UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

DIETARY COMPLEMENTS IN THE UTILIZATION OF ALTERNATIVE LIPIDS TO FISH OIL IN MARINE AQUACULTURE DIETS: A TOOL TO MAXIMIZE PATHOGEN RESISTANCE POTENTIAL OF FISH

D. Fernando Rivero Ramírez

Tesis para el grado de *Doctor*

Julio, 2019

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UNIVERSIDAD DE LAS PALMAS
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D. DANIEL MONTERO VÍTORES COORDINADOR DEL PROGRAMA DE DOCTORADO ACUICULTURA SOSTENIBLE Y ECOSISTEMAS MARINOS DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "DIETARY COMPLEMENTS IN THE UTILIZATION OF ALTERNATIVE LIPIDS TO FISH OIL IN MARINE AQUACULTURE DIETS: A TOOL TO MAXIMIZE PATHOGEN RESISTANCE POTENTIAL OF FISH"- COMPLEMENTOS DIETÉTICOS EN LA UTILIZACIÓN DE LÍPIDOS ALTERNATIVOS AL ACEITE DE PESCADO EN ACUICULTURA MARINA: UNA HERRAMIENTA PARA MAXIMIZAR EL POTENCIAL DE RESISTENCIA A PATÓGENOS presentada por el doctorando **D. Fernando G. Rivero Ramírez** y dirigida por el Doctor D. Daniel Montero Vítores y la Doctora Dña. Silvia Torrecillas Burriel.

Y para que así conste, y a efectos de lo previsto en el Art. nº11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a de mayo de dos mil diecinueve.

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
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A mi familia: los que están y los que se han ido.

Always choose the hardest way

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Appendix I

LIST of ABBREVIATIONS

AA (Amino acids)

Ace (Angiotensin-converting enzymes)

ACPH (Alternative complement pathway)

ALA (18:3 n-3, alpha-linolenic acid)

ANFs (Antinutritional factors)

ANOVA (Analysis of variance)

Anxa-1 (Annexin a1)

APC (antigen presenting cells)

ARA (20:4 n-6, arachidonic acid)

BOX (Total palmitoyl CoA Beta-oxidation)

Calr (calreticulin)

Cat (Catalase)

CAO (Camelina oil)

Cd3 (Cluster of differentiation 3)

Cd4 (Cluster of quadruple differentiation)

Cd8α (Cluster of differentiation 8 alpha)

cDNA (Complementary DNA)

CDR (Carbohydrate recognition domain)

Cel (Carboxyl ester lipase)

CFU (Colony-forming unit)

Cstb (Cathepsin B)

CO (Canola oil)

Cox-2 (Cyclooxygenase 2)

Cpt1α (*C*arnitine palmitoyl transferase I alpha)

CSIC (Centro Superior de Investigaciones Científicas/Spanish National Research Council)

DAMPs (damage-associated molecular patterns)

Dc-sing (Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin)

DHA (22:6 n-3, docosahexaenoic acid)

DI (Distal intestine)

DPA (22:5 n-3, docosapentaenoic acid)

DM (Dry matter)

Ech (Enoyl-CoA hydratase)

Ef-1α (Elongation factor-1 alpha)

EFA(s) (Essential fatty acid(s))

EPA (20:5 n-3, eicosapentaenoic acid)

Elovl (Elongation of very long chain fatty acids protein)

FA(s) (Fatty acid(s))

Fabp (Fatty acid binding protein)

Fads2 (Fatty acyl desaturase 2)

FAMES (Fatty acid methyl esters)

Fas (Fatty acid synthase)

FCR (Feed conversion ratio)

FM (Fish meal)

FO (Fish oil)

FOS (Fructo-oligosaccharides)

FXO (Flaxseed oi)

GALT (Gut associated lymphoid tissue)

Gck (Glucokinase)

Gh (Growth hormone)

Ghr-II (Growth hormone receptor II)

GIA (Grupo de Investigación en Acuicultura/ Aquaculture research group)

GOS (Galacto-oligosaccharides)

GP(s) (Glycerophospholipid(s))

GPsARA (ARA in GPs)

Gpx (Glutathione peroxidase)

Gr (Glutathione reductase)

Gr1 (Glucocorticoid receptor 1)

Gsh (Reduced glutathione levels)

Gssg (Gluthathione disulfide/oxidized glutathione levels)

HK (Head kidney)

Hmgcr (3-hydroxy-3-methylglutaryl-coenzyme A reductase)

HPTLC (High-performance thin layer chromatography)

HSI (Hepatosomatic index)

Hsp (Heat shock protein)

HUFA (Highly unsaturated fatty acids, used as synonymous of LC-PUFAs)

i.e. (Id est, that is)

Ifn (Type I interferon)

Ig (Immunoglobulin)

Igf-1 (Insulin-like growth factor -1)

IHQ (Immunohistochemistry)

i.e. (*id est*; that is)

Il10 (Interleukin 10)

Il-1ß (Interleukin -1 beta)

iNos (Inducible nitric oxide synthase)

ISH (*In situ* hybridization)

ISM (Spleen-somatic index)

K (Fulton's condition factor)

LA (18:2 n-6, linoleic acid)

LAB (Lactic acid bacteria)

LC-PUFAs or HUFAs (Long-chain PUFAs)

LDL (Low-density lipoprotein)

LO (Linseed oil)

Lox (Lypooxygenase)

LPC (Lysophosphatidylcholine)

Lpo (Hepatic and intestinal lipid peroxidation)

LPS (Lipopolysaccharides)

LTs (Leukotrienes)

LXs (Lipoxins)

Lyo (Lyophilized)

Lyz (Lysozyme)

MALT (Mucosal associated lymphoid tissue)

MAMPs (Microbe-associated molecular patterns)

MBL (Mannose-binding lectin)

Mc2r (Melanocortin 2 receptor)

Mda (Malondialdehyde)

Mhcii-ß (Major histocompatibility complex ii-beta)

Mhci-α (Major histocompatibility complex i alpha)

MINECO (Spanish Ministry of Economy and Competitiveness)

MOS (Mannan-oligosaccharides)

Muc (Mucins)

Mul1b (Mitochondrial ubiquitin ligase activator of NFκB1)

Naga (alpha-N-acetyl-galactosaminidase)

NBT (Nitro-blue tetrazolium)

ND (No differences)

Nf-κβ (Nuclear factor kappa beta)

NO (Nitric oxide)

OO (Olive oil)

P/S/G (Penicillin / streptomycin / gentamicin)

P38 Mapk (p38 mitogen-activated protein kinase)

PAMPs (Pathogen-associated molecular patterns)

PAS (Periodic acid-Schiff)

PBS (Phosphate buffered saline)

PC (Phosphatidylcholine)

PCA (Principal component analysis)

PE (Phosphatidylethanolamine)

PER (Protein efficiency ratio)

PERMANOVA (permutational multivariate analysis of variance).

PFA (Paraformaldehyde)

PFI (Perivisceral fat index)

PGE (Prostaglandin E)

PGF2α (Prostaglandin F2 alpha)

PGs (Prostaglandins)

PhD (Philosophiae Doctor)

PI (Phosphatidylinositol)

Pla2 (Phospholipase 2)

PO (Palm oil)

PP (Plant protein)

Ppar (Proliferator-activated receptors)

ppm (Parts per million)

PRPs (Pattern recognition proteins)

PRRs (Pattern recognition receptors)

PS (Phosphatidylserine)

Psma5 (Proteasome subunit alpha type-5)

PUFAs (Polyunsaturated fatty acids)

R&D (Research and development)

RO (Rapeseed oil)

ROS (Reactive oxygen species)

RT (Reverse transcription)

RT-qPCR (Quantitative real-time polymerase chain reaction)

SAFO (Safflower oil)

SB (Soybean)

SBO (Soybean oil)

SCFA (Short-chain fatty acids)

SD (Standard deviation)

SEM (Standard error of mean)

SFO (Sunflower oil)

SGR (Specific growth rate)

SM (Sphingomyelin)

Sod (Superoxide dismutase)

Srebp (Sterol regulatory element binding transcription factor)

TAGs (Triacylglycerides)

TAS (Total antioxidant status)

Tcr-α (T-cell receptor alpha)

TFA (Total fatty acids)

Tgf-β (Transforming growth factor beta)

Tlr4 (Toll like receptor 4)

TM (Terrestrial meals)

Tnfα (Tumor necrosis factor alpha)

Treg (Regulatory T-cells)

Trp1 (Trypsin-1)

TXs (Thromboxanes)

UN (Unvaccinated)

VC (Vaccinated)

VCCS (Vertebral column compression syndrome)

VD (Vegetable diet)

Veg (Vegetative)

VLDL (Very low-density lipoprotein)

VO (Vegetable oils)

VSI (Viscerosomatic index)

^{o}C (Celsius degrees)

β-Actin (beta actin)

Δ6-d (Delta 6-desaturase)

Appendix II

LIST OF COMMON AND SCIENTIFIC NAMES

Aeromonas salmonicida (A. salmonicida)

Angelfish *(Pterophyllum scalare)*

Artemia (*Artemia franciscana*)

Atlantic cod (*Gadus morhua*)

Atlantic herring *(Clupea harengus)*

Atlantic salmon *(Salmo salar)*

Bacillus subtilis (B. subtilis)

Bacillus clausii (B. clausii)

Blue whiting *(Micromesistius poutassou)*

Brown trout (*Salmo trutta fario*)

Capelin *(Mallotus villosus)*

Chilean jack mackerel (*Trachurus murphyi)*

Clostridium spp.

Common carp *(Cyprinus carpio)*

European seabass *(Dicentrarchus labrax)*

Gilthead seabream *(Sparus aurata)*

Gobid *(Synechogobius hasta)*

Grass carp *(Ctenopharyngodon idellus)*

Green terror *(Aequidens rivulatus)*

Guppies *(Poecilia reticulate)*

Japanese anchovy *(Engraulis japonicus)*

Japanese eel *(Anguilla japonica)*

Japanese flounder *(Paralichthys olivaceus)*

Japanese sea bass *(Lateolabrax japonicus)*

Lactobacillus farciminis (*L. farciminis*)

Lactobacillus rhamnosus (*L. rhamnosus*)

Lactobacillus delbrueckii delbrueckii (*L. delbrueckii delbrueckii)*

Mortierella alpina (M. alpina)

Mozambique tilapia (*Oreochromis mossambicus*)

Nile tilapia (*Oreochromis niloticus*)

Northern snakehead (*Channa argus*)

Pacific sardine *(Sardinops sagax)*

Pediococcus acidilactici (P. acidilactici)

Peruvian anchovy/anchoveta *(Engraulis ringens)*

Pollack (*Pollachius pollachius*)

Rainbow trout *(Oncorhynchus mykiss)*

Rd hybrid tilapia (*Oreochromis sp.*)

Red tilapia *(Oreochromis niloticus)*

Round sardinella *(Sardinella aurita)*

Saccharomyces cerevisiae (S. cerevisiae)

Senegalese sole (*Solea senegalensis*)

Shrimp (*Litopenaeus stylirostris*)

Staphylococcus aureus (S. aureus)

Streptococcus iniae (S. iniae)

Striped bass *(Morone saxatilis)*

Tetrahymena sp.

Turbot (*Scophtalamus maximus; synonymy: Psetta maxima*)

Vannamei shrimp *(Litopenaeus vannamei)*

Vibrio anguillarum (V. anguillarum)

Vibrio SKT-b (V. SKT-b)

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DIETARY COMPLEMENTS IN THE UTILIZATION OF ALTERNATIVE LIPIDS TO FISH OIL IN MARINE AQUACULTURE DIETS: A TOOL TO MAXIMIZE PATHOGEN RESISTANCE POTENTIAL OF FISH

D. FERNANDO GUSTAVO RIVERO RAMÍREZ

Chapter 1

1. GENERAL INTRODUCTION

1.1. THE GROWING SECTOR OF FARMED FISH: FIGURES OF EUROPEAN SEABASS PRODUCTION

Since the late 1980s up to the present the fish catches from wild fisheries have remained stationary or even diminished (Sargent & Tacon, 1999; Pauly & Zelles, 2016; FAO, 2018) whereas the aquaculture sector has experimented an average growth of 8% yearly during the last three decades (Tacon & Metian, 2017). Indeed, in 2016, aquaculture production represented 47% of total global captures, a 3% increase compared to 2014 data (FAO, 2018). Considering only fish for human consumption, since 2014 aquaculture has provided more fish than wild fisheries (53% in 2016) and it is now responsible for a global increase in fish availability (FAO, 2016 and 2018). For instance, in 2016, farmed European seabass (*Dicentrarchus labrax*) represented the 97.1% (+0.9% than in 2015) of consumptions of this species in Mediterranean region compared to 2.9% from wild caught (APROMAR, 2018). Besides, in 2016, farmed European seabass was the 73rd species in quantity in global aquaculture, with a value of 720 million euro, and the 42.5% was produced in European Union, being the 3rd species by value in this territory (APROMAR, 2018). In Spain, the third main producer of this species, European seabass reached in the year 2016 the second position in the list of main cultivated aquatic species and the first position as the more profitable species for the national aquaculture sector (APROMAR, 2017).

1.2. RAW MATERIALS AS AQUATIC FEEDSTUFFS

1.2.1. *Current situation and sustainable alternatives*

The intensification of the activity of farming of aquatic organisms requires a responsible use of natural resources for a sustainable production of feedstuffs for the cultivated species (FAO, 2018). In 2017, more than 129 metric tons of feedstuffs were necessary

for Spanish aquaculture (APROMAR, 2018). Globally, farming of European seabass together with gilthead seabream (*Sparus aurata*) accounted for 2% of global feedstuffs supplies in 2017 (APROMAR, 2018). For decades aquaculture depended on external feed inputs from wild fish, in form of fish meal (FM) and oil (FO), especially small pelagic species as blue whiting *(Micromesistius poutassou)*, capelin *(Mallotus villosus)*, Atlantic herring *(Clupea harengus)*, Chilean jack mackerel *(Trachurus murphyi)*, round sardinella *(Sardinella aurita)*, Peruvian anchoveta *(Engraulis ringens)* or Japanese anchovy *(Engraulis japonicus)*, among others (Naylor *et al.*, 2009; Tacon & Metian, 2015; SEAFISH, 2018). These raw sources have adequate levels and quality of amino acids (AA), essential fatty acids (EFAs), and trace minerals (Sargent & Tacon, 1999; Hasan *et al.*, 2007; NRC, 2011), in addition to the advantage of having an acceptable taste and texture for farmed fish (Sargent & Tacon, 1999).

However, the production of FM and FO has had important variations, with a global reduction in the last decades (FAO, 2018) related to the great pressure that wild fisheries have suffered due to bad practices, pollution, climate change, and overfishing (FAO, 2016; Barange *et al.*, 2018). In 2013 more than 30% of fish stocks were estimated to be under overfishing pressure, so the stock abundance is under the maximum sustainable yield, and these figures increased in 2016 (FAO, 2018). With respect to climate change, it is having increasing effects in fisheries and by extension into aquaculture. An example of it is an adverse climate event called El Niño. The Peruvian anchovy and Pacific sardine (*Sardinops sagax*) fisheries are located in the eastern Pacific Ocean, in one of the most productive upwelling area in the world, responsible for the highest FM and FO worldwide production (Evans & Tveteras, 2011; Tacon *et al.*, 2011; Cashion *et al.*, 2017; FAO, 2018). This ecosystem is under the occurrence of El Niño which has decreased the production of FM and FO (FAO, 2016), demonstrating the fragility of aquatic ecosystems and the deep relations between climatic challenges and economic and social structures (Fernandes *et al.*, 2016). Consequently, the marine raw materials are now mainly obtained from endangered wild fisheries (Naylor *et al.*, 2009; Pauly & Zeller, 2016; Golden *et al.*, 2016; FAO, 2016 and 2018), producing negative ecological, economic and social consequences (FAO, 2016 and 2018).

Another cause of FO and FM scarcity is because they are also excellent sources in other markets as for feeding farmed land-animals as pigs or chickens, for human consumption, production of paints, pesticides, cosmetics or medicaments (Tacon *et al.*, 2011; Pike & Jackson 2010; SEAFISH, 2018).

According to FAO (2011), in 2008 aquaculture consumed 60.8% and 73.8% of global FM and FO production respectively, with a reduction of around 8% from year 2007 (Tacon *et al.*, 2011). However, those consumptions were outperformed in 2016 when reached 69% and 75% of FM and FO respectively (IFFO, 2017), indicating a growing demand.

The main consumptions of FM and FO (in percentage) during 2009 compared to 2016, is indicated in Table 1.

Table 1. Comparative of global fish meal and fish oil consumed in aquaculture in 2009 and 2016*.

Species	%FM 2009	%FM 2016	%FO 2009	%FO 2016
Tilapias	6	9	3	6
Cyprinids	4	3	0	0
Salmonids	27	23	68	60
Crustaceans	26	31	6	6
Eels	5	13	1	3
Marine fish	26	15	19	18
Other freshwater fish	6	6	3	7

*Data from Chamberlain (2011) and SEAFISH (2018).

Thereby, the high pressure over FM and FO due to the increasing demand leads to variable production figures and explains the increased price of these marine feed ingredients, especially meals, questioning the sustainability of marine sources as raw materials for the industry of fish feed production (Tacon & Foster, 2003; FAO, 2018). Thus, there is a real need to increase the use of sustainable ingredients for feedstuffs in the sense of being economically viable and environmentally friendly (Pickova & Mørkøre, 2007; Tacon & Foster, 2003; FAO, 2018).

As prices for marine raw materials tend to rise, terrestrial sources become a good alternative for aquafeeds due to the reasonably affordable cost-effective ratio of those ingredients experimented since the last decade, particularly those from plants, in contrast to those from fish and, beyond that, their production is steady and can increase

at the same rhythm than aquaculture (Gatlin *et al.*, 2007; Turchini *et al.*, 2009; Hardy *et al.*, 2010; Tacon *et al.*, 2011).

1.2.2. *Considerations of including terrestrial sources in aquafeeds*

During the last decades, different types of oils and meals obtained from plants (including their seeds or treated derivatives) such as palm, soy, camelina, canola, sunflower, cotton, groundnut, corn, linen, lupin or wheat, among others, have been tested as alternative ingredients in fish feed formulation (Gatlin *et al.*, 2007; Turchini *et al.*, 2009). Some of these sources have presented different problems to be included in fish diets as the presence of antinutritional factors (ANFs), reduced palatability, low AA content and protein level content and quality, lack of micronutrients, low glycerophospholipid (GP) level, and variations in the fatty acid (FA) type/content to cope fish requirements. Nowadays, some of those factors are no longer a problem since great economical and research efforts have been done during the last decades in order to reduce/or attenuate them, implementing techniques such as plant-breeding and plant-genetic modifications (Krogdahl *et al.*, 2010; Betancor *et al.*, 2015a) or by adequate preparation of sources to remove or deactivate harmful compounds (Francis *et al.*, 2001; Hardy *et al.*, 2010; Krogdahl *et al.*, 2010).

Besides, different feeding strategies as the use of blends of vegetal sources have been indicated by different authors to be overall more adequate for substitutions than singular administration to get a better inclusion level of different FAs (Montero *et al.*, 2005; Mourente *et al.*, 2007; Jobling *et al.*, 2008; Montero & Izquierdo, 2010). Similarly, supplementation of GPs, micronutrients or essential AA in substituted diets can avoid nutritional imbalances (Li *et al.*, 2014; Domínguez *et al.*, 2017) and then, important substitutions with terrestrial sources can be made in different species as European seabass without affecting growth (Gomes *et al.*, 1995; Dias *et al.*, 1997; Berge *et al.*, 2002; Kaushik *et al.*, 2004; Bonaldo *et al.*, 2011; Torrecillas *et al.*, 2017a, 2017b and 2017d) but taking into account the negative effects of EFA reductions. In this line, it is well-known that, when administered together, dietary administration of arachidonic acid (ARA, 20:4 n-6) and eicosapentaenoic acid (EPA, 20:5 n-3) produces their esterification in polar lipids in a competitive manner (Bell *et al.*, 1995; Reitan *et al.*, 1994). This means that the inclusion of vegetable oils (VO) in diets for marine fish may require not

only supplementation of long-chain polyunsaturated fatty acids (LC-PUFAs) but to adjust the FA profile to ensure the minimal and the optimal presence of EFAs as EPA, ARA or docosahexaenoic acid (DHA, 22:6 n-3) (Tocher, 2010 and 2015). Thus, the minimal EFA level will be that which allows to avoid the symptoms of deficiencies that can lead the animal to dead, whereas the optimal level allows good growth and health (Tocher, 2010). However, EFA requirements depend on fish species and change in quality and quantity along fish life (Tocher, 2015). Hence, the efficient use of different singular vegetal sources or blends depends on the information on lipid metabolism and nutritional requirements for the species to be fed, which has produced a considerable increase of research in the last decades (Sargent *et al.*, 1995, 1999a, 1999b; Izquierdo, 1996 and 2005; Izquierdo *et al.*, 2000; Frøyland *et al.*, 2000; Sargent *et al.*, 2002; Bell & Sargent, 2003; Tocher, 2003, 2010 and 2015; Tocher *et al.*, 2008; Montero & Izquierdo, 2010; Lim *et al.*, 2011; Torrecillas *et al.*, 2017a and 2017b; Ma *et al.*, 2018; Xu *et al.*, 2018). In this regard, requirements of n-3 LC-PUFAs for European seabass juveniles have been set in a 0.7% DM (DHA:EPA ratio 1.5:1 and 18% lipid content) (Skalli & Robin, 2004). As mentioned before, requirements can be quite variable when considering not only fish species, but age, cycle of life or interactions with other nutrients. As an example, for gilthead seabream juveniles, n-3 LC-PUFAs requirements have been settled in 1.5-1.8% of the dry diet (with 12-15% dietary lipid content) when fish are fed for short periods (Montero *et al.*, 1998) whereas the 2.5% DM in commercial diets has been found to be a correct level for the on-growing period in juveniles of this species (Izquierdo, 2005).

1.3. A QUICK SIGHT INTO FISH INTESTINAL HEALTH

Fish mucosal tissues, such as fish gut, are composed by an epithelial physical barrier, an extrinsic barrier composed mainly by mucus and a diffuse and widespread network of immune cells in its inner part, the mucosal associated lymphoid tissues (MALT) (Uribe *et al.*, 2011; Van Muiswinkel & Nakao, 2013). In this sense, fish gastrointestinal tract constitutes a defensive barrier that is highly exposed to dietary changes, being one of the main interfaces for interactions environment-microorganisms-immune system (Bernard *et al.*, 2006, Dimitroglou *et al.*, 2011; Van Muiswinkel & Nakao, 2013).

Mucus, mainly composed by O-glycosylated glycoproteins, provides a medium for microorganisms to attach, feed, and growth (Gomez *et al.*, 2013) but paradoxically, is recognized as a passive part of the defensive barrier due to its antiadhesive elements and antibacterial components as lysozyme, complement proteins or immunoglobulins (Ig) (Ellis, 2001; Dimitroglou *et al.*, 2011; Salinas, 2015), avoiding intestinal surfaces of being colonized by pathogens, particularly those non-specialists. Then, an increased mucus production can reduce bacterial attachment, help to flush out pathogens and reduces the friction forces by food passage during the digestive process, avoiding or decreasing damage of the tissue integrity (Ellis, 2001; Torrecillas *et al.*, 2011a). Indeed, increasing mucus production in European seabass has been related to the enhancement of disease-resistance capacity when fish were challenged against intestinal pathogens (Torrecillas *et al.*, 2011a).

In relation to health, intestinal barrier integrity is crucial. Diets with high levels of FM/FO substitutions can lead to disruptions of epithelial cell unions in the intestine (Kano-Sueoka *et al.*, 2001), affecting the integrity of intestinal barrier and consequently, increasing the probabilities of bacterial translocation (Torrecillas *et al.*, 2013). In part for those reasons, fish intestinal health is frequently affected by diets with high substitutions of marine raw materials and the inclusion of terrestrial sources and therefore, increasing gut bacterial translocation rates and reducing fish disease resistance (Khroghdal *et al.*, 2010; Torrecillas *et al.*, 2017a).

But digestive tract not only must act as a passive barrier to reduce bacterial translocation through epithelia but diminish bacterial survival capacity (Secombes & Wang, 2012) which is done by the local immune system. In this sense, fish immune responses are divided into the innate, with a rapid and unspecific reaction against threats, and the acquired, which requires more time to develops specific ways to face menaces (Tort *et al.*, 2003). Despite this difference, both pathways are not independent because both interact in the defense of fish health state and then, both can be active at the same time (Kum & Sekkin, 2011). Thus, to reduce bacterial survival after a successful attachment and entry into fish tissues, the intestinal tract houses a special and highly reactive type of MALT called gut associated lymphoid tissue (GALT) that will be in charge of intestinal defense by the action of humoral and cellular immune mechanisms (Secombes & Wang, 2012). The GALT constitutes a local immune system that will react to

variances of homeostasis as those that occur during an infectious process or stressful situations as substitutions of marine sources in diet (Torrecillas *et al.*, 2014; Salinas, 2015).

For GALT to accomplish its mission, antigens must be detected and classified between those potentially harmful and those frequent and non-harmful, and this require the communication between microbiota, epithelial cells and inner leucocytes (Pelaseyed *et al.*, 2014). Antigen recognition and presentation to specialist cells, mainly intraepithelial lymphocytes (IELs), is mainly the way of connection between both innate and acquired immunity, although some of these IELs can act independently of antigen presenting cells (Rombout *et al.*, 2014). Besides, detection of frequent and non-damaging antigens, different than self-antigens, in the intestine, is also related to T-cells interactions with resident microbiota, which will help to educate the immune system in a similar way as the process to avoid self-recognition, what is called immune-tolerance (Nutsch & Hsieh, 2010). In the intestine of higher vertebrates, as part of the detection and processing systems of antigens and the subsequent immunoprotection, small mucus-secreting cells are believed to act taking up small intestinal luminal material and delivering to immune cells, as dendritic cells, in lamina propria (Pelaseyed *et al.*, 2014). This antigen-capturing capacity is known to occur in the intestine of teleosts by other cells (Ellis, 1995). Indeed, in Atlantic salmon and European seabass intestine, some cells have been identified as M-like cells to perform this function, through the interaction with IELs (Fuglem *et al.*, 2010; Torrecillas *et al.*, 2017d). This stimulation of the local immunity will influence the development of a systemic immune response (Kim *et al.*, 2012) and therefore, gut is also considered an important place to achieve oral immune-protection (Le Breton, 2009; Dimitroglou *et al.*, 2011; Rombout *et al.*, 2011).

1.4. IMPORTANCE OF ESSENTIAL FATTY ACIDS FOR FISH

1.4.1. *The n-3 and n-6 series*

Depending on the feeding pattern or, most generally, on the environment in which each fish species lives, two different scenarios, namely freshwater or marine milieu, can be found, the latter conditioned by the relative abundance of an extensive variety of FA (Monroig *et al.*, 2013). For most freshwater species, the need of LC-PUFAs can be partially met with linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3)

from dietary origin (Sargent *et al.*, 1995; Mourente *et al.*, 2005b). In contrast, marine species, as European seabass, live and have evolved in an ambient fulfill with n-3 and, to a lesser extent, with n-6 LC-PUFAs (Monroig *et al.*, 2013). In consequence, most of the marine species have barely ability, if at all, to elongate and desaturate FA chains even when they have the whole set of genes to carry out this function (Mourente & Dick, 2002; Sargent *et al.*, 2002; Mourente *et al.*, 2005b; Kabeya *et al.*, 2018). Then, LC-PUFAs are essential to marine species and, consequently, they have specific requirements (Higgs & Dong, 2000; Oliva-Teles, 2000).

Among this essential group of FAs, the most important belonging to n-3 LC-PUFAs are EPA and DHA. Both are essential and abundant in fish cell membranes and then, dietary requirements of them are larger than those of the n-6 series as ARA (Bell & Sargent, 2003; Izquierdo, 1996; Yaqoob & Calder, 2007). Low dietary EPA and DHA levels conduct to poor fish growth (Izquierdo, 2005). However, the important roles of these n-3 LC-PUFA lead to selective deposition in some tissues when dietary deficiencies are present. Thus, when low dietary levels of DHA, selective retentions of this EFA has been described in phosphoglycerides of different tissues of marine species, including nervous system (Skalli *et al.*, 2006; Tocher, 2010), liver, muscle, intestine or immune cells (Farndale *et al.*, 1999; Izquierdo *et al.*, 2003; Montero *et al.*, 2001 and 2005; Benítez-Dorta, *et al.*, 2013). Selective incorporation of EPA over dietary levels in membranes has also been found in head kidney (HK) macrophages of gilthead seabream (Montero *et al.*, 2003) and similar results have been found in plasma of that species (Ganga *et al.*, 2005) or blood leukocytes in European seabass (Farndarle *et al.*, 1999). Thus, beyond poor growth, in marine species such as European seabass, reductions in n-3 LC-PUFAs can produce other adverse effects such as high mortality, alterations in health status, variations in other FAs levels in tissues, altered tissue morphology or increased stress parameters (Izquierdo, 2005; Montero *et al.*, 2001 and 2005).

1.4.2. *Arachidonic acid*

Regarding to the n-6 series of LC-PUFAs, the fact that fish possess low ARA levels in tissues compared to n-3 LC-PUFAs has directly induced to pay less attention to determine its dietary requirements. Indeed, ARA levels present in fillet, perivisceral fat and liver of farmed European seabass have been reported to be reduced with respect to those from wild-caught fish probably due to lower dietary levels (Alasalvar *et al.*, 2002;

Bell *et al.,* 2007; Bhouri *et al.,* 2010; Fuentes *et al.,* 2010; Lenas *et al.,* 2011). However, this situation is changing in the last years due to studies that have proved important positive roles of ARA in fish lipid metabolism (Martins *et al.,* 2012; Ma *et al.,* 2018; Xu *et al.,* 2018), growth (Bessonart *et al.,* 1999; Koven *et al.,* 2003; Lund *et al.,* 2007; Bae *et al.,* 2010; Carrier *et al.,* 2011; Luo *et al.,* 2012; Montero *et al.,* 2015c; Torrecillas *et al.,* 2018a; Ding *et al.,* 2018; Ma *et al.,* 2018), survival (Bessonart *et al.,* 1999; Koven *et al.,* 2001; Atalah *et al.,* 2011; Montero *et al.,* 2015c), immune system (Hughes *et al.,* 1996; Sanderson *et al.,* 1997; Huang *et al.,* 1992; Xu *et al.,* 2010; Dantagnan *et al.,* 2017; Ding *et al.,* 2018), stress resistance (Koven *et al.,* 2001; Van Anholt *et al.,* 2004; Ganga *et al.,* 2006, 2011a and 2011b; Carrier *et al.,* 2011; Atalah *et al.,* 2011; Montero *et al.,* 2015c), smoltification and osmoregulation (Bell & Sargent, 2003; Oxley *et al.,* 2010; Van Anholt *et al.,* 2012), pigmentation (Estevez *et al.,* 1997; Villalta *et al.,* 2005; Lund *et al.,* 2007 and 2008) or egg quality (Furuita *et al.,* 2003), among others.
As for n-3 LC-PUFAs, the importance of ARA leads to its preferential retention under dietary deficiencies in different tissues where functions seem to be influenced by this EFA (Castell *et al.,* 1994; Farndale *et al.,* 1999; Montero *et al.,* 2003; Fountoulaki *et al.,* 2003; Ganga *et al.,* 2005).

Therefore, reductions of dietary ARA can expose fish to undesirable effects on growth performance and health, in particular when submitted to stress conditions as those from commercial high-density rearing, increasing the probability of significant economic losses (Farndale *et al.,* 1999). Moreover, to date, studies of optimum dietary ARA levels in Mediterranean species, such as European seabass or gilthead seabream, have been mainly made in larval stages which optimal dietary levels of ARA are settled at 1.2% for the first and up to 1% for the second, when dietary DHA and EPA levels are 1.3% and 0.7% respectively (Atalah *et al.,* 2011; Bessonart *et al.,* 1999). Whilst no specific information of specific ARA requirements exists for European seabass juveniles regarding deposition in fish tissues or effects on growth performance or immune function, those optimal levels observed for larvae of this species have been used in diets for early juveniles and larger sizes.

Furthermore, as the deficiencies cause negative physiological effects, special care must be taken when supplementing EFAs due to the fact that too high dietary inclusions of LC-PUFAs could give rise to a toxic outcome (Watanabe, 1982; Furuita *et al.,* 2003;

Blanchard *et al.*, 2008; Xu *et al.*, 2010; Luo *et al.*, 2012). On this point, ARA has demonstrated to inhibit β-oxidation when supplemented in high dietary levels, leading to fat deposition (Whelan, 1996; Xu *et al.*, 2018). ARA excess can also negatively affect growth or egg and larval quality (Xu *et al.*, 2010) probably by the already mentioned competition with EPA (Furuita *et al.*, 2003; Norambuena *et al.*, 2016). It has been demonstrated that ARA can affect insulin-like growth factor-1 (Igf-1) and growth hormone (Gh) levels which can occur through its derived eicosanoids (Montero *et al.*, 2015c). Additionally, experiments in mammals have shown that ARA-derived eicosanoid prostaglandin E2 (PGE2) has inhibitory effects in muscle fibre formation and stimulate protein degradation (Bell & Sargent, 2003).

Therefore, the lack of information concerning ARA minimal and optimal levels for European seabass juveniles increases the risks of making dietary FM/FO substitutions.

1.5. EFFECTS OF DIETARY FISH OIL SUBSTITUTIONS IN FISH

Plant sources are notably rich in 18 carbons FAs, but they lack larger FAs, which implies potential nutritional deficiencies when utilized to substitute FO in farmed fish diets (Monroig *et al.*, 2013). Nevertheless, substitutions of FO are less problematic in freshwater fish because of the increment in gene expression of desaturase and elongase enzymes provoked by nutritional regulation likely stimulated due to the lack of dietary LC-PUFAs and/or the increased intake of short PUFAs (Bell *et al.*, 2002; Tocher, 2003; Zheng *et al.*, 2004). This situation is different for marine species. Thus, in European seabass the dietary influence of VO on elongation-desaturation has been shown to be null/undetectable or minimal depending of cell type (Mourente & Dick, 2002; Mourente *et al.*, 2005b, Geay *et al.*, 2010; Torrecillas *et al.*, 2017b). A synthesis of associated effects of FO substitutions by VO in main farmed species of European aquaculture and inclusion levels is detailed in Table 2.

Different approaches have been made to provide fish with tools that allow them to exploit their genetic ability to biosynthesize LC-PUFA, as breeding selection (Bell *et al.*, 2010; Le Boucher *et al.*, 2011; Teoh *et al.*, 2011). Besides, completely new strategies, as genetic modifications, are being studied currently but they have not been able to induce high levels of these FAs in farming conditions yet, so nutritional supplementation is the most reliable instrument nowadays (Kabeya *et al.*, 2014; Tocher,

2015). As a result, introduction of vegetable oils (VO) in fish diets must be realized with care to avoid EFAs deficiencies, altered n-3/n-6 ratios and the many related changes in fish tissues and their functions (Turchini *et al.*, 2009; Montero & Izquierdo, 2010).

1.5.1. *A view on dietary fish oil substitutions effects in fish growth*

1.5.1.1. Freshwater species

As previously mentioned, one of the most important limitations of FO replacements by alternative sources in farmed fish is not to cope EFA requirements for achieving an adequate growth performance. On one hand, high levels of FO substitutions up to 50-100% can be done for salmonids as rainbow trout (*Oncorhynchus mykiss)* or Atlantic salmon (*Salmo salar*), using different blends of VO without affecting growth or survival due to their ability to synthesize LC-PUFA (Bell *et al.*, 2001 and 2002; Caballero *et al.*, 2002; Zheng *et al.*, 2004; Torstensen *et al.*, 2000 and 2005). Similarly, for Atlantic cod (*Gadus morhua*) and Nile tilapia (*Oreochromis niloticus*), dietary VO inclusion levels up to 100%, did not reduce fish growth performance compared to fish fed total FO based diets (Bell *et al.*, 2006; Hixson *et al.*, 2013; Ayisi *et al.*, 2018) and even promoted growth performance in tilapia (Ayisi *et al.*, 2018). On the other hand, some studies have demonstrated that different VO sources, levels or experimental conditions can indeed negatively affect growth of freshwater species as Atlantic cod, Mozambique tilapia (*Oreochromis mossambicus*), red hybrid tilapia (*Oreochromis sp.*) or Nile tilapia (Hixson & Parrish, 2014; Demir *et al.*, 2014; Teoh & Ng, 2016; Ayisi *et al.*, 2018; Apraku *et al.*, 2017). Therefore, even when most freshwater species can utilize more efficiently 18:C PUFAs, it is important to balance adequately the dietary level of n-3/n-6 FA (Bransden *et al.*, 2003). This is because of the different FA utilization and the competitions between FA to be used by Δ6 desaturase (Rosenlund *et al.*, 2001; Bell *et al.*, 2001 and 2002; Caballero *et al.*, 2002). Eventually, the n-3/n-6 balance can also affect protein utilization for growth (Torstensen *et al.*, 2005). Moreover, digestibility of FA is directly affected by the grade of unsaturation which is known to be increased with the number of double bonds and the shorter length of carboxylic chains (Francis *et al.*, 2007). Besides, the imbalance of unsaturated-saturated FA has showed to produce a negative effect on feed conversion ratio (FCR) in trout (Caballero *et al.*, 2002).

1.5.1.2. Marine species

For more susceptible species to VO, as European seabass, substitution levels up to 60% of FO have been successfully tested using different singular sources or blends and considering the adequate lipid content and EFA levels (Izquierdo *et al.*, 2003; Montero *et al.*, 2005; Mourente *et al.*, 2005a; Richard *et al.*, 2006). For gilthead seabream, 70% of FO substitution by VO was tested without negatives effects on growth performance whereas higher levels and the inclusion of different sources resulted in reduced weight gain (Izquierdo *et al.*, 2005; Mourente & Bell, 2006; Montero *et al.*, 2005, 2008 and 2010). In other species, as Senegalese sole (*Solea senegalensis*), growth performance was not affected when fish were fed with a plant protein-based diet supplemented with 50% or 100% of a VO mix (Conde-Sieira *et al.*, 2018) or with 100% using two different singular VO (Montero *et al.*, 2015b).

For European seabass, total FO substitution using a VO for 60 days did not affect growth performance and it was correlated with better energy/meal content and satisfactory LC-PUFA supplementation in diets (Torrecillas *et al.*, 2016).
As pointed out by the authors, another factor that explains inconsistencies seems to be the time of feeding. Indeed, previous experiments with supplementation levels of 80% have found growth differences in gilthead seabream after 6 months but not before (Caballero *et al.*, 2004). Similarly, for this species, over 80 days of total depletion of FO, provoked deleterious effects in fish performance (Montero *et al.*, 2008 and 2010).
These results demonstrated the importance of the selected source, level, EFA requirements, and time of supplementation, to avoid deleterious effects on growth of high levels of FM and FO substitution in marine fish diets.

1.5.2. *Effects of dietary fish oil substitutions in fish health: from eicosanoids to cellular and humoral immunity*

Changes in tissue FAs by alternative oils can affect cellular morphology as it has been shown in blood cells membranes in trout or enterocytes in gilthead seabream (Leray *et al.*, 1986; Caballero *et al.*, 2003). Reductions of FO and the inclusion of VO implies the induction of changes in the FA composition of cell membranes which are connected to fish health as the storage place of LC-PUFAs in its constituent lipids. These changes can affect different organs. It is known that EPA, DHA, and ARA exert their actions by

controlling structure, fluidity and functionality of cell membranes and as precursors of carboxylic acid chains called eicosanoids that act as paracrine hormones (Tocher, 2003). Eicosanoids, including prostaglandins (PGs), leukotrienes (LTs), lipoxins (LXs) or thromboxanes (TXs), have a short life and cannot be stored by cells (Smith & Murphy, 2016). These hormones are synthesized principally from ARA and EPA, influencing a broad range of physiological functions and constitute a link between nutrition and immunity (Lieb *et al.,* 2001). It is also noteworthy that, as for the esterification on lipids, ARA competes against EPA for the same enzymes to synthesise eicosanoids and additionally, their derived-eicosanoids compete for the same cell membrane receptors (Sargent *et al.,* 1999a). The occurrence of EFA imbalances in substituted diets would affect n-3/n-6 ratios, especially EPA/ARA ratios, which it is known to affect the equilibrium in eicosanoid synthesis (Bell *et al.,* 1998; Sargent *et al.,* 1999a; Montero *et al.,* 2008; Montero & Izquierdo, 2010; Norambuena *et al.,* 2016). This has been demonstrated for European seabass (Mourente *et al.,* 2007) and other species (Ganga *et al.,* 2005; Baltzegar *et al.,* 2013). These modifications can be different depending on substitutions level (Gjøen *et al.,* 2004) and on source (Bell *et al.,* 1996a). Affections of organ morphology induced by dietary FA changes can also produce health-side effects. For instance, liver steatosis was found related to reduced immune parameters as complement activity since it has been shown that fish hepatocytes, as in mammals, produce some complement components and a reduction of this production is related to a reduction of total plasma proteins (Abelseth *et al.,* 2003; Sitjá-Bobadilla *et al.,* 2005).

Therefore, even when growth is not affected, health side effects of VO must be considered. In this line, Senegalese sole fed with two different VO as the only oil source have a different performance in the relative expression of different immune-related genes (Montero *et al.,* 2015b). For the same species, a plant protein-based diet supplemented with 50% or 100% of a VO mix affected humoral immune parameters as plasma lysozyme levels and reduced peroxidase activity when administered altogether the application of a stressor, whereas the complement activity was differently influenced by experimental parameters (Conde-Sieira *et al.,* 2018).

For gilthead seabream, long feeding periods with two different VO sources affected humoral and/or cellular immunity by reducing serum alternative complement activity and phagocytic activity of HK macrophages (Montero *et al.,* 2003). In that study,

authors did not find effects for short feeding periods or using mixes of these VO, indicating that FA profile is better in blends of sources (Montero *et al.*, 2003). Thus, impaired function was related to altered FA profiles of immune cell membranes (Montero *et al.*, 2003).

Moreover, preferential retention of EPA and ARA in HK macrophages membranes, to maintain EPA/ARA ratios, denotes a great importance for cellular functions (Montero *et al.*, 2003) in agreement with previous findings in European seabass leukocytes (Farndale *et al.*, 1999).

But FA profiles from vegetal sources can alter other parameters of fish health. Hence, terrestrial oils are related to reduced immune cell activity as it has been demonstrated for European seabass (Mourente *et al.*, 2007). Equally, partial replacement of FO using a mix of VO in diets for European seabass affected the number of circulating leukocytes and the HK macrophages respiratory burst (Mourente *et al.*, 2005a). In gilthead seabream, FO replacement at 70-100% can reduce bactericidal activity of serum during feeding period or after challenge assay likely due to low LC-PUFA levels and/or high n-6 PUFA levels in immune cells membranes both related to health negative effects, whereas fish fed LO supplemented diets showed higher activity of serum lysozyme after infection (Montero *et al.*, 2010). In salmonids, as Atlantic salmon, a blend of vegetal sources can potentially reduce the number of circulating leucocytes/granulocytes and decrease serum lysozyme activity (Petropoulos *et al.*, 2009).

The alteration of immune-cell function can be reflected in the expression of proinflammatory cytokines. Thus, intraperitoneal stimulation in gilthead seabream with polyinosinic:polycytidylic acid or a bacterial suspension after feeding with VO supplemented diets can modify the immunological regulation of type I interferon (Ifn) which is reflected by the hepatic expression of the protein of resistance against myxovirus, an interferon-induced protein (Montero *et al.*, 2008). Furthermore, the expression of proinflammatory cytokines analyzed in HK and intestine, showed that VO tended to increase tumor necrosis factor alpha (Tnfα) and reduced interleukin 1 beta (Il-1β) in both tissues (Montero *et al.*, 2010). Moreover, after infection, those cytokines showed an affected patter of expression in the intestine and HK (Montero *et al.*, 2010).

Table 2. Some results of FO substitutions with VO in main farmed species of European aquaculture.

Farmed species	Sources and substitution level	Length of feeding period	Results	References
Atlantic salmon (*Salmo salar*)	100% of SFO or LO	12 weeks	VO ↕ FA profile in heart & blood cells. ↓ TXB2 & PGE2 in LO fed fish. SFO → cardiac lesson & ↑ *pla2*. LO ↓ PC levels & ↕ FA in heart.	Bell *et al.*, 1993a
	100% of SBO or LO	12 weeks	Dietary FA ↕ FA profile in tissues & GPs. VO → ↕ eicosanoids.	Bell *et al.*, 1993b
	100% of SBO or LO	12 weeks	SBO → ↓ *pla2*. SBO → ↓ PGF3α & ↑ PGF2α & PGF2α/PGF3α in gills. SBO → ↑ PGE2, PGE3 & PGE2/PGE3 in HK.	Bell *et al.*, 1996a
	100% SFO	Trial I: 150 days; trial II: 112 days.	Trial I: SFO → ↓ weight & K but ↑ HSI & ISM. Trial II: growth ≡. SFO → ↓ plaquet-forming cells in kidney & spleen. Immunological parameters ≡. Challenge I: with *Aeromonas salmonicida*, ½ group VC/ ½ group UN; SFO-UN ↑ mortalities & SFO-VC ↓ mortalities (even than FO-VC). Challenge II: with *Vibrio anguillarum*. SFO → ↓ mortalities than FO. SFO → ↓ B cells activity in HK & spleen.	Thompson *et al.*, 1996
	100% or 50% SFO; 100% PO	21 weeks	Growth ≡. ↕ FA in lipoproteins & ↕ plasma levels. ↑ lipid content in liver. ↕ FA profile & level in tissues. PO ↓ apparent digestibility of FA.	Torstensen *et al.*, 2000
	10%RO, 25%RO, 50%RO or 100% RO	17 weeks	Growth & survival ≡. VO → ↓ muscle lipid, ↑ hepatic desaturation & elongation activities. 110%RO → ↑ lipid liver level. VO ↕ FA in muscle. + 50%VO → ↓ n-3/n-6 PUFA ratio, EPA & DHA in flesh.	Bell *et al.*, 2001
	25%PO, 50%PO or 100%PO	30 weeks	Growth & survival ≡. 50%PO & 100%PO → ↓↓ lipid deposition. ↕FA profile in liver & muscle. 100%PO ↑ hepatic desaturation. VO → ↑ elongation activities. +50%VO ↓ n-3 LC-PUFA in flesh.	Bell *et al.*, 2002
	4-100% SFO	63 days	Growth ≡. ↕ FA profile in tissues. +4% SFO (muscle & liver) & 20% SFO (carcass) ↕ n-3/n-6 ratios. Challenge mortalities not correlated with VO.	Bransden *et al.*, 2003
	25-100% LO	40 weeks	↑ VO levels after 20 weeks ↑ desaturate & elongase activities in liver. LO for 40 weeks ↑ LC-PUFA biosynthesis.	Zheng *et al.*, 2004

	75 or 100% with a mix of RO, PO, LO (3.7:2:1)	~1 year	Overall same results in groups from 2 different locations. 100% VO ↑ growth & protein utilization in fish fed during seawater winter period. VO → ↕ FA profile.	Torstensen *et al.*, 2005
	50 or 100% SBO	5°C (27 weeks) & 12°C (11 weeks)	Growth & survival ≡. VO ↕ FA profile of HK & liver. *In vitro*, 50% SBO ↕ Lox (LTB4 production) activity in HK macrophages of fish fed at 5°C.	Gjøen *et al.*, 2004
	100%LO, 100%RO, 100%SBO	16 weeks	VO ↕ FA profile in the whole fish. LO ↓ final weight. LO ↓ whole body lipid & ↑ the protein:lipid ratio. RO ↑ liver malic enzyme activity & hepatic malic enzyme gene expression, LO and RO ↑ hepatic BOX. VO ↑ genes of cholesterol biosynthesis pathway, relative percentage of protein in the whole fish & LC-PUFA synthesis in liver microsomes.	Leaver *et al., 2008*
	100% with a mix of CAO, RO & PO (1:2.5:1.5)	42 weeks	Trial with a (1) commercial stock, (2) "lean" or a (3) "fat" strain (lipid content in muscle). Growth ≡. VO → ↑ liver deposition & ↓ hematocrit. VO → ↕ FA in tissues. Strain (2) with ↑ Lyz activity (independently of food). VO → ↓ circulating leucocytes in (3) & ↑ granulocyte number in (1) than (2).	Petropoulos *et al.*, 2009
	100% with a mix of CAO, RO & PO (5:3:2)	55 weeks	Interaction between diet & fish family ↕ weight & lipid content. VO ↓ of n-3 LC-PUFA.	Bell *et al.*, 2010
	43% (SBM and wheat)	47 days	SBM → enteritis. ↑ *mhcii-β*-reactive cells in the lamina propria and submucosa, & ↓ in the epithelium and brush border.	Romarheim *et al.*, 2013
	80% with a blend in three diets where major components were either OO, RO or SBO	28 weeks	VO ↓ folds in mid intestine. SBO ↓ height of complex folds in distal intestine. SBO & OO ↓ wall thickness in distal intestine. VO → ↕ *cd3, mhcii-β* & *tgf-β* in pyloric caeca & *cd3, IgM, IgT* in distal intestine.	Moldal *et al.*, 2014
Rainbow trout (*Oncorhynchus mykiss*)	100% SBO or 100% SB lecithin	25 days	SBO → lipid droplet accumulation in enterocytes with cellular damage in anterior & middle intestine. SBO ↓ free cholesterol. VO ↓ the n-3/n-6 ratio in all digestive tissues. VO → ↕ PC & PE levels.	Olsen *et al.*, 2003
	(1) 50%SBO, (2) 61% RO, (3) 30% OO+60%lard, (4) 40%RO+40%PO	64 days	Growth ≡. FCR ↑ & apparent lipid digestibility ↓ by (2) & ↑ by (3). ↑ number of lipid droplets in enterocytes of (1), (2), (4) & hepatocytes of all groups.	Caballero *et al.*, 2002
	100% SAFO or LO	18 months	LO ↑↑ reactive oxygen production.	Kiron *et al.*, 2011

	100% CO; 100% SAFO; 100% LO; 100% with a blend of 40%CO:30%SAFO:30%LO	60 days	SO & LO ↓ growth. CO↓ goblet cells; SAFO & LO ↑ goblet cells. SAFO & LO ↑ height of intestinal epithelial cells. SAFO ↓ but CO & LO ↑ height of intestinal folds. CO, LO & blend ↑ diameter of intestinal folds. CO, SAFO & blend ↑ thickness of tunica mucosa. SAFO & LO ↓ tunica compactum. SAFO & LO ↓ but blend ↑ thickness of tunica submucosa. CO & SAFO ↑ but blend ↓ thickness of tunica muscularis.	Shahrooz *et al.*, 2018
	D1=100%CO; D2=100%FXO; D3=50%CO, D4=50%FXO, D5=50%CO-50%FXO, D6=33%FO-33%FXO-33%CO	8 weeks	VO ↕ FA in fillet. D2 & D4 ↓ K. D2 ↑ PER. D4 ↑ protein & lipid in fillet.	Masiha *et al.*, 2013
Gilthead seabream (*Sparus aurata*)	EI & EII: 60%SBO, 60%RO, 60%LO & 60% of SBO+RO+LO EII: 80%SBO & 80% LO	Experiment I:101 days Experiment II: 204 days	EI: No effects. EII: 80% ↓ growth. SBO ↓ serum ACPH activity & HK phagocytic activity. RO ↓ HK phagocytic activity. VO changed patterns of stress response. VO → ↕ FA profile of HK macrophages. Selective incorporation of EFAs in HK macrophages & circulating erythrocytes. 60%SBO & 60% LO ↓ number of circulating erythrocytes.	Montero *et al.*, 2003
	60%SBO, 60%RO, 60%LO & a 60% of a mix including SBO, RO, LO (1:3:6)	101 days	Growth ≡. VO → ↕ FA in liver & muscle. Preferential retention of DHA in liver.	Izquierdo *et al.*, 2003
	60% or 80% with LO or SO: 60%LO, 80%LO, 60%SO, 80%SO	7 months	LO & 80%SO ↓ weight. 80%SO ↓ SGR & ↑ FCR. VO ↓ HSI & ↕ FA profile in liver & muscle. SO ↑ liver lipid deposition. SO ↓ peroxidation in muscle. VO ↓ lipogenesis in liver & increased β-oxidation in muscle, proportionally to inclusion level.	Menoyo *et al.*, 2004
	60%-80%SBO; 60%-80% LO; 60% RO	Short period: 3 months Long period: 6 months	Survival ≡. In the long period, 80%VO ↓ growth than FO. 60%SBO → ↑↑ steatosis.	Caballero *et al.*, 2004
	60%-80%SBO; 60%-80% LO; 60% RO	6-7 months	80%VO ↓ growth. VO → ↕ FA profile in muscle.	Izquierdo *et al.*, 2005
	60% or 100% substitution with a mix of 15RO:60LO:25PO [diets 60LO & 100LO] & 60% with a mix 40RO:40LO:20PO [diet 60RO]	281 days	VO → ↕ FA profile plasma. 100LO ↓ PGE3. Leptin plasma levels were negatively correlated with PGE2.	Ganga *et al.*, 2005

60%SBO, 60%RO, 80%SBO	3 months	Glycerol-3-phosphate pathway related to GPs synthesis. Monoacylglycerol pathway related to TAG synthesis. RO ↓ reacylation activity & ↑ lipid droplets in enterocytes. SBO ↑ PC synthesis & ↑ VLDL.	Caballero *et al.*, 2006
33%, 66% & 100% (1RO:3.4LO: 1.5PO)	11 weeks	Survival ≡. 100%VO ↓ growth & feed intake. 33%VO ↑ plasma *igf-I* & ↓ circulating *gh* with the ↓. 100%VO ↓ *igf-I* & ↑ *gh*. 66%VO → ↑↑ *ghr-II* expression.	Benedito-Palos *et al.*, 2007
	8 months	Survival ≡. 100%VO ↓ SGR, feed intake & weight. VO → signs of lipoid liver disease & ↕ FA profiles in muscle with selective incorporation of LC-PUFA in polar lipids.	Benedito-Palos *et al.*, 2008
100%LO, 100% SBO, 100% of a mix with LO & SBO (1:1)	6 months	100%VO ↓ growth. 100%LO & 100%SBO ↓ HK phagocytic activity & serum ACPH activity. VO ↑ basal levels of protein of resistance against mixovirus.	Montero *et al.*, 2008
70 or 100% LO, 70 or 100% SBO	80 days	Feeding trial: 100%VO ↓ growth. In intestine, proinflammatory cytokines expression ≡. SBO ↑ *tnfα* HK. Challenge: LO ↑ & SBO ↓ serum bactericidal activity. 100%VO ↑ proinflammatory cytokines in the intestine. 100%SBO ↑ *tnfα* levels in HK at the beginning & the end. SBO & 100%LO ↑ *il-1β*.	Montero *et al.*, 2010
70 or 100% LO; 70-100% SBO	26 weeks	VO → ↕ FA profile of HK membranes. LO ↑ cortisol stimulation factor. *In vitro* eicosanoid inhibition ↓ cortisol of fish fed LO & 70% SBO ↑ its level.	Ganga *et al.*, 2011a
(1) 70%LO, (2) 70%SBO, (3) 20%LO+50%SBO, (4) 50%LO+20%SBO, (5) 100%LO, (6) 100%SBO, (7) 100% LO+SBO (1:1)	8 months	VO ↕ FA in HK. Diets (2), (4), (5) & (7) ↑ basal cortisol. Crowding challenge, LO ↑ cortisol levels & SBO ↕ pattern of response.	Ganga *et al.*, 2011b
66% (17%RO58%LO25%PO)	9 months of feeding & 102 days of infection	Growth ≡. VO ↑ symptoms of parasitosis (↓ growth, K factor, hematocrit but ↑ anorexia, intensity & extension of infection). VO in not infected group ↑ activity of serum complement & TAS. VO in infected group ↓ hepatic synthesis of glutathione & hepatic *gsh*/*hssh* ratio.	Estensoro *et al.*, 2011
66% (17%RO58%LO25%PO)	9 months of feeding & 102 days of infection	VO in infected fish ↑ genes & ↑ degree of fold-change variation of gene expression.	Calduch-Giner *et al.*, 2012

		10 weeks	Growth & survival ≡. VO during stress challenge ↑ plasma cortisol & glycaemia & rapid ↓ of cortisol level after stress challenge. VO ↕ gene expression in liver.	Pérez-Sánchez *et al.*, 2013
	70% (20%RO50%LO30%PO)	81 days	Growth & survival ≡. VO ↓ plasma GPs & cholesterol VO → ↕ FA in tissues. VO ↓ *fads2* in intestine & ↑ in liver. VO + high dietary carbohydrate ↓ *pparβ* & ↑ *elovl5* in the intestine.	Castro *et al.*, 2016
	D1= 100VO/0FO, D2= 88.5VO/11.5FO, D3= 83.1VO/16.9FO, D4= 71.2VO/28.8FO, D5= 47.4VO/52.6FO, D6= 0VO/100FO	18 weeks	↑ VO content ↓ weight & SGR. VO ↕ total lipid content in liver, mid intestine & viscera. VO → ↕ FA content in liver & mid-intestine. VO ↕ differently metabolism-related genes (*pparα1, cpt1α & fabp1, srebp1, srebp2*, *fas* & *fads2*) in liver & mid intestine.	Houston *et al.*, 2017
	EI= D1: 33%(1RO:3.4LO: 1.5PO) EII= DD1: FM25%FO100% DD2: FM5% FO42% DD3: FM5%FO16%	EII: 20 months	VO ↓ microbiota diversity, with ↓↓↓ of *Vibrio spp.* in DD3. VO ↑ parasite infection, disease signs & inflammation. VO ↓ gut integrity & function and → ↕ in gut proteome.	Piazzon *et al.*, 2017
	3% DM or 6% DM with a blend (LO, RO, PO) in two different proportions (1:0.05:0.45) & (0.64:0.51:1)	8 weeks	Growth ≡. VO → ↕ FA in tissues. ↓ FO level ↑ liver gene expression of *Δ6-d, pparα, ech* & *cel.*	Torno *et al.*, 2018
European seabass (*Dicentrarchus labrax*)	60% RO; 60% LO; 60% OO	34 weeks	Survival & growth ≡. 60%RO ↓ liver mass & HSI. RO & LO ↓ desaturation levels. VO → ↓ nutritional regulation of FA desaturation in hepatocytes.	Mourente & Dick, 2002
	60%SBO, 60%RO, 60%LO & a 60% of a mix including SBO, RO, LO (1:3:6)	89 days	Growth ≡. VO → ↕ FA in liver & muscle. Preferential retention of DHA in liver.	Izquierdo *et al.*, 2003
	60%SBO, 60% RO, 60% & 80%LO	8 months	80%LO & 60%RO ↓ growth. VO → ↕ FA profiles in flesh & liver.	Montero *et al.*, 2005
	60%RO; 60%LO; 60%OO	34 weeks	Growth & survival ≡. VO → ↕ FA profiles. Selective deposition of DHA. VO ↓ total circulating leucocytes & burst respiratory activity of HK macrophages. RO ↓ absorptive vacuoles in intestine. LO & RO livers with ↑ fat levels & ↓ n-3 LC-PUFA in liver.	Mourente *et al.*, 2005a
	60% using DB=LO35%PO15%RO10%, or DC= LO24%PO12%RO24%	64 weeks	Growth & survival ≡. VO → ↕ FA profiles & → FA desaturation in hepatocytes. VO → ↑ desaturation in pyloric caeca enterocytes than in liver. VO → ↑ β-oxidation rates than desaturation rates without effects. DC ↓ β-oxidation of 18:3 n-3 in enterocytes.	Mourente *et al.*, 2005b

		DC ↓ lipoprotein lipase white muscle. DB & DC ↓ VLDL, LDL & cholesterol levels in plasma.	Richard *et al.*, 2006
		DB ↓ growth & liver mass. VO → ↕ FA profiles in muscle & liver with selective retention of DHA in both tissues.	Mourente & Bell, 2006.
		DB ↓ live mass, liver mass & plasma PGE2. DC ↑ flesh dry mass. DB & DC ↕ FA composition of perivisceral blood leucocytes. DB & DC ↓ respiratory burst activity. VO → ↕ FA profiles in liver & blood leucocytes.	Mourente *et al.*, 2007
VD= 100PP/100VO	9 months	VD ↓ growth. Survival ≡. VD → ↕ FA profiles in flesh lipid classes. VD ↓ plasma Lyz activity & ↑ plasma complement activity. VD ↕ genes of lipid metabolism, protein/AA metabolism, carbohydrate metabolism, immune function & blood coagulation.	Geay *et al.*, 2011
70% (20%RO50%LO30%PO)	73 days	Growth & survival ≡. VO ↕ FA profiles in liver & muscle. VO ↓ plasma GPs. Lipid source ↕ gene expression in liver.	Castro *et al.*, 2015a
	36 days	Intestine: VO ↑ Gpx and Gr activities & ↓ LPO activity.	Castro *et al.*, 2015b
	73 days	Growth, survival & feed utilization ≡. VO → ↕ FA profiles in muscle & liver. VO ↓ GPs. VO ↑ hepatic genes of cholesterol metabolism.	Castro *et al.*, 2015c
		VO ↓ intestinal lipid peroxidation & ↑ *gpx* & *gr* in the intestine.	Castro *et al.*, 2015d
100% SBO	60 days	Growth ≡. SBO ↕ immune-related genes (↓ *cox-2, IgM, il-1β, tgf-β* and *il-10*, but ↑ *mhcii-β*), ↓ intestinal fold length, ↕ FA profiles in the intestine, ↑ goblet cells size & ↓ cell density in anterior intestine.	Torrecillas *et al.*, 2015b
		Growth ≡. VO → ↕ FA profiles in muscle, liver & anterior intestine.	Torrecillas *et al.*, 2016
75% (58/15); 94% (20/6; 10/6 & 5/6) 97% (20/3; 10/3 & 5/3); 100% & 100%+LC-PUFA. A blend (1LO:2RO:2PO)	90 days	Growth ↕ by FM level. Inclusion level ↕ morphology and FA profiles in anterior and distal intestine. Number of goblet cells correlated to meal and oil sources. Diets ↕ *il-1β, tnfα* genes expressions but *il-10* ≡. Microbiota richness and diversity ≡.	Torrecillas *et al.*, 2017a

			VO ↓ growth, the ↓↓ in 100% without LC-PUFA. VO ↑ liver to body ratios, liver deposition & hepatocellular size. VO → ↕ FA profiles in liver & muscle. VO ↑ activities of Elovl6 & Elovl5.	Torrecillas *et al.*, 2017b
	94% (20/6 & 5/6) 97% (20/3 & 5/3)	153 days	94 to 97%VO ↑ *ex vivo* & *in vivo* gut bacterial translocation.	Torrecillas *et al.*, 2017d
	70% (20%RO50%LO30%PO)	73 days	VO ↑ Lyz activity. VO + carbohydrate ↑ peroxidase activity & ↓ NO & antiproteases activity. VO in interaction with stress & carbohydrates influenced plasma antiproteases & bactericidal activity. VO + carbohydrates ↓ *mc2r*. VO + stress ↕ *gr1* & *cox-2*.	Machado *et al.*, 2019

CAO = camelina oil, CO = canola oil, FXO = flaxseed oil, LO = linseed oil, OO = olive oil, PO = palm oil, RO = rapeseed oil, SAFO = safflower oil, SB =soybean, SBO = soybean oil, SFO = sunflower oil. ≡ = unaffected; ↕ = altered/affected; ↑ = high/increased; ↓ = low/decreased; → = produced/induced.

ACPH = alternative complement pathway. BOX = total palmitoyl CoA Beta-oxidation. DHA = docosohexaenoic acid. EPA = eicosapentaenoic acid. FA = fatty acid. HK = head kidney. FO = fish oil. FM = fish meal. LC-PUFA = long chain polyunsaturated fatty acids. LT = leukotriene. NO = nitric oxide. PC = phosphatidylcholine. PE = phosphatidylethanolamine. PG = prostaglandin. PP = plant protein. TAG = triacylglycerides. TAS = total antioxidant status. TX = thromboxane. UN = unvaccinated. VC = vaccinated. VD = vegetable diet. VO = vegetable oil.

Parameters and indexes: FCR = feed efficiency ratio. HSI = hepatosomatic index. ISM = spleen-somatic index. K = condition factor. PER = protein efficiency ratio. SGR = specific growth rate.

Genes: *cd3* = cluster of differentiation *3*. *cel* = carboxyl ester lipase; *cox-2* = cyclooxygenase 2; *cpt1α* = carnitine palmitoyl transferase I alpha; *Δ6-d* = delta 6-desaturase; *ech* = enoyl-CoA hydratase; *elovl* = elongation of very long chain fatty acids protein. *fabp1* = fatty acid binding protein; *fads2* = fatty acid desaturase 2; *fas* = fatty acid synthase; *gh*= growth hormone*; ghr-II* = growth hormone receptor II; *igM* = immunoglobulin M*; igT* =immunoglobulin T*; igf-I* = insulin-like growth factor; *il-1β* = interleukin 1 beta; *il-10* = interleukin 10; *mc2r* = melanocortin 2 receptor; *mhcii-β* = major histocompatibility complex ii beta. *pla2* = phospholipase A2. *ppar* = peroxisome proliferator-activated receptor; *srebp* = sterol regulatory element binding protein; *tgf-β* = transforming growth factor beta.

Proteins: Gpx = glutathione peroxidase. Gr = glutathione reductase. Gr1= glucocorticoid receptor 1. LDL = low-density lipoprotein. Lox = lipoxygenase. Lpo = hepatic and intestinal lipid peroxidation. Lyz = lysozyme. VLDL = very low-density lipoprotein.

1.5.3. *Critical combinations of very high dietary fish meal/fish oil substitutions on European seabass*

FM can contain an important percentage of LC-PUFAs (Turchini *et al.,* 2009), contributing to meet fish nutritional requirements, so reducing its levels in combination with a reduction of FO, can magnify deficiencies. Thus, in European seabass, 100% substitutions of FM and FO with alternative sources for 90 days induced severe reductions in weight gain and fish length, but when LC-PUFAs were supplemented, specific growth rate (SGR) was improved (Torrecillas *et al.,* 2017a), indicating the already-described importance of these macronutrients. The affectation in growth agreed with previous results in this species fed FM/FO free diets (Geay *et al.,* 2011, Le Boucher *et al.,* 2011 and 2013). More, the presence of dietary LC-PUFAs was associated with the existence of a more adequate environment for healthy microbiota (Torrecillas *et al.,* 2017a). However, when dietary FO was reduced from 6% down to a 3% (percentage of total dietary ingredients) in diet, growth was not affected if FM was included up to a 10%, but it decreased with lower FM levels (Torrecillas *et al.,* 2017a, 2017d), indicating the great importance of protein sources for a proper performance. Similarly, in Atlantic salmon, protein sources demonstrated to affect greatly growth in the long term (Torstensen *et al.,* 2008). In both species, reductions of FM led to diminished feed intake in the short term (Torrecillas *et al.,* 2017b, Torstensen *et al.,* 2008).

Apart from growth, total replacement of FO and FM in European seabass affected anterior and distal intestine morphology distinctly and it was related to lipid absorption processes and low FM in each region respectively (Torrecillas *et al.,* 2017a). Likewise, affectation in distal intestine morphology was correlated with a defensive response and changes in natural microbiota, pointing out the high sensitivity of this tissue to alternative sources and the strong relation with the immune system (Van den Ingh *et al.,* 1991; Miao *et al.,* 2017; Vigneulle & Laurencin, 1991; Torrecillas *et al.,* 2011a and 2013). However, in another experiment, FM reductions did not affect liver composition, an indicator of AA mismatches, but indeed FO replacement affected lipid metabolism, altering the content and producing lipid accumulation (Torrecillas *et al.,* 2017b). In a similar way, European seabass fed totally FM/FO substituted diets, showed

modifications of hepatic lipid metabolism as well as negative effects on health (Geay *et al.*, 2011).

Eventually, high FO/FM dietary reductions lead to affections of the immune response and infection resistance. Hence, extreme FO reductions down to 3% (percentage of total dietary ingredients) were related to increased bacteria trespassing through epithelial barrier whereas FM reductions down to 5% produced a proinflammatory response after feeding period that makes fish less competent to face an intestinal bacterial infection (Torrecillas *et al.*, 2017d).

1.6. ADDING PREBIOTICS AND PROBIOTICS AS FUNCTIONAL INGREDIENTS IN AQUAFEEDS

As detailed above, high levels of FM and FO substitutions can be made up to a certain level for European seabass with adequate supplementation of several nutrients as micronutrients or GPs, among others, without growth or health problems under experimental conditions.

Nevertheless, when fish are fed with high substituted diets under commercial conditions, they can not only develop poor growth but functional and morphological tissue alterations that may reduce their capacity to face or counteract homeostasis changes and consequently they will be predisposed to the appearance of generalized infection outbreaks (Izquierdo, 2005; Montero *et al.*, 2001 and 2005).
In addition, the usual response against infections in aquaculture has been the use of antibiotics and chemicals, but those can induce bacterial resistances and modify natural microbiota profiles thus predisposing fish to future complications, including new infections, as it has been demonstrated (Becattini *et al.*, 2016; Falony *et al.*, 2016; Zhernakova *et al.*, 2016).

Therefore, functional ingredients can be used to counteract or reduce somehow undesirable consequences of low FM/FO based diets in fish health and growth performance, and in microbiota ecosystems (Panigrahi & Azad, 2007; Kiron, 2012). Those ingredients are considered functional due to their capacity to influence different functions at the same time (Roberfroid, 2000). Thus, during the last decade, prebiotics, probiotics and other compounds such organic acids or essential oils, have been proposed as promising candidates to enhance fish immune status and growth performance of fish

fed plant-based diets (Torrecillas *et al.*, 2015b and 2016; Rimoldi *et al.*, 2016, Terova *et al.*, 2016).

1.6.1. *Prebiotics: mannan oligosaccharides in diets for marine fish species*

There are many official definitions of prebiotics but, in general, the term can be explained as feeding elements, non-digestible by the host, with probed positive effects for resident *biota* and on diverse aspects of fish physiology as growth and/or immune system (Gibson & Roberfroid, 1995; Burr *et al.*, 2005; Dimitroglou *et al.*, 2011; Song *et al.*, 2014). For that last reason, some of them are usually included in the group of immunostimulants. To accomplish their mission, prebiotics must arrive intact to the intestinal tract and there, some of these ingredients can be fermented by resident microbiota, constituting an instrument to stimulate and to reinforce beneficial bacteria populations as an additional mechanism of action (Guerreiro *et al.*, 2017; Dimitroglou *et al.*, 2011; Torrecillas *et al.*, 2014). On this regard, prebiotics have demonstrated to modulate microbial communities in fish intestine of different species such as rainbow trout, Nile tilapia, gilthead seabream or European seabass (Dimitroglou *et al.*, 2009 and 2010; Torrecillas *et al.*, 2012; Levy-Pereira *et al.*, 2018). This is particularly interesting due to substitutions can alter microbial populations in fish gut (Green *et al.*, 2013).

Prebiotics are mainly carbohydrates as inulin, β-glucans, and different types of oligosaccharides (i.e.: galacto-oligosaccharides, GOS; fructo-oligosaccharides, FOS; mannan oligosaccharides, MOS) among others, that can act directly or through intermediaries on the enhancement of passive and probably the active immunity (Dimitroglou *et al.*, 2011; Song *et al.*, 2014).

Particularly for MOS, which is the prebiotic studied in the present PhD thesis, it seems that its positive effects on fish defenses are in part derived from a nonspecific response against its presence. This could be explained by one of the modes of action of prebiotics, that consists in acting as microbe/pathogen-associated molecular patterns (MAMPs or PAMPs) to interact with pattern recognition receptors/proteins (PRRs/PRPs), which are placed on body fluids, cell cytoplasm, and on membranes of some different cells as macrophages, among others (Tort *et al.*, 2003; Russell & Lumsden, 2005; Lee & Kim, 2007). On cell surfaces, PRRs/PRPs are intermembrane

molecules, and their external activation constitutes the first step for triggering the major pathways of innate immunity through the activation of the nuclear factor kappa beta (Nf-κβ) (Aoki *et al.*, 2008; Dam & Brewer 2010) among others. These receptors present a non-catalytic carbohydrate recognition domain (CDR), actin as lectins, and recognize characteristic molecular patterns from the outer cells wall of gram-negative and gram-positive bacteria, which presence is widespread among microorganisms and then this mechanism serve to warn nonspecific immunity components about their arrival (Brown *et al.*, 2002; Russell & Lumsden, 2005; Song *et al.*, 2014). In this way, prebiotics emulate the same first action in the encounter between a pathogen and the host defenses (Huang *et al.*, 2009; Areschoug & Gordon, 2008). Thus, MOS can attach to specific carbohydrates from enterocytes surface and mimic part of the structure of some lectins as the inducible mannose-binding lectin (MBL) which can start the activation of the complement pathway via lectins pathway and the opsonization by phagocytosis (Russell & Lumsden, 2005). Very interesting reviews about prebiotics effects in fish, including a compilation of MOS effects and possible mechanism of action, have been published in the last years (Dimitroglou *et al.*, 2011; Vetvicka *et al.*, 2013; Song *et al.*, 2014; Torrecillas *et al.*, 2014; Carbone & Faggio, 2016; Guerreiro *et al.*, 2017).

A great number of pathogens use adhesins to attach MBL from gut epithelial cells as a first step of infection (Arason, 1996; Redondo & Álvarez-Pellitero, 2010). Consequently, MOS can be recognized and bound by a great number of pathogenic bacteria, which will be later flushed out with feces, interrupting colonization of animal tissues (Arason, 1996; Redondo & Álvarez-Pellitero, 2010; Torrecillas *et al.*, 2014).

Beyond immunity, dietary administration of MOS has been extensively studied in the last few years in different marine-fish species as European seabass or gilthead seabream, with positive effects in growth performance, survival, stress response, feed intake and nutrient utilization, lipid metabolism, intestinal morphology (particularly increment of absorptive surface), gut-wall integrity and increased mucus secretion even under commercial farming conditions (Salze *et al.*, 2008; Dimitroglou *et al.*, 2009 and 2010; Gültepe *et al.*, 2011 and 2012; Torrecillas *et al.*, 2007, 2011a, 2012, 2013, 2015a and 2015b, 2016; Salem *et al.*, 2015; Eryalçyn *et al.*, 2017; Gelibolu *et al.*, 2018a and 2018b).

Nowadays and despite the adequate vaccination protocols, vibriosis outbreaks still cause great economic losses and important fish mortalities in aquaculture (Jun & Woo, 2003; Toranzo *et al.*, 2005; Novriadi *et al.*, 2016), including in Western Mediterranean aquaculture (Vendramin *et al.*, 2016). It is known that those outbreaks occur mainly due to stress or ecological factors (Sorroza *et al.*, 2012). European seabass is susceptible to harmful intestinal pathogens that cause *vibriosis*, as *V. anguillarum* (Mosca *et al.*, 2014, Sepulcre *et al.*, 2007) among other *Vibrio* species. Developing new ways to deal with this bacterial pathogen has been a growing concern in recent years (Sorroza *et al.*, 2012), complementary to the use of vaccines. This *Vibrio* strain, widespread in marine environment, is commonly found on fish surfaces and/or gastrointestinal tract (Jun & Woo, 2003; Novriadi, 2016) and its increased pathogenicity has been described in the posterior section of fish intestine, probably related to pH (Ransom *et al.*, 1984). In this sense, the improvement in gut integrity, produced by the enhancement of unions between cells in the intestinal mucosal layer, has been found to reduce bacterial paracellular translocation (Ellis, 2001) and MOS have been found capable to increase this aspect of intestinal morphology (Torrecillas *et al.*, 2011a and 2011b). Therefore, the use of functional ingredients as prebiotics is also a way to complement the immunoprotection given by vaccination (Uribe *et al.*, 2011).

However, effects of prebiotics are dependent on form of presentation, dose, time of administration, fish species, age or culture conditions (Dimitroglou *et al.*, 2010 and 2011; Torrecillas *et al.*, 2014) and then, contradictory results have been reported during the last decades (Azeredo *et al.*, 2017) when one or several of these factors change. For this reason, it is very interesting to follow results of a series of experiments in the species of our interest, European seabass, with minor changes in those factors. Thus, when European seabass are fed diets with 100% of SBO, MOS reduced liver lipid content and reduced hepatocyte cytoplasmatic vacuolization (Torrecillas *et al.*, 2016). The same outcome has been found in European seabass fed FO diets supplemented with MOS, and it was related to an increased capacity of glucose production in liver and reduced lipogenic activity, so authors suggested a better use of dietary energy, feed utilization, LC-PUFA retention and, consequently, improved growth performance (Torrecillas *et al.*, 2011a, 2015a). But more, the complete substitution of FO by SBO in European seabass affected the morphology of posterior gut and its homeostasis in relation to the expression of different immune-related genes and both effects were

partially compensated, after a short period of feeding (60 days) by the inclusion of MOS that, besides, increased the number and area of mucus-secreting cells (Torrecillas *et al.*, 2015b). Therefore, MOS has demonstrated to compensate some of the frequent negative effects that high dietary SBO levels produces in marine species as European seabass gut health. Moreover, MOS (or derivatives as Bio-MOS® or c-MOS®) effects on European seabass immunity are not only restricted to the local immune system in the digestive tract, where they are administered, but have been also demonstrated to affect European seabass systemic immunity (Torrecillas *et al.*, 2007, 2011a, 2013, 2015a, 2015b).

The positive response activated by MOS in this species has been related to dietary supplementation of increased levels from 0.2 to 0.6%, finding that the overall best dietary level in relation to its functional actions in different European seabass physiological aspects as immunostimulation and growth performance optimization, is 0.4% (Torrecillas *et al.*, 2007, 2011a, 2011b, 2012, 2013, 2015b, 2016).

1.6.2. *Probiotics: the use of Pediococcus acidilactici in aquaculture*

Microbiota, mainly composed by bacteria and yeast (Gatesoupe, 2007), is highly variable between individuals from the same species raised in different locations, since fish larvae are presumably colonized not only from maternal transmission (Gómez *et al.*, 2013) but also from their immediate environment, being these ones affected by fluctuations in ecosystem features (Balcazar *et al.*, 2006a, Gatesoupe *et al.*, 2007, Dimitroglou *et al.*, 2011). Therefore, microorganisms penetrate to the digestive tract immediately after the opening of the mouth (Gómez & Balcazar, 2008) and maybe before (Ringø *et al.*, 1995). From the instant of colonization, intestinal microbiota will be a key factor that leads to the development and maturation of immune system and it means that they will be part of the defensive barriers and will contribute to the homeostasis and to the correct functioning of protective mechanisms against pathogens (Gómez & Balcázar, 2008; Dimitroglou *et al.*, 2011). Besides, it is known that the net of resident microorganisms contributes to the intestinal barrier integrity (Ashida *et al.*, 2011).

Gut microbiota populations constitute an ecosystem that will be configurated and modulated through mechanisms of competition and exclusion between the different species, during fish life (Gómez and Balcázar, 2008). These interactions must be done

without alerting the fish immune system and therefore avoiding its response at the same time that those bacteria and yeasts regulate host gene expression to transform this tract in a favorable environment (Gómez and Balcázar, 2008). The mucosal immune-tolerance is implicated in this process of settlement, being the mechanism that avoids the immune system to react against common and non-damaging antigens from food or microbiota (Kim *et al.*, 2012). Besides, microbiota can ferment some fibers from the diet to produce short-chain fatty acids (SCFA) which can modulate several immune parameters through G protein coupled receptors (Maslowsky & Mackay, 2011). It has been demonstrated that bacteria can stimulate cellular and humoral immunity of the host (Irianto & Austin, 2002; Gómez & Balcázar, 2008; Balcazar *et al.*, 2006a & 2006d) through its influence on host gene expression to induce a state of immune-tolerance which is mediated by T-cells (Nutsch & Hsieh, 2012; Tafalla *et al.*, 2016).

Bacterial species from outside, when introduced in a natural microbiota, can also have positive effects on the health of host fish (Balcázar *et al.*, 2006a). Those strains are recognized as probiotics. The term probiotic was defined elsewhere (Parker, 1974) but is mainly explained as an alive beneficial microorganism to the host that acts as a new component into the microbiota during the administration period, to stop pathogen adhesion and the consequent damage, but not only (Viera *et al.*, 2013; Hamilton *et al.*, 2015; Miao *et al.*, 2017). It must be highlighted that indigenous microbiota is very competitive and the presence and dominance of a probiotic strain will last only during the administration of high doses (Balcázar *et al.*, 2006a).

Moreover, microbial interactions and host gene regulations must not avoid an effective immune response against pathogens. Stablishing a symbiosis status is a great opportunity where host obtains important benefits as the integration of this new resident's resource into that of its autochthonous microbiota (Balcázar *et al.*, 2006a, Dimitroglou *et al.*, 2011). On this subject, it is known that lactic acid bacteria (LAB) produce inhibitory substances as bacteriocins to compete for binding sites and nutrients, avoiding colonization by other bacteria (Balcázar *et al.*, 2006a, Dimitroglou *et al.*, 2011). The continued administration of probiotic ends up inducing favorable changes in resident microbial populations, as reduction of potential harmful species (Sun *et al.*, 2011), which is one of the beneficial effects of a competent probiotic strain.

Another of the known effects of probiotics is to improve growth (Ringø *et al.,* 2016 and 2018), FA absorption and, in general, digestion processes (Ringø *et al.,* 1995). To perform the enhancement of growth, probiotics could help by producing vitamins as biotin or vitamin B12 or even neutralizing some potentially harmful substances from diet (Irianto & Austin, 2002) which is particularly interesting when using alternative meals and oils. On this subject, it has been demonstrated for European seabass that *Lactobacillus delbrueckii delbrueckii* can influence genes related to growth as *igf-1* and myostatin (Carnevali *et al.,* 2006). In other study, the oral administration of two forms of the same commercial probiotic composed by two strains of *Lactobacillus* (*L. farciminis* and *L. rhamnosus*), one form including live bacteria and the other inactivated bacteria, for 103 days, did not affect growth of European seabass juveniles (Frouël *et al.,* 2008) but both forms of the probiotic increased endocytic vacuoles in relation to a better digestive capacity through increased acid phosphatase and trypsin activities (Frouël *et al.,* 2008). Besides, in this study, *L. farciminis* and *L. rhamnosus* reduced skeletal malformations incidence and improved survival in European seabass (Frouël *et al.,* 2008).

Besides, it has been proved that probiotics as *L. delbrueckii delbrueckii* have the capacity of modulating stress by reducing whole-body cortisol levels (Carnevali *et al.,* 2006) which is of special interest in aquaculture for daily situations as handling, which can compromise immune response in fish.

Nevertheless, even when temporary, the close relationship created between the probiotic and microbiota during the continuous supplementation can lead to a dependence. Indeed, it has been described that a sudden interruption in the administration of a probiotic can affect homeostasis and to induce infection susceptibility (Liu *et al.,* 2016). Similarly than for prebiotics, the use of probiotics requires the consideration of different conditions as the strain, viability, dose, mode of supplementation, period, *etc*., (Nayak, 2010). The use of the lactic strain *P. acidilactici* has demonstrated to induce beneficial effects in growth, survival, correct body development, microbiota modulations, cellular and humoral immune parameters, and disease resistance in different aquatic species (Aubin *et al.,* 2005; Merrifield *et al.,* 2010 and 2011b; Ferguson *et al.,* 2010; Neissi *et al.,* 2013; Ramos *et al.,* 2013, Standen *et al.,* 2013; Vasanth *et al.,* 2015; Mondaloo *et al.,* 2017; Hoseinifar *et al.,* 2015 and 2017).

In a marine species as green terror (*Aequidens rivulatus*), *P. acidilactici* improved growth performance (weight, weight gain, SGR and FCR) and immunity (lysozyme and alternative complement activities, and total Ig (Neissi *et al.,* 2013). In red tilapia, *P. acidilactici* improved survival and it could be related with an increase in blood leucocytes and serum lysozyme activities (Ferguson *et al.,* 2010), immune cells mobilization, a trend to elevate the number of goblet-cells in the intestine and modulation of several immune-related gene expression in the intestine, overall indicating a better health status in this species (Standen *et al.,* 2013). In common carp, this probiotic increased lysozyme activity in skin mucus, incremented complement activity and skin mucus Ig and downregulated *tnfα* expression (Mondaloo *et al.,* 2017). In rainbow trout, *P. acidilactici* increased survival after intraperitoneal infection with *Streptococcus iniae* (Hoseinifar *et al.,* 2017). In this fish species, probiotic increased humoral and cellular defenses (serum lysozyme and complement activities, respiratory burst, bactericidal activity of skin mucus) and disease resistance of rainbow trout (Hoseinifar *et al.,* 2015). Maybe, some of these changes were due to intestinal microbial modulations that this probiotic strain can produce in rainbow trout (Ramos *et al.,* 2013). In this fish species, the long-period administration with *P. acidilactici* is also responsible for the reduction in deformity incidence, that is believed to be primary caused by a pathogen (Aubin *et al.,* 2005). Besides, *P. acidilactici* contributes to recover homeostasis after induced enteritis in Atlantic salmon by suppressing proinflammatory-cytokine induction through Nf-κβ activation and a sustained induction of annexin a1 (*anxa-1*), a gastro-protective and anti-inflammatory-related gene (Vasanth *et al.,* 2015). As the prebiotics, also *P. acidilactici* has the capacity of increasing mucus-secreting cells number, contributing to protect intestinal mucosa (Balda & Matter, 2003). In Atlantic salmon, this cell increment has been associated with the protein calreticulin, induced by the probiotic, which is also associated with cell proliferation, wound-recovering, and cellular adhesion (Balda & Matter, 2003). In trout, *P. acidilactici* has been visualized associated to epithelial cells and it was related to findings in enhanced morphology, activity and better status of enterocytes (Merrifield *et al.,* 2010a and 2011b). As a resume, Table 3 summarizes main effects of dietary *P. acidilactici* administration in different fish and shellfish species.

Table 3. Main effects of *Pediococcus acidilactici* dietary supplementation in fish and shellfish.

Fish species	Dose	Administration	Period	Parameters	Reference
Pollack (*Pollachius pollachius*)	10^7 CFU * ml^{-1}	In self-emulsifying concentrate of DHA as follows: for 14 h, treatments with brewer's dried yeast (BP) or probiotic *S. cerevisae* (LP) & a second step of 14 h with *P. acidilactici*.	18 hours	↑ food consumption in BP & LP. ↑ larval growth in BP & LP, ↑↑ in LP.	Gatesoupe, 2002
Rainbow trout (*Oncorhynchus mykiss*)	(1.5 ± 0.4) x10^6 of pellets	Alone in feedstuffs	B1: 20 days & B5: 5 months	Growth ≡. B5 ↓ prevalence of malformations during VCCS.	Aubin *et al.*, 2005
	10^7 CFU * g^{-1}	Alone in feedstuffs	5 weeks	*P. acidilactici*-like cells found in both intestines of supplemented fish. Probiotic ↑ microvilli length in proximal intestine but not in distal. Probiotic ↑ endocytic activity in distal intestine.	Merrifield *et al.*, 2010
	log 7 CFU * g^{-1}	Alone in feedstuffs	2 weeks	Anterior intestine isolated for *ex-vivo* challenge (*V. anguillarum*). *P. acidilactici* populated mucosa. Probiotic ↑ IELs and goblet cells.	Harper *et al.*, *2011*
	Veg cells [10^7 (low) or 10^8 (high) CFU * g^{-1}] or Lyo [10^7 (low) or 10^8 (high) CFU * g^{-1}]	Alone in feedstuffs	10 weeks	Both lyophilized (low & high) ↓ K. Veg(low) ↑ total number of circulating leucocytes.	Merrifield *et al.*, 2011b
	B1= 0.1 g kg^{-1} (2.6x10^4 CFU) or B2= 0.2 g kg^{-1} (7.2x10^4 CFU)	Alone in feedstuffs	56 days for feeding trial. 96 days for microbiota analyses.	Growth ≡. B1 ↑ intestinal bacteria community diversity.	Ramos *et al.*, 2013

	A1= 1.5 g kg^{-1} ($8.6x10^5$ CFU) or A2= 3 g kg^{-1} ($1.6x10^6$ CFU)	In a multi-species (*Bacillus sp.*, *Pediococcus sp.*, *Enterococcus sp.*, *Lactobacillus sp.*) probiotic	56 days for feeding trial. 96 days for microbiota analyses.	A1 ↑ weight gain, SGR, bacterial richness & diversity in the intestine.	
	log 7.57 CFU * g^{-1}	Alone in feedstuffs	8 weeks	Probiotic ↑ serum Lyz activity, ACPH50 activity, respiratory burst activity of leucocytes, bactericidal activity of skin mucus, skin mucus protein, & disease resistance.	Hoseinifar *et al.*, 2015
		Synbiotic (1% GOS in combination with *P. acidilactici*).	8 weeks	Synbiotic ↑↑ serum Lyz activity, ACPH50 activity, respiratory burst activity of leucocytes, bactericidal activity of skin mucus, skin mucus protein, & disease resistance.	
		Alone in feedstuffs	8 weeks	Probiotic ↑ antioxidant enzyme activity (Cat, glutathione S-transferases & *gr* gene-expressions), & infection resistance.	Hoseinifar *et al.*, 2017
		Synbiotic (1% GOS in combination with *P. acidilactici*).	8 weeks	Synbiotic ↑↑ antioxidant enzyme activity (Cat, glutathione S-transferases & *gr* gene-expressions), & infection resistance.	
Atlantic salmon (*Salmo salar*)	10^{10} CFU * kg^{-1}	Alone in feedstuffs	3 weeks prior induction of inflammation & 3 weeks after that.	Probiotic ↑ number of goblet cells, intraepithelial lymphocytes in the intestine, supranuclear vacuoles in the villi & immune cells in the lamina propria. Probiotic → delayed signs of inflammation in distal intestine & faster recovery. At 24 h ↓ *mul1b* & *tnfα* in distal intestine. At the end ↑ *carl*, *psma5*, *trp1* & ↓ *ctsb* & *naga* in distal intestine.	Vasanth *et al.*, 2015
Common carp (*Cyprinus carpio*)	$0.9x10^7$ CFU * g^{-1}	Alone in feedstuffs	8 weeks	Probiotic ↑ serum ACPH50 activity, skin mucus Lyz activity, & serum/skin total Ig levels. Probiotic ↑ Lyz & *il-1β* expression & ↓ *tnfα* expression.	Mondaloo *et al.*, 2017
		Synbiotic (10g/kg GOS in combination with lyophilized *P. acidilactici*).	8 weeks	Synbiotic ↑↑ serum ACPH50, serum total Ig level, skin mucus Lyz activity, total skin mucus Ig level, & ↓↓ *tnfα* expression in the intestine.	
Nile/red Tilapia, (*Oreochromis*	10^8 CFU * g^{-1}	Alone in feedstuffs	83 days	Probiotic ↑ weight gain.	Shelby *et al.*, 2006

niloticus)	10^7 CFU * g^{-1}	Alone in feedstuffs	32 days	Growth parameters ≡. Probiotic ↓ hematocrit & ↑ blood leucocyte level & serum Lyz activity after 14 days. Richness & diversity at the end of the experiment ≡, but ↕ during the trial, with ↓ in both parameters in some points. Survival ↑. Probiotic strain remained in fish intestine for 17 days after supplementation cessation.	Ferguson *et al.*, 2010
	$2.81x10^6$ CFU * g^{-1}	Alone in feedstuffs	6 weeks	Probiotic ↑ IELs in the middle intestine at the end of feeding period & ↑ *tnfα* at week 6. Trend to ↑ number of goblet cells in the middle intestine at week 6.	Standen *et al.*, 2013
Channel catfish (*Ictalurus punctatus*)	$1x10^8$ CFU * g^{-1} (EI) & $1x10^6$ CFU * g^{-1} (EII & EIII)	Alone in feedstuffs	EI (35 days), EII (56 days), EIII (56 days)	Growth, immune parameters (total serum protein, total Ig, ACPH50 activity, Lyz activity) or disease resistance ≡.	Shelby *et al.*, 2007
Turbot (*Psetta maxima*)	$1x10^8$ CFU * ml^{-1}	Via enriched rotifers Via seawater of rearing tanks.	A) Rotifers enriched for 1h or 24 h. B) In sea water for 8 days.	≡ survival or weight gain. ↑ bacterial recovery in fish larvae intestine when administration was via seawater.	Villamil *et al.*, 2010
Green terror (*Aequidens rivulatus*)	$0.9x10^7$ CFU * g^{-1}	Alone in feedstuffs	56 days	Probiotic ↑ weight, weight gain, SGR & ↓ FCR. Survival ≡. ↑ serum immune parameters (Lyz activity, total Ig & complement activity).	Neissi *et al.*, 2013
Angelfish (*Pterophyllum scalare*)	10^{10} CFU * g^{-1}	As *Artemia* enriched with lyophilized probiotic	7 weeks	Probiotic ↑ weight & SGR, & ↓ total viable counts but ↑ levels of adherent heterotrophic intestinal bacteria levels. ↑ skin mucus Lyz activity & skin mucus protease activity.	Azimirad *et al.*, 2016
European seabass (*Dicentrarchus labrax*)	10^7 CFU * g^{-1}	Alone in feedstuffs	41 days	Probiotic ↑ *il-1β* & ↓ *cat* expressions at 20 days post hatchery, & ↑ *hsp70* expression, at 41 days.	Lamari *et al.*, 2016
Shrimp (*Litopenaeus stylirostris*)	$(9.7±1.1)x10^6$ CFU * g^{-1}	Alone in feedstuffs	10 weeks	Weight & biomass ≡. ↑ survival & FCR. ↑ adjusted dry weight of digestive gland. ↑ total & specific activities of trypsin & α-amylase at different times (before & after eating). Bacteriostatic effect in gut commensal bacteria & *Vibrio spp.*	Castex *et al.*, 2008

	$1.8x10^7$ CFU * g^{-1} (trial I) & $9.5x10^6$ CFU * g^{-1} (trial II)	Alone in feedstuffs	22 days each trial	Trial I: ↑ TAS & in hemolymph & digestive gland & *gpx* in digestive gland (only measured there). Survival ≡. Trial II: ↑ infection resistance. ↓ prevalence of infected shrimps. ↑ *tas* & in hemolymph & digestive gland & *gpx* in digestive gland (only measured there). Reduced *sod*, *cat*, & oxidative stress level (*gssg/gsh* ratio) in digestive gland (only measured there). ↓ indicator of free radicals' damage (carbonyl protein).	Castex *et al.*, 2009
	$0.9x10^7$ CFU * g^{-1}	Alone in feedstuffs	1 month	Probiotic ↓ prevalence of infection & ↑ resistance to infection. In digestive gland before infection, ↑ TAS, *sod* & *gpx*, & ↓ *cat*. In blood, before infection, ↑ *tas*. ↓ oxidative stress level by *gssg/gsh* ratio. ↑TAS, *gpx* & *gssg/gsh* & ↓ *mda* at 24h post-infection in challenged shrimps with respect to infected/non-supplemented shrimps. ↑ *cat* at 24h in challenged shrimps than non-infected/non-supplemented shrimps. After infection, ↓ tissue damage (*mda* & carbonyl protein).	Castex *et al.*, 2010

≡ = unaffected, ↑ = high/increased, ↓ = low/decreased, → = produced/induced.
ACPH = alternative complement pathway. IELs = intraepithelial leucocytes. Lyo = Lyophilized. TAS = total antioxidant status. VCCS = vertebral column compression syndrome. VEG = vegetative.
Parameters and indexes: FCR = feed conversion ratio. K = condition factor. SGR = specific growth rate.
Genes: *calr* = calreticulin. *gpx* = glutathione peroxidase; *gr* = glutathione reductase; *gsh* = reduced glutathione levels; *gssg* = gluthathione disulfide/oxidized glutathione levels; *hsp* = heat shock protein; *il-1β* = interleukin 1 beta; *mda* = malondialdehyde; *mul1b* = mitochondrial ubiquitin ligase activator of NFκB1; *naga* = alpha-N-acetyl-galactosaminidase; *psma5* = proteasome subunit alpha type-5; *tnfα* = tumor necrosis factor alpha; *trp1* = trypsin-1.
Proteins: Cat = catalase. Cstb = cathepsin B. Lyz = lysozyme. Sod= superoxide dismutase.

1.6.3. *Combined dietary administration of prebiotics* and *probiotics: a 'synbiotic' approach*

The term 'synbiotic', first described by Gibson & Roberfroid (1995), refers to the synergistic use of different types of functional ingredients with the potentiality of enhancing each other functions. Thereby, synbiotics constitute functional additives and although we can get an idea of its potentiality from the individual evaluation of its components, when they are supplemented together may emerge interactions that can lead to enhance or to diminish its potential beneficial effects. In fact, synbiotics implies to introduce probionts with a competitive advantage when compared to the microbiota, which can result in greater possibilities of attachment and survival in the intestine (Gibson & Roberfroid, 1995). The successful attachment of probiotic bacteria to intestinal epithelia is a frequent problem that affect the efficacy of these functional additives (Balcázar *et al.*, 2007).

As mentioned for prebiotics and probiotics, the modes of use (dose, strain, period, *etc.*) will restrict the effectiveness of the synbiotic to the experimental design conditions and to the fish species in which it is tested (Huynh *et al.*, 2017).

The use of synbiotics has been extensively reviewed by Cerezuela *et al.* (2011), Hoseinifar *et al.* (2016) or Ringø & Song (2016), among others, exposing many successful cases in aquaculture. However, it is very useful to analyze studies that investigate synbiotics and their individual components to compare under the same conditions the effects of the singular use of both functional additives and the improvements by their synbiotic application. Hence, it has been demonstrated that the supplementation of enriched Artemia (*Artemia franciscana*) with *P. acidilactici* (700 mg * l^{-1}) and FOS (100 mg * l^{-1}) for 7 weeks increased weight gain of angelfish (*Pterophyllum scalare*) compared to control diet or individual administration of both functional products (Azimirad *et al.*, 2016). Similarly, in rainbow trout, inactivated *Enterococcus faecalis* (2.5 g/kg or 5 g/kg) and its combination with MOS (2.5 g/kg or 5 g/kg) for 12 weeks, demonstrated to improve fish performance and disease resistance when administered individually, but their synbiotic combination extended the positive effects with better feed gain and protein efficiency ratios (Rodríguez-Estrada *et al.*, 2013). Besides, in Japanese flounder (*Paralichthys olivaceus*) the use of different synbiotics using *Bacillus clausii* (10^7 cells * g^{-1}) as the probiotic plus FOS and/or MOS

(2.5 g/kg or 5 g/kg) as prebiotics, for 56 days, increased not only fish growth performance but also health status over the singular administration of prebiotics, probiotics, and control diet (Ye *et al.,* 2011). In rainbow trout, feeding diets with *P. acidilactici (*7.57 log CFU * g^{-1}) or GOS (1% in diet) for 8 weeks, improved serum lysozyme and serum complement activity, respiratory burst activity of leucocytes, skin mucus protein level, skin mucus bactericidal activity, and disease resistance (Hoseinifar *et al.,* 2015 and 2017). Furthermore, these results were enhanced with the synbiotic combination made of the same doses of functional additives for the same period, (Hoseinifar *et al.,* 2015 and 2017). Similarly, in vannamei shrimp (*Litopenaeus vannamei),* the use of a synbiotic combination with probiotic strain *Vibrio SKT-b* (10^6 CFU * ml^{-1}) and oligosaccharides from sweet potatoes (2% in diet) for 28 days, enhanced immune response (hemocyte count, phagocytic and phenol-oxidase activities) after their individual supplementation in both healthy and infected animals (Arisa *et al.,* 2015). In common carp fingerlings, individual or synbiotic administration of *P. acidilactici* ($0.9x10^7$ CFU * g^{-1}) and GOS (10g * kg^{-1}) for 8 weeks increased different humoral parameters as serum ACH50, serum total Ig level, skin mucus lysozyme activity and total skin mucus Ig level and among them, total Ig was significantly enhanced by the synbiotic than the rest of treatments (Mondaloo *et al.,* 2017). In Nile tilapia, the use of *Bacillus subtilis* (1g * kg^{-1}) and MOS (1g * kg^{-1}) in synbiotic combination only induced different results than singular effects of both functional ingredients in carcass crude protein and ether extract content, being the higher and the lower respectively (Vieira de Azevedo *et al.,* 2016). In the same line than for singular administration of prebiotics and probiotics, not all synbiotics can induce positive effects (Geng *et al.,* 2011; Mouriño *et al.,* 2012) and can even increase the susceptibility of infection (Cerezuela *et al.,* 2012). Therefore, as described for prebiotics and probiotics, results with the use of synbiotics depend on species, dose or time of administration but it is also important to evaluate the individual components of this product.

Thus, although the use of prebiotics as MOS and probiotics as *P. acidilactici* have demonstrated to be a secure and a valuable strategy to improve performance, health, and welfare for fish, the synergistic combination of both functional ingredients can potentially increase their individual effects. Therefore, synbiotic is revealed as a reality that must be studied in species of special interest as European seabass to face the present and future challenges of aquaculture as the important reductions of dietary FM and FO.

1.7. OBJECTIVES

The present PhD thesis aims to define nutritional tools and strategies to improve European seabass performance and health when feeding low FM/FO based diets. For that purpose, it has been evaluated: **(1)** the effects of different dietary levels of arachidonic acid (20:4 n-6), including those that can occur during dietary fish oil substitutions, on growth, content and fatty acid composition of glycerophospholipids of distal intestine, fatty acid profiles of tissues and also the effects on humoral and cellular immune parameters and disease resistance after a bacterial challenge test, for European seabass (*Dicentrarchus labrax*) juveniles; **(2)** the effectiveness of (prebiotic) mannan oligosaccharides, (probiotic) *Pediococcus acidilactici* and their synbiotic combination, as dietary complements for European seabass juveniles fed low fishmeal/fish oil based diets; and **(3)** the evaluation of different supplementation feeding strategies with those functional ingredients, either in single or in synbiotic supplementation, with alternate or continuous supplementation strategies to maximize pathogen resistance potential of fish.

To cover the main aims mentioned, a series of specific objectives were defined and carried out in different experiments:

1.- To evaluate the necessity of arachidonic acid supplementation in diets for European seabass juveniles. This evaluation was done in terms not only of fish growth, but also in terms of fish health (Chapter 3), lipidic metabolism (Chapter 4) and gut integrity and lipid class composition (Chapter 5).
2.- To balance European seabass juveniles gut health through the use of functional additives (prebiotics -mannan oligosaccharides-, probiotics -*Pediococcus acidilactici*- and synbiotic) in diets based on a low fishmeal/fish oil content (Chapter 6).
3.- To determine the best feeding strategies to improve the beneficial action of functional ingredients in European seabass juveniles fed low fish meal/fish oil based diets (Chapter 7).

The specific objectives were distributed through the following chapters:

- ***Chapter 3:*** supplementation of arachidonic acid rich oil in European seabass juveniles (*Dicentrarchus labrax*) diets: effects on leucocytes and plasma fatty acid profiles, selected immune parameters and circulating prostaglandins levels.

- ***Chapter 4:*** supplementation of arachidonic acid rich oil in European seabass juveniles (*Dicentrarchus labrax*) diets: effects on growth performance, tissue fatty acid profile and lipid metabolism.

- ***Chapter 5:*** effects of dietary arachidonic acid in European seabass (*Dicentrarchus labrax)* distal intestine lipid classes and gut health.

- ***Chapter 6:*** feeding European seabass (*Dicentrarchus labrax*) juveniles with a functional synbiotic additive (mannan oligosaccharides and *Pediococcus acidilactici*): an effective tool to reduce low fishmeal and fish oil gut health effects?

- ***Chapter 7:*** synbiotics in diets for European seabass (*Dicentrarchus labrax*): feeding strategies to improve the beneficial action of functional ingredients in low fishmeal and fish oil bases diets.

Chapter 2

2. GENERAL MATERIALS AND METHODS

In order to achieve the objectives of the present PhD thesis in the context of PROINMUNOIL project (ref. AGL2012-39919), three experiments were carried out:

- **Experiment I:** ARA supplementation assay (Chapters 3, 4 and 5)
- **Experiment II:** functional ingredients supplementation assay (Chapter 6)
- **Experiment III:** feeding strategies with functional ingredients assay (Chapter 7)

2.1. FACILITIES AND FISH FOR EXPERIMENTAL ASSAYS

The experiments with alive animals were conducted at the facilities of Marine Science-Technological Park (PCTM), within the activities carried out by the University Institute of Sustainable Aquaculture and Marine Ecosystems (IU-EcoAqua®), at the University of Las Palmas de Gran Canaria (ULPGC). The PCTM is located in Taliarte, in the city of Telde (Gran Canaria, Canary Islands, Spain). Animals were manipulated in compliance of guidelines under European Union Council (86/609/EU) and Spanish legislation (RD 53/2013). Protocols used were approved by Bioethical Committee of the ULPGC (Reference 007/2012 CEBA ULPGC).

European seabass (*Dicentrarchus labrax*, Linnaeus, 1758) juveniles were reared in commercial farms and selected by weight to be transferred to PCTM facilities. For 4 weeks before Experiments I and II, fish were acclimatized in fiberglass tanks of 2000 liters (l) and fed a commercial diet for European seabass juveniles. Experiment III was performed as an extension of Experiment II.

During acclimatization and experimental periods, fish were fed 3 times a day, 6 days a week. When fish achieved the expected weight, they were anesthetized with clove natural essential oil (5 ml / 100 l; Guinama S.L.U., La Pobla de Vallbona, Spain), 15 fish were collected to initial analyses and the rest were then weighed and measured. Before all samplings, fish were fasted for 24 hours.

Tanks were provided with filtered seawater in an open water system at a natural photoperiod (12L / 12D). The temperature of the water fluctuated daily according to the seasonal average (21-25 Celsius degrees (ºC)) during the time of the experiments. Oxygen concentration was daily measured and ranged between 7.0 and 8.0 parts per million (ppm).

2.2. EXPERIMENT I

2.2.1. *Experimental design*

For Experiment I, fish were randomly allocated in 15 tanks at a final density of 4 kg * m^{-3} to assay by triplicate each treatment. The procedures of this Experiment I are outlined in Figure 1. Two sampling points were determined at 30 and 70 days. At both points, growth parameters were calculated, and mortality was recorded daily. At the end of feeding period, 70 days, samples were taken for biochemical, morphological, and gene-expression analyses.

At the end of feeding period, fish were submitted to an infection trial against an intestinal pathogen. At the end of this infection trial, samples were taken for gene-expression analyses.

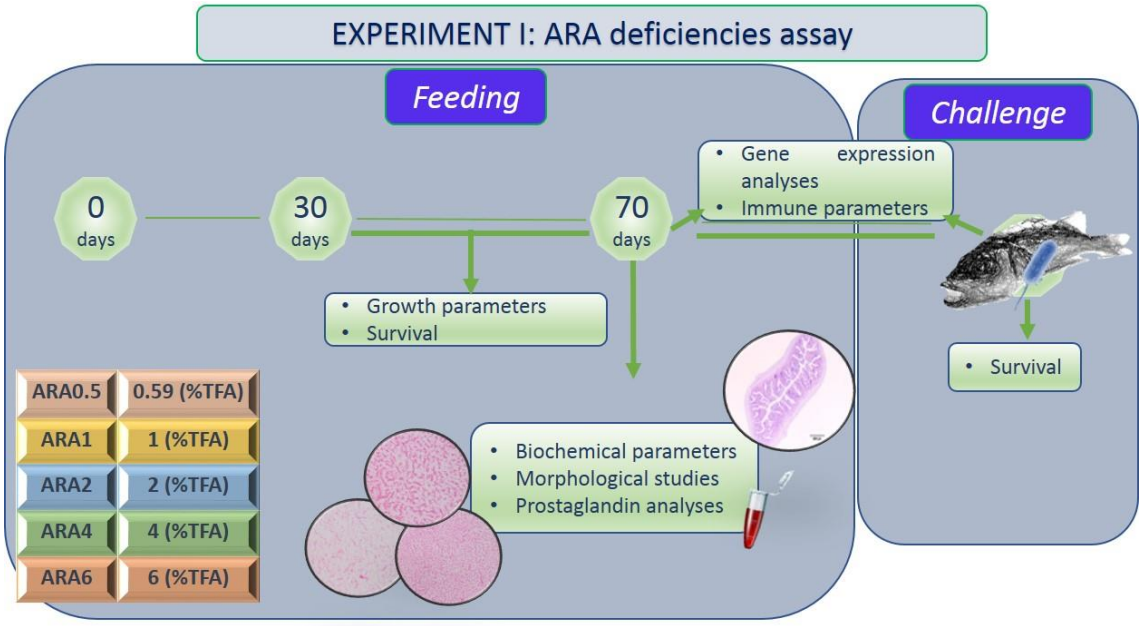

Fig. 1. Schematic representation of proceedings in Experiment I.

2.2.2. *Experimental infection assay in Experiment I*

For the experimental infection assays in Experiment I, fish were transferred to the biosecurity facilities at the PCTM-ULPGC. Along the two weeks of adaptation to the new experimental conditions, fish were fed their corresponding diets at the already mentioned frequency. Afterwards, fish were challenged against *V. anguillarum* (strain 507, isolated from a culture after a clinical outbreak in Canary Islands) with a sublethal dose (10^7 CFU * ml^{-1}). Bacterial inoculation was made by intestinal inoculation, following the method described by Torrecillas *et al.*, 2007. Fish survival was daily recorded along the bacterial challenge assay.

For dead fish, the presence of bacteria in the internal organs was determined by routine methods of the biosecurity laboratory of the aquaculture research group (GIA). At the end of time exposure, all surviving fish were sacrificed by anesthetic overdose.

2.2.3. *Diets in Experiment I*

Five isolipidic and isoproteic experimental dry pelleted diets were formulated and produced at the IU-EcoAqua experimental feed production plant to contain graded levels of ARA (% of ARA in diet) as follows: 0.59% (ARA0.5; considered as negative control), 1% (ARA1), 2% (ARA2), 4% (ARA4) and 6% (ARA6). The desired ARA content was achieved using a commercially available ARA oil obtained from *Mortierella alpina* (Vevodar®, DSM Food Specialties, the Netherlands). DHA50 and EPA50 (CRODA, East Yorkshire, UK) were used for supplementation of DHA and EPA. Diet ingredients, biochemical composition and fatty acid profile are detailed in Tables 4 and 5.

Table 4. Ingredients (as g * kg^{-1}) and biochemical analysis of Experiment I diets.

DIETS*	ARA0.5	ARA1	ARA2	ARA4	ARA6
Ingredients					
Fish meal[a]	–	52.5	52.5	52.5	52.5
Fish oil[a]	–	14.5	12.6	11.4	10.1
Defatted fish meal[b]	46.5	–	–	–	–
Corn meal[c]	7.0	6.0	6.0	6.0	6.0
Soy 44 meal[c]	10.0	10.0	10.0	10.0	10.0
Wheat meal[c]	5.5	5.5	5.5	5.5	5.5
Wheat gluten[c]	7.0	7.0	7.0	7.0	7.0
Vegetable fats and oils[c]	14.5	–	–	–	–
Vitamins mix[d]	2.0	2.0	2.0	2.0	2.0

Mineral mix[e]	2.0	2.0	2.0	2.0	2.0
CMC[f]	0.5	0.5	0.5	0.5	0.5
ARA[g]	–	–	0.5	1.5	2.5
DHA and EPA[h]	5.0	–	1.4	1.6	1.9
Biochemical composition (g kg^{-1}; d.w.)					
Crude lipids	20.8	21.3	20.9	21.1	22.0
Crude protein	43.7	43.3	44.9	44.6	45.1
Ash	9.8	10.5	10.5	10.4	10.5
Moisture	8.9	6.6	7.6	7.3	7.4

[a] Fish meal and oil, South American origin, (65% protein, 12% lipid); [b] Defatted fish meal by 3 × chloroform extraction (73% protein, 2% lipid); [c] Vegetable ingredients locally found (soy 44 meal 46% protein, 3% lipid); [d] Vitamin premix contains (mg * kg^{-1} or IU * kg^{-1} of dry diet): thiamine 40 mg, riboflavin 50 mg, pyridoxine 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, biotin 1 mg, folic acid 10 mg, cyanocobalamin, 0.5 mg, choline chloride 2700 mg, myo-inositol 2000 mg, ascorbic acid 5000 mg, menadione 20 mg, cholecalciferol 2000 IU, ethoxyquin 100 mg, retinol acetate 5000 IU; [e] Mineral premix contains (g * kg^{-1} of dry diet): calcium orthophosphate 1.60 g, calcium carbonate 4 g, ferrous sulphate 1.5 g, magnesium sulphate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminum sulphate 0.02 g, zinc sulphate 0.24 g, copper sulphate 0.20 g, manganese sulphate 0.08 g, potassium iodate 0.02 g; [f]Carboxymethyl cellulose (sodium salt, Sigma-Aldrich, Munich, Germany); [g] Vevodar®, DSM Food Specialties, the Netherlands; [h] DHA50 and EPA50, CRODA, East Yorkshire, UK. * In Chapter 4, ARA level in diets was expressed as (g fatty acid * g^{-1} diet (dry weight)); according to Chapter 4: diet ARA0.5=ARA0.1; ARA1=ARA0.2; ARA2=ARA0.4; ARA4=ARA0.8; ARA6=ARA1.4.

Table 5. Fatty acid composition (as g fatty acid * g^{-1} diet (dry weight)) of the experimental diets.

	ARA0.5	ARA1	ARA2	ARA4	ARA6
14:0	0.04	0.97	1.04	1.01	0.94
16:0	1.28	3.53	3.78	3.78	3.67
18:0	0.66	0.84	0.95	1.03	1.11
Total saturates[a]	2.04	5.62	6.07	6.12	6.02
16:1 n-7	0.06	1.23	1.26	1.21	1.14
18:1 n-9	3.60	3.70	3.82	3.81	3.76
18:1 n-7	0.18	0.64	0.66	0.64	0.61
20:1 n-7	0.15	0.54	0.54	0.51	0.48
22:1 n-11	0.04	0.48	0.44	0.42	0.38
Total monoenes[b]	4.14	6.95	7.08	6.93	6.68
18:2 n-6	2.90	1.25	1.25	1.28	1.35
18:3 n-6	0.01	0.07	0.09	0.12	0.16
20:2 n-6	0.03	0.08	0.08	0.09	0.09
20:3 n-6	0.01	0.03	0.05	0.09	0.13
20:4 n-6	0.12	0.22	0.42	0.85	1.40
22:5 n-6	0.11	0.10	0.10	0.10	0.12
Total n-6 PUFA[c]	3.21	1.79	2.03	2.57	3.30

18:3 n-3	8.78	0.34	0.33	0.29	0.28
18:4 n-3	0.02	0.30	0.23	0.22	0.22
20:3 n-3	0.05	0.04	0.04	0.03	0.03
20:4 n-3	0.03	0.14	0.12	0.11	0.11
20:5 n-3	0.43	2.06	1.60	1.55	1.68
22:5 n-3	0.12	0.39	0.29	0.28	0.31
22:6 n-3	1.90	3.12	2.54	2.51	2.86
Total n-3 PUFA[d]	11.33	6.60	5.33	5.16	5.68
n-3 LC-PUFA	2.52	5.75	4.59	4.48	5.00
ARA/EPA	0.29	0.11	0.26	0.55	0.83
EPA/ARA	3.49	9.37	3.78	1.82	1.20
DHA/ARA	15.49	14.22	6.00	2.95	2.05
n-3/n-6	3.53	3.69	2.62	2.01	1.72

[a] 15:0, 17:0 and 20:0; [b] 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-5, 20:1n-9 and 22:1n-9; [c] 16:2n-6 and 22:4n-6; [d] 16:3n-3 and 16:4n-3.

2.3. EXPERIMENT II

2.3.1. *Experimental design*

For Experiment II, fish were randomly allocated in 18 tanks at a final density of approximately 4 kg * m^{-3} to assay experimental diets by triplicate for each experimental treatment. Procedures of Experiment II are outlined in Figure 2. Two sampling points were set at 60 and 90 days. At both points, growth parameters were calculated. At the end of feeding period, 90 days, samples were taken for histological and molecular (*in situ* hybridization and gene-expression) studies. Along the feeding trial, survival was daily recorded.

At the end of feeding period, fish were submitted to an infection trial against an intestinal pathogen. At the end of this infection trial, samples were taken for gene-expression analyses.

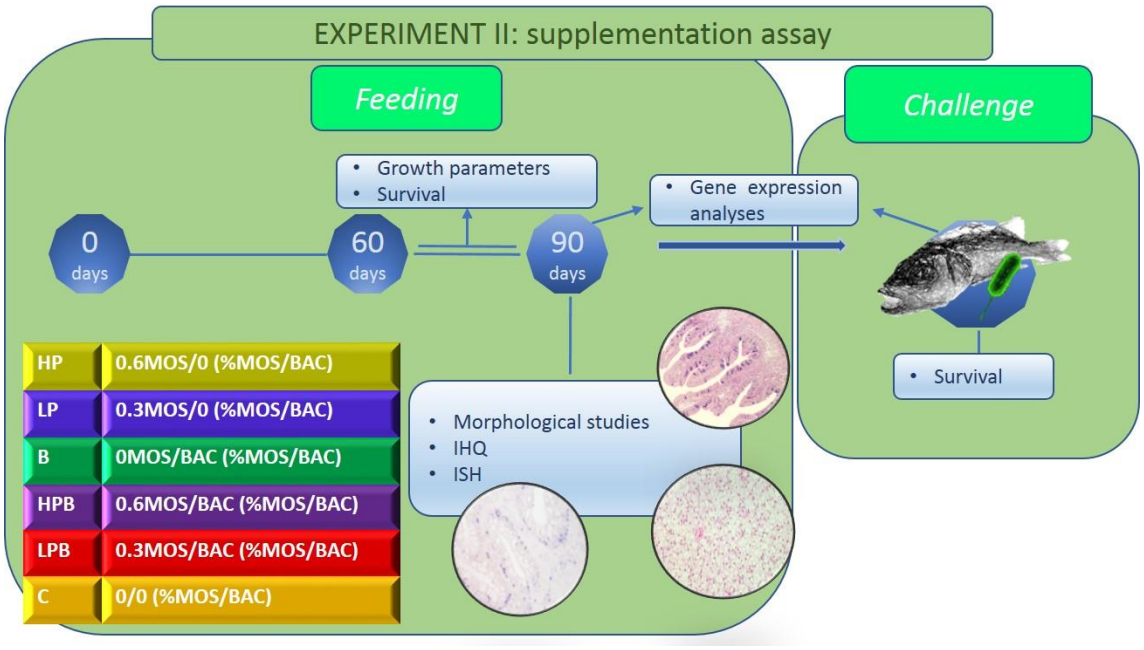

Fig. 2. Schematic representation of proceedings in Experiment II.

2.3.2. *Experimental infection assay in Experiment II*

For the experimental infection assays in Experiment II, fish were transferred to the biosecurity facilities at the PCTM-ULPGC. Along the two weeks of adaptation to the new experimental conditions, fish were fed their corresponding diets at the already mentioned frequency. Afterwards, fish were challenged against *V. anguillarum* (strain 507, isolated from a culture after a clinical outbreak in Canary Islands) with a sublethal dose (10^6 CFU * ml^{-1} per fish). Bacterial inoculation was made by intraperitoneal injection. Fish survival was daily recorded along the bacterial challenge assay.

For dead fish, the presence of bacteria in the internal organs was determined by routine methods of the biosecurity GIA laboratory. At the end of time exposure all surviving fish were sacrificed by anesthetic overdose.

2.3.3. *Diets in Experiment II*

Six isoenergetic and isonitrogenous experimental and extruded diets were formulated to contain 5% FM and 6% FO. Dietary standard carbohydrates were replaced using mannan oligosaccharides (MOS, Bio-Mos® and Actigen©; Alltech, Inc., Kentucky, USA) and *Pediococcus acidilactici* (BAC, Bactocell®; Lallemand Inc., Cardiff, UK) as follows (%MOS/BAC): HP=0.6/0, LP=0.3/0, B=0/BAC, HPB=0.6/BAC, LPB=0.3/BAC, C=0/0. BAC was added to diets according to commercial recommendations. Diets were

manufactured in the BioMar Tech-Centre (Brande, Denmark). Ingredients and proximate composition of diets are detailed in Table 6.

Table 6. Ingredients (as %) of experimental diets used on Experiment II and III, and proximate composition of experimental diet used on Experiment II.

Fish meal[a]	5.0
Blood meal (spray-dried)[b]	7.0
Soy protein concentrate[c]	20.0
Com gluten meal[d]	22.0
Wheat gluten[d]	5.5
Rapeseed meal[e]	11.4
Wheat gluten[f]	6.8
Fish oil[g]	6.0
Rapeseed oil[e]	4.0
Linseed oil[h]	2.0
Palm oil[d]	4.0
Microingredients[i]	5.6
Vitamin and mineral premix[j]	0.8
Antioxidant[k]	0.1

Proximate composition (% of dry matter)	**HP**	**LP**	**B**	**HPB**	**LPB**	**C**
Crude lipids	25.8	25.9	27.9	28.1	27.1	25.9
Crude protein	47.2	46.8	47.7	46.9	46.1	47.2
Moisture	5.8	5.9	7.6	6.5	6.4	5.8
Ash	5.6	5.6	5.7	5.7	5.7	5.8

[a] South-American, Superprime – Feed Service Bremen, Germany; [b] Daka, Denmark; [c] Svane Shipping, Denmark; [d] Gargill, Netherland; [e] Emmelev, Denmark; [f] Hedegaard, Denmark; [g] South American fish oil, LDN Fish Oil, Denmark; [h] Ch. Daudruy, France; [i] Contains lysine, methionine, mono-calcium phosphate, choline, inositol, phospholipids (Emulthin G35); [j] Commercial Biomar A/S vitamins and minerals Premix; [k] BAROX BECP, Ethoxyquin, Vilomix (Denmark).

2.4. EXPERIMENT III

2.4.1. *Experimental design*

For Experiment III, fish were fed the experimental diets for a total period of 39 weeks (Total period; TP), distributed in 3 sequential periods: Period 1 consisting of 25 weeks of feeding (P1; 0-25 week), followed by Period 2 consisting of 6 weeks (P2; 25-31 week) and ending with a Period 3 consisting of 8 weeks (P3; 31-39 week) (detailed in Table 2).

In order to cope with the strategies designed for sequential periods 2 and 3 (P2 & P3), supplementation of both functional products was alternated as follows: P/PB/P, P/PB/PB, PB/P/PB and PB/PB/P, as reflected in Table 2, corresponding P to only MOS, and PB to MOS + *P. acidilactici* supplementation. Additionally, a whole period (TP) of continuous dietary supplementation of MOS (P), *P. acidilactici* (B) or their combination (PB) was also evaluated altogether a control diet non-supplemented (C) (Table 2).

For the first period, P1, fish were randomly allocated in 12 tanks at a final density of approximately 4 kg * m^{-3} to assay four experimental diets by triplicate. At the end of P1, fish of each diet, P, B, PB and C, were first pooled and then redistributed in 16 tanks according to the schematic representation detailed in Figure 3, to assay eight experimental treatment by duplicate, for the second and third periods (P2 and P3, respectively). Different strategies and periods of feeding are summarized in Table 7.

Table 7. Periods and feeding strategies during Experiment III.

	Total period (P1/P2/P3; 39 weeks)			
	Period 1 (0-25 week)	Period 2 (25-31 week)	Period 3 (31-39 week)	Strategy acronym
Diets during corresponding period	**P**	**P**	**P**	**P**
		PB	P	P/PB/P
		PB	PB	P/PB/PB
	PB	**PB**	**PB**	**PB**
		P	PB	PB/P/PB
		PB	P	PB/PB/P
	B	**B**	**B**	**B**
	C	**C**	**C**	**C**

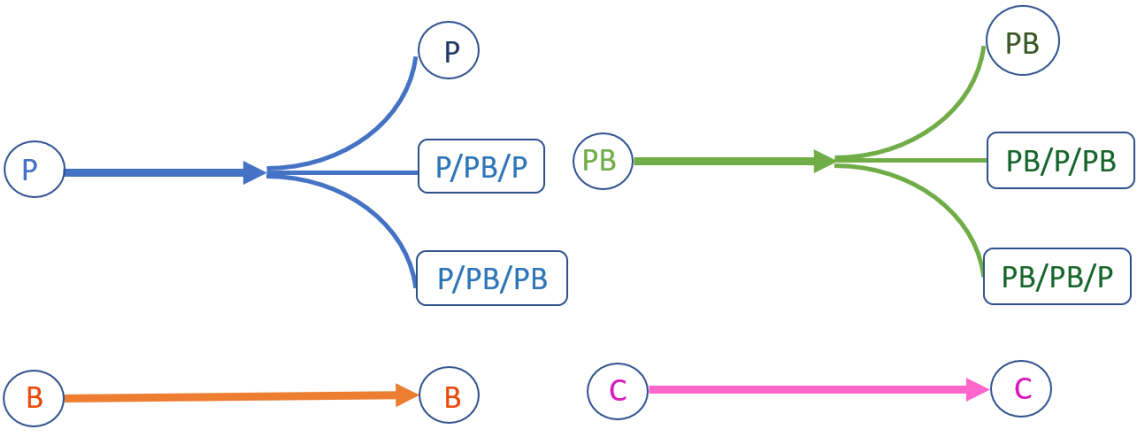

Fig. 3. Schematic representation of original diets and derived strategies in Experiment III.

Procedures of Experiment III are outlined in Figure 4. A sampling point was set at the end of each period (P1, P2 and P3). At these points, growth parameters and somatic indexes were calculated. Besides, at the end of P3, blood and tissue samples were collected for immunological, morphological and gene expression analyses. Fish were then submitted to an experimental bacterial infection against *V. anguillarum* by intestinal inoculation. At the end of the challenge test, samples were taken for gene-expression analyses. Along the feeding trial, survival was daily recorded.

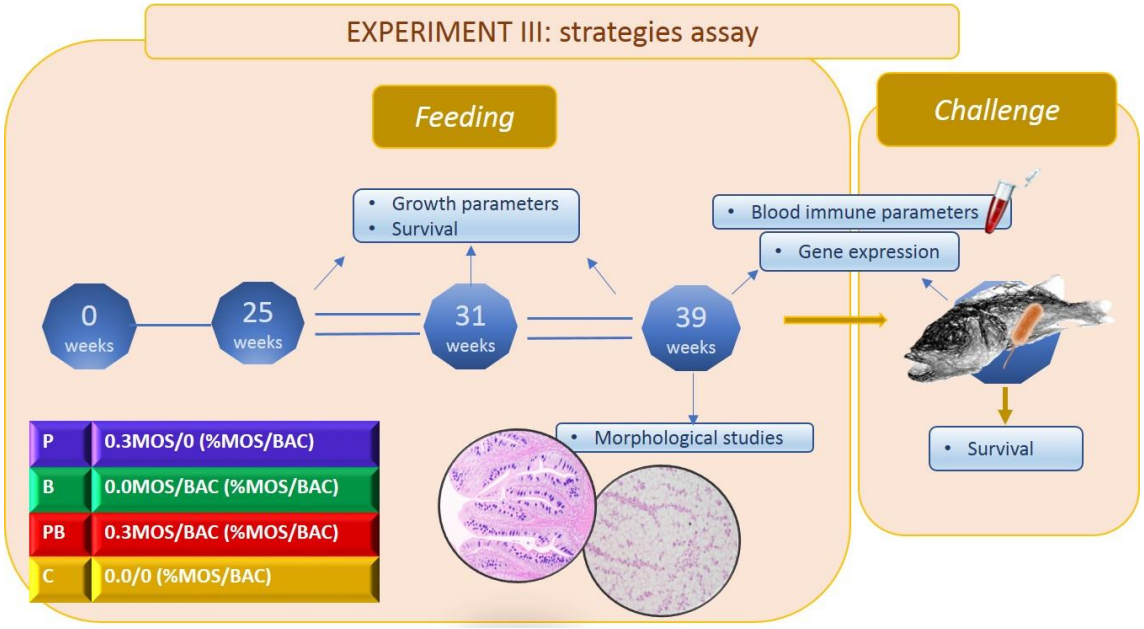

Fig. 4. Schematic representation of proceedings in Experiment III.

2.4.2. *Experimental infection assay in Experiment III*

For the experimental infection assays during Experiment III, fish were transferred to the marine biosecurity facilities at the PCTM-ULPGC. Along the two weeks of adaptation to the new experimental conditions, fish were fed their corresponding diets at the already mentioned frequency. Afterwards, fish were challenged against *V. anguillarum* (strain 507, isolated from a culture after a clinical outbreak in Canary Islands) with a sublethal dose (10^3 CFU * ml^{-1} per fish in chapter 7). Bacterial inoculation was made by anal cannulation following the method described by Torrecillas *et al.*, 2007. Fish survival was daily monitored along the bacterial challenge assay.

For dead fish, the presence of bacteria in the internal organs was determined by routine methods of the biosecurity GIA laboratory. At the end of time exposure all surviving fish were sacrificed by anesthetic overdose.

2.4.3. *Diets in Experiment III*

Four selected diets from Experiment II were tested in this assay: (MOS (%) / BAC (presence +/ absence -)): only prebiotic (P) = 0.3/-, only probiotic (B) = 0/+, prebiotic plus probiotic (PB) = 0.3/+, and control diet (C) = 0/-. BAC levels were set based on commercial recommendations. Ingredients of corresponding diets are summarized in Table 6, whereas proximate composition is included in Table 8.

The following feeding strategies were organized and assayed by duplicated: strategy only prebiotic, P; P/PB/P; P/PB/PB; strategy prebiotic plus probiotic, PB; PB/P/PB; PB/PB/P; strategy only probiotic, B; control diet, C.

Table 8. Proximate composition of diets in Experiment III.

Proximate composition (% of dry matter)				
Diets[1]	P	B	PB	C
Crude lipids	24.1	27.9	24.7	24.7
Crude protein	44.6	47.6	44.2	44.5
Moisture	6.6	7.5	6.1	6.1
Ash	5.3	5.7	5.4	5.4

2.5. GROWTH PARAMETERS

Individual whole-body fish weight (g; W = whole body weight) and length (cm; L= length from the tip of the head to the end of the caudal fin) from all animals in each tank were measured along all carried out experiments.

2.5.1. *Performance indices*

- ***Relative growth (RG)***

This parameter is calculated using the increment in biomass (g; final whole-body weight [Wf] – initial whole-body weight [Wi]) in relation to Wi (g). Relative growth can be expressed in percentage and it is corrected in relation to the individual fish weight through the following equation:

$$\textbf{Relative growth} = \left(\frac{W_F - W_i}{W_i}\right) \cdot 100$$

- ***Specific growth rate (SGR)***

This parameter is calculated using the relationship between Wi and Wf (g) and indicates the increase weight gain in relation to feeding period, as days (D). The final parameter is expressed as a percentage with the following equation:

$$\textbf{SGR} = \left(\frac{Ln\, W_F - Ln\, W_i}{D}\right) \cdot 100$$

- ***Viscerosomatic index (VSI)***

This parameter is expressed as a percentage and is utilized to discuss the metabolic state of fish. Viscera package (V (g); whole intestine + pyloric caeca + stomach + hepatopancreas + liver weight) is used in relation to Wf (g) to calculate this parameter as follows:

$$\textbf{VSI} = \left(\frac{V}{W}\right) \cdot 100$$

- ***Hepatosomatic index (HSI)***

This parameter is calculated with the relationship between liver weight (g; Li) and W (g) to discuss the metabolic state of this important organ. The final parameter is expressed as a percentage with the following equation:

$$\mathbf{HSI} = \left(\frac{\boldsymbol{Li}}{\boldsymbol{W}}\right) \cdot \mathbf{100}$$

- ***Perivisceral fat index (PFI)***

This parameter is expressed as a percentage and is calculated by using the relationship between the fat accumulated around the visceral package (g; PF) and W (g) of the fish, as follows:

$$\mathbf{PFI} = \left(\frac{\boldsymbol{PF}}{\boldsymbol{W}}\right) \cdot \mathbf{100}$$

- ***Fulton's condition factor (K)***

This parameter calculates the approximate fish shape to estimates changes in nutritional condition through minimal changes in the relation between W and L. Final parameter is expressed as a percentage with the following equation:

$$\mathbf{K} = \left(\frac{\boldsymbol{W}}{\boldsymbol{L^3}}\right) \cdot \mathbf{100}$$

2.6. BIOCHEMICAL ANALYSES

All parameters of proximal analysis were calculated following standard procedures from Official Methods of Analysis (A.O.A.C., 2000 and 2016). For these analyses, tissues of 5 fish * tank (3 tanks / diet) were pooled (N=3).

2.6.1. *Ash*

Ash content was determined in a muffle furnace. Sample weight (0.2 to 2g) was recorded before combustion at 600ºC overnight. After this time, the sample was extracted and rested in a desiccator until achieving ambient temperature and a constant weight.
Ash content is expressed as a percentage and was calculated as follows:

$$\%\boldsymbol{Ash} = \left(\frac{\boldsymbol{C} - \boldsymbol{A}}{\boldsymbol{B} - \boldsymbol{A}}\right) \cdot \mathbf{100}$$

Where:

A = Weight of empty porcelain crucible, previously heated.

B = Weight of sample + porcelain crucible pre-combustion

C = Weight of sample + porcelain crucible post-combustion

2.6.2. *Moisture*

Moisture content was determined by drying in a thermal oven at 110ºC to constant weight. Sample weight (approximately 100 mg) was recorded before drying. After a drying period of 24 hours and following the cooling in a desiccator, weight was registered again. This operation was repeated after 1-hour periods until a constant weight was attained.

This parameter is expressed as a percentage and calculated using the following equation:

$$\%\boldsymbol{Moisture} = \left(\frac{\boldsymbol{B} - \boldsymbol{C}}{\boldsymbol{B} - \boldsymbol{A}}\right) \cdot \mathbf{100}$$

Where:

A = Weight of empty flask

B = Weight of wet sample + flask

C = Weight of dry sample + flask

2.6.3. *Proteins*

Proteins were estimated from total nitrogen present in the sample, using the Kjeldhal method. After registering weight (0.2 to 0.4 g), sample was digested at a temperature of 420ºC with concentrated sulphuric acid [37% H_2SO_4] in the presence of a copper catalyst to convert molecular nitrogen [N_2] into ammonium sulfate [$(NH_4)_2SO_4$]. Afterward, ammonia [NH_3] was released by the addition of 40% sodium hydroxide [NaOH] in excess. This ammoniac acid was steam distilled in boric acid [H_3BO_3] 1% and quantified by titration with hydrochloric acid [HCl] 0.1 N.

Total nitrogen content was converted to total crude protein value by multiplying by the empirical factor 6.25.

This parameter is expressed as a percentage and calculated using the following formula:

$$\%\boldsymbol{Protein} = \left(\frac{[\boldsymbol{A} - \boldsymbol{B}]}{\boldsymbol{C}}\right) \cdot \mathbf{0.1} \cdot \mathbf{14.007} \cdot \mathbf{6.25} \cdot \mathbf{100}$$

Where:

A = HCl consumed by the sample (ml)

B = HCl consumed by the blank (ml)

C = Weight of sample (mg)

2.6.4. *Total crude lipid content*

Crude lipid content were extracted from approximately 200mg of fish tissue following the method described by Folch *et al.*, (1957) using a chloroform:methanol [$CHCl_3$/CH_3OH] (2:1 v/v) mixture containing 0.01% butylated hydroxytoluene [$C_{15}H_{24}O$]. After adding potassium chloride [KCl], lipids were centrifugated and filtered. Extracted lipids were then diluted in $CHCl_3$ and stored at -80°C under nitrogen atmosphere until analysis to avoid oxidation.

2.6.5. *Fatty acid methyl esters (FAMES)*

Total crude lipids were transmethylated with 1% H_2SO_4 in methanol according to the method described by Christie, (1982 and 2003). The procedure was performed in two different steps. Firstly, transmethylation reaction was conducted in dark conditions under nitrogen atmosphere for 16h at 50°C. Secondly, FAMES were diluted in hexane:diethyl ether [C_6H_{14}/$(C_2H_5)_2O$] (1:1v/v) and purified by adsorption chromatography on amino [NH_2] Sep-Pak cartridges (Waters S.A., Massachusetts, USA). FAMES were separated following conditions described by Izquierdo *et al.* (1992) and, afterwards, were quantified by flame ionizator detector and identified using external standards (EPA 28, Nippai, Ltd Tokyo, Japan).

2.7. HISTOLOGICAL STUDIES

Histological studies were performed on hepatic and intestinal tissues obtained from the experimental fish by dissection after euthanