusion of functional additives on fish growth and conversion efficiency, relevant responses were normalized to remove the effect of important confounding variables (i.e., fish size and temperature) employing the formula:

$$normalized\quad trait = \frac{measured\quad raw\quad trait}{maximumtrait\quad value}$$

where the *measured raw trait* consisted of the value obtained from the direct calculation of the different parameters as follows:

$$SGR\ (day^{-1}) = [\ln(FBW) - \ln(IBW)] / days,$$

$$FI\ (\%\ body\ weight/day) = [\ (individual\ feed\ intake)/\ (IBW + FBW)/2/\ days] \times 100,$$

$$FCR\ (g\ feed\ intake/g\ weight\ gain) = individual\ feed\ intake\ /\ (FBW - IBW),$$

and the *maximum trait value* consisted of the value obtained from three

Table 7.2.
Effects of different functional ingredients on growth performance and feed efficiency of European sea bass (*Dientrarchus labrax*) juveniles.

Group	Functional additive	Inclusion method	Dose	Duration	Dietary crude protein content (%)	Dietary Protein/ Energy ratio	Effects	Reference
Probiotics								
	Bactocel PA10 (Lamelland SAS, Canada) (*Pediococcus acidilactici*, strain CNCM I-4622)	Grounded and mixed before extrusion	2, 2.5 or 3 g/kg ~ 1×10^{10} CFU	100 days	45.5	2.25	– ↑ FBW, WG, SGR – No differences in FCR	Eissa et al. (2022)
	AquaStar Growout (BIOMIN Holding GmbH) (commercial probiotic blend of *Bacillus* sp., *Lactobacillus* sp., *Enterococcus* sp. and *Pediococcus* sp.)	Added as mash feed	3 g/kg ~ 5.23×10^{8} CFU/kg.	100 days	47.3	2.14	– No differences on FBW, DGI or protein efficiency ratio	Pereira et al. (2018)
	MIX-AVI® pro(IVS-Wynco LLC, Springdale, AR, USA) (*L. plantarum*)	Sprayed after extrusion	Sprayed 10×10^{9} CFU/ kg	90 days	42.25	2.10	– ↑ Survival – No differences on FBW, SGR or FCR	Piccolo et al. (2015)
	Bactocell PA10 (Lamelland SAS, Canada) (*Pediococcus acidilactici*, strain CNCM I-4622)	-	-	60 days	47.20	1.97	– No differences on FBW and length	Torrecillas et al. (2018)
Prebiotics								
	Fructo-oligosaccharide or xylo-oligosaccharide	-	1 g/kg	49 days	45.95	2.09	– ↑ FBW – ↑ WG on low protein diets	Guerreiro et al. (2015)
	Mannan-oligosaccharides (Bio-Mos, Alltech Inc.)	-	2,4 or 6 g/kg	60 days	48.71	-	– No differences on FBW, RG, K or SGR – ↓ FCR on fish fed 4 and 6 g/kg – ↓ FI	Torrecillas et al. (2011)
	MOS (Biomos® and Actigen© second and generation of MOS; Alltech, Inc., Kentucky, USA)	-	3 or 6 g/kg MOS	60 days	47.20	1.97	– ↑ FBW and length	Torrecillas et al. (2018)
	Galactomannan-oligosaccharides (GMOS) (Delacon, Austria)	Grounded and mixed before extrusion	5 g/kg	63 days	47.87	2.04	– No differences on FBW, SGR or FCR – ↑ Resistance *V.anguillarum*	Torrecillas et al. (2019)
Others								
	Anise (*Pimpinella anisum* L.)	Powdered and mixed with fish oil	1.5, 2.5 and 3.5 g/kg	120 days	44.06	2.06	– ↑ FBW, WG and SGR – ↑ FBW, WG and protein efficiency ratio with increasing levels of supplementation	Ashry et al. (2022)
	SSF-BSG (solid-state fermentation of brewer's spent grain) (Unicer-Bebidas de Portugal, S.A. (Matosinhos, Portugal))	Grounded and mixed before extrusion	4 and 8 g/kg	64 days	47.90	1.98	– No differences on FBW or body composition – Lower feed intake	Fernandes et al. (2022)
	Digestarom PEP MGE150 (Biomin Holding GmbH, Austria) (Anise, citrus and oregano essential oils)	Added as mash feed	2 g/kg	60 days	47.30	2.14	– ↑ SGR	Gonçalves et al. (2019)
	Cinnamon	Powder	10, 15 or 20 g/kg	90 days	46	2.15	– ↑ FBW, WG, and protein efficiency ratio – ↑ SGR on fish fed 10 g/kg – ↓ FCR on fish fed 15 and 20 g/kg	Habiba et al. (2021)
	Garlic meal	Grounded and mixed before extrusion	20, 40 and 60 g/kg	60 days	43.03	2.25	– ↑ FBW on fish fed 40 g/kg	I˙rkin and Yig˘it (2015)
	Yucca schidigera	Grounded and mixed before extrusion	0.25, 0.5 or 1 g/kg	45 days	44.82	2.21	– ↑ FBW, WG and SGR – ↓ FCR with increasing supplementation	Mansour et al. (2021)
	Synbiotic [(MOS, Biomos® and Actigen© (second generation of MOS; Alltech, Inc., Kentucky, USA) + *Pediococcus acidilactici* (BAC, Bactocell®; Lallemand Inc., Cardiff, UK)		3 or 6 g/kg Biomos	60 days	47.20	1.97	– ↑ FBW and length	Torrecillas et al. (2018)
	Mixture of garlic and labiate plant essential oils (PHYTO) (Delacon, Austria)	Vacuum coating	5 g/kg	63 days	47.87	2.04	– No differences on FBW, SGR or FCR – ↑Resistance *V.anguillarum*	Torrecillas et al. (2019)
	Carvacrol (5-isopropyl-2-methylphenol) (cod. 282197; Sigma-Aldrich, Milan, Italy)	Diluted in fish oil	2.5 and 5 g/kg	63 days	51.30	2.30	– No differences on FBW, WG, SGR or FCR	Volpatti et al. (2013)

(*continued on next page*)

Table 2 (*continued*)

Group	Functional additive	Inclusion method	Dose	Duration	Dietary crude protein content (%)	Dietary Protein/ Energy ratio	Effects	Reference
	Dried leaf powder of thyme (*Thymus vulgaris* L.) or rosemary (*Rosmarinus officinalis* L.) or seed powder of fenugreek (*Trigonella foenum graecum* L.)	Powder	10 g/kg	45 days	48.43	2.23	– No differences on FBW, SGR, FCR or fat retention – Thyme increased protein efficiency ratio, protein retention, energy retention and fillet protein composition	Yılmaz et al. (2012)

↑ increased in comparison to not supplemented diet. ↓ reduced in comparison to not supplemented diet. FBW (final body weight); WG (weight gain); SGR (specific growth rate); FCR (feed conversion ratio); DGI (daily growth index); FI (feed intake)

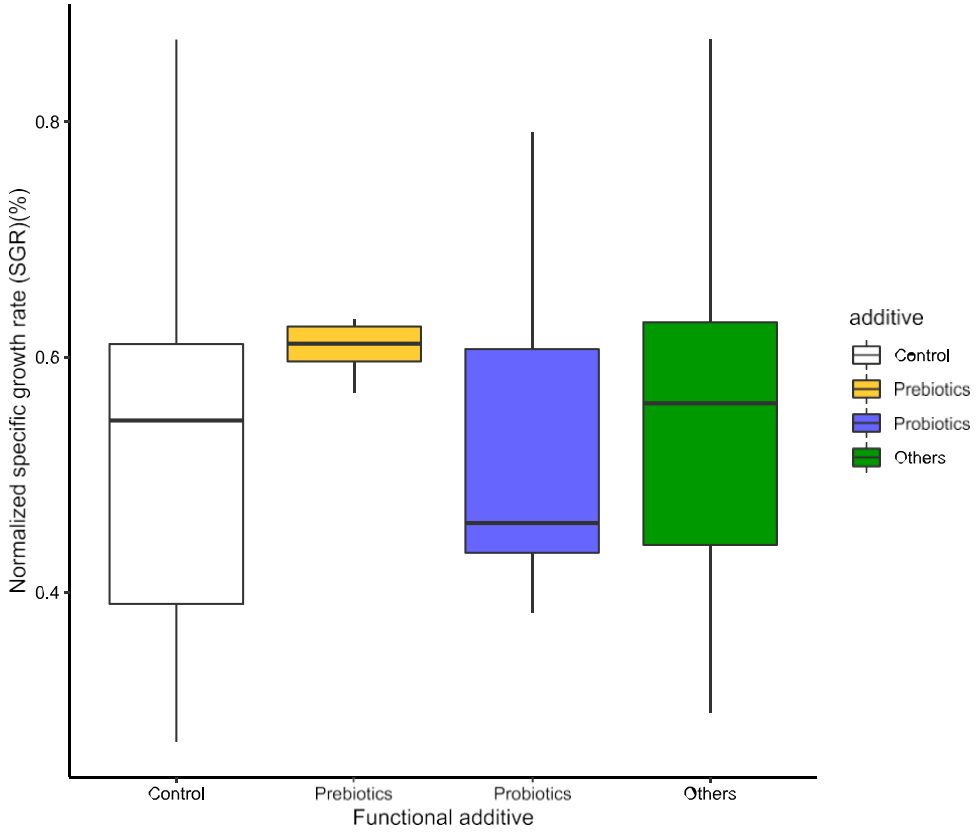


Fig. 7.1. Boxplot of functional ingredients dietary inclusion effects on normalized specific growth rate

Table 7.3.
Coefficients of linear regression obtained for the normalized specific growth rate (SGR_norm) observational models.

Specific growth rate model	Equation	R^2	Adj R^2	Variable *p-value*	Model *p-value*
Simple model[a]	Exp ($5.65e^{-2}$ *(Temp) + $6.94e^{-5}$ * (norm_FI)^2 + 0.71 * (diet_CP /diet_GE))	0.67	0.65	-	$9.20e^{-10}$
Simple model w/ Prebiotics[b]	Exp ($6.1e^{-2}$ *(Temp) + $7.1e^{-5}$ * (norm_FI)^2 + 0.81 * (diet_CP /diet_GE) + 0.32 * (diet_Prebiotics))	0.75	0.73	$8.52e^{-4}$	$5.3e^{-9}$
Simple model w/ Probiotics[c]	Exp ($5.65e^{-2}$ *(Temp) + $6.94e^{-5}$ * (norm_FI)^2 + 0.71 * (diet_CP /diet_GE) – $4.22e^{-3}$ * (diet_Probiotics))	0.67	0.63	0.96	$5.3e^{-9}$
Simple model w/ Others[d]	Exp ($6.58e^{-2}$ *(Temp) + $7.1e^{-5}$ * (norm_FI)^2 + 0.73 * (diet_CP /diet_GE) – 0.1 (diet_Others))	0.7	0.66	0.11	$1.49e^{-9}$

Temp (water temperature °C); norm_FI (normalized fish individual feed intake (g /fish per day)); diet_CP /diet_GE (Protein – Energy ratio (g /MJ)); diet_Prebiotics (dietary prebiotics content (g /kg)); diet_Probiotics (dietary probiotics content (g /kg)); diet_Others (dietary Others content (g /kg)).

[a] SGR simple model (trimmed simple model).

[b] SGR simple model with prebiotics dietary inclusion as quantitative variable.

[c] SGR simple model with probiotics dietary inclusion as quantitative variable.

[d] SGR simple model with Others dietary inclusion as quantitative variable.

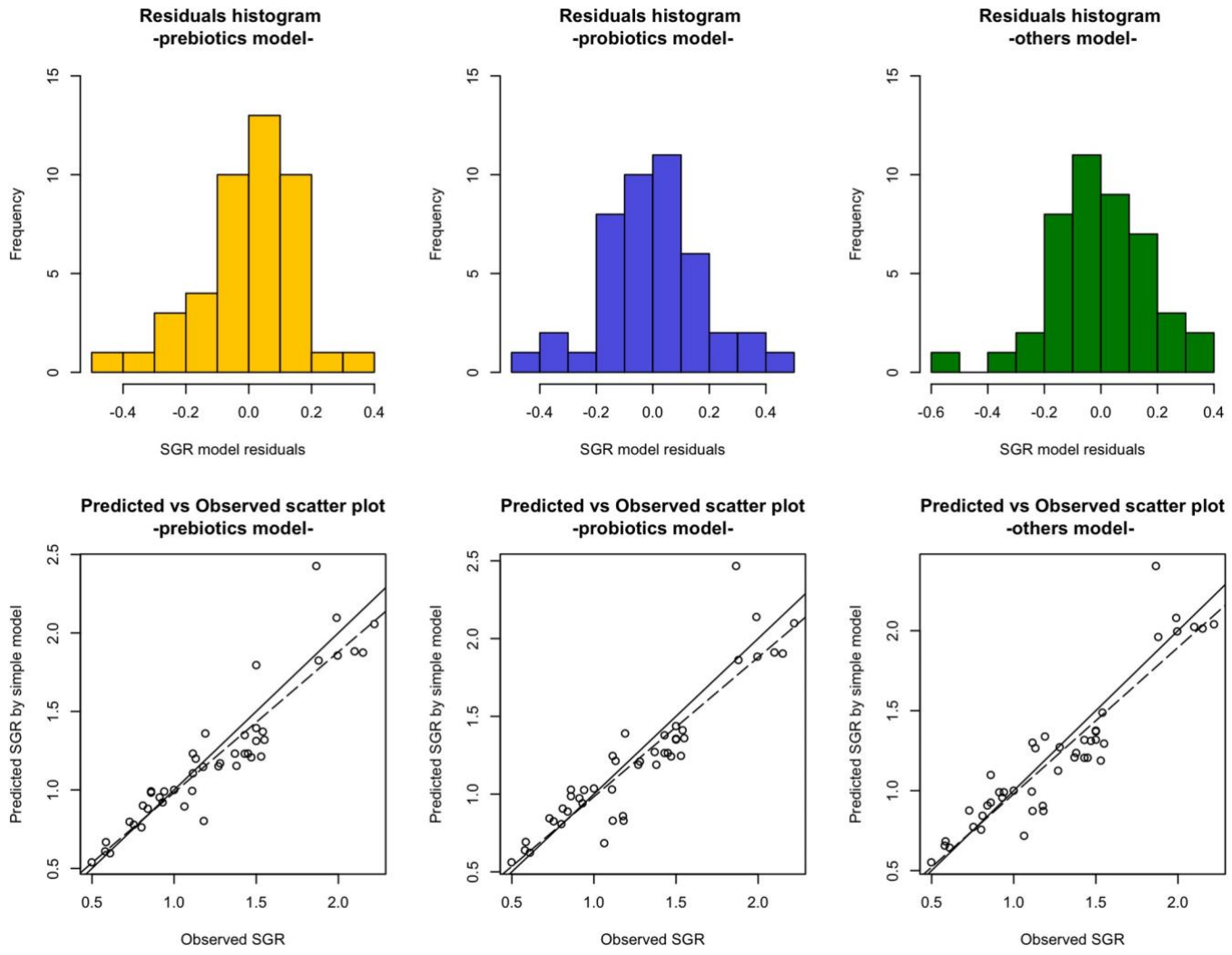


Fig. 7.2. European sea bass (*Dicentrarchus labrax*) specific growth rate (SGR) distribution for a) prebiotics simple model residuals; b) probiotics simple model residuals; c) others simple model residuals; d) prebiotics simple model fitted values; e) probiotics simple model fitted values; f) others simple model fitted values.

reference models developed in the context of the AquaIMPACT EU project (Horizon 20/20) for the prediction of European sea bass growth, feed intake and feed conversion, which were provided by Sparos Lda. (Olh˜ao, Portugal). The models used are described by the following equations:

$$\ln(SGR_{max}) = -7{\cdot}93079 + [0{\cdot}50781 \times \ln(ABW)] - [0{\cdot}00133 \times ABW] - [0{\cdot}09766 \times (\ln(ABW)^2)] + [0{\cdot}2524 \times Temp] - [0{\cdot}0041 \times (Temp)^2]$$

$$\ln(FI_{max}) = 5{\cdot}11608 + [0{\cdot}61529 \times \ln(ABW)] + [0{\cdot}14896 \times Temp] - 0{\cdot}00136 \times (Temp)^2,$$

$$FCR_{typical} = 0{\cdot}9036676 \times (ABW)^{0{\cdot}1082725}.$$

*ABW (average body weight (g)) = $(IBW * FBW)^{0.5}$.
*Temp (temperature (°C)).
These models were obtained using quantile regression (to estimate quantiles 0.95 for SGR_max and FI_max, and quantile 0.50 for FCR_typical) of log transformed responses, based in an aggregated data base including information about European sea bass growth trials from 37 sources.

After model fitting employing the explanatory quantitative variables presented in Table 1, a stepwise backward selective regression was performed by iteratively adding and removing coefficients in order to find the simplest and best performing model (Agostinelli, 2002; Wang et al., 2007). The obtained equations were evaluated in reference to the model selection criteria and residuals analysis. (Sanquetta et al., 2018).

3. Results

3.1. Data base overview

Fifteen studies (Table 1) passed the minimum requirements in order to be eligible for the model database, adding up to a total of 61 dietary treatments to be used to define the present descriptive model. From the total 61 dietary treatments registered, 12 addressed the study of prebiotics, 13 of probiotics, 21 of "others" group and 15 treatments were void of supplementation (control diets). The data set covered a wide range of culture conditions, with temperatures ranging between 17 and 28 °C and oxygen concentrations ranging from 5.4 to 9.7 ppm. The database presented a range of fish body weight values between 4.69 and 130.30 g. The chemical composition of the diets used in the listed studies presented the following ranges of values: crude protein (42.00–51.30 % dry weight); crude lipids (12.56–28.90 % dry weight); gross energy (18.30–24.46 MJ/kg dry weight) and moisture (2.92–12.00 % wet weight). The duration of the experiments ranged from 45 to 120 days.

The final database included four studies focusing on the effects of dietary inclusion of probiotics (Piccolo et al., 2015; Pereira et al., 2018; Torrecillas et al., 2018; Eissa et al., 2022), four studies focusing on the

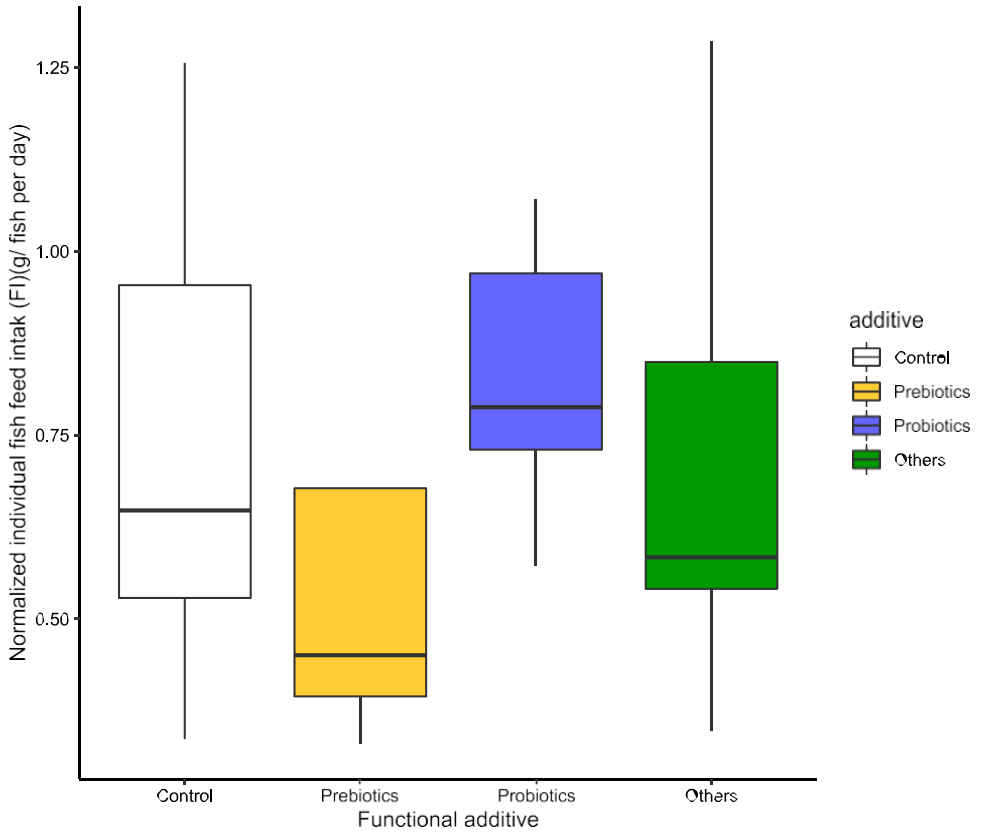


Fig. 7.3. Boxplot of functional ingredients dietary inclusion effects on normalized fish individual feed intake

Table 7.4.
Coefficients of linear regression obtained for the normalized feed intake (FI_norm) observational models.

Feed intake model	Equation	R^2	Adj R^2	Variable *p-value*	Model *p-value*
Simple model[a]	Exp (- 5.1e^{-2} *(Temp) - 4.14e^{-3} * (ABW) + 1.91 * (diet_CP) - 3.54 * (diet_GE) - 38.1 * (diet_CP /diet_GE) + 5.75e^{-2} * (diet_moisture))	0.75	0.7	-	6.45e^{-8}
Simple model w/ Prebiotics[b]	Exp (- 3.85^{-2} *(Temp) – 3e^{-3} * (ABW) + 2,1 * (diet_CP) – 4.28 * (diet_GE) – 41.5 * (diet_CP /diet_GE) + 5.33e^{-2} * (diet_moisture) – 0.44 * (diet_Prebiotics))	0.86	0.82	6.27e^{-5}	1.1e^{-10}
Simple model w/ Probiotics[c]	Exp (- 4.37e^{-2} *(Temp) – 4.37e^{-3} * (ABW) + 1.87 * (diet_CP) - 3.84 * (diet_GE) - 37.13 * (diet_CP /diet_GE) + 5.12e^{-2} * (diet_moisture) + 0.2 * (diet_Probiotics))	0.80	0.72	0.13	8.6e^{-8}
Simple model w/ Others[d]	Exp (- 5.51^{-2} *(Temp) – 4.1e^{-3} * (ABW) + 1.91 * (diet_CP) - 4 * (diet_GE) – 38.21 * (diet_CP /diet_GE) + 5.73e^{-2} * (diet_moisture) + 3e^{-2} * (diet_Others))	0.75	0.7	0.70	2.5e^{-7}

Temp (temperature °C); ABW (average body weight (g)); diet_CP (dietary protein content (%)); diet_GE (dietary energy content (MJ/kg)); diet_CP /diet_GE (Protein – Energy ratio (g /MJ)); diet_moisture (diet mositure content (%)); diet_Prebiotics (dietary prebiotics content (g/kg)); diet_Probiotics (dietary probiotics content (g/kg)); diet_Others (dietary Others content (g/kg)).

[a] FI simple model (trimmed simple model).
[b] FI simple model with prebiotics dietary inclusion as quantitative variable.
[c] FI simple model with probiotics dietary inclusion as quantitative variable.
[d] FI simple model with Others dietary inclusion as quantitative variable.

effects of dietary inclusion of prebiotics (Torrecillas et al., 2011; Guerreiro et al., 2015; Torrecillas et al., 2018; Torrecillas et al., 2019), and eleven studies focusing on the effects of dietary inclusion of plant derived compounds or synbiotics (Torrecillas et al., 2011; Yılmaz et al., 2012; Volpatti et al., 2013; İrkin and Yiğit, 2015; Torrecillas et al., 2018; Torrecillas et al., 2019; Habiba et al., 2021; Mansour et al., 2021; Ashry et al., 2022; Fernandes et al., 2022).

Half of the studies reported positive effects on fish growth parameters, associated to dietary supplementation with probiotics, prebiotics or others (i.e., plant derived compounds or synbiotics) (Table 2). Regarding feed utilization, 37.5 % of the selected studies described beneficial effects of functional ingredients on feed intake or FCR. Two studies reported lower feed intake rates associated to dietary supplementation (Torrecillas et al., 2011; Fernandes et al., 2022).

3.2. Correlation between dietary supplementation and specific growth rate (SGR)

The experiments employing prebiotics as functional ingredients typically presented higher normalized SGR than those employing other dietary treatments (Fig. 1).

Normalized individual SGR modelling with a complex multiple-regression obtained a total R-squared (R^2) = 0.96, an adjusted R- squared (*adj* R^2) = 0.92 and a *p-value* = 7.213e-08. The full model fol- lowed the equation:

Ln (SGR_norm) =0·44 *(Temp) -1.52e-2*(Temp)^2 + 0·33*(Oxygen) – 5·91 e^{-3}*(ABW) + 0·28*ln (ABW) - 1·46e^{-2}*(FI_norm) + 1·49e^{-4}* (FI_norm)^2 + 0.5*(diet_CP) – 1·12*(diet_GE) – 98 (diet_CP /diet_GE) – 0·16*(diet_CL) + 3 1 e-2*(diet_moisture) -2 23e-2*(diet_ash) + 8.57e-2* (diet_Probiotics) + 0 12 (diet_Prebiotics) + 7 02e-2 (Others)

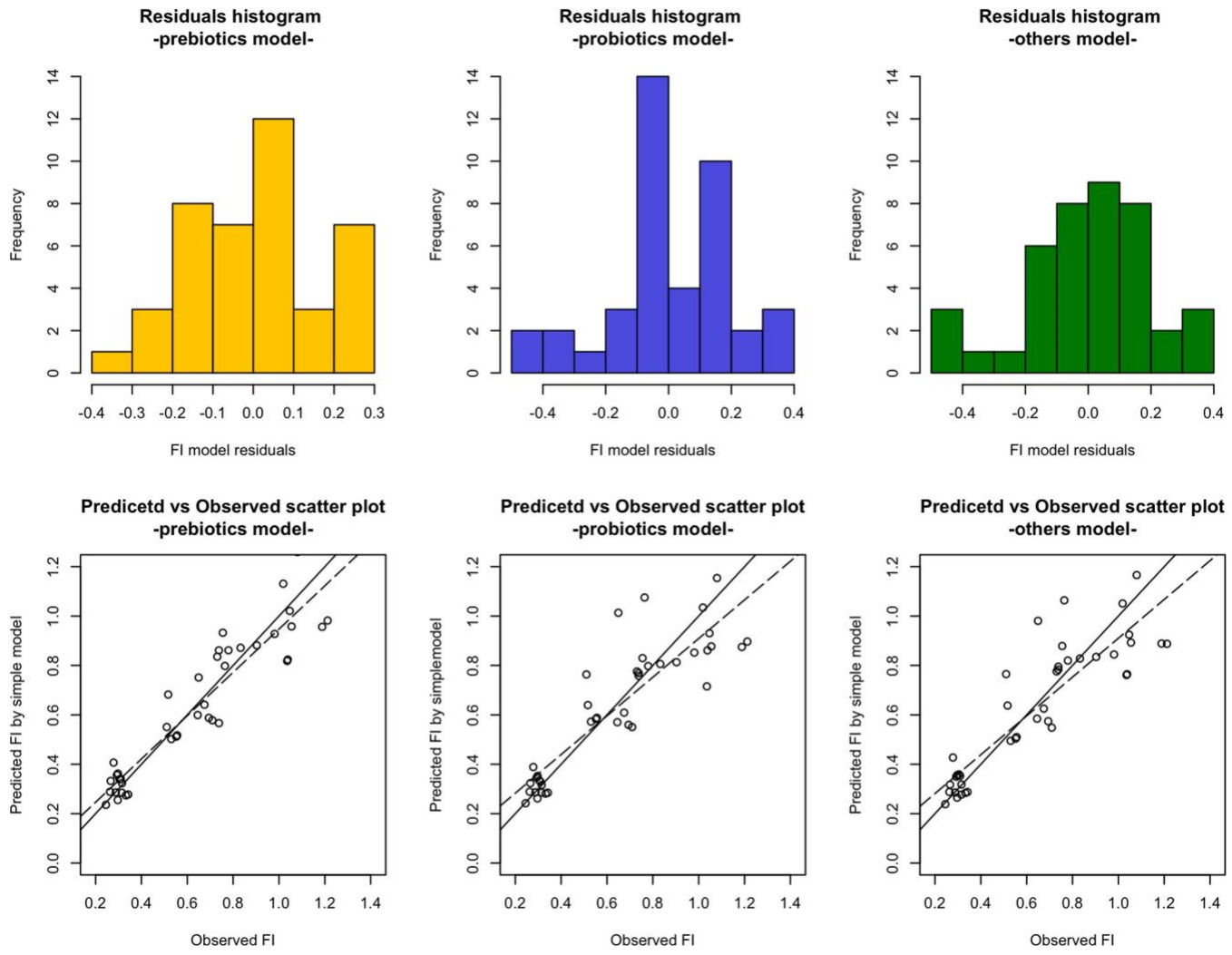


Fig. 7.4. European sea bass (*Dicentrarchus labrax*) individual feed intake (FI) distribution for a) prebiotics simple model residuals; b) probiotics simple model residuals; c) others simple model residuals; d) prebiotics simple model fitted values; e) probiotics simple model fitted values; f) others simple model fitted values.

in which "diet_Prebiotics", "diet_Probiotics" and "diet_Others" are quantitative variables.

Model trimming resulted in a *Simple model* with a (R^2) = 0.67, an adjusted R-squared (*adj* R^2) = 0.65 and a *p-value* = 9.20E-10 (Table 3). The addition of dietary prebiotics ("diet_Prebiotics") as descriptive variables significantly improved the *Simple model* selection criteria (Table 3) (Fig. 2).

3.3. Correlation between dietary supplementation and individual feed intake (FI)

The experiments employing prebiotics as functional ingredients presented significantly lower normalized FI values ($p < 0.05$; one way ANOVA (presence /absence)) than those employing dietary treatments supplemented with probiotics (Fig. 3).

Normalized individual FI modelling with a complex multiple-regression obtained a total R-squared (R^2) = 0.97, an adjusted R-squared (*adj* R^2) = 0.95 and a *p- value* = 5.42E-12. The full model followed the equation:

$$\text{Ln (FI\_norm)} = -1{\cdot}88*(\text{Temp}) + 4\text{e-}2*(\text{Temp})^2 + 0{\cdot}21*(\text{Oxygen}) - 9{\cdot}1\text{e}^{-3}*(\text{ABW}) + 0{\cdot}23*\ln(\text{ABW}) + 0{\cdot}7*(\text{diet\_CP}) - 1{\cdot}22*(\text{diet\_GE}) - 13{\cdot}5$$

$$(\text{diet\_CP}/\text{diet\_GE}) + 0{\cdot}16*(\text{diet\_CL}) + 0{\cdot}06*(\text{diet\_moisture}) + 0{\cdot}13*(\text{diet\_ash}) + 1{\cdot}76*(\text{diet\_Probiotics}) - 0{\cdot}16*(\text{diet\_Prebiotics}) - 0{\cdot}07*(\text{Others})$$

in which "diet_Prebiotics", "diet_Probiotics" and "diet_Others" are quantitative variables.

Model trimming resulted in a *Simple model* with a (R^2) = 0.75, an adjusted R-squared (*adj* R^2) = 0.7 and a *p-value* = 6.45E-08 (Table 4). The addition of dietary prebiotics ("diet_Prebiotics") as a model descriptor, significantly improved the *Simple model* selection criteria (Table 4) (Fig. 4).

3.4. Correlation between dietary supplementation and feed conversion ratio (FCR)

The experiments employing prebiotics as functional ingredients typically presented lower normalized FCR than those employing the other dietary treatments (Fig. 5).

Normalized feed conversion ratio modelling with a complex multiple-regression obtained a total R-squared (R^2) = 0.90, an adjusted R-squared (*adj* R^2) = 0.80 and a *p-value* = 2.02e-05. The full model followed the equation:

$$\text{Ln (FCR\_norm)} = 0{\cdot}46*(\text{Temp}) - 6{\cdot}7\text{e-}3*(\text{Temp})^2 - 0{\cdot}31*(\text{Oxygen}) - 1{\cdot}3\text{e}^{-2}*(\text{ABW}) + 0{\cdot}2*\ln(\text{ABW}) + 0{\cdot}3*(\text{diet\_CP}) - 0{\cdot}6*(\text{diet\_GE}) - 5{\cdot}25\ (\text{diet\_CP})/\text{diet\_GE}) + 0\ \ 13*(\text{diet\_CL}) - 3\ \ 4\text{e-}2*(\text{diet\_moisture}) + 4\ \ 3\text{e-}2*(\text{diet\_ash}) - 6\ \ 13\text{e-}2*(\text{diet\_Probiotics}) - 0.27*(\text{diet\_Prebiotics}) - 0\ 13*(\text{Others}$$

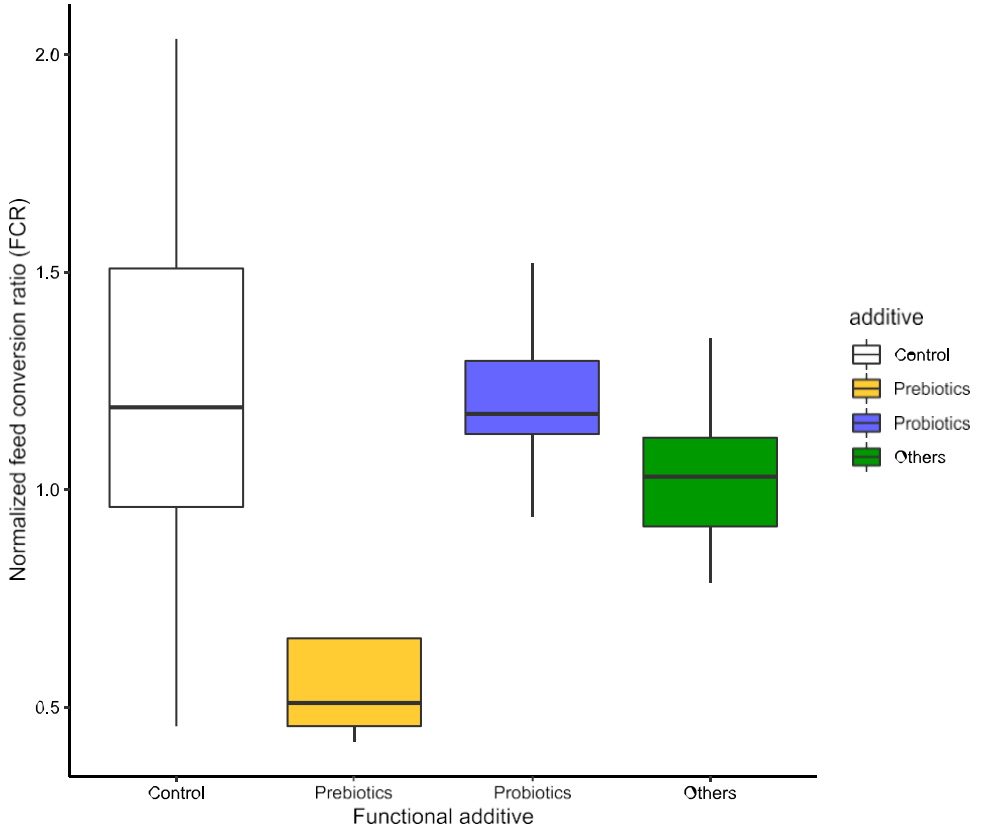


Fig. 7.5. Boxplot of functional ingredients dietary inclusion effects on normalized feed conversion ratio

in which "diet_Prebiotics", "diet_Probiotics" and "diet_Others" are quantitative variables.

Model trimming resulted in a *Simple model* with a (R^2) = 0.63, an adjusted R-squared (*adj* R^2) = 0.54 and a *p- value* = $1.01e^{-4}$ (Table 5). The addition of dietary prebiotics ("diet_Prebiotics") as a model descriptor, significantly improved the *Simple model* selection criteria (Table 5) (Fig. 6).

4. Discussion

The models developed in the present study presented significant *p-values*, validating the regression coefficients between the descriptor variables and the different modelled traits (Sanquetta et al., 2018). The experimental models presented acceptable R^2 scores (between 0.90 and 0.97), adjusted R^2 scores (between 0.80 and 0.95) and normal residuals distribution. SGR was positively correlated (0.71 regression coefficient) to the dietary protein to energy ratio, meanwhile FI was negatively correlated (-38.1 regression coefficient) to this descriptive variable. The FCR was negatively correlated (-0.14 regression coefficient) to the dietary energy contents. In this sense, the linear models confirmed the elevated importance of dietary nutrient and energy balance on fish growth and feed utilization (Azevedo et al., 2002; Oliva-Teles, 2012; M´endez-Martínez et al., 2021).

Regarding the inclusion of functional additives, the analysis performed in the present study showed a pattern by which those dietary treatments employing prebiotics as functional additives presented higher normalized SGR values (Fig. 1) and lower individual FI and FCR normalized values (Figs. 3 and 5) than those employing probiotics or other functional additives. Model trimming revealed that prebiotics were the only functional additives group inducing significant effects on SGR, FI and FCR models outcome. Interestingly, prebiotic inclusion presented the same patterns of correlation with the modeled traits as those presented by the dietary energy contents.

The prebiotic inclusion was positively correlated to SGR (0.32 regression coefficient) and negatively correlated to FI (-0.44 regression coefficient) and FCR (-0.44 regression coefficient), altogether pointing to a beneficial effect of prebiotic supplementation on fish growth and feed efficiency as suggested Torrecillas and co-authors in 2011. After feeding European sea bass juveniles with diets supplemented with 4 and 6 g/kg mannan-oligosaccharides (MOS), the authors observed a significant improvement on fish FCR, with lower feed intake and similar growth performance than fish fed a diet void of supplementation. MOS dietary inclusion led to reduced fish liver lipid vacuolization and decreased glucose-6-phosphate dehydrogenase (G6PD) and malic enzyme (ME). The authors proposed an effect of dietary prebiotic inclusion on the promotion of hepatic glycolytic activity, providing internal energy for body tissues and reducing feed intake through the induction of neural signals modulating appetite and satiation systems (Torrecillas et al., 2011). Similarly, in 2005 Laíz-Carrion and co-authors reported increased hepatic glycogenolysis and gluconeogenesis together with a reduced G6PD activity in sea bream (*Sparus aurata*) fed with immunostimulants (Laiz-Carrio´n et al., 2005).

Several studies have reported beneficial effects of prebiotic functional ingredients on fish growth and feed utilization. A study carried out with rainbow trout (*Oncorhynchus mykiss*) juveniles reported better growth performance in groups of fish fed diets supplemented with inulin or fructo-oligosaccharides (FOS) at either 5 or 10 g/kg than those fish fed not supplemented diets (Ortiz et al., 2013). Similarly, Soleimani and collaborators (Soleimani et al., 2012) fed Caspian roach (*Rutilus rutilus*) with diets supplemented with FOS at a concentration of 20 and 30 g/kg. After a 7-week feeding trial, fish fed the functional diets presented higher growth and better FCR compared to fish fed a reference diet. This increased growth performance could be associated to the increased

Table 7.5.
Coefficients of linear regression obtained for the normalized feed conversion ratio (FCR_norm) observational models.

Feed conversion ratio model	Equation	R^2	Adj R^2	Variable *p-value*	Model *p-value*
Simple model[a]	Exp ($3.7e^{-2}$ *(Temp) − 0.12 * (Oxygen) − $4.5e^{-3}$ * (ABW) − $1.23e^{-2}$ * (diet_CP) − 0.14 * (diet_GE) - 0.11 * (diet_moisture))	0.63	0.54	-	$1.01e^{-4}$
Simple model w/ Prebiotics[b]	Exp ($2.45e^{-2}$ *(Temp) − 0.1 * (Oxygen) − $3.34e^{-3}$ * (ABW) − $1.61e^{-2}$ * (diet_CP) − 0.1 * (diet_GE) - $9.33e^{-2}$ * (diet_moisture) − 0.41 * (diet_Prebiotics))	0.80	0.75	$8.96e^{-5}$	$2.22e^{-7}$
Simple model w/ Probiotics[c]	Exp ($3.57e^{-2}$ *(Temp) − 0.13 * (Oxygen) − $4.6e^{-3}$ * (ABW) − $1.47e^{-2}$ * (diet_CP) − 0.14 * (diet_GE) - 0.11 * (diet_moisture) − $6.74e^{-2}$ * (diet_Probiotics))	0.63	0.53	0.61	$2.87e^{-4}$
Simple model w/ Others[d]	Exp ($2.38e^{-2}$ *(Temp) − 0.12 * (Oxygen) − $4.18e^{-3}$ * (ABW) − $2,13e^{-2}$ * (diet_CP) − 0.13 * (diet_GE) − 0.11 * (diet_moisture) + 0.11 * (diet_Others))	0.66	0.56	0.17	$1.33e^{-4}$

Temp (water temperature °C); Oxygen (dissolved oxygen (ppm)); ABW (average body weight (g)); diet_CP (dietary protein content (%)); diet_GE (dietary energy content (MJ/kg)); diet_moisture (diet moisture content (%)); diet_Prebiotics (dietary prebiotics content (g/kg)); diet_Probiotics (dietary probiotics content (g/kg)); diet_Others (dietary Others content (g/kg)).

[a] FCR simple model (trimmed simple model).

[b] FCR simple model with prebiotics dietary inclusion as quantitative variable.

[c] FCR simple model with probiotics dietary inclusion as quantitative variable.

[d] FCR simple model with others dietary inclusion as quantitative variable.

concentrations of short chain fatty acids (SCFAs) as by-product of prebiotic fermentation by intestinal bacteria (Rastall and Gibson, 2015; Rivera-Piza and Lee, 2020). Between the different SCFAs derived from these indigestible fibers, butyrate and propionate are known to stimulate the intestinal gluconeogenesis leading to metabolic advantages on host intestinal health and growth. The propionate is directly absorbed, triggering the *de novo* synthesis of glucose acting as internal energy source and thus enhancing host growth performance. Butyrate meanwhile, can be metabolized by the intestinal cells, playing an important role stimulating the growth and differentiation of enterocytes and colonocytes leading to higher absorptive surface and an enhanced gut homeostasis (Rivera-Piza and Lee, 2020). Zhou et al. (2010), reported increased pyloric caeca and intestinal microvilli height in red drum (*Sciaenops ocellatus*) fed 4 different functional diets supplemented with prebiotics (Zhou et al., 2010). Similarly, Torrecillas and co-authors (2013) reported significant longer and more densely distributed microvilli on the posterior intestinal enterocytes surface in European sea bass juveniles fed with a 4 g/kg MOS supplemented diet in comparison to a control diet void of supplementation (Torrecillas et al., 2013).

An enhanced intestinal health and functionality will directly benefit host by increasing nutrient absorption (Butt and Volkoff, 2019; Dawood, 2021) even under unfavorable conditions such as those derived from the nutritional imbalances derived from high FM/FO replacement on diet formulation. As reported by Guerreiro and co-authors in 2015, the inclusion of either 1 g/kg of xylo-oligosaccharides (XOS) or 1 g/kg of fructo-oligosaccharides (FOS) led to higher body weight in European sea bass fed low protein based diets (Guerreiro et al., 2015). Similarly, in 2018, Torrecillas and co-authors studied the effects of prebiotics (MOS), probiotics (*Peridococcus acidilactici*) and their combination in fish growth and immune response of European sea bass juveniles fed low FM (5 %) and FO (6 %) based diets. The authors reported higher final body weight in those fish fed prebiotics and the symbiotic compound (MOS + *P. acidilactici*). On the contrary the probiotic alone did not induced significant differences on fish growth in comparison to the control diet, void of supplementation. Nevertheless, the symbiotic compound attenuated the MOS-induced gut humoral pro-inflammatory response. Those results may suggest a role of probiotic compounds as immune modulators rather than as growth enhancer products (Nayak, 2010; Lazado and Caipang, 2014; Huang and Lee, 2018; Firmino et al., 2021), supporting the lack of influence of this compounds inclusion in the final outcome of the models developed in the present study. In the same way, P´erez-Sanchez et al., analyzed the effects of a combination of phytogenic compounds in the growth performance of the sea bream. The phytogenic compounds did not induced significant effects on fish growth performance, but reduced their gut inflammatory response leading to an improved absorptive capacity (P´erez-Sa´nchez et al., 2015).

The observational models developed in the present study met the conditions necessary to be validated. Nevertheless, full models required a trimming treatment in order to split the full model into local models with lower R^2 values but simpler equations (Chicco et al., 2021). Model simplification allowed the identification of dietary prebiotics inclusion as a determinant factor on fish growth and feed utilization. Nevertheless, considering the wide variety of functional ingredients and their ways of action, further studies are required in order to clarify these mechanisms and employ prebiotics as effective tools in order to increase aquaculture production yields.

CRediT authorship contribution statement

All authors contributed to the study conception and design. Data collection and curation was performed by Antonio Serradell. Data validation was performed by Tome´ Silva and Filipe Soares. The first draft of the manuscript was written by Antonio Serradell and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding

This work was founded by the European Union's Horizon 2020 research and innovation program under grant agreement no. 818367; AquaIMPACT—Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture. Authors want to thank the University of Las Palmas de Gran Canaria (ULPGC) for the funding for Antonio Serradell through the call "ERASMUS + PRÁCTICAS (SMT)" 2020-2021.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antonio Serradell Pastor reports financial support was provided by University of Las Palmas de Gran Canaria. Daniel Montero Vitores reports financial support was provided by European Commission. Daniel Montero Vitores reports a relationship with Sparos Lda that includes: non-financial support.

Data Availability

The datasets generated during and/or analyzed during the current study are publicly available at https://data.mendeley.com/datasets/bhgmxs3g2d.

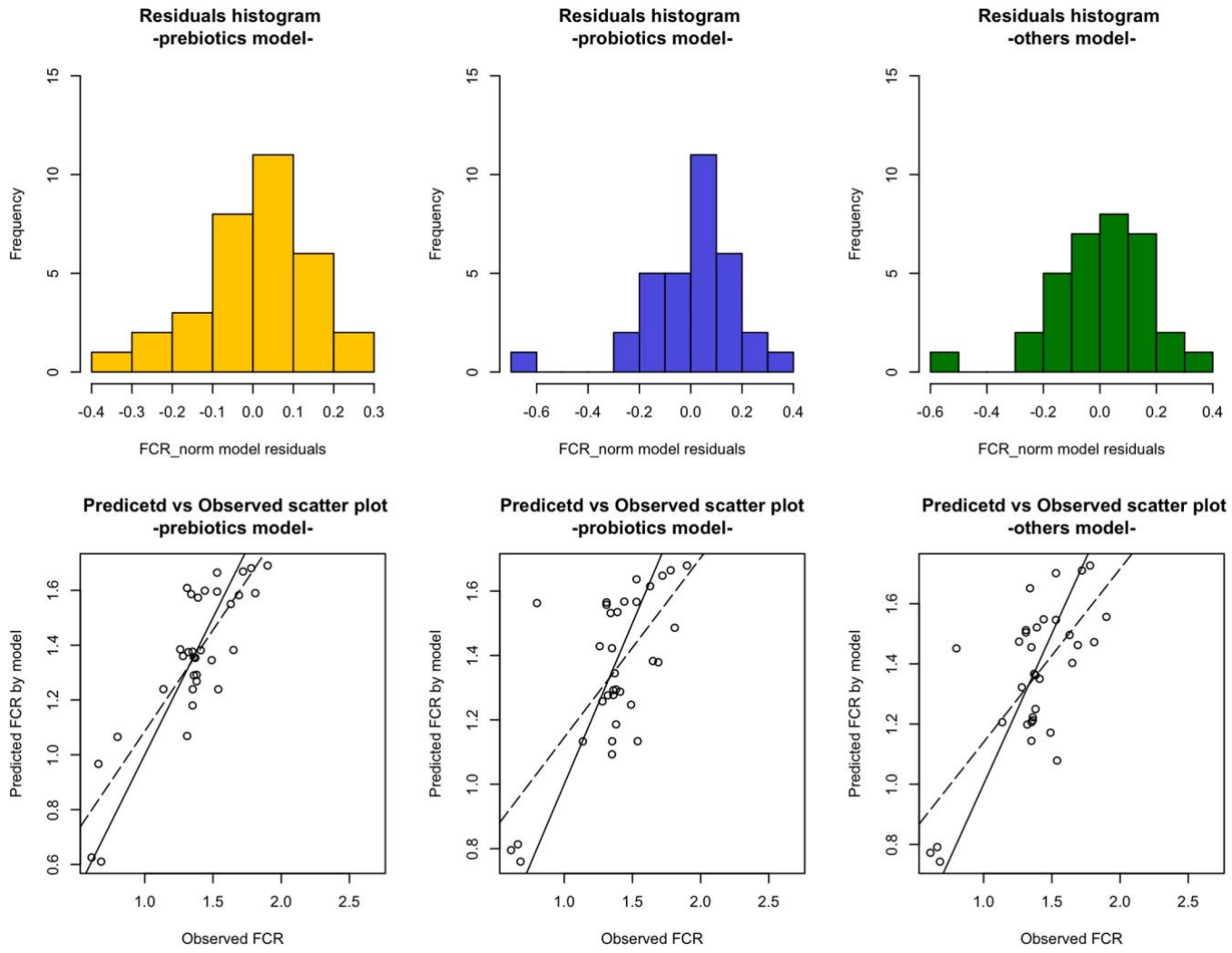
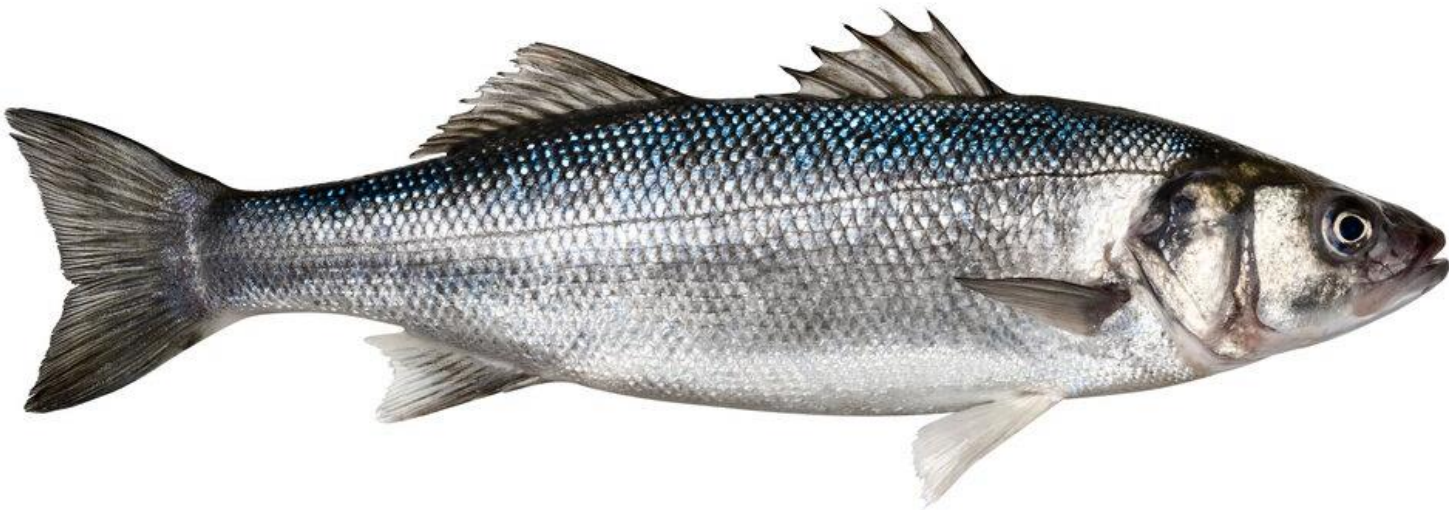


Fig. 7.6. European sea bass (*Dicentrarchus labrax*) feed conversion ratio (FCR) distribution for a) prebiotics simple model residuals; b) probiotics simple model re- siduals; c) others simple model residuals; d) prebiotics simple model fitted values; e) probiotics simple model fitted values; f) others simple model fitted

Acknowledgments

Authors want to thank the European Union's Horizon 2020 research and innovation program under grant agreement no. 818367; Aqua-IMPACT—Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture. Authors want to thank to the University of Las Palmas de Gran Canaria (ULPGC) for the funding for SA through the call "ERASMUS + PRÁCTICAS (SMT)" 2020–2021.

Chapter VIII
General Discussion



There is an increasing demand of aquaculture products as the population grows and increases the demand of high-quality food. Cultured fish are considered to be one of the most efficient protein sources, and aquaculture fish production is increasing in response to this demand. However, this increase must take place in the context of efficient and sustainable fish feeds. The aquafeeds must face the challenge to reduce the dietary fishmeal (FM) and fish oil (FO) content as much as possible and it is conditioned by the target species and its development age. Even though the reduction of FM and FO has been shown to be feasible in some marine species, in some cases this strategy is associated with different side effects on growth performance and health. Those effects will be dependent not only on the fish species and developmental stage, but also on the amount of FM/FO replaced and the type of ingredients used for the replacement. In European sea bass (*Dicentrarchus labrax*), a high replacement of marine raw materials is associated to different gut alterations such as engrossment of the *lamina propria* and submucosa, intestinal up-regulation of several inflammation related genes or increased mucus production among others. The nutritional consequences derived from low FM/FO dietary inclusion may also induce a chronic marginal-stress status in fish impairing their capacity to cope with subsequent stress processes. All this potential negative effects can compromise the competitiveness of the European sea bass production when feeding a very low FM/FO diet.

Which zootechnical tools could be used to avoid the negative effects of feeding a low FM/FO based diet in European sea bass?

Among different strategies, including the formulation of appropriate diets with sustainable ingredients, and the supplementation of ingredients that can be deficient in a low FM/FO diet, there are two strategies that can be used to improve European sea bass performance and robustness to face the dietary challenge of Low FM/FO. *i*) Functional additives dietary supplementation, which may play an important role as stress relieving and immune modulator substances, improving European sea bass health status and performance against common aquaculture stressors; and *ii*) genotype selection using multitrait strategies, which may induce important advantages on the utilization of low FM/FO based diets leading to an improved growth performance and robustness against possible stressors.

Functional additives dietary supplementation is an effective strategy for boosting fish immune response and health status. These ingredients contain bioactive compounds with the ability to modulate fish response during stress processes, such as environmental stress or disease infections [Soleimani et al., 2012; Hoseinifar et al., 2013; Herrera et al., 2019]. Functional additives have shown modulatory effects on inflammatory processes and oxidative status via interaction with a a wide arrange of cellular signaling pathways [Soares et al., 2018; Filippone et al., 2020; Porter et al., 2022]. Besides, different functional additives have been reported to exert beneficial effects on fish gut microbiome by favouring homeostasis, benefiting fish health status and digestive performance [Yaramahdi et al., 2016; Gonçalves and Gallardo-Escárate, 2017; Soares et al., 2018; Butt and Volkoff, 2019; Caipang et al., 2020]. Thus, functional additives dietary supplementation may result in a generalized enhanced health status leading to an increased growth performance and/or the capacity to cope with the nutritional unbalances derived from low FM/FO diets.

In the present thesis, the use of functional additives has shown the potential to improve fish health performance and coping ability in response to different stressors

(Chapters III, IV, V and VI) [Serradell et al., 2020; Serradell et al., 2022; Serradell et al., 2023; Rimoldi et al., 2023]. Nevertheless, the functional additives employed did not induced any change on fish growth performance or feed utilization when supplemented in FM/FO diets. Despite fish key performance indicators (KPI) were not improved, functional additives successfully offset the inflammatory processes associated to low FM/FO diets. For example, Torrecillas and co-authors in 2019 in a parallel study to Chapters III and IV, reported an effect of GMOS supplementation increased fish rectum microvilli height and reduced submucosa width. Those results support the observations reported in Chapter VII of the present thesis, in which the descriptive functional additives model detected beneficial effects of prebiotics dietary supplementation on European sea bass growth performance and feed utilization. Thus, in the present thesis, it has been demonstrated that the use of functional additives dietary inclusion is an effective strategy in order to offset the negative side effects associated to low FM/FO instead of the promotion of fish growth performance in comparison to fish fed non-supplemented diets.

Multitraits selective breeding programs are also an effective strategy increasing fish performance in terms of growth and feed utilization [de Verdal et al., 2018; Knap and Kause, 2018; Besson et a., 2019; Vandeputte et al., 2022]. A higher capacity to obtain and utilize nutrients may suppose a significant advantage to increase fish tolerance to nutritional unbalances derived from high FM/FO replacement by alternative raw materials [Gjedrem et al., 2012, Overturf et al., 2013; Abernathy et al., 2017; de Verdal et al., 2018; Besson et al., 2020; Vandeputte et al., 2022]. An increased capacity to perform even under low FM/FO nutritional conditions may also translate into a better capacity to cope with other stressors, resulting in healthier and more robust fish. [Hermesch et al., 2015; Perera et al., 2019; Piazzon et al., 2020]. In the present thesis, genotype selection significantly improved feed conversion ratio and growth performance of European sea bass despite being fed extremely low FM/FO levels (Chapters V and VI). In Chapter V, selective breeding resulted in significant higher body weight at 300 days post hatch (dph), even before starting the feeding experience. In chapter VI, fish belonging to selected genotype presented the ability to shape gut microbiome and resulting in a higher tolerance to low FM/FO conditions and resulting in higher survival rate to the pathogen V.anguillarum. These results highlight the potential of selective breeding as an effective strategy to increase the dietary tolerance of fish to novel protein and lipid sources, leading to successful high levels of FM/FO replacement without compromising fish growth performance or health status.

Are functional additives a useful tool to improve European sea bass robustness and welfare status?

In the present thesis the use of functional additives has successfully improved European sea bass fish capacity to cope with different stressors and the general health status (Chapters III, IV, V, VI [Serradell et al., 2020; 2022; 2023a; Rimoldi et al., 2023]). Along the present thesis, different mechanisms were proposed in order to explain the different ways of action of the functional additives employed. For instance, due to their elevated concentration on flavonoids, phytogenic feed additives were proposed as a potential strategy in order to improve fish antioxidant status and as immune modulation substances. Certain phytogenic compounds, such as flavonoids, elicit a modulatory effect on fish pro-inflammatory response and antioxidant status through the interaction with different cellular signaling factors such as MAPKS, and thus playing a fundamental role

on cellular homeostasis [Shen and Liu, 2006; Chakraborty et al., 2011; Mansuri et al., 2014]. Besides flavonoids, have been reported to exert powerful sedative activities by directly interacting with fish central nervous system [de Souza et al., 2016; Souza et al., 2019; Hoseini et al., 2019; Caipang et al., 2020]. These bioactive compounds are highly liposoluble, facilitating their diffusion through biological membranes. Once in the brain, flavonoids modulate the gamma-aminobutyric acid receptor complex (GABA) inhibiting neurotransmission impulse and inducing its sedative effects [Saccol et al., 2017; Sangeetha et al., 2016; Saccol et al., 2017]. On the other hand, prebiotic dietary supplementation was proposed to exert an indirect effect on fish stress response and health status based on the modulation of fish gut homeostasis. The main mechanism proposed was the stimulation of host microbiome production of short chain fatty acids with powerful antioxidant [Leonel and Álvarez-Leite, 2012; Filippone et al., 2020] and anti-inflammamtory properties [Buentello et al., 2010; Leonel andÁlvarez-Leite, 2012, Filippone et al., 2020]. The ability of such compounds in order to modulate fish microbiome and gut health status, might benefit fish by improving their performance under stressful conditions [Yaramahdi et al., 2016; Soares et al., 2018; Butt and Volkoff, 2019] through the modulation of the gut-brain axis leading to an attenuated reactivity of fish HPI axis [Cryan and Dinan 2012; Soleimani et al., 2012; Hoseinifar et al., 2013; Herrera et al., 2019; Porter et al., 2022].

Dietary supplementation of functional additives attenuated European sea bass stress response offsetting the possible physiological unbalances derived the concomitant occurrence of different stressors; a soft chronic stress derived from low FM/FO dietary supplementation and acute stress processes such as pathogen outbreaks, crowding stress and chemicals exposure.

Are selective breeding programs and effective tool to improve fish health and welfare when fed los FM/FO based diets?

Genotype selection has shown the ability to improve European sea bass stress tolerance (Chapter V [Serradell et al., 2023a]) and survival against the pathogen V. anguillarum (Chapter VI [Rimoldi et al., 2023]).

Within populations, individuals often display different grades of stress tolerance and copying styles, allowing to their classification as high (reactive) or low (proactive) stress responders [Martins et al., 2011; Castanheira et al., 2017;Alfonso et al., 2019]. The proactive individuals have low cortisol basal levels and lower HPI axis activation in response to stress [Martins et al., 2011; Castanheira et al., 2017; Ruiz-Gomez et al., 2015]. Reduced, but not impaired, activity of the HPI axis is associated with a lower sensitivity to environmental changes and an increased tendency to establish routines [Silva et al., 2010; Martins et al., 2011; Castanheira et al., 2017]. In addition, proactive fish are characterized by a more efficient feed utilization and robustness [Martins et al., 2011; Castanheira et al., 2017; Alfonso et al., 2019]. In natural populations copying styles are not polarized, with individuals displaying intermediate features of both proactive and reactive strategies [Martins et al., 2011]. Nevertheless, the cumulative effects of breeding programs may induce this polarization process leading to a progressive domestication [Vandeputte et al., 2009; Jensen, 2014; Vandeputte et al., 2016]. Lower stress susceptibility will traduce into higher performance even under the stressful conditions associated to aquaculture production [Tort et al., 2011; Ellis et al., 2012]. For instance, Trenzado and co-authors in 2006, detected a beneficial effect of rainbow trout genotype selection for low-cortisol responsiveness on fish growth performance under crowding

conditions [Trenzado et al., 2006]. Low responsive fish, presented higher weight gain, SGR, feed efficiency and feed intake than high responsive fish regardless the culture density, pointing the important role of stress tolerance on fish growth performance under culture conditions.

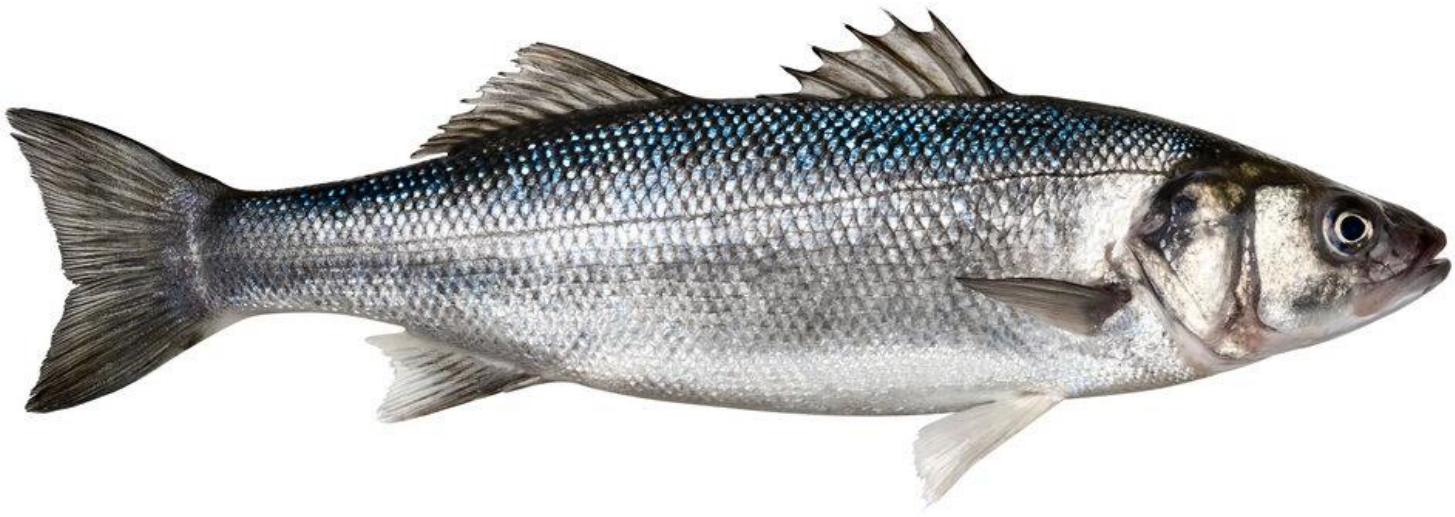
Selective breeding strategies offer great potential for improving the robustness of fish, allowing high production performance regardless of environmental conditions [Agha et al., 2003; Knap, 2005; Hermesch et al., 2015]. In the present thesis, compared to wild type fish, GS European sea bass presented better growth performance when fed low FM/FO diets and a higher capacity to cope with different stressors.

Is the combination of genotype selection and dietary supplementation with functional additives a potential tool to optimize European sea bass juvenile culture?

Apparently, in the present thesis, both strategies exerted individual and separated effects on European sea bass performance. Nevertheless, it must be taken into account non-genetically selected fish start from a worse health status and a lower performance, as they are less robust than GS fish. Wild type fish with a lower capacity to utilize low FM/FO based diets, presented higher sensitivity and reactivity to the different stressors employed, resulting in lower survival rates. The beneficial effects induced by functional additives dietary supplementation were easier to observe in comparison to non-selected fish fed non-supplemented low FM/FO based diets. GS fish response to functional additives is conditioned by the better basal health status, resulting in a lower responsiveness to the beneficial effects of the dietary stimulus when compared to wild type fish. In fact, in Chapter V the basal cortisol levels presented by the GS European sea bass were lower to those expected for this fish species [Samaras et al., 2023] which may be directly related to improved tolerance to the stressful conditions associated with rearing conditions and tolerance to very low FM/FO based diets. To our knowledge there are no more studies investigating the combined effects of this three factors (very low FM/FO dietary regime, functional additives supplementation on European sea bass juveniles growth performance and feed utilization. MLR model trimming, allowed the detection of a significant positive correlation between dietary prebiotics supplementation and SGR. On the contrary, prebiotic supplementation presented a negative correlation with fish FI and FCR. Those results suggest the possibility to use prebiotics to improve fish performance. However, not enough information on the effects on fish health can be included in the model, as the literature does not provide enough data, as the different published experiments analyzed a wide variety of health indicators, and repetition among different studies is very low to be included in the model. Same occurs with the data coming from different selective breeding programs for this species.

For this reason, an integrative approach could be done in future, when more selective breeding programs will be implemented in the industry, in order to describe the most efficient synergies of those strategies to gain competitiveness in the industry of European sea bass aquaculture production. In special, the development of more complex nutritional models may suppose a powerful tool in order to combine all the possible factors conditioning the maximum performance of European sea bass under culture conditions leading to fast improvements on this sector of aquaculture industry.

Chapter IX
General conclusions



9. General Conclusions

1. The use of dietary galactomannan oligosaccharides (GMOS) at a 0.05% attenuated the stress response of European sea bass juveniles, reducing plasma cortisol concentrations post crowding stress and down-regulating stress-related gene expression in the head kidney in fish fed a low fishmeal fish oil based diet. Similarly, the use of 200 ppm of a mixture of garlic and *Labiatae* plants essential oils induced lower plasma cortisol levels after stress by crowding.

2. The use of a dietary mixture of garlic and *Labiatae* plants essential oils has a protective role against apoptosis in head kidney cells in response to crowding stress.

3. The use of 200 ppm of a mixture of garlic and *Labiatae* plants essential oils or 0.05% of GMOS increased the resistance of European sea bass juveniles to *V. anguillarum* during a stress challenge by crowding.

4. The use of either of 200 ppm of a mixture of garlic and *Labiatae* plants essential oils or 0.05% of GMOS in 10% FM/6% FO diets induced a down-regulation of the gill pro-inflammatory response under stress challenge, as a result of a modulation of the inflammatory response against the stressor.

5. Functional additives dietary supplementation induced an attenuated stress response, resulting in stable energy metabolism and improved antioxidant status under stressful conditions caused by crowding stress.

6. Both functional additives improved the antioxidant status of European sea bass gills by upregulating catalase gene expression in response to an environmental chemical stress (H_2O_2).

7. Selected European sea bass juveniles presented lower basal cortisol levels and a lower reactivity to stress processes when compared to wild type fish, indicating a better ability to cope with the stress processes associated with aquaculture production.

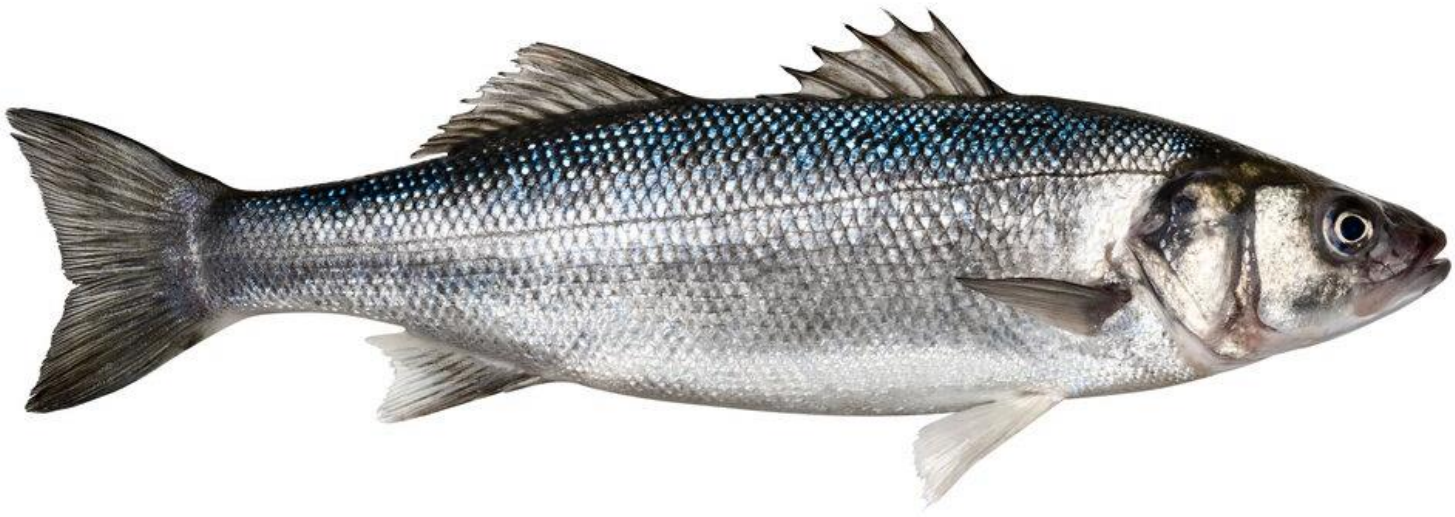
8. The better growth performance and improved basal health status of the European sea bass selected genotype, resulted in a better survival rate against the pathogen *V. anguillarum* compared to wild type sea bass.

9. The effect of functional additives was both additive- type, additive concentration and genotype dependent.

10. A probiotic mixture (INVE, Belgium - containing three *Bacillus* species: *B. subtilis*, B. *licheniformis*, and *B. pumilus*) activated the gut local immune response of selected European sea bass.

11. Despite in the present thesis dietary supplementation with functional additives did not improve fish growth or feed utilization when fish were fed low FM/FO based diets, the descriptive multiple linear regression model developed pointed to prebiotic dietary inclusion as a potential strategy in order to improve European sea bass growth performance and feed efficiency.

Resumen en español



1. Introducción

1.1. Lubina europea (*Dicentrarchus labrax*): producción acuícola

Los alimentos de origen marino se han convertido en una de las principales fuentes de nutrientes para consuno humano, llegando a suponer hasta el 20% del total de proteína de origen animal consumida en el mundo. En 2020, se produjeron un total de 178 millones de toneladas de peso vivo de pescados y mariscos, con un rendimiento económico total de 406 billones de dólares (USD). El 51% de la producción total provino de la pesca extractiva (90.3 millones de toneladas) y el 48% restantes (87.7 millones de toneladas) fueron producidos por el sector acuícola [SOFIA, 2022]. Sin embargo, esta tendencia se va a ver revertida a la larga debido a la rápida expansión y tecnificación del sector acuícola y el estancamiento de la producción extractiva debido al agotamiento de los caladeros [Carvalho y Guillen, 2021]. Desde finales de la década de 1980, la pesca extractiva genera de media anual entre 86 y 90 millones de toneladas de peso vivo. El sector acuícola por el contrario, ha tenido un incremento en la producción del 609% en las últimas 30 décadas, pasando de producir 14.4 millones de toneladas en los 90s a los 87.7 millones actuales [FEAP, 2021; SOFIA, 2022].

La lubina europea (*Dicentrarchus labrax*) es un pez teleósteo marino de hábitos carnívoros que presenta una amplia distribución geográfica, pudiendo encontrarse a lo largo del Atlántico noroeste y toda la cuenca mediterránea y mar Negro. Con ecología euriterma(2-32°C) y eurihalina (2-40 ppt), la lubina puede encontrarse en aguas costeras por encima de los 100 metros de profundidad y en cuerpos de agua salobres como estuarios y lagunas costeras [Kousoulakis et al., 2015; Vandeputte et al., 2019]. La lubina es un claro ejemplo del desarrollo y tecnificación de la producción acuícola. En los 80s con el desarrollo de técnicas modernas en reproducción y cuidado larvario asegurando un suministro continuo de individuos, la producción acuícola de lubina comenzó su expansión. Ya en 1992, la producción acuícola mediterránea generaba el 96% del total de la lubina consumida a nivel mundial alcanzando en 2020 una producción anual media de 233.900 toneladas en peso vivo. Los mayores productores de lubina en el mar Mediterráneo son Egipto, Turquía, Grecia e Italia sumando el 86% de la producción acuícola [Stavrakidis-Zachou et al., 2019; Carvalho y Guillen, 2021].

El ciclo de producción de la lubina está dividido en dos fases, con una duración total de aproximadamente 24 meses [Vandeputte et al., 2009]. Un fase de criadero desarrollada desde la eclosión del huevo hasta aproximadamente los 20g de peso corporal (de 3 a 8 meses) y una fase de engorde hasta su peso de mercado de 250-400 g (12 a 20 meses) [Vandeputte et al., 2019].

1.2. Nutrición de la lubina europea: desafíos de producción

Los requerimientos nutricionales de la lubina han sido bien establecidos, asegurando el mayor rendimiento y estado de salud posible de los animales bajo condiciones de cultivo [Cerdá et al., 1994; Rizzo et al., 1996; Dias et al., 1998; Zambonino y Cahu, 1999; Lupatsch et al., 2001; Kaushik et al., 2002; Boujard et al., 2004; Oliva-Teles y Pimentel-Rodrigues, 2004; Enes et al., 2011]. Al ser una especie carnívora, la lubina presenta elevados requerimientos proteicos con un perfil de amino ácidos esenciales muy específico [Kaushik et al., 1998; Tibaldi y Kaushik, 2005; Kousoulaki et al., 2015] (Tabla 1). Por otra parte, la lubina también presentan requerimientos muy específicos en ácidos grasos esenciales ya que los carnívoros marinos no son capaces o

presentan una capacidad muy reducida para la de elongación y desaturación de ácidos grasos poliinsaturados (PUFA) a ácidos grasos de cadena larga poli insaturados (LC-PUFA). En el caso de la lubina, los requisitos nutricionales en ácidos grasos esenciales estarán cubiertos con la inclusión de un 1% en la dieta de LC-PUFA omega 3 [NRC, 2011; Oliva-Teles et al., 2015; Kousoulaki et al., 2015].

Tabla 1. Composición proximal media y perfil de amino ácidos esenciales en dietas comerciales para lubina europea (*D. labrax*).

Peso corporal (g)	Tamaño del grano (mm)	Contenido proteínas (%)	Contenido lípidos (%)	Contenido fibras (%)	Ceniza (%)	Fósforo total (%)	Energía digerible (MJ kg^{-1})
< 0.1	< 0.3	57	12	0.7-10	10	1.9	17.8-20
0.3-20	0.3-1.9	52-60	11-18	0.4-1.9	10-13	1.4-1.9	17.4-20.2
20-250	2.2-5	45-50	11.5-24	1-3.2	8.2-13	10-2	17.1-21.6
250-600	5-7	35-45	10.5-26	1.7-2	8.2-12	0.9-2	18-21.6
> 600	6-8	33.8-45	10.5-26	1.7-2	9-11	0.9-2	18-21.6

Amino ácidos esenciales					
ARG	3.9 - 4.6	LYS	4.4 - 4.8	MET + CYS	4 – 4.4
HIS	1.6*	THR	2.3 - 2.6	PHE + TYR	2.6*
ILE	2.6*	VAL	2.9*		
LEU	4.3*				

ARG (arginina); HIS (histidina); ILE (isoleucina); LEU (leucina); LYS (lisina); THR (treonina); VAL (valina); MET+CYS (metionina + cisteina); PHE+TYR (fenilalanina + tirosina); n-3 LC-PUFA (ácidos grasos de cadena larga poliinsaturados omega 3). *Valores estimados para lubina europea por Kaushik, 1998.

Las harinas (HP) y aceites de (AP) pescado son la principal fuente de proteínas y lípidos en la producción de dietas acuícolas. Esto se debe a su excepcional composición nutricional en micronutrientes, sus buenos perfiles de amino ácidos y ácidos grasos esenciales y una elevada digestibilidad y biodisponibilidad de nutrientes. Además, estos ingredientes presentan potentes características organolépticas, estimulando el apetito de los peces [Hardy, 2002; Turchini et al., 2009; Monterol y Izquierdo, 2010; Oliva-Teles, 2015].

Sin embargo, la producción de HP/AP de pescado recae totalmente sobre la pesca extractiva, suponiendo un importante inconveniente en sus sostenibilidad. En el año 2020, del total de 90.6 millones de toneladas en peso vivo producidas por la industria pesquera, 16 millones fueron destinadas a la producción de HP/AP para producción animal [FAO, 2020]. El colapso de los caladeros naturales ha dado lugar a la escasez de harinas y aceites de pescado y por tanto al incremento en la volatilidad de sus precios. En 2012, las harinas y aceites de pescado alcanzaron respectivamente el valor de 1600 y 1800 dólares (USD) por tonelada [Hodar et al., 2020]. El elevado coste de las HP/AP en combinación con su creciente demanda por parte del sector acuícola, está dando lugar a la necesidad de encontrar ingredientes alternativos y sostenibles para la producción de piensos para acuicultura [Turchini et al., 2009; Hardy et al., 2010; Olsen y Hasan, 2012; Oliva-Teles, 2015; Turchini et al., 2019].

En las últimas décadas, un gran esfuerzo de investigación se ha llevado a cabo para la identificación de fuentes alternativas de proteínas [Torstensen et al., 2008; Oliva-Teles et al., 2015; Salze y Davis, 2015; Hamre et al., 2016; Hemre et al., 2016; Lock et al., 2018; Benedito-Palos et al., 2016; Hua et al., 2019; Lazzarotto et al., 2018; Turchini et al., 2019;

Carvalho et al., 2020; Parma et al., 2020; Carvalho et al., 2021; Gesto et a., 2021; Luthada et al., 2021] y lípidos [Turchini et al., 2009; Turchini et al., 2010; Lenihan-Geels et al., 2013; Oliva-Teles et al., 2015; Gasco et al., 2018; Carvalho et al., 2020; Hodar et al., 2020; Carvalho et al., 2021]. Sin embargo, el empleo de estos nuevos ingredientes puede dar lugar a desequilibrios nutricionales como deficiencias en los perfiles de amino ácidos o desajustes en los ratios de ácidos omega 3 y omega 6, o afectando negativamente el crecimiento y la salud de los peces. [Turchini et al., 2009; Montero y Izquierdo, 2010; Oliva-Teles et al., 2015]. El uso de determinados ingredientes alternativos, como los de origen vegetal, puede dar lugar a la presencia de antinutrientes reduciendo la digestibilidad y disponibilidad de nutrientes y empeorando su palatabilidad [Kousoulaki et al., 2015; Oliva-Teles, 2015; Daniel, 2018].

Bajo el marco del proyecto de investigación europeo ARRAINA (N288925: "Advanced Research Initiatives for Nutrition & Aquaculture" financiado por EU7FP), en 2017, Torrecillas y coautores investigaron los efectos de la alta sustitución de HP/AP por ingredientes de origen vegetal en la salud y crecimiento de juveniles de lubina europea. Los investigadores llevaron a cabo una experiencia de engorde de 90 días de duración, en la que juveniles de lubina con un peso aproximado de 9.78 ± 1.50 g fueron alimentados con dietas con distintos grados de sustitución de HP%/AP%: 58/15, 20/6, 20/3, 10/6, 10/3, 5/6, 5/3, 0/0. Los autores observaron que la sustitución de HP/AP era posible hasta un 10%/3% respectivamente, sin afectar al crecimiento de los peces en comparación a una dieta control (HP58%AP15%) [Torrecillas et al., 2017 a,b]. Niveles superiores de la sustitución de estos ingredientes dieron lugar a menores tasas de ingesta por parte de los peces y por lo tanto a menores tasas de crecimiento. Además, estos niveles de sustitución dieron lugar a alteraciones en el microbioma intestinal y un engrosamiento de la submucosa del intestino posterior, dando lugar a una mayor producción de mucus intestinal y altos niveles de expresión génica de las citoquinas pro inflamatorias IL-1β y TNF-α [Torrecillas et al., 2017a,b]. En lo referente a la sustitución de AP por aceites de origen vegetal, la sustitución de AP hasta el 3% y 0% dio lugar a un incremento de la deposición de ácidos grasos en el hígado, indicando una reducción de la utilización de lípidos alimenticios y por tanto menor eficiencia alimenticia. En el intestino el empleo de aceites vegetales dio lugar a la acumulación de lipoproteínas en la *lamina propria* del intestino anterior, una posible consecuencia de alteraciones en la reacilación y transporte de lípidos digeridos [Torrecillas et al., 2017 b,c]. La reducción total de las harinas de pescado por debajo del 6% también dio lugar a cambios en la morfología e integridad intestinal de los peces. Todas estas alteraciones dieron lugar a la pérdida de la integridad y funcionalidad del epitelio estomacal, resultando en una mayor susceptibilidad frente al patógeno *V. anguillarum* [Torrecillas 2017 c].

De este modo, el éxito en la tarea de reducir la dependencia en HP/AP para la producción de pienso acuícolas deberá considerar el estrés fisiológico generado por los posibles desequilibrios nutricionales y sus efectos negativos en la salud y rendimiento de los peces [Ashley, 2007; Montero y Izquierdo, 2010; Oliva-Teles, 2012].

1.3. Fisiología de la lubina europea: Susceptibilidad al estrés

1.3.1 Fisiología del estrés.

Los peces, del mismo modo que otros organismos, presentan la capacidad de mantener un equilibrio interno de carácter dinámico conocido como *homeostasis.* Cuando este equilibrio se ve desestabilizado por algún agente interno o externo, conocido como *agente estresante*, el organismo inicia la *respuesta al estrés* que consiste en una cascada de cambios fisiológicos y de comportamientos con la finalidad de

restablecer la homeostasis y sobrevivir [Aluru et al., 2009; Ellis et al., 2012; Tort, 2011; Schreck and Tort, 2016; Mateus et al., 2017]. El coste energético total para hacer frente y sobrevivir a un proceso de estrés se denomina *carga alostática* y será directamente proporcional al grado de severidad del agente estresante [Aluru et al., 2009; Tort, 2011; Mateus et al., 2017].

La respuesta al estrés es un proceso altamente demandante de energía, que consiste en el desvío y restablecimiento de las reservas energéticas del organismo con la finalidad de hacer frente al agente estresante. Este proceso se puede dividir en tres fases; (*i*) la fase de alarma, en al que las reservas energéticas son rápidamente destinadas a escapar o combatir el agente estresante (huida o lucha). A continuación se iniciará la segunda fase (*ii*), de resistencia, que consiste en la reorganización de los recursos energéticos con la finalidad de restablecer las condiciones previas al proceso estresante. Cuando un proceso de estrés es muy severo o se repite en el tiempo, el organismo puede sufrir una *sobrecarga alostática*, dando lugar a la tercera fase (*iii*) de agotamiento. En esta fase de la respuesta al estrés el rendimiento general del organismo se ve afectado, dando lugar a una reducción en las funciones de crecimiento y reproducción, la supresión inmune e incluso la muerte (Figura 1) [Ellis et al., 2012; Nardocci et al., 2014; Schreck and Tort, 2016].

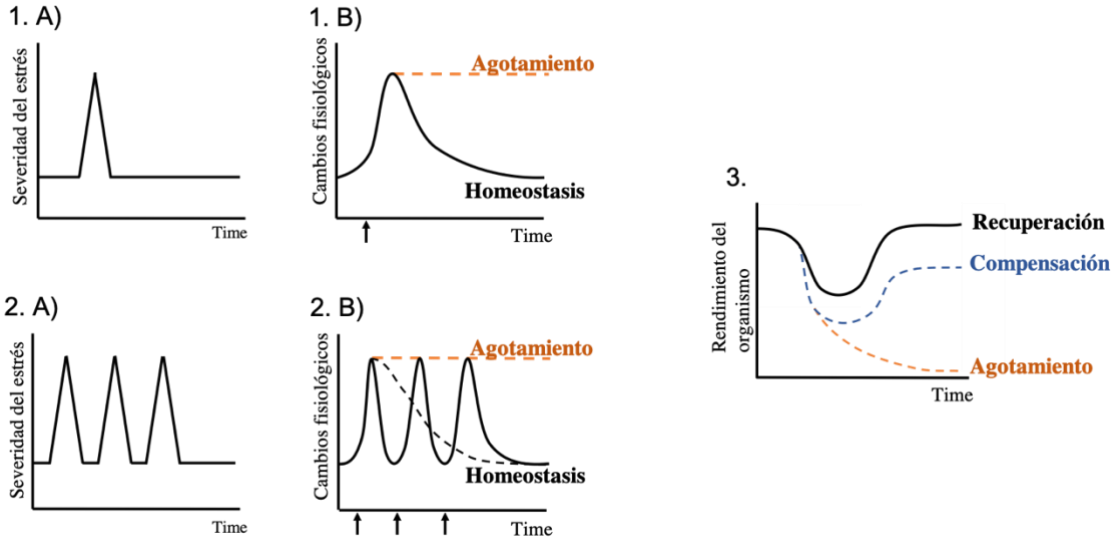


Figura 1. Patrones de respuesta frente al estrés. (1A) Un único agente estresante agudo; (1B) Estrés agudo o puntual indicado por ↑: un proceso de estrés leve o moderado resultará en el restablecimiento de la homeostasis, mientras que un proceso de estrés severo puede dar lugar a la fase de agotamiento; (2.A) Diversos agentes estresantes consecutivos; (2.B) Estrés crónico, con cada proceso de estrés indicado por ↑: Diferentes procesos de estrés consecutivos, simultáneos o repetitivos pueden dar lugar a la fase de agotamiento; (3) Un proceso de estrés puede resolverse de diferentes formas dependiendo de la capacidad del organismo para recuperar la homeostasis previa al proceso de estrés. Si el proceso de estrés se resuelve satisfactoriamente pero el organismo no recupera las condiciones iniciales, se producirá la compensación. La incapacidad para superar un proceso de estrés o una mala recuperación de la homeostasis dará lugar al agotamiento. Adaptado de Schreck and Tort, 2016.

Inmediatamente después de la percepción de un agente estresante, la respuesta al estrés es iniciada y regulada por dos ejes neuro-endocrinos, el eje simpático-cromafín (SC) y el eje hipotálamo-pituitaria-interrenal (HPI), activando la síntesis y liberación de catecolaminas (CA) y glucocorticoides (GC) respectivamente. La función principal de estas hormonas es la provisión de energía, sin embargo cada una ejerce sus efectos en momentos distintos de la respuesta al estrés [Reid et al., 1998; Ellis et al., 2012; Schreck et al., 2016].

Las CA, adrenalina y noradrenalina, son sintetizadas en cuestión segundos por estimulación nerviosa del proceso de Blachsko en las células cromafines interrenales [Reid et al., 1998; Gorissen and Flik, 2016]. La liberación de CA al torrente sanguíneo dará lugar a un incremento en las tasas cardíaca y respiratoria, además de la movilización de las reservas energéticas atendiendo las demandas metabólicas del proceso de estrés [Schreck et al., 2016; Rodnick and Planas, 2016]. Las CA darán lugar a un rápido incremento de los niveles de glucosa plasmática circulante a través de la estimulación de la glucogenólisis hepática y la glucolisis anaeróbica. A nivel cardiovascular, las CA darán lugar a un incremento en la tasa cardíaca y estimularán contracciones del bazo, dando lugar a un incremento en las poblaciones de eritrocitos sanguíneos y facilitando el transporte de oxígeno y glucosa a los diferentes tejidos involucrados en la respuesta de "huida o lucha" [Pottinger, 2008; Peter, 2011]. En lo referente al sistema respiratorio, las CA darán lugar a una mayor permeabilidad de las branquias, incrementado la toma de oxígeno [Rodnick and Planas, 2016]. Las CA también estimularán la respuesta inmune innata, incrementando la producción de proteínas de fase aguda, péptidos y citoquinas pro inflamatorias. En el riñón, las CA estimularán la producción y actividad de macrófagos y células T de vigilancia, favoreciendo los procesos inflamatorios [Sarkar et al., 2011; Yada y Tort, 2016; Urbinati et al., 2020].

Todos estos cambios ejercidos por las CA están acompañados de una serie de desequilibrios fisiológicos que deberán ser reestablecidos para poder volver a las condiciones pre estrés [Ellis et al., 2012; Tort, 2011; Schreck and Tort, 2016]. Un ejemplo claro de estos desequilibrios es el fenómeno conocido como "compromiso osmorregulatorio", que consiste en la pérdida del balance osmótico e hidromineral a consecuencia directa del incremento de la tasa cardíaca y el incremento de la capacidad difusiva branquial. Por otra parte, a consecuencia de una mayor actividad muscular, el incremento de la liberación de lactato y protones al torrente sanguíneo resultará en el desequilibrio del pH sanguíneo [Rodnick y Planas, 2016]. El incremento de la actividad inmune en respuesta a las CA también supondrá una importante carga energética, con procesos como la proliferación celular y la síntesis de proteínas [Aluru et al., 2009; Tort, 2011; Yada y Tort, 2016].

El restablecimiento de la homeostasis corporal está dirigido por los GC, principalmente el cortisol [Ellis et al., 2012; Schreck and Tort, 2016]. La síntesis del cortisol también se inicia inmediatamente después de la percepción de un agente estresante, sin embargo este proceso está mediado a través de una cascada de señales químicas en un periodo de tiempo que va de minutos a horas [Schreck and Tort, 2016]. Una vez activado, el hipotálamo sintetiza el factor de liberación de cortisol (CRF) que a su vez estimulará la pituitaria para la síntesis y liberación de hormonas adrenocorticotrópicas (ACTH). Una vez en el torrente sanguíneo, las ACTH llegarán hasta el riñón anterior, donde estimularán la esteroidogénesis de cortisol [Ellis et al., 2012; Schreck and Tort, 2016; Mateus et al., 2017]. El cortisol será sintetizado en la mitocondria a través de una serie de isomerizaciones e hidroxilaciones del cortisol con un paso final catalizado por la enzima P450 11β-hidroxilasa, que es un citocromo codificado por la expresión génica del gen *cyp11β* [Vukelic et al., 2011]. La síntesis de cortisol estará limitada por la disponibilidad de colesterol en la mitocondria, que será regulado por la proteína reguladora de esteroidogénesis aguda (StAR) [Stocco et al., 2005]. Una vez el cortisol llega a un tejido diana, sus efectos serán mediados por los receptores de glucocorticoides (GR). Estos receptores se pueden encontrar en la superficie de las membranas celulares, mediando los procesos no genómicos inducidos por el cortisol, o en el citoplasma formando un complejo con las chaperonas moleculares hsp70 y hsp90 [Balasch y Tort, 2019]. Tras penetrar en la célula, el cortisol se unirá al GR provocando la separación del

complejo GR-hsp. Acto seguido, el complejo cortisol-GR se traslocará al núcleo induciendo la expresión de genes en respuesta al cortisol [Terova et al., 2005; Ellis et al., 2012; Nardocci et al., 2014] (Figura 2).

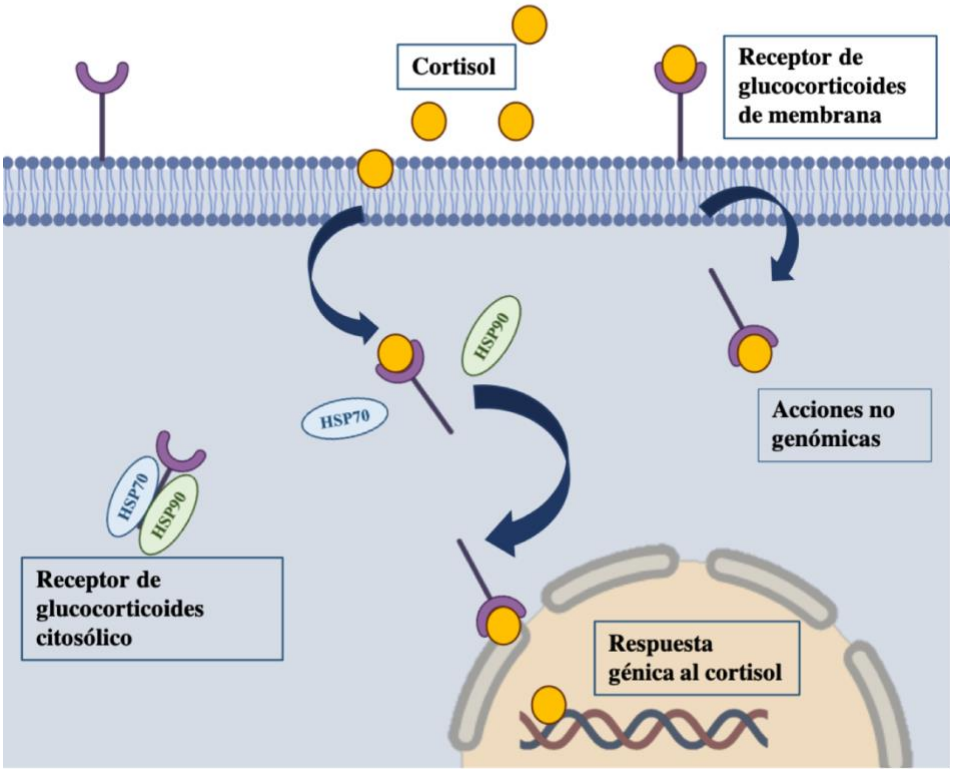


Figura 2. Mediación de efectos del cortisol por receptores de glucocorticoides. HSP70 (chaperona molecular hsp70); HSP90 (chaperona molecular hsp90).

La función principal del cortisol es el abastecimiento de energía efectiva y duradera para abastecer las demandas metabólicas de la respuesta la estrés. El cortisol estimula el metabolismo aerobio, incrementando los procesos de fosforilación oxidativa produciendo ATP (aprox. 34 ATP por ciclo) [Ballard and Towarnicki, 2020] de forma mucho más efectiva que los procesos de glucogenólisis y glucolisis (3 y 2 ATP por glucosa, respectivamente) [Rodnick y Planas, 2016]. Además, el cortisol regula los niveles de glucosa hepática, el metabolismo proteico y la actividad gluconeogénica [Kuo et al., 2015; Faught y Vijayan, 2016]. La energía disponible abastecerá la actividad de las bombas Na^+K^+ ATPasas, que reestablecerán los balances osmóticos e hídricos [Nardocci et al., 2014; Rodnick y Planas, 2016, Schreck and Tort, 2016]. El cortisol también jugará un importante papel en la resolución de procesos inflamatorios, inhibiendo la liberación de citoquinas pro inflamatorias y estimulando las anti inflamatorias a través de la modulación del factor nuclear kappa beta (NFkβ) [Baker et al., 2011; Liu et a., 2017]. La actividad del cortisol también resultará en una reducción generalizada de las poblaciones

circulantes de leucocitos y su concentración en tejidos afectados por el proceso de estrés. La reducción de las poblaciones de leucocitos será mediada a través de la inhibición de procesos de mitosis y la promoción de la apoptosis [Aluru et al., 2009; Tort, 2011; Yada y Tort, 2016; Mateus et al., 2017].

1.3.2. Estrés crónico

En una situación ideal, el organismo será capaz de enfrentar el agente estresante y posteriormente recuperar las condiciones previas al estrés. Sin embargo cuando un agente estresante es demasiado severo, se prolonga en el tiempo o adquiere un carácter repetitivo, el organismo puede sufrir una sobrecarga alostática dando lugar a la fase de agotamiento de la respuesta al estrés (Figura 3). Es más, la coincidencia de diferentes agentes estresantes o poca separación entre varios procesos de estrés pueden presentar efectos aditivos, superando la capacidad de respuesta del organismo. La activación continuada del eje HPI tendrá graves consecuencias en el metabolismo y la capacidad inmune del organismo debido a los efectos ejercidos por el cortisol [Ellis et al., 2012; Nardocci et al., 2014; Schreck et al., 2016]. Uno de los efectos más característicos del cortisol es la supresión del apetito, posiblemente debido señales neuronales en respuesta a los altos niveles de glucosa plasmática circulante [Ellis et al., 2012; Rodnick and Planas, 2016]. Está condición hará recaer todos los costes metabólicos de la respuesta al estrés sobre reservas endógenas, privando de energía a procesos secundarios como el crecimiento o la capacidad reproductiva [Rodnick and Planas, 2016; Gorissen and Flik, 2016] (Figura 3). Los procesos de estrés crónicos también traerán asociados la inmunosupresión del organismo, con la reducción de las poblaciones de células inmunes. En particular, el cortisol reduce drásticamente las poblaciones de células B afectando negativamente a la capacidad fagocítica y la producción de anticuerpos B [Tort, 2011; Schreck and Tort, 2016; Yada and Tort, 2016]. Una exposición prolongada al cortisol también puede dar lugar a la formación de radicales libres de oxígeno (ROS) como resultado de un metabolismo aerobio acelerado, dando lugar a procesos de estrés oxidativo [Spiers et al., 2015; Mittler, 2017].

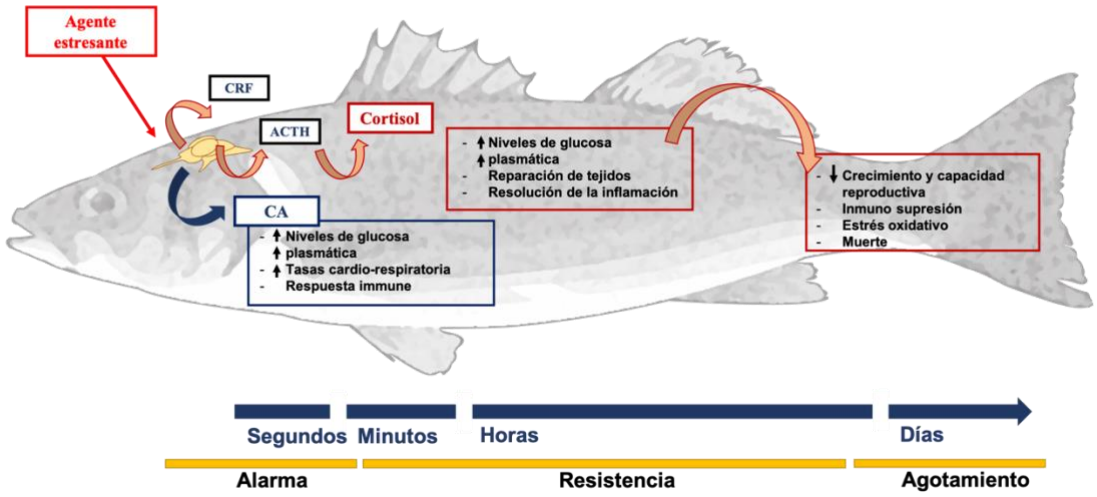


Figure 3. Descripción del desarrollo temporal de la respuesta al estrés y sus efectos fisiológicos. Agente estresante (Estrés percibido); CRF (factor de liberación de cortisol); ACTH (hormona

adrenocorticotrópica); CA (catecolaminas); Cortisol (cortisol plasmático circulante); Alarma (Fase (*i*) de alarma de la respuesta al estrés); Resistencia (Fase (*ii*) de resistencia de la respuesta al estrés); Agotamiento (Fase (iii) de agotamientos de la respuesta al estrés).

1.3.3. Estrés oxidativo

Entre otros, el cortisol es capaz de unirse al receptor de membrana mitocondrial BCL-2, activando la cadena electrónica de transporte mitocondrial (ETC). La ETC está compuesta por una serie de proteínas transmembrana (Complejos I, II, III y IV) que generan un flujo de protones impulsado a partir de un gradiente de electrones a través de la membrana mitocondrial interna [Orrenius et al., 2007; Spiers et al., 2015; Zhao et al., 2019] (Figura 4). La energía generada abastecerá la síntesis de ATP por parte de la ATP sintasa (Complejo V) [Cash et al., 2007; Spiers et al., 2015]. Sin embargo este proceso no es totalmente eficiente y en un proceso llamado goteo de electrones, entre el 1 y el 3% de los electrones escapa de la ETC [Brand et al., 1994; Brand et al., 2010; Spiers et al., 2015; Zhao et al., 2019]. Estos electrones fugados pueden reaccionar con moléculas de oxígeno (O_2), tanto en el espacio mitocondrial intermembrana como en el citoplasma mitocondrial, dando lugar a la formación de radicales superóxido ($O2^-$) [Brand et al., 2010; Zhao et al., 2019]. Estos radicales serán detoxificados por las enzimas antioxidantes cobre-zinc superóxido dismutasa (Cu, Zn-SOD) dando lugar a la formación de peróxido de hidrógeno (H_2O_2), el cual escapará de la mitocondria por difusión pasiva [Grivennikova and Vinogradov, 2006; Brand et al., 2010]. El H_2O_2 a su vez será detoxificado por dos enzimas antioxidantes, la catalasa (CAT) y la glutatión peroxidasa (GPX). Las ROS son un residuo normal del metabolismo aeróbico y son detoxificadas por las defensas antioxidantes enzimáticas y no enzimáticas [Martínez-Álvarez et al., 2005; Zafir et al., 2009; Pamplona and Constantini, 2011]. Sin embargo, una capacidad antioxidante insuficiente puede dar lugar a la formación de radicales hidroxilo (OH^-) que es la forma más inestable de ROS y no pueden ser detoxificados por ningún mecanismo de defensa [Abele and Puntarulo, 2004; Birben et al., 2012; Spiers et al., 2015; Bahn et al., 2017]. Los radicales OH^- se forman a consecuencia de la oxidación por parte del H_2O_2 de los metales de transición Fe^{2+} y Cu^{2+} presentes en el citoplasma, a través de las reacciones de Haber-Weiss y Fenton respectivamente [Birben et al., 2012; Marciano and Vajro, 2017; Demirci-Cekic et al., 2022]. Los daños oxidativos generados por la presencia de radicales libres dará lugar a un bucle de retroalimentación positiva dando lugar a mayor producción de ROS [Ballard and Towarnicki, 2020; Marciano and Vajro, 2017; Zhao et al., 2019].

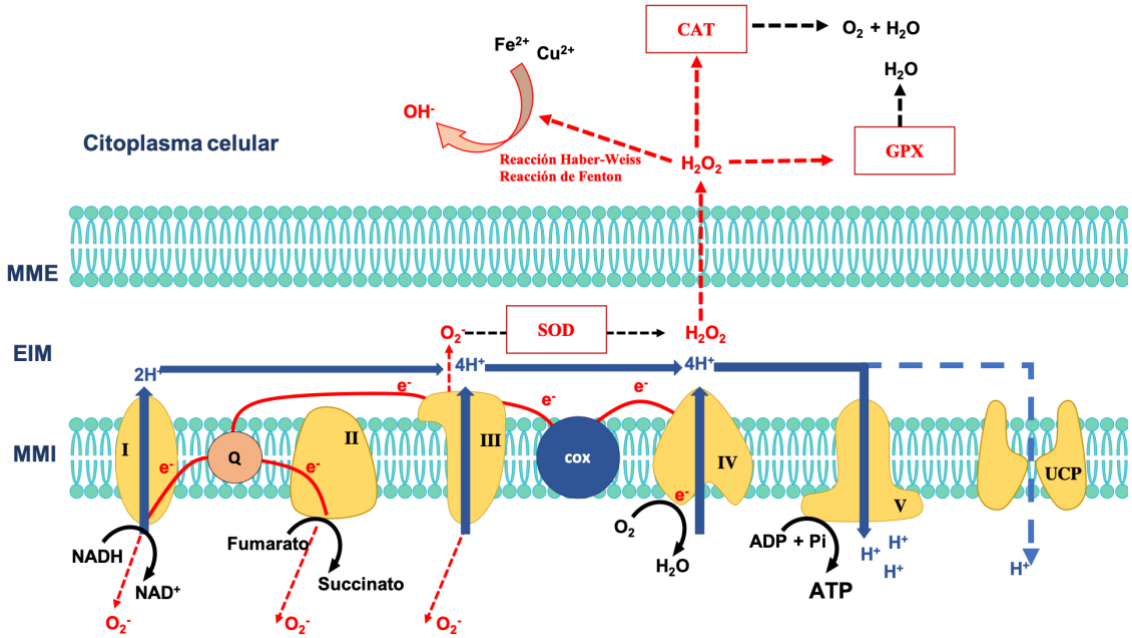


Figura 4. Representación esquemática de la cadena mitocondrial de transporte electrónico (ETC). Los complejos I, II, III y IV generan un flujo de electrones derivado de compuestos oxidables, generan el bombeo de protones a la EIM. La energía generada en el bombeo de protones será consumida por el complejo V (ATP sintetasa) o las proteínas de desacople (UCPs). El O^{2-} generado será detoxificado por la SOD formando H_2O_2 que difundirá pasivamente a través de la MME al citoplasma celular. El H_2O_2 será detoxificada por la CAT y la GPX, dando lugar a la formación de O_2 y H_2O. El H_2O_2 puede reaccionar con los metales de transición, Fe^{2+} o Cu^{2+}, dando lugar a la formación de OH^- a través de las reacciones de Haber-Weiss o Fenton. Las líneas rojas indican la transferencia de electrones. Las líneas azules indican el flujo de protones. Las líneas discontinuas negras indican detoxificación. I, II, III, IV y V (complejos de proteínas intermembrana); Q (ubiquinona); cox (Citorcomo C); SOD (superóxido dismutasa) ; CAT (catalasa); GPX (glutation peroxidasa); O_2 (oxígeno); H_2O (agua); H_2O_2 (peróxido de hidrógeno); MME (membrana mitocondrial externa); EIM (espacio intermembrana mitocondrial); MMI (membrana mitocondrial interna). Adaptado de Zhao et al., 2019.

Cuando las defensas antioxidantes del organismo se ven superadas por las tasas de formación de ROS, el organismo sufre un proceso de estrés oxidativo dando lugar a importantes daños como la peroxidación de lípidos y proteínas o daños al ADN [Burton et al., 2011; Preiser, 2012; Sies et al., 2017]. Estos daños pueden dar lugar a la pérdida de la estructura y funcionalidad celular, resultando en la muerte celular. La mitocondria contiene una gran variedad de factores activadores de la apoptosis, como el citocromo c (cox), jugando un papel fundamental en la regulación de los procesos de muerte celular programada [Moll and Zaika, 2001]. Los daños resultantes del estrés oxidativo pueden dar lugar a un mal funcionamiento de la mitocondria, resultando en una pérdida de su potencial de membrana y en consecuencia su permeabilización y liberación de estos factores proapoptóticos [Orrenius et al., 2007; Ott et al., 2007; Sinha et al., 2016].

A pesar de que la mitocondria es la principal fuente de formación de ROS interno, otros procesos biológicos pueden dar lugar a su formación. Entre ellos, el metabolismo lipídico y la actividad del sistema inmune también son fuentes endógenas de ROS y RONS (especies reactivas de nitrógeno). Por este motivo aquellos órganos y tejidos con funciones de biosíntesis, transporte de iones, función contráctil o defensa inmune serán susceptibles a sufrir proceso de estrés oxidativo [Lushchak, 2014; Rodnick and Planas, 2016; Chowdhury and Saikia, 2020].

Las branquias de los peces interactúan directamente con el medio externo, actuando como barrera física y química semipermeable con importantes funciones en el intercambio gaseoso, el balance hidromineral y la respuesta inmune [Hwang et al., 2011; Li et al., 2019].

El tejido epitelial de la branquia esta compuesto por dos tipos principales de células, las células pavimentosas (PVCs) y las células ricas en mitocondrias o ionocitos (MRCs). Las MRCs cumplen un importante papel en el transporte iónico, siendo claves en el balance osmoregulatorio e hidromineral de los peces [Hwang et al., 2011; Hiroi et al., 2012]. Estas células se caracterizan por presentar un elevada concentración de mitocondrias, suministrando energía para la actividad de las bombas Na^+K^+ ATPasas [Hwang et al., 2011; Torrecillas et al., 2019]. El tejido branquial también presenta un importante papel en la respuesta inmune de los peces, ejercido a través de su tejido linfático asociado (GIALT). El GIALT, al igual que otros tejidos mucosos, está compuesto principalmente por células B, células T, macrófagos, neutrófilos, granulocitos eosinófilos, células calciformes y diversos componentes humorales como lisozimas, bactericinas y complemento proteico [Salinas et al., 2011; Torrecillas et al., 2021]. Debido a la gran variedad de funciones regulatorias del tejido branquial, será altamente susceptible a procesos de estrés oxidativo.

1.3.4. Estrés y condiciones de cultivo.

Bajo condiciones de cultivo los peces están sometidos a una gran variedad de estímulos que pueden ser interpretados como peligros potenciales, dando lugar a una activación continuada de la respuesta al estrés. Procesos estresantes como aquellos derivados de prácticas comunes en la industria acuícola, incluyendo manipulación, vacunación y exposición a biocidas, densidades de cultivo altas, relaciones jerárquicas entre individuos de un misma población [Ashley, 2007; Gabriel and Akinrotimi, 2011; Faouraki et al., 2011; Samaras et al., 2017; Rehman et al., 2017] o problemas nutricionales asociados a una dieta inadecuada [Ashley, 2007; Montero y Izquierdo, 2010; Oliva-Teles, 2012]. Además, hay que tener en cuenta que los peces están en contacto directo con el medio, siendo altamente susceptibles a cambios en parámetros físico-químicos del agua [Gabriel y Akinrotimi, 2011; Rehman et al., 2017; Abisha et al., 2022] y brotes patógenos de bacterias, virus, hongos y parásitos [Paperna, 1991; Yanong, 2003; Johnson et al., 2004; Dos Santos y Howgate, 2011; Pridgeon and Klesius, 2012; Gozlan et al., 2014; Zhang y Gui, 2015; Kibenge, 2019].

La lubina europea es una especie con una alta susceptibilidad al estrés [Rotllant et al., 2003; Di Marco et al., 2008; Fanouraki et al., 2011] y en especial a procesos asociados a las condiciones de cultivo [Samaras et al., 2017]. En 2011, Fanouraki y colaboradores investigaron la respuesta al estrés de distintas especies de acuicultura sometidas a pruebas de persecución y exposición al aire. La lubina fue la especie con mayor susceptibilidad al estrés, con niveles de cortisol hasta veinte veces superiores a los del mero (*Epinephelus marginatus*), la corvina (*Argyrosomus regius*) y el dentón común (*Dentex dentex*) y hasta entre dos y cuatro veces superiores a los niveles de cortisol de la dorada (*Sparus aurata*), el sargo picudo (*Diplodus puntazo*) y la breca (*Pagellus erythrinus*). Es más, la lubina también requirió más tiempo para recuperar las condiciones pre-estrés [Fanouraki et al., 2011].

Esta elevada susceptibilidad al estrés está asociada a otros factores negativos, como una alta incidencia de patógenos oportunistas. De la gran variedad de patógenos perjudiciales para la lubina europea [Pridgeon and Kresius; 2012; Zhang y Gui, 2015], la especie *Vibrio* sp. es conocida por ocasionar pérdidas de hasta el 50% de población de las granjas en pocos días tras un brote patógeno [Arab et al., 2020; Regev et al., 2020]. Los patógenos oportunistas pertenecientes a la familia *Vibrioceae* son una de las mayores casusas de enfermedades en peces marinos y estuarinos, y pueden ser encontrados libres en el ambiente acuático o asociados a tejidos mucosos y tracto gastrointestinal de los peces [Laganà et al., 2011; Regev et al., 2020].

Tras la infección, la vibriosis da lugar a la pérdida del apetito y a la decoloración de la librea. Estadios más avanzados de la infección se caracterizan por úlceras epiteliales y la aparición de petequias alrededor de boca y branquias. En los estadios finales, cuando la infección alcanza el grado de sistémica, puede producir exoftalmia e inundación del tracto gastrointestinal con sangre y otro fluidos [Regev et al., 2020]. Está situación se ve empeorada por la prohibición del empleo de compuestos antibióticos desde la entrada en vigor de la ley 1831/2003/EC de la Unión Europea. Esta ley fue aprobada en 2006, como consecuencia de la aparición de especies bacterias resistentes a diversos antibióticos procedentes de las instalaciones acuícolas [Giannenas et al., 2012; Monzón-Atienza et al., 2023].

Considerando la elevada prevalencia de procesos estrés en las granjas de producción acuícola y los potenciales efectos negativos del uso de dietas bajas en harinas y aceites de pescado, el desarrollo de estrategias efectivas para el refuerzo de la capacidad inmune y antioxidante de los peces así como la atenuación de los procesos de estrés cobrará una elevada importancia en la productividad y rendimiento de la producción acuícola de lubina europea.

1.4. Ingredientes funcionales: mejorando la salud y bienestar de los peces.

En relación a la necesidad previamente expuesta, los proyectos "PROINMUNOIL (AGL2012-39919) y PROINMUNOIL PLUS (AGL2016-79725-P): Functional additives and alternative oils to fish oils in Aquaculture: an effective tool to increase fish disease resistance" fueron desarrollados con la finalidad de investigar el uso potencial de aditivos funcionales (FA) para reforzar el estado de salud y el bienestar de lubinas alimentadas con dietas bajas HP/AP. Los FA son nutrientes con una amplia variedad de efectos beneficiosos para los peces, capaces de estimular la respuesta inmune e integridad de tejidos, la atenuación de la respuesta al estrés, el refuerzo de el estado antioxidante y la mejora de la digestibilidad y utilización de nutrientes [Hoseinifar 2015; Encarnação, 2016; Dawood et al., 2016]. Los proyectos PROINMUNOIL investigaron el empleo de probióticos, prebióticos y productos fitogénicos. Esta tesis doctoral se desarrolló bajo el marco del proyecto PROINMUNOIL + (AGL2016-79725-P), con especial énfasis en la utilización prebióticos y fitogénicos.

1.4.1. Compuestos prebióticos

Los compuestos prebióticos son fibras indigestibles de origen vegetal que pueden beneficiar la salud del huésped a través de la estimulación del crecimiento y actividad de un número determinado de especies bacterianas ya presentes en el tracto digestivo del huésped [Gibson, 2004; Hoseinifar et al., 2015; Guerreiro et al., 2017]. Los prebióticos han mostrado la habilidad de mejorar el crecimiento de los peces y su utilización de los alimentos [Ringø et al., 2010, 2014; Guerreiro et al., 2017; Torrecillas et al., 2015a,b; Torrecillas et al., 2018] a través de la modulación de la secreción de enzimas digestivas por parte del microbioma intestinal, incrementando la biodisponibilidad de nutrientes [Guerreiro et al., 2017]. Además, los prebióticos presentan la habilidad de mejorar la integridad epitelial del intestino, dando lugar a una mayor capacidad de absorción de nutrientes [Ringø et al., 2010; Guerreiro et al., 2015; Torrecillas et al., 2018; Butt et al., 2019; Dawood et al., 2021].

A nivel de respuesta inmune y resistencia a patógenos, los prebióticos también han mostrado un elevado potencial como sustancias moduladoras de la respuesta inmune.

Entre otros mecanismos, la promoción de las poblaciones bacterianas autóctonas puede facilitar la competitividad exclusiva contra patógenos o la producción de compuestos químicos con actividad bacteriolítica y bacteriostática, compuestos como ácidos orgánicos, H_2O_2, antibióticos, bacteriocinas y lisozimas [Buentello et al., 2010; Roberfroid et al., 2010; Zhou et al., 2010; Akrami et al. 2013; Guerreiro et al. 2016]. Los prebióticos también pueden interactuar con los sistemas de reconocimiento de patógenos (PAMPs) del huésped, estimulando el sistema inmune [Torrecillas et al., 2011a; Cerezuela et a., 2013; Song et al., 2014] (Tabla 2). Además, la mejora de la integridad y funcionalidad del tejido intestinal a consecuencia del uso de prebióticos puede dar lugar a menores tasas de translocación bacteriana, incrementando la resistencia del huésped a patógenos [Torrecillas et al., 2011b; Hoseinifar et al.,

La suplementación dietética de prebióticos también ha mostrado un elevado potencial en el refuerzo del estado antioxidante de los peces, minimizando los daños oxidativos [Guerreiro et al., 2017; Hoseinifar et al. 2017]. Los compuestos prebióticos presentan un núcleo fenólico con propiedades antioxidantes capaces de detoxificar ROS [Carbone et al., 2016; Guerreiro et al., 2016; Guerreiro et al., 2017; Hoseinifar et al., 2017; Abasubong et al., 2022]. Por otra parte, la fermentación de los compuestos prebióticos por las bacterias intestinales puede dar lugar a la producción de ácidos grasos de cadena corta (SCFAs), como el butirato, el propionato y el acetato, que son capaces de estimular la actividad enzimática antioxidante del huésped (Tabla 2) [Yuan et al., 2018; Cuciniello et al., 2023].

Tabla 2. Efectos de distintos compuestos prebióticos en el crecimiento, utilización del alimento y salud en peces

Prebiótico	Especie objetivo/ Peso corporal inicial	Dosis	Resultados	Referencia
MOS (Bio-Mos®)	*S. aurata* PI ≈ 24 g	0.2 y 0.4% 9semanas	Incremento en riqueza y diversidad de la microbiota intestinal	Dimitroglou et al., 2010
MOS (Bio-Mos®)	*Sciaenops ocellatus* PI ≈ 7 g	1% 8semanas	Incremento actividad lisozima	Zhou et al., 2010
MOS (Bio-Mos®)	*S. aurata* PI ≈ 170 g	0.2 y 0.4 %, 12 semanas	Mejora en incremento de peso final y ganancia de peso, incremento digestibilidad de proteínas, lípidos y energía y FCR optimizado	Gültepe et al. (2011)
MOS (Bio-Mos®)	*D. labrax* PI ≈ 116 g	0.4%, 8 semanas	Incremento en longitud y anchura del microvilli intestinal, incremento secreción de mucinas, incremento en la concentración de granulocitos eosinófilos	Torrecillas et al., 2011a
FOS	Rutilus rutilus PI ≈ 0.67 g	1,2 y 3% 7 semanas	Incremento producción Ig, incremento actividades lisozima y ACH50.	Soleimani et al., 2012
XOS	*D. labrax* PI ≈ 4.8 g	0.5 y 1% 12 semanas	Mejora en incremento de peso final y longitud final, optimización FCR	Abdemlaek et al., 2015

cMOS	*D. labrax* PI ≈ 20 g	0.16%, 8 semanas	Mejoras en incremento de longitud, SGR y crecimiento relativo	Torrecillas et al., 2015 a,b
MOS (Bio-Mos®)	*S. ocellatus* PI ≈ 11 g	1% 6semanas	Incremento de la actividad lisozima	Buentello et al., 2017
GOS (Quingdao FTZ United International Inc.)	*D. sargus* PI ≈ 53 g	1% 15 días y 12 semanas	Incremento en tripsina intestinal (15 días) y reducción en amilasa (12 semanas) intestinal	Guerreiro et al., 2017
scFOS (PROFEED®)	*D. sargus* PI ≈ 53 g	1% 15 días y 12 semanas	Incremento actividad proteasa alkalina intestinal (15 días), reducción actividad amilasa intestinal (12 semanas)	Guerreiro et al., 2017
MOS (Bio-Mos®)	*D. labrax* PI ≈ 20 g	0.3 y 0.6 % 13 semanas	Mejora en incremento de peso final (todas), mayor resistencia a *V. anguillarum* (dosis 0.3%)	Torrecillas et al., 2018

PI (Peso inicial); SGR (tasa de crecimiento específico); Ig (inmunoglobulinas); ACH50 (complemento de actividad alternativa 50); FCR (tasa de conversión de alimento).

La correcta suplementación de prebióticos será determinante en su capacidad para potenciar el estado de salud y crecimiento del huésped [Hoseinifar et al., 2015; Encarnação et a., 2016]. La dosis requerida será altamente dependiente de factores como la especie del huésped y su tamaño, las condiciones de cultivo y la composición y propiedades del prebiótico en cuestión [Guerreiro et al., 2017].

1.4.3. Productos fitogénicos

Los productos fitogénicos (PF) son un grupo heterogéneo de compuestos de origen vegetal que pueden proceder de hojas, raíces, tubérculos, hierbas y frutas. Estos compuestos presentan una gran variedad de efectos beneficiosos para la salud de los peces [Chakraborty et al., 2011; Encarnação, 2016; Firmino et al., 2021; Reverter et al., 2021]. Los PF compuestos pueden ser incluidos en la dieta en forma de solidos o como aceites esenciales y extractos [Encarnação, 2016]. De entre los distintos PF, los más empleados como ingredientes funcionales en acuicultura son los pertenecientes a la familia *Lamiaceae* y *Allium* spp. [Firmino et al., 2021].

Los PF presentan actividad antibacteriana [Firmino et al., 2021; Kazempour, 2022], especialmente los derivados de plantas de la familia *Lamiaceae*, que presentan potentes propiedades citotóxicas debido a su alta concentración en timol y carvacrol [Memar et al., 2017; Kachur ySuntres, 2020]. En varias especies de producción acuícola, el uso de PF incrementó la resistencia a brotes patógenos [Volpatti et al., 2013; Menanteau-Ledouble et al., 2015; Torrecillas et al., 2019; Caipang et al., 2021; Reverter et al., 2021]. Además, los PF también presentan núcleos fenólicos que pueden interactuar con el NF-kB y las rutas de señalización controladas por las proteínas quinasas activadas por mitógeno (MAPKs), modulando la expresión génica de citoquinas inflamatorias [Zhou et al., 2014; Huang and Lee, 2018; Liu et al., 2019]. El uso de PF también ha mostrado propiedades antiestrés y sedativas, atenuando la respuesta al estrés de los peces [Chakraborty et al., 2014; Choubey et al., 2015; Yilmaz and Ergün, 2015;

Gbadamosi et al., 2016; Mohiseni et al., 2017; Pahor-Filho et al., 2017; Hoseini and Yousefi, 2019; Yonar et al., 2019; Firmino et al., 2021].

Debido a su composición en fenoles, los PF presentan la capacidad de inhibir la producción y detoxificación de ROS, mejorando el estado antioxidante de los peces [Alloui et al., 2014; Irkin et al., 2014; Firmino et al., 2021; Kazempour, 2022]. Por otra parte, los PF también han mostrado la capacidad de modular de forma selectiva la expresión génica de enzimas antioxidantes, modulando de forma directa el estado antioxidante y la respuesta inmune de los peces [Giannenas et al., 2012; Chowdhury et al., 2020; Chowdhury et al., 2021; Firmino, 2021; Firmino et al., 2021; Ibrahim et al., 2021; Liu et al., 2022; Magouz et al., 2022; Poolsawat et al., 2022] (Tabla 3).

El empleo de PF puede estimular la secreción de enzimas digestivas, mejorando la eficiencia alimenticia de los peces y por tanto estimulando su crecimiento [Peterson et al., 2014; Habiba et al., 2021; Mansour et al., 2021; Kazempour et al., 2022]. Es más, la suplementación de PF puede dar lugar a mejoras en las propiedades organolépticas del filete [Peterson et al., 2014; Steiner and Syed, 2015; Firmino, 2021].

Tabla 3. Efectos de distintos productos fitogénicos en el crecimiento, utilización del alimento y salud en peces.

Producto fitogénico	Especie objetivo/ Peso corporal inicial	Dosis	Resultados	Referencia
Allium sativum (Extracto)	*Oreochromis. niloticus* PI ≈ 100 g	1,4 y 8 % 2 semanas	Incremento resistencia a *Trichodina* sp y *Aeromonas hydrophila*	El Deen and Razin, 2009
Allium sativum (Extracto)	*Lates calcarifer* PI ≈ 21 g	1, 2, 3 ,4 y 5 % 2 semanas	Mejora en incremento de peso, optimized FCR yincreased resistance to *Vibrio harveyii*	Talpur and Ikhwanuddin, 2012
Thymus vulgaris o *Rosmarinus officinalis* o *Trigonella foenum graecum* L. (Polvo)	*D.labrax* PI ≈ 21 g	1% 7 semanas	Mejora del ratio de eficiencia proteica, incremento nivel de proteínas en filete, mejora retención de proteínas y energía	Yilmaz et al., 2012
Thymol (25 %) ycarvacrol (25 %) (Aditivo commercial)	*S. aurata*	0.01 % 9 semanas	Mejora de salud y función tejido del intestinal, mayor expresión génica de enzimas antioxidantes y factores antiproliferativos	Pérez-Sánchez et al., 2015
Origanum vulgare (Aceites esenciales)	*Onchorhynchus keta*	0.01, 0.02, 0.05 y 0.1 % 8 semanas + 28 días immune challenge	Mejora eficiencia alimenticia, mejora de la resistencia a *Ichthyobodo salmonis* y *Trichodina truttae*.	Mizuno et al., 2018
Thymus vulgaris (Extracto)	*Onchorhynchus mykiss*	0.5, 1 y 2 % 2 semanas	Incremento actividad enzymatica antioxidante en intestine y reducción en niveles de malondialdehido	Hoseini and Yousefi, 2019

Allium sativum + carvacrol (Aceites esenciales) y thymol (Aditivos comerciales)	*S. aurata*	0.5 % 9 semanas + 39 días immune challenge	Modulación de la expression génica proinflamatoria, expression génica antiinflamatoria y antioxidante prolongada en branquias e incremento de concentración de glucoproteínas con contenido en ácido siálico en epitelio branquial y mucus	Firmino et al., 2020
Origanum vulgare (Extracto)	*O. niloticus*	0.2 y 0.5 % 9 semanas + 7 días immune challenge	Mejora en incremento de peso, ganancia de peso y SGR, incremento en Ig total en mucus, mejora resistencia a *A. hydrophyla*	Mohammadi et al., 2020
Origanum vulgare (Aceites esenciales)	*Cyprinus carpio* PI ≈ 16 g	0.5, 1.5 y 4.5 % 8 semanas	Incremento actividad enzimas digestivas, mejora de la capacidad antioxidante y mayor resistencia a *A. hydrophyla*	Zhang et al., 2020
Origanum vulgare (Polvo)	*Danio rerio*	0.5, 1 y 2 % 8 semanas	Mejora de peso final, ganancia de peso y SGR, incremento lisozima en mucus, incremento actividad fosfatasa alcalina y proteasa, mayor resistencia a *A. hydrophyla*	Rashidian et al., 2021

PI (Peso inicial); SGR (tasa de crecimiento específico); FCR (tasa de conversión del alimento).

A pesar de la gran variedad de efectos beneficiosos asociados a la suplementación de PF en la salud y bienestar de los peces, estos compuestos también conllevan desventajas importantes. Como ingredientes de origen vegetal, pueden inducir desequilibrios nutricionales como la presencia de antinutrientes o la reducción de la palatabilidad del alimento [Guerreiro et al., 2017]. Además, estos compuestos son altamente voláties, reduciendo su disponibilidad durante los procesos digestivos o incluso presentando cambios en la dosis de administración [Firmino et al., 2021].

1.4.3. Compuestos probióticos

Los probióticos son organismos bacterianos vivos que pueden beneficiar la salud del pez balanceando su homeostasis interna o mejorando el ambiente de cultivo [Lazado et al., 2014; Merrifield y Carnevalli, 2014; Merrifield and Ringo, 2014; Encarnação, 2016; Dawood et al., 2016]. Los probióticos pueden ser suministrados a través de la vacunación, añadidos directamente a la columna de agua o suplementados como ingredientes

funcionales, siendo este último el método mas empleado en la acuicultura [Lazado et al., 2014; Monzón-Atienza et al., 2023].

El objetivo principal de la suministración oral de probióticos es la modulación de la salud intestinal, incremento potencialmente las respuesta inmune de los peces y su crecimiento. El empleo de probióticos ha demostrado la habilidad de estimular la producción bacteriana de enzimas digestivas, facilitando la digestibilidad del alimentos y la obtención de nutrientes resultando en una mayor utilización del alimento y por tanto mejores tasas de crecimiento [Avella et al., 2011; Pérez-Sánchez et al., 2014; Eissa et al., 2022]. Los probióticos también han mostrado capacidad para modular el microbioma intestinal y las respuestas inmunes local y sistémica de los peces [Balcázar et al., 2006; Lazado et al., 2014; Dawood et al., 2016]. Esta modulación del microbioma intestinal, puede darse a través de la competencia por nutrientes esenciales, la adhesión competitiva, la inhibición de la expresión génica virulenta y la disrupción del *quorum sensing* de bacterias patógenas [Hasan et al., 2023; Monzón-Atienza et al., 2023; Moroni et al., 2023]. El empleo de probióticos también puede dar lugar a la producción de compuestos inhibidores de crecimiento bacteriano, como lisozimas y bactericinas además de la modulación de citoquinas inflamatorias [Burbank et al., 2011; Dawood et al., 20017; Monzón-Atienza et al., 2023] (Tabla 4).

Entre la gran variedad de compuesto probióticos con efectos beneficiosos para a salud de los peces Merrifield et al., 2010], las bacterias lácticas (*Lactobacillus* spp., *Peridococcus* spp., *Enteroccoccus* spp.) son uno de los grupos más empleados [Lazado et al., 2014; Encarnação, 2016]. Las bacterias del ácido láctico tienen una serie de características que las hacen propicias como cepa probiótica, entre ellas, la alta capacidad para su adhesión y crecimiento en el tracto intestinal de los peces, no presentan resistencia a antibióticos, no modifican ningún rasgo heredable el huésped y presentan una elevada capacidad para fomentar el crecimiento del huésped y estimular su respuesta inmune contra patógenos [Monzón-Atienza, 2023].

Tabla 4. Efectos de distintos compuestos probióticos en el crecimiento, utilización del alimento y salud en peces.

Probiótico	Especie objetivo/ Peso corporal inicial	Dosis	Resultados	Referencia
Lactobacillus delbrueckii delbrueckii	*D. labrax* Larva	10^5 bacteria ml^{-1} 25 or 59 días	Mejora en incremento de peso (81% dosis larga y 28% dosis corta), reducción niveles cortisol, incremento de la expresión génica de IGF-1	Carnevali et al., 2006
L. lactis sbsp. lactis, *L. sakei* or *Leuconostoc mesenteroides*	*Salmo trutta* PI ≈ 70 g	10^6 cfu g^{-1} diet 2 semanas	Incremento en la actividad de lisozima y mayor concentración de inmunoglobulinas y complemento en suero	Balcázar et al., 2007
L. delbrueckii delbrueckii or a multispecies probiotic (*L. fructivorans* + *L. plantarum*	*D.labrax* y *S.aurata* Larva	10^5 bacteria mL^{-1} 63 días	IIncremento crecimiento, atenuación de la respuesta al estrés y mejora de la repsuesta inmune. En *D.labrax* incremento corporal de TCR-β, incremento en recuentos de granulocitos acidófilos y menor expresión génica pro	Abelli et al., 2009

			inflamatoria. En *S.aurata* incremento en el recuento *de* immunoglobulinas (Ig+) y granulocitos acidófilos	
Enterococcus faecalis	*O. mykiss* PI ≈ 13 g	1% diet 12 semanas	Incremento niveles de hematocrito, mayor índice fagocítico y producción de mucus, mayor resistencia a *V.anguillarum*	Rodríguez-Estrada et al., 2009
Debaryomyces hansenii	*D.labrax* Larvaa (2 dpe)	4.3% live yeast 48 días	Mejora en incremento de pesoy mayor expresion génica de las enzimas antioxidantes SOD y GPX	Tovar-Ramírez et al., 2010
Pdp11	*S.aurta* PI ≈ 38 g	10^9 cfu g^{-1} diet 116 días	Incremento crecimiento, mayor tolerancia a alta densidad de cultivo	Varela et al., 2010
Debaryomyces hansenii	*D.labrax* Larvaa (2 dpe)	4.3% live yeast 48 días	Mejora en incremento de pesoy mayor expresion génica de las enzimas antioxidantes SOD y GPX	Tovar-Ramírez et al., 2010
Multispecies probiotic (*Bacillus sp., Pediococcus sp., Enterococcus sp., L. sp.*) or single *Pediococcus acidilactici*	*O. mykiss* PI ≈ 16 g	10^9 cfu g^{-1} dry powder 96 días	Incremento crecimiento, modulación microbioma intestinal	Ramos et al., 2013
L. plantarum	*D.labrax* PI ≈ 75 g	10x10^9 CFU kg^{-1} diet 90 días	Mayor tasa de supervivencia y mayores niveles de cholesterol y trigliceridos	Piccolo et al., 2015
Multispecies probiotic (Aqua Star® Growout: *Bacillus* sp., *P.* sp., *Enterococcus* sp., *L.* sp)	*Solea senegalensis* PI ≈ 33 g	1.34 ×10^{10} cfu kg^{-1}diet 73 días	Mejor estado antioxidante, con incremento en la expression génica de CAT y GPX en peces alimentados con dietas bajas en harinas y aceites de pescado	Batista et al., 2016
Bacillus velezensis strain V4 CGMCC 10149 y *Rhodotorula mucilaginosa* strain CGMCC 1013	*S.salar* PI ≈ 180 g	*B.velezensis* V4 5×10^6 cfu g^{-1}, *R.mucilaginosa* 5×10^7 cfu g^{-1}; *B.velezensis* V4 1.5 × 10^7 cfu^{-1}, *R.mucilaginosa* 1.5×10^8 cfu g^{-1}; *B.velezensis*	Mejora en incremento de pesoy mejora de la utilización del alimento, respuesta inmune y estado antioxidante mejorados, mayor resistencia a *Aeromonas salmonicida*	Wang et al., 2019

		V4 2.5×10^{7} cfu g^{-1}, *R.mucilaginosa* 2.5×10^{8} cfu g^{-1} 62 días		
Debaryomyces hansenii strain BCS004	*S.aurata* PI ≈ 80 g		Incremento capacidad fagocítica, mayor actividad peroxidasa y estallido respiratorio en leucocitos, incremento en la actividad del complemento hemolítico e IgM total en suero sanguíneo, incremento de la expresión génica de TNFα, C3 y IgM en intestino.	Reyes-Becerril et al., 2021
P. acidilactici	*D.labrax* PI ≈ 16 g	2, 2.5 or 3 g/ kg ~ 1 × 1010 CFU 100 días	Incremento crecimiento, incremento longitud de microvilli intestinal, reducción de la excreción de amonio y mayor tasa de supervivencia (n.s.s.)	Eissa et al. 2022

PI (Peso inicial); cfu (unidades formadoras de colonias); IGF-1 (Factor insulínico de crecimeinto 1); TCR-β (Receptor β de células T); SOD (superoxido dismutasa); CAT (catalasa); GPX (glutation peroxidasa).

Las tasas de supervivencia de los compuestos probióticos se pueden ver perjudicadas por las elevadas temperaturas a las que son sometidos durante los procesos de extrusión y peletización durante los procesos de fabricación del pienso, suponiendo un inconveniente en el empelo de estos ingredientes funcionales en la industria acuícola [Rokka et al., 2010; Encarnação, 2016; Dawood et al., 2016; Guerreiro et al., 2017].

1.5. Programas de selección génica

La selección génica (SG) es una estrategia con elevado potencial para la obtención de peces más vigorosos y capaces de tolerar los desequilibrios nutricionales derivados del empleo de dietas con bajo contenido en HP/AP. La selección génica se basa en el cálculo de los valores de heredabilidad de un carácter basándose en mediciones de rasgos fenotípicos e información genética [Louro et al., 2014; Boudry et al., 2021].

La selección génica puede estar enfocada a una gran variedad de rasgos fenotípicos, como el crecimiento y la utilización del alimento, la resistencia a patógenos o la tolerancia al estrés [Gjedrem et al., 2012; Stear et al., 2012; Das et al., 2014; Hermesch et al., 2015]. También existen programas de selección múltiple, con la selección simultánea de distintos rasgos [Sae-Lim et al., 2013; Thodesen et al., 2013; Boudry et al., 2021]. La selección de una serie de rasgos concretos puede conllevar la selección indirecta de otros rasgos. Un ejemplo es la selección indirecta de mejores tasas de conversión del alimento cuando los programas están enfocados al crecimiento rápido o al mayor crecimiento [Knap and Kause, 2018; Besson et al., 2019; Vandeputte et al., 2019]. La selección génica también puede dar lugar a procesos de domesticación, que consisten en la adaptación de esa población a la condiciones de cautividad debido a la modificación de su genética [Vandeputte et al., 2009; Vandeputte et al., 2019].

En la actualidad existen un total de siete programas de selección génica de lubina europea, pertenecientes a compañías privadas de Italia, Turquía, Grecia (Niveuus) y Francia (Ecloserie Marine de Graveline, Ferme Marine du Douhet) [Vandeputte et al., 2019]. La lubina presenta niveles de heredabilidad medios a altos para rasgos de crecimiento, presentando incrementos de entre el 23 y el 42% por generación [Vandeputte et al., 2019].

El proyecto AquaIMPACT (EU Horizon 2020 no. 818367): "Genomic and nutritional innovations for genetically superior farmed fish to improve efficiency in European aquaculture", ha sido desarrollado para integrar el empleo de programas de selección génica y nuevas técnicas nutricionales, mejorando la competitividad de la producción acuícola europea. Esta tesis doctoral también está desarrollada bajo la red de trabajo del proyecto AquaIMPACT.

1.6. Objetivos

El objetivo principal de esta tesis es investigar las posibles sinérgias entre las estrategias selección génica y la suplementación dietética de ingredientes funcionales para minimizar el impacto de las dietas con reducidos niveles de HP/AP en la salud y el bienestar de la lubina Europea. Con este propósito, se los siguientes objetivos específicos fueron abordados:

- Evaluación del potencial del uso de ingredientes funcionales para reducir los efectos negativos asociados a las dietas bajas en HP/AP en los indicadores de estrés y salud de juveniles de lubina europea.

- Evaluación de los efectos del empleo de ingredientes funcionales como potenciadores del estado antioxidante en branquia de juveniles de lubina europea alimentados con dietas bajas en HP/AP y sometidos a pruebas de estrés físicas y biológicas.

- Evaluar las posibles sinergias entre las estrategias de selección multi-rasgo de y la suplementación de ingredientes funcionales para contrarrestar los desequilibrios fisiológicos derivados del uso de dietas bajas en HP/AP y reforzar la capacidad antioxidante en branquia de juveniles de lubina sometidas a una prueba de estrés por exposición a H_2O_2.

- Determinar si las líneas de selección multi-rasgo de lubina europea se pueden beneficiar la suplementación de ingredientes funcionales para contrarrestar los problemas derivados de la reducción de HP/AP en términos de mejora de crecimiento, salud intestinal y resistencia a patógenos.

- El desarrollo de un modelo linear múltiple capaz de caracterizar los efectos derivados de la suplementación dietética de distintos ingredientes funcionales en el crecimiento y eficiencia alimenticia de la lubina

2. Material y métodos

2.1. Capítulo III. Diseño experimental

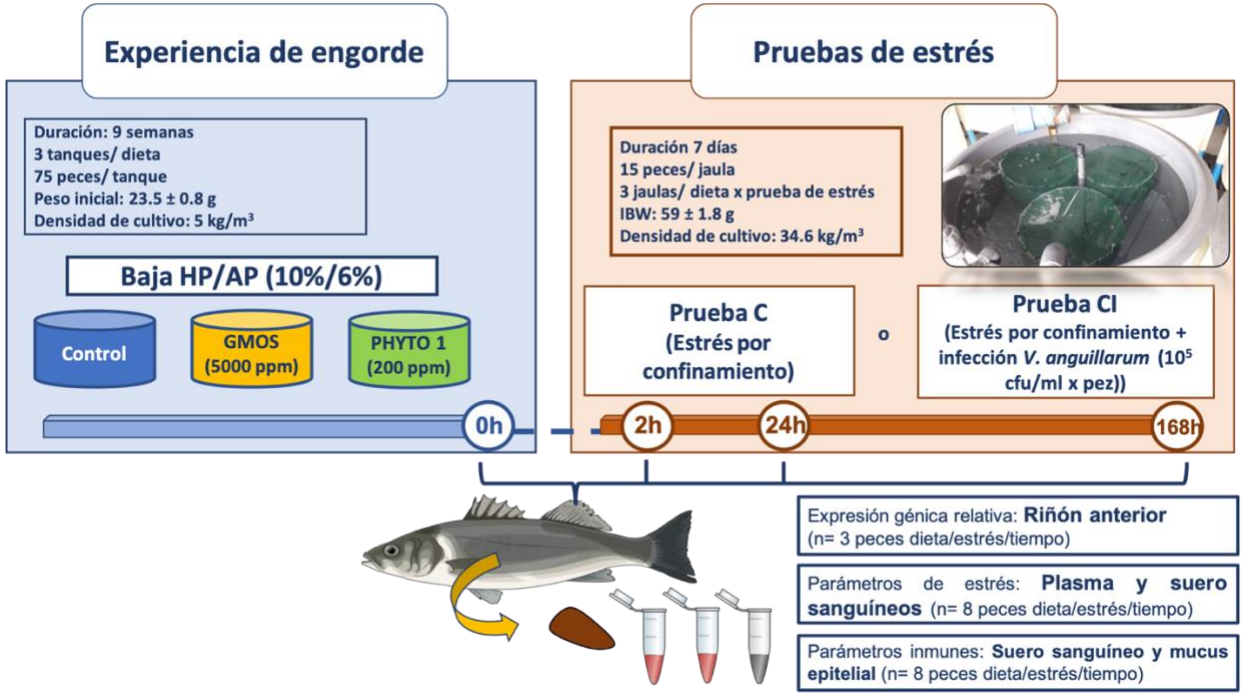


Figura 2.1. Esquema experimental del capítulo III. Nueve semanas de experiencia de engorde (tres dietas con 10% HP/ 6% AP experimentales administradas en triplicado: Control (sin suplementación); GMOS (suplementada con 5000ppm GMOS); PHYTO (suplementada con 200ppm PHYTO 1); 3 veces al día, 6 días a la semana, a saciedad aparente; n=75 peces/tanque, peso inicial = 23.5 ± 0.8 g). Prueba de estrés con dos tratamiento experimentales: prueba C (prueba de estrés por confinamiento; 3 jaulas/ dieta; 15 peces/ jaula; peso inicial = 59 ± 1.8 g) o prueba CI (prueba de estrés por confinamiento + infección intestinal con el patógeno *V. anguillarum* (10^5 cfu/ml x pez); 3 jaulas/ dieta; 15 peces/ jaula; peso inicial = 59 ± 1.8 g). Puntos de muestreo después de la experiencia de engorde (t= 0h) y a t= 2h, 24h y 168h después de las pruebas de estrés; Muestras para expresión génica relativa (riñón anterior, n= 3 muestras dieta/ prueba de estrés/ punto de muestreo); Parámetros de estrés (plasma y suero sanguíneo, n= 8 muestras dieta/ prueba de estrés/ punto de muestreo); Parámetros de inmunes (suero sanguíneo y mucus epitelial, n= 8 muestras dieta/ prueba de estrés/ punto de muestreo).

2.2. Capítulo IV. Diseño experimental

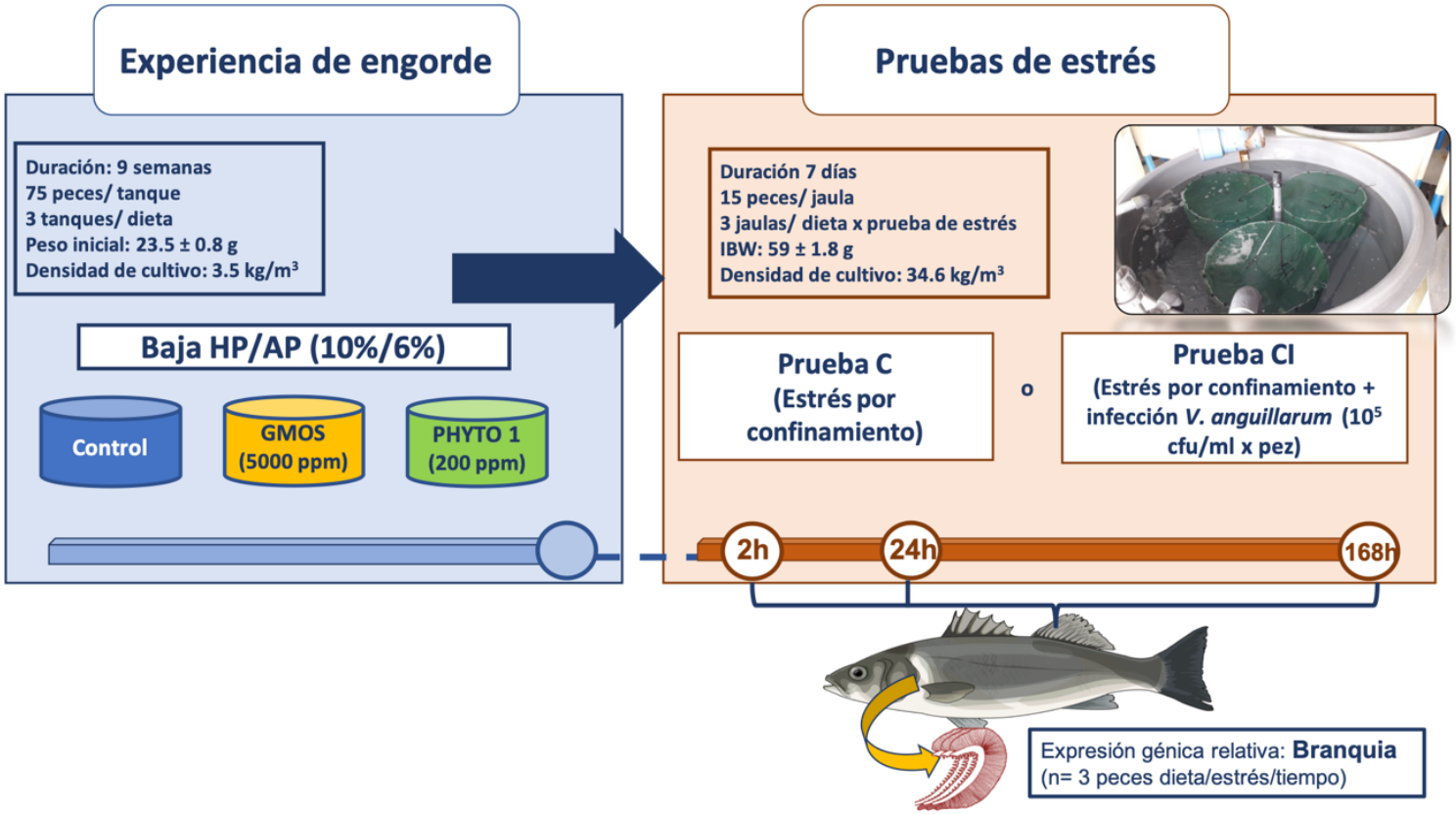


Figura 2.2. Esquema experimental del capítulo IV. Nueve semanas de experiencia de engorde (tres dietas experimentales con 10% HP/ 6% AP administradas en triplicado: Control (sin suplementación); GMOS (suplementada con 5000ppm GMOS); PHYTO (suplementada con 200ppm PHYTO 1); 3 veces al día, 6 días a la semana, a saciedad aparente; n=75 peces/tanque, peso inicial = 23.5 ± 0.8 g). Prueba de estrés con dos tratamiento experimentales: prueba C (prueba de estrés por confinamiento; 3 jaulas/ dieta; 15 peces/ jaula; peso inicial = 59 ± 1.8 g) o prueba CI (prueba de estrés por confinamiento + infección intestinal con el patógeno *V. anguillarum* (10^5 cfu/ml x pez); 3 jaulas/ dieta; 15 peces/ jaula; peso inicial = 59 ± 1.8 g). Puntos de muestreo a t= 2h, 24h y 168h después de las pruebas de estrés; Muestras para expresión génica relativa (branquia, n= 3 muestras dieta/ prueba de estrés/ punto de muestreo).

2.3. Capítulo V. Diseño experimental

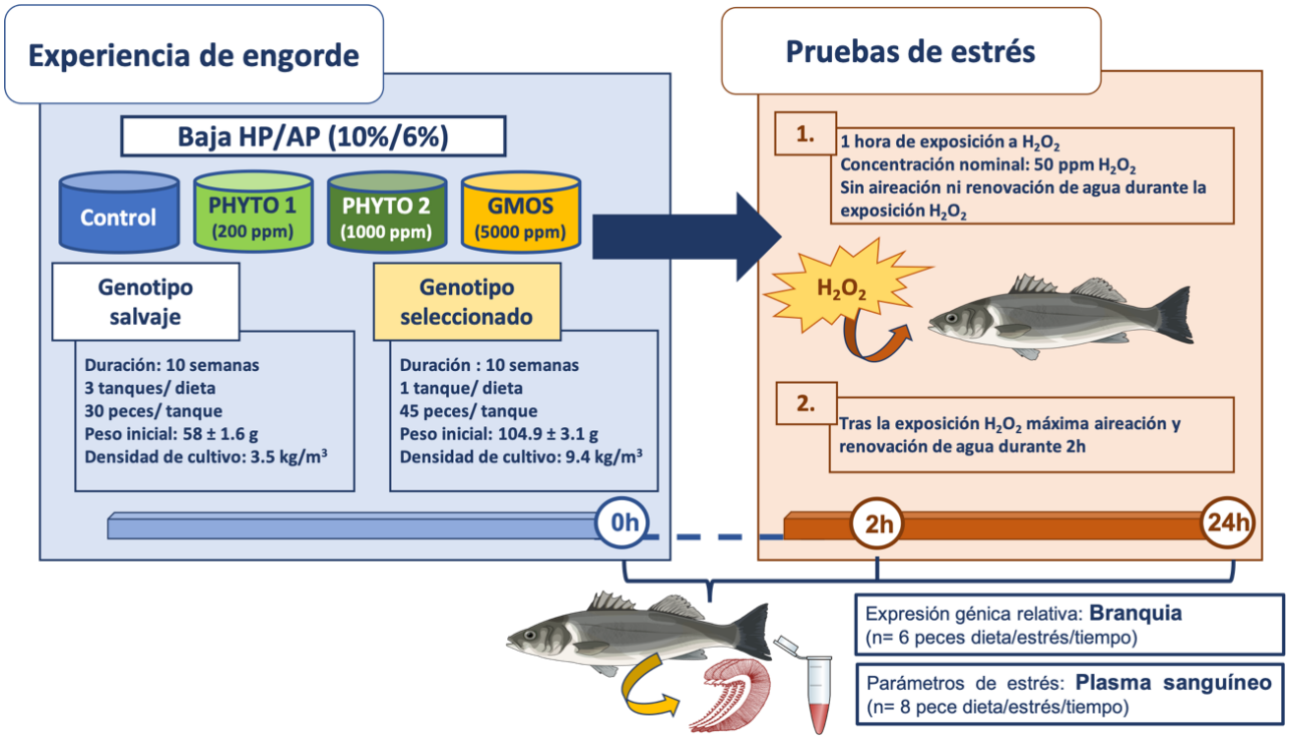


Figura 2.3. Esquema experimental del capítulo V. Diez semanas de experiencia de engorde con dos líneas genéticas de lubina europea: Genotipo salvaje (4 dietas experimentales con 10% HP/ 6% AP administradas en triplicado (Control (sin suplementación); PHYTO0.02 (suplementada con 200 ppm PHYTO 1); PHYTO0.1 (suplementada con 1000 ppm PHYTO 2); GMOS0.5 (suplementada con 5000 ppm GMOS); 3 veces al día, 6 días a la semana, a saciedad aparente; n=35 peces/tanque, peso inicial = 58 ± 1.6 g) y Genotipo seleccionado para alto crecimiento (4 dietas experimentales con 10% HP/ 6% AP administradas a un solo tanque cada una (Control (sin suplementación); PHYTO0.02 (suplementada con 200 ppm PHYTO 1); PHYTO0.1 (suplementada con 1000 ppm PHYTO 2); GMOS0.5 (suplementada con 5000 ppm GMOS); 3 veces al día, 6 días a la semana, a saciedad aparente; n=45 peces/tanque, peso inicial = 104.9 ± 3.1g). Prueba de estrés (en cada tanque) por exposición a H_2O_2 (1 hora de exposición a 50 ppm, sin aireación o renovación de agua) seguido de 2h de recuperación (máxima aireación y renovación de agua). Puntos de muestreo a t= 0h antes de exposición y t= 2h y24h después de la exposición; Parámetros de estrés (plasma sanguíneo, n= 8 muestras dieta/ punto de muestreo). Muestras para expresión génica relativa (branquia, n= 6 muestras dieta/ punto de muestreo).

2.4. Capítulo VI. Diseño experimental.

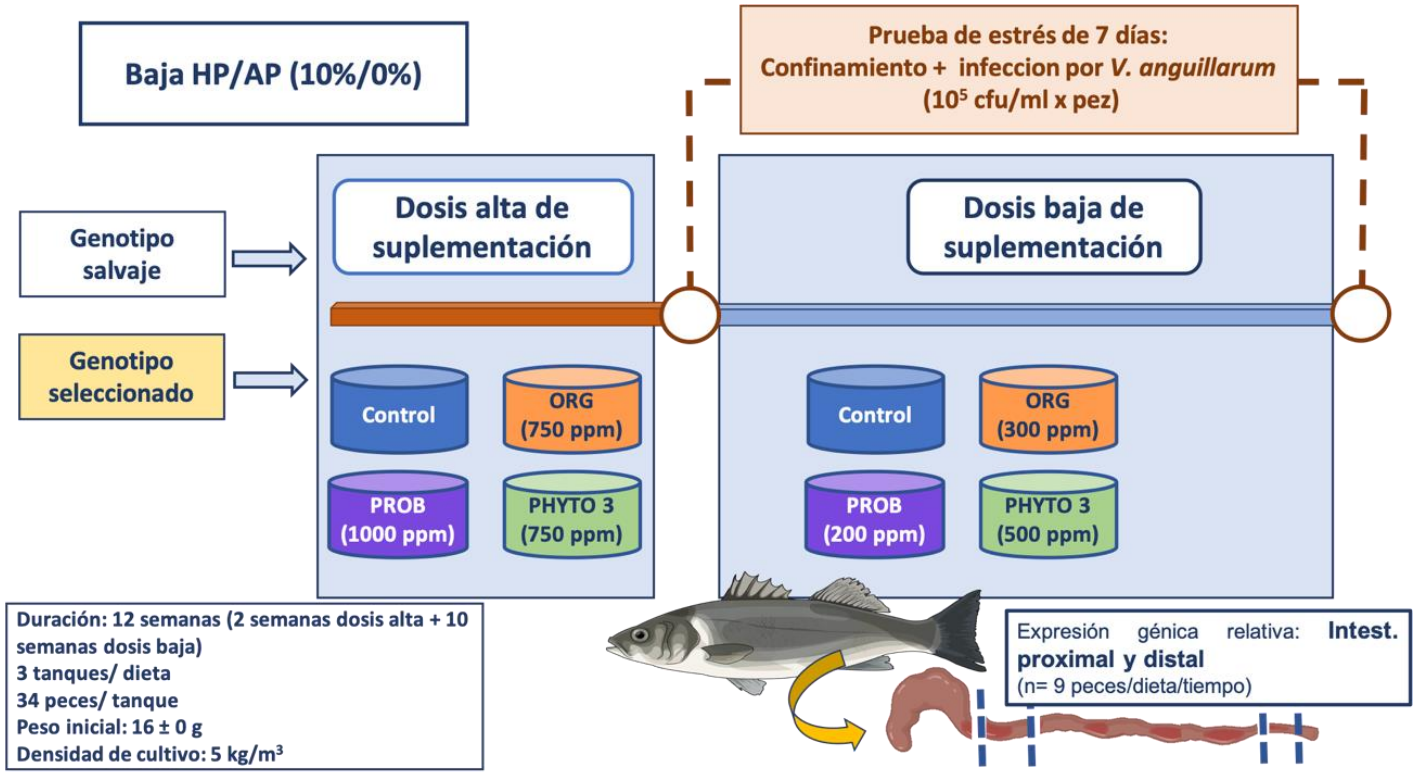
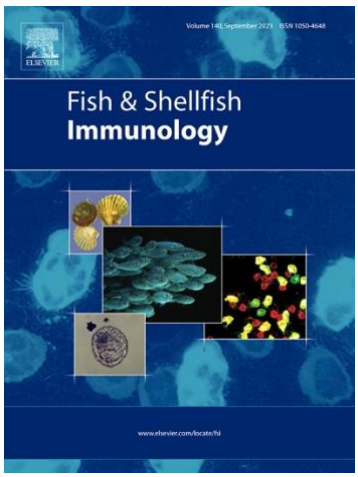


Figure 2.3. Esquema experimental del capítulo VI. Doce semanas de experiencia de engorde (2 semanas con alta dosis de suplementación + 10 semanas con baja suplementación). Dos genotipos: Genotipo salvaje y Genotipo seleccionado. Cuatro dietas experimentales con 8% HP/ 0% AP suplementadas con distintas dosis de ingredientes funcionales (Control (sin suplementación); PROB (dosis alta 1000 ppm/ dosis baja 200 ppm), ORG (dosis alta 750 ppm/ dosis baja 300 ppm), PHYTO 3 (dosis alta 750 ppm/ dosis baja 500 ppm) alimentadas en triplicado; 3 veces al día, 6 días a la semana, a saciedad aparente; n= 34 peces/tanque, peso inicial = 16 ± 0.1g). Prueba de estrés por confinamiento (3 jaulas/ dieta; 15 peces/ jaula) + infección intestinal con el patógeno *V. anguillarum* (10^5 cfu/ml x pez). Puntos de muestreo a t= 7d después de prueba de estrés; Muestras para expresión génica relativa (fragmentos de intestino distal y posterior, n= 9 muestras dieta/ punto de muestreo).

3. Resultados

3.1. Capítulo III. Prebiotics and phytogenics functional additives in low fish meal and fish oil based diets for European sea bass (*Dicentrarchus labrax*): Effects on stress and immune responses

Fish and Shellfish Inmunology 100 (2020) 219-229



En el presente estudio se investigaron los efectos de la suplementación de dos ingredientes funcionales en la tolerancia al estrés y la salud de juveniles de lubina alimentados con dietas bajas en HP/AP. Para ello, dos dietas isoenergéticas e isoproteicas con bajos niveles de HP y AP (10% y 6% respectivamente) fueron suplementadas con 5000 ppm de galactomananoligosacáridos (dieta GMOS) o 200 ppm de una mezcla de aceites esenciales (dieta PHYTO en el capítulo/ PHYTO 1 en material y métodos). Se empleó como Control una dieta con los mismos niveles de HP/AP pero sin suplementación de ningún tipo de ingrediente funcional. Los peces fueron alimentados 9 semanas en triplicado con las distintas dietas experimentales y posteriormente sometidos a una prueba de estrés por confinamiento (prueba C) o estrés por confinamiento combinado con una infección intestinal del patógeno *V. anguillarum* (prueba CI). Al inicio (t= 0h) y a las 2h, 24h y 168h tras el inicio de las pruebas de estrés se evaluaron indicadores de respuesta al estrés y parámetros inmunes. Los indicadores de estrés seleccionados fueron los niveles circulantes de cortisol y glucosa plasmática y la expresión génica relativa en riñón de los genes cyp11β hidroxilasa, el factor inducido de hipoxia, la proteína reguladora de esteroidogenesis aguda, y las proteínas de choque térmico 70 y 90 (*cyp11β, hif-1α, StAR, hsp70* y *hsp90*). Los marcadores inmunes analizados fueron las actividades lisozima, bactericida y peroxidasa en mucus epitelial y suero sanguíneo y la expresión génica relativa en riñón de los genes caspasa 3 e interleuquina 1β (*casp-3, il-1β*).

El uso de ingredientes funcionales redujo significativamente ($p < 0.05$) los niveles de cortisol de los peces sometidos a la prueba de estrés C. La suplementación de PHYTO redujo ($p < 0.05$) los niveles de expresión génica de *cyp11β, hif-1α, il-1β y casp-3* e incremento ($p < 0.05$) la expresión de *StAR* a las 2h después del estrés. Por el contrario, cuan los peces fueron sometidos a la prueba CI, el uso de PHYTO redujo los niveles de expresión de *StAR y casp-3* a las 2h después del estrés indicando un papel de protección de los leucocitos interrenales contra procesos apoptóticos a pesar de una mayor expresión de *il-1β* en respuesta a la infección. De forma adicional, la suplementación de GMOS y PHYTO incrementó la actividad lisozima en suero en respuesta a la infección. Ambos ingredientes funcionales dieron lugar a una mejor resistencia de los peces al patógeno *V. anguillarum* a pesar de estar alimentados con dietas bajas en HP/AP.

3.2. Capítulo IV. Gill Oxidative Stress Protection through the Use of Phytogenics and Galactomannan Oligosaccharides as Functional Additives in Practical Diets for European Sea Bass (*Dicentrarchus labrax*) Juveniles

Animals 2022; 12, 3332.

El objetivo de este estudio fue la evaluación del potencial de dos ingredientes funcionales para reforzar la capacidad antioxidante de la branquia de juveniles de lubina europea alimentados con dietas bajas en HP/AP y sometidos a procesos de estrés físico y biológico. Para ello, dos dietas isoenergéticas e isoproteicas con bajos contenidos en HP (10%) y AP (6%) fueron suplementadas con 5000 ppm de galactomananoligosacáridos (GMOS) o 200 ppm de una mezcla de ajo y aceites esenciales de plantas labiadas (PHYTO en el capítulo/ PHYTO 1 en material y métodos). Una tercer tratamiento con la misma composición pero sin suplementar fue utilizada como dieta Control. Tras una experiencia de engorde de 9 semanas, los peces fueron sometidos a una prueba de estrés por confinamiento (prueba C) o estrés por confinamiento combinado con una infección intestinal del patógeno *V. anguillarum* (prueba CI). A las 2h, 24h y 168h tras el inicio de las pruebas de estrés se evaluó la expresión génica relativa en branquia de: *nfκβ2, hif-1α, gr, nd5, cox, sod, cat, gpx, zo-1, ocln, hsp70* y *hsp90*. La suplementación de ingredientes funcionales atenuó la respuesta al estrés de los peces, dando lugar a un metabolismo energético más estable con menor expresión ($p < 0.05$) de los componentes de la cadena de transporte mitocondrial *nd5 y cox*, lo que resultó en una mejor capacidad antioxidante de los juveniles de lubina en respuesta a las pruebas de estrés.

3.3. Capítulo V. Functional Additives in a Selected European Sea Bass (*Dicentrarchus labrax*) Genotype: Effects on the Stress Response and Gill Antioxidant Response to Hydrogen Peroxide (H2O2) Treatment

Animals 2023; 13, 2265.

El objetivo de esta investigación fue determinar la capacidad de una línea de juveniles de lubina seleccionados para alto crecimiento para utilizar distintos ingredientes funcionales como productos anti estrés y potenciadores de la capacidad antioxidante en dietas con bajos niveles de HP/AP. Con este objetivo, tres dietas isoenergéticas e isoproteicas (10% HP/ 6% AP) fueron suplementadas con 200 ppm de una mezcla de ajo y aceites esenciales (PHYTO0.02 en el capítulo/ PHYTO 1 en material y métodos), 1000 ppm de una mezcla de flavonoides cítricos y aceites esenciales de plantas labiadas y asteráceas (PHYTO0.1 en el capítulo/ PHYTO 2 en material y métodos) o 5000 ppm de galactomannanoligosacaridos (GMOS0.5 en el capítulo/ GMOS en material y métodos). Una cuarto tratamiento con la misma composición pero sin suplementar fue utilizado como dieta Control. Tras una experiencia de engorde de 72 días de duración, los peces experimentales fueron sujetos a una prueba de estrés por exposición a 50 ppm de H_2O_2. La respuesta al estrés de los peces fue evaluada a través de los niveles circulantes de cortisol plasmático y la respuesta antioxidante en branquia con la evaluación de expresión

génica relativa de *nfκβ2*, il-1*β*, hif-1*α*, nd5, cyb, cox, sod, cat, gpx, tnf-1*α* and caspasa 9. La selección génica para alto crecimiento afecto significativamente a la tolerancia al estrés de los peces, presentando la línea seleccionada niveles basales de cortisol inferiores ($p < 0.05$) y un mejor patrón de recuperación de las condiciones previas al estrés. Todos los ingredientes funcionales incrementaron ($p < 0.05$) la expresión génica en branquia de *cat* en comparación a la dieta Control e independientemente del genotipo de los peces. En conjunto, estos resultados indicaron una mejor capacidad de los peces pertenecientes a la línea de selección génica para afrontar los potenciales efectos negativos de una exposición a un agente oxidante.

3.4. Capítulo VI. Genetically superior European sea bass (*Dicentrarchus labrax*) and nutritional innovations: Effects of functional feeds on fish immune response, disease resistance, and gut microbiota

Aquaculture reports 33 (2023)101747

El objetivo de este estudio fue determinar si juveniles de lubina europea pertenecientes a un programa de selección génica para alto crecimiento se podrían beneficiar de la suplementación de ingredientes funcionales como promotores del crecimiento, la salud del microbioma intestinal, la respuesta inmune y la resistencia a patógenos cuando son alimentados con dietas basadas en ingredientes alternativos. Dos lotes distintos de juveniles de lubina europea, línea genética seleccionada o genotipo salvaje, fueron alimentados con 3 dietas isoenergéticas e isoproteicas (“dieta Futura” (8% HP/ 0% AP)) suplementadas con ingredientes funcionales en una experiencia de engorde de 12 semanas. Una primera fase de 2 semanas con altos niveles de suplementación y una segunda fase de 10 semanas con bajos niveles de suplementación. Los ingredientes funcionales empleados fueron PROB (dosis alta 1000 ppm/ dosis baja 200 ppm), ORG (dosis alta 750 ppm/ dosis baja 300 ppm), PHYTO (PHYTO 3 material y métodos de esta tesis; dosis alta 750 ppm/ dosis baja 500 ppm). Una cuarto tratamiento con la misma composición pero sin suplementar fue utilizada como dieta Control. Después de cada experiencia de engorde se sometió a los peces prueba de estrés por confinamiento combinado con una infección intestinal con el patógeno *V. anguillarum* (10^5 cfu/ml x fish). Tras las experiencias de engorde, los peces genéticamente seleccionados presentaron mayor rendimiento ($p < 0.05$) en términos de eso corporal, crecimiento relativo, SGR e índice de crecimiento diario. La exposición al patógeno tuvo efectos significativos en la supervivencia de los peces independientemente del genotipo. Los análisis de expresión génica relativa de en el tejido linfoide asociado al estómago revelaron una interacción entre el genotipo de los peces y la dieta afectando a la expresión de *il-1β*. En lo referente al microbioma intestinal, los análisis de discriminación de poblaciones no mostraron una separación clara entre tratamientos alimenticios. Sin embargo, la abundancia relativa de determinados taxones bacterianos varió entre grupos experimentales. Por ejemplo, los peces de ambas líneas genéticas alimentados con la dieta ORG presentaron mayor abundancia de *Streptococcos*. Mientras que los peces alimentados con la dieta PHYTO presentaron mayores abundancias de

lactobacilariales. Finalmente, los peces alimentados con la dieta PROB presentaron menor abundancia de *Pseudomonas* and *Acinetobacter*.

3.5. Capítulo VII. Modelling the effect of prebiotics, probiotics and other functional additives on the growth, feed intake and feed conversion of European sea bass (*Dicentrarchus labrax*) juveniles

Aquaculture reports 32 (2023)101729

Está investigación se centró en el desarrollo de un modelo de regresión lineal múltiple (MLR) capaz de identificar y describir los posibles efectos de distintos grupos de ingredientes funcionales en el crecimiento y la eficiencia alimenticia de la lubina europea. Con este fin, se elaboro una base de datos compuesta por los resultados experimentales de un total de 61 experiencias científicas. Los ingredientes funcionales se clasificaron en tres grandes grupos; "probióticos", "prebióticos" y "otros", incluyendo este último productos fitogénicos y compuestos simbióticos. Se obtuvieron y validaron un total de tres MLR, describiendo los efectos de los distintos ingredientes funcionales en la tasa de crecimiento específica (SGR) (con un R cuadrado (R^2) = 0.96, un R^2 ajustado (adj R^2) = 0.92 y un p-valor = 7.21E-08)), la tasa de ingesta individual (FI) (con un R cuadrado (R^2) = 0.97, un R^2 ajustado (adj R^2) = 0.95 y un p-valor = 5.42E-12)) y la tasa de conversión del alimento (FCR) (con un R cuadrado (R^2) = 0.90, un R^2 ajustado (adj R^2) = 0.80 y un p-valor = 2.02E-05)). La simplificación de los distintos MLRs permitió la detección de una correlación positiva entre la inclusión de prebióticos y el SGR de la lubina europea (cor = 0.32, p-valor = 8.52E-04). Por el contrario la inclusión de estos mismos ingredientes funcionales presentó una correlación negativa con el FI (cor = - 0.44, p-valor = 6.27 -05) y el FCR (cor = - 0.41, p-valor = 8.96E-05) de la lubina.

4. Discusión general

Con el incremento de la población mundial y la demanda de alimentos de alta calidad se está produciendo un incremento paralelo de la demanda de productos acuícolas. El pescado de acuicultura está considerado como una de las fuentes de proteínas más eficiente, y en respuesta a esta demanda la producción acuícola está creciendo. Sin embargo, este crecimiento debe producirse en un contexto de piensos para peces más eficientes y sostenibles. Los piensos acuícolas deben lograr la mayor reducción posible de los niveles de harinas (HP) y aceites (AP) de pescado, un desafío condicionado por la especie objetivo y su estadio de desarrollo. A pesar de que se ha demostrado que es posible reducir los niveles de HP y AP en algunas especies marinas, en algunos casos esta reducción se ha asociado a efectos negativos en el crecimiento y la salud de los peces. Estos efectos secundarios serán dependientes no solo de la especie y edad del animal, sino de los niveles de HP/AP sustituidos y del tipo de ingredientes empleados en la sustitución. En el caso de la lubina europea (*Dicentrarchus labrax*), altos niveles de sustitución de estos ingredientes están asociados a distintos desordenes intestinales como el engrosamiento de la *lamina propia* y la submucosa, el incremento de la expresión de

genes proinflamatorios o incrementos en la producción de mucus intestinal. Estos desequilibrios nutricionales derivados de la alta sustitución de HP/AP también pueden inducir un estado crónico de estrés marginal, mermando la capacidad del animal para afrontar procesos de estrés futuros. Este conjunto de alteraciones pueden resultar potencialmente perjudiciales, comprometiendo la productividad de la lubina europea alimentada con dietas bajas en HP/AP.

¿Qué herramientas zootécnicas podrían ser empleadas para evita los efectos negativos de alimentar lubinas con dietas bajas de HP/AP?

De entre varias estrategias, como la formulación apropiada de una dieta a base de ingredientes sostenibles o la suplementación de ingredientes deficientes en una dieta baja en HP/AP, hay dos estrategias que pueden ser empleadas con la finalidad de mejorar el rendimiento y el vigor de la lubina Europea para afrontar los desequilibrios derivados de las dietas con bajo contenido en HP/AP. *i*) Suplementación dietética de ingredientes funcionales (FI), los cuales pueden jugar un importante papel como sustancias anti estrés e inmunomoduladoras, mejorando el estado de salud de la lubina europea frente a procesos de estrés asociados a la producción acuícola; y *ii*) selección génica multi-rasgo, la cual puede inducir importantes ventajas en la utilización de dietas con bajos niveles de HP/AP y resultando en un mayor crecimiento y mayor vigor para afrontar posibles procesos de estrés.

El empleo de FI es una estrategia efectiva en la potenciación del sistema inmune y la salud de los peces. Estos ingredientes presentan compuestos activos con la habilidad de modular la respuesta de los peces frente a procesos de estrés, como cambios ambientales o infecciones patógenas [Soleimani et al., 2012; Hoseinifar et al., 2013; Herrera et al., 2019]. Los FI han mostrado la capacidad de modular procesos inflamatorios y el estado antioxidante de los peces mediante la interacción con distintos proceso de señalización celular [Soares et al., 2018; Filippone et al., 2020; Porter et al., 2022]. Además, distintos FI han mostrado la capacidad de favorecer la estabilidad del microbioma intestinal, beneficiando la salud y la capacidad digestiva del huésped [Yaramahdi et al., 2016; Gonçalves and Gallardo-Escárate, 2017; Soares et al., 2018; Butt and Volkoff, 2019; Caipang et al., 2020]. Por lo tanto, el empleo de ingredientes funcionales puede resultar en un mayor crecimiento y/o una mayor capacidad para tolerar los desequilibrios nutricionales de derivados de dietas bajas en HP/AP.

En esta tesis, el uso de ingredientes funcionales ha mostrado potencial para mejorar el estado de salud y la capacidad de respuesta frente a procesos de estrés (Capítulos III, IV, V y VI) [Serradell et al., 2020; Serradell et al., 2022; Serradell et al., 2023; Rimoldi et al., 2023]. Sin embargo, ninguno de los ingredientes funcionales indujo mejoras en el crecimiento o la utilización de dietas bajas en HP /AP. A pesar de que los parámetros indicadores de rendimiento no mejoraron, el uso de ingredientes funcionales pudo contrarrestar los procesos inflamatorios asociados a los bajos niveles de HP/AP. Por ejemplo, en un estudio paralelo a los Capítulos III y IV [Serradell et al., 2020; Serradell et al., 2022] y desarrollado por Torrecillas y coautores en 2019, se observó que la suplementación de GMOS indujo un incremento en el tamaño del microvilli del recto y redujo el grosor de la submucosa. Estos resultados apoyan las observaciones descritas en el capítulo VII [Serradell et al., 2023b] de esta tesis, en el cual, el modelo descriptivo en ingredientes funcionales detectó un efecto beneficioso del empleo de prebióticos en el crecimiento y la eficiencia alimenticia de la lubina europea. Por lo tanto, en esta tesis se ha demostrado que el empleo de ingredientes funcionales es una estrategia efectiva para

contrarrestar los posibles desequilibrios asociados a los bajos niveles de HP/AP en lugar de la promoción de su crecimiento en comparación a peces alimentados con dietas sin suplementar.

Los programas de selección génica multi-rasgo también son una herramienta eficaz para incrementar el rendimiento de los peces en términos de crecimiento y eficiencia alimenticia [de Verdal et al., 2018; Knap and Kause, 2018; Besson et a., 2019; Vandeputte et al., 2022]. Una mayor capacidad para obtener y utilizar nutrientes puede significar una ventaja para tolerar los desequilibrios nutricionales derivados de la alta sustitución de HP/AP por ingredientes alternativos [Gjedrem et al., 2012, Overturf et al., 2013; Abernathy et al., 2017; de Verdal et al., 2018; Besson et al., 2020; Vandeputte et al., 2022]. Por otra parte, una mayor capacidad para rendir en condiciones de bajos niveles de HP/AP se puede traducir en una mejor capacidad para afrontar otros agentes estresantes, y por tanto resultando en animales mas sanos y vigorosos [Hermesch et al., 2015; Perera et al., 2019; Piazzon et al., 2020]. En esta tesis, la selección génica mejoró la tasa de conversión del alimento y el crecimiento de la lubina europea a pesar de emplear dietas extremadamente bajas en HP/AP (Capítulos V, VI) [Serradell et al., 2023a; Rimoldi et al., 2023]. En el capítulo V, con tan solo 300 días de edad y sin haber empezado siquiera la experiencia de engorde, los peces genéticamente seleccionados ya presentaban un tamaño dos veces mayor a los peces del genotipo salvaje. En el capítulo VI, los peces pertenecientes al genotipo seleccionado presentaron la habilidad de modular su microbioma intestinal para adaptarse a las dietas con bajo contenido en HP/AP, lo que resultó en una mayor supervivencia al patógeno *V. anguillarum*. Estos resultados remarcan el potencial de los programas de selección génica para incrementar la tolerancia de los peces a las nuevas fuentes de proteínas y grasas, dando lugar a la sustitución de HP/AP sin afectar al rendimiento y salud de los peces.

¿Son los ingredientes funcionales una herramienta efectiva para mejorar el estado de salud y vigor de la lubina europea?

En esta tesis el empleo de ingredientes funcionales ha mejorado el estado de salud de la lubina europea su la capacidad para afrontar distintos procesos de estrés (Capítulos III, IV, V, VI) [Serradell et al., 2020; Serradell et al., 2022; Serradell et al., 2023; Rimoldi et al., 2023].

A lo largo de la tesis, se han propuesto varios mecanismos para explicar las distintas formas de actuación de los ingredientes funcionales empleados. Por ejemplo, debido a su elevada concentración en flavonoides, los compuestos fitogénicos han sido propuestos como una herramienta efectiva en la mejora del estado antioxidante y la modulación de la respuesta inflamatoria de los peces. Los flavonoides ejercen sus efectos en la modulación del estado antioxidante y la respuesta infamatoria a través de la interacción con factores de señalización celular como las MAPKS, y por tanto jugando un papel fundamental en la homeostasis celular [Shen and Liu, 2006; Chakraborty et al., 2011; Mansuri et al., 2014]. Además, los flavonoides también han mostrado importantes propiedades anestésicas, debido a su capacidad para interactuar directamente con el sistema nervioso central [de Souza et al., 2016; Souza et al., 2019; Hoseini et al., 2019; Caipang et al., 2020]. Estos compuestos activos son altamente liposolubles, facilitando su difusión a través de membranas biológicas. Una vez en el cerebro, los flavonoides modulan el complejo receptor de ácido gamma-aminobutírico (GABA), inhibiendo la transmisión del impulso nervioso e induciendo efectos sedantes [Sangeetha et al., 2016; Saccol et al., 2017]. En lo referente a los compuestos prebióticos, el mecanismo de actuación

propuesto para estos ingredientes funcionales es la modulación indirecta de la respuesta al estrés y el estado de salud del huésped a través de su homeostasis intestinal. El mecanismo principal por el cual podría ejercer estos efectos, es la estimulación de la producción de ácidos grasos de cadena corta (SCFAs) con potentes efectos antioxidantes [Leonel and Álvarez-Leite, 2012; Filippone et al., 2020] y antiinflamatorios [Buentello et al., 2010; Leonel and Álvarez-Leite, 2012, Filippone et al., 2020]. La habilidad de estos compuestos para modular el microbioma y la salud intestinal podría beneficia al huésped incrementando su rendimiento en condiciones de estrés [Yaramahdi et al., 2016; Soares et al., 2018; Butt and Volkoff, 2019] a través de la modulación del eje cerebro-estómago, dando lugar a una menor reactividad del eje HPI [Cryan and Dinan 2012; Soleimani et al., 2012; Hoseinifar et al., 2013; Herrera et al., 2019; Porter et al., 2022]. La suplementación de ingredientes funcionales atenuó la respuesta al estrés de la lubina europea (Capítulos III y IV) [Serradell et al., 2020; Serradell et al., 2022], contrarrestando los desequilibrios fisiológicos derivados de varios procesos de estrés simultáneos; un estrés leve pero crónico asociado a los bajos niveles de HP/AP y procesos de estrés agudo como infecciones patógenas, altas densidades de cultivo y la exposición a agentes químicos.

¿Son los programas de selección génica una herramienta efectiva para mejorar el estado de salud y vigor de la lubina europea?

La selección génica de lubina europea ha sido capaz de mejorar la tolerancia de la lubina europea al estrés (Capítulo V) [Serradell et al., 2023a] y su supervivencia al patógeno *V.anguillarum* (Capítulo VI) [Rimoldi et al., 2023].

Dentro de una población, los individuos habitualmente muestran distintos grados de tolerancia y respuesta frente a un proceso de estrés, lo que permite su clasificación como altamente (reactivos) o poco (proactivos) sensibles [Martins et al., 2011; Castanheira et al., 2017; Alfonso et al., 2019]. Los individuos proactivos presentan bajos niveles de cortisol basal y menores tasas de activación del eje HPI en respuesta al estrés [Martins et al., 2011; Castanheira et al., 2017; Ruiz-Gomez et al., 2015]. Una actividad reducida, pero no dañada, del eje HPI se asocia a una menor sensibilidad a los cambios ambientales y una mayor tendencia a establecer rutinas [Silva et al., 2010; Martins et al., 2011; Castanheira et al., 2017]. Adicionalmente, los individuos proactivos están caracterizados por una utilización mas eficiente del alimento y un mayor vigor [Martins et al., 2011; Castanheira et al., 2017; Alfonso et al., 2019]. En las poblaciones naturales, reactividad al estrés no está polarizada, habiendo individuos con características intermedias [Martins et al., 2011]. Sin embargo, el efecto acumulativo de la selección génica puede dar lugar a la polarización de este rasgo generando un proceso de domesticación [Vandeputte et al., 2009; Jensen, 2014; Vandeputte et al., 2016]. Una menor susceptibilidad al estrés se traducirá en un mejor rendimiento incluso bajo condiciones de cultivo [Tort et al., 2011; Ellis et al., 2012]. Por ejemplo, Trenzado y coautores en 2006, observaron que la selección génica de trucha (*O. mykiss*) para baja sensibilidad al cortisol resultó en mejoras en el crecimiento de los peces incluso en condiciones de alta densidad de cultivo [Trenzado et al., 2006]. Los peces con baja sensibilidad presentaron mayor ganancia de peso, SGR, eficiencia alimenticia e ingesta de pienso, independientemente de la densidad de cultivo. Esto demuestra el importante papel que puede jugar la tolerancia al estrés en la productividad bajo condiciones de cultivo.

La selección génica ofrece un elevado potencial aumentando el vigor de los peces, permitiendo un mayor crecimiento independientemente de las condiciones ambientales [Agha et al., 2003; Knap, 2005; Hermesch et al., 2015]. En esta tesis, en comparación a los peces pertenecientes al genotipo salvaje, los peces seleccionados presentaron mayor

crecimiento en un régimen de bajos niveles de HP/AP y una mayor capacidad para afrontar distintos procesos de estrés.

¿La combinación de los programas de selección génica y la suplementación dietética de ingredientes funcionales es una herramienta eficaz para optimizar el cultivo de lubina europea ?

Aparentemente, en esta tesis ambas estrategias ejercieron efectos individuales y separados en el rendimiento de la lubina europea. Sin embargo, se debe tener en consideración que los peces no seleccionados parten de un estado de salud peor y una capacidad de rendimiento menor, al ser menos vigorosos que los peces genéticamente seleccionados. Los peces no seleccionados, con una menor capacidad de utilización de las dietas bajas en HP/AP, presentaron una mayor sensibilidad y reactividad frente al estrés lo que resultó en menores tasas de supervivencia. Los beneficios inducidos por el empleo de ingredientes funcionales fueron más evidentes en peces no seleccionados. La respuesta de los peces seleccionados al uso de ingredientes funcionales está condicionada por un mejor estado de salud basal, resultando en unos beneficios cualitativamente inferiores a los observados en peces salvajes. De hecho, en el capítulo V [Serradell et al., 2023a], los niveles basales de cortisol de las lubinas seleccionadas fueron inferiores a los valores esperados para esta especie [Samaras et al., 2023]. Esto puede estar directamente relacionado con una mayor tolerancia a las condiciones de cultivo y las dietas bajas en HP/AP. A nuestro saber, no hay más estudios investigando el efecto combinado de estos tres factores (niveles extremadamente bajos de HP/AP, el empleo de ingredientes funcionales y las estrategias de selección génica) sobre la salud y rendimiento de la lubina europea.

¿Es posible el desarrollo de un modelo predictivo que determine la efectividad de la combinación de ambas herramientas zootécnicas?

En el capítulo VII [Serradell et al., 2023b], el modelo de regresión linear múltiple (MLR) fue desarrollado para identificar los efectos de la suplementación dietética de distintos ingredientes funcionales en el crecimiento y la eficiencia alimenticia de la lubina europea. El MLR, permitió la detección de una correlación positiva entre el empleo de prebióticos y el SGR de los peces. Por el contrario, la suplementación de prebióticos presentó una correlación negativa con el FCR y la ingesta de alimento de los animales. Estos resultados sugirieron la posibilidad de emplear prebióticos como potenciadores del rendimiento de la lubina. Sin embargo, en este modelo no se pudo incluir suficiente información sobre la salud de los animales, ya que ninguno de los estudios proveía suficiente información. En las diferentes experiencias se analizaron distintos indicadores de salud, pero la baja homogeneidad en las pruebas realizadas en los distintos estudios dificultaba su inclusión en el modelo. Con los programas de selección génica la situación es la misma para esta especie.

Por este motivo, en el futuro cuando haya más información disponible acera de los programas de selección génicas, se podría optar por un enfoque integrativo de ambas estrategias, con la finalidad de elucidar sus posibles sinergias en la optimización la producción acuícola de lubina europea. Especialmente, el desarrollo de modelos nutricionales más complejos podría suponer una potente herramienta para combinar todos los factores condicionantes del rendimiento máximo de la lubina europea en condiciones de cultivo, dando lugar al rápido avance de este sector de la industria acuícola.

5. Conclusiones Generales

1. El uso de galactomannanoligosacáridos (GMOS) como aditivo funcional al 5000 ppm atenuó la respuesta al estrés de los juveniles de lubina europea, reduciendo las concentraciones de cortisol plasmático tras las prueba de estrés por confinamiento y redijo la expresión de genes relacionados con la respuesta al estrés en el riñón anterior en peces alimentados con dietas bajas de harinas y aceites de pescado. Del mismo modo, el uso de 200 ppm de una mezcla de ajo y aceites esenciales de plantas labiadas indujo niveles menores de cortisol plasmático después del estrés por confinamiento

2. El uso de una mezcla de ajo y aceites esenciales de plantas labiadas tiene un efecto protector contra los procesos apoptóticos de las células interrenales en respuesta al estrés por confinamiento.

3. El uso de 200 ppm de una mezcla de mezcla de ajo y aceites esenciales de plantas labiadas o 5000 ppm de GMOS aumentó la resistencia de los juveniles de lubina europea frente a una infección de *Vibrio anguillarum* durante el estrés por confinamiento.

4. El uso de 200 ppm de una mezcla de mezcla de ajo y aceites esenciales de plantas labiadas o 5000 ppm de GMOS en dietas con 10% HP/6% AP redujo los niveles de expresión génica proinflamatoria en branquia en respuesta al proceso de estrés.

5. El empleo de aditivos funcionales indujo una respuesta al estrés atenuada, dando lugar a un metabolismo energético más estable y mejorando el estado antioxidante en condiciones de estrés asociadas al confinamiento

6. Ambos ingredientes funcionales mejoraron el estado antioxidante en branquia de los juveniles de lubina Europea estimulando la expresión génica de catalasa en respuesta a un estrés ambiental por exposición a H_2O_2

7. El genotipo seleccionado de lubina Europea presentó niveles basales de cortisol inferiores y una reactividad menor al proceso de estrés en comparación a los peces salvajes, indicando una mejor capacidad para lidiar con los procesos de estrés asociados a la producción acuícola

8. El mayor crecimiento y un estado de salud basal mejorado e los juveniles de lubina genéticamente seleccionados dio lugar a mayores tasas de supervivencia al patógeno *V. anguillarum* en comparación a los peces salvajes.

9. El efecto de los aditivos funcionales fue dependiente del tipo de aditivo, su concentración en la dieta y el genotipo de los juveniles de lubina.

10. Una mezcla de probióticos (INVE, Bélgica – compuesto por tres especies de *Bacillus: B. subtilis, B. licheniformis y B. pumilus*) activó la respuesta inmune intestinal de los juveniles de lubina genéticamente seleccionados.

11. A pesar de que en esta tesis la suplementación dietética de aditivos funcionales no mejoró el crecimiento y la utilización de las dietas bajas en HP/AP, el modelo de regresión lineal múltiple desarrollado identificó el uso de prebióticos como una estrategia con potencial para mejorar el crecimiento y la eficiencia alimenticia de la lubina europea.

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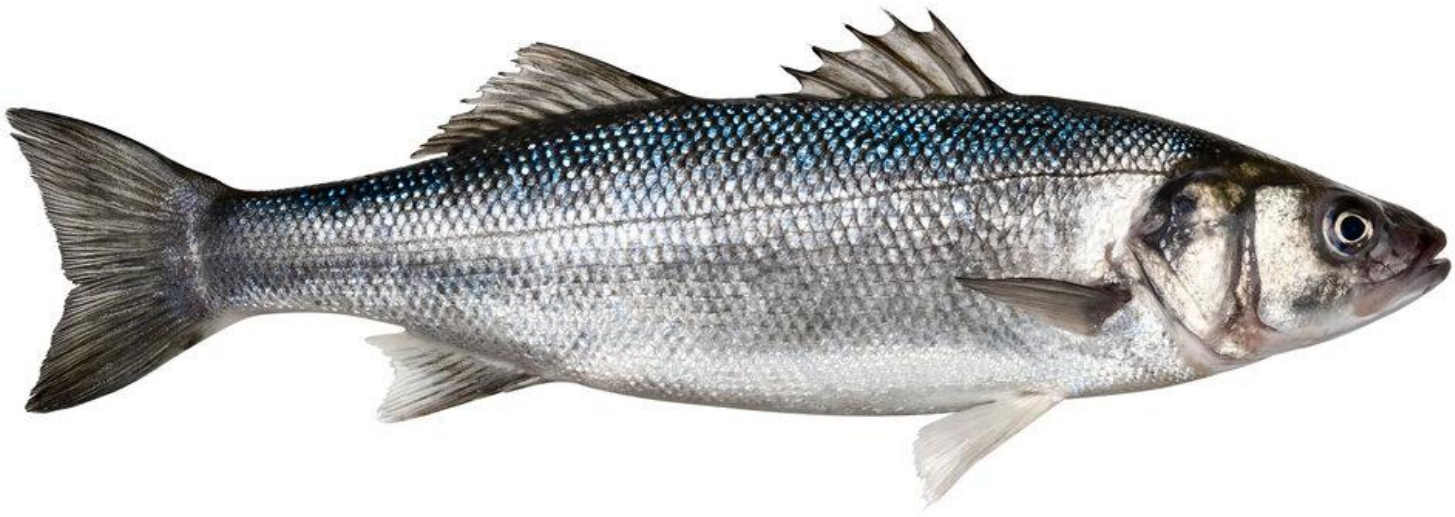

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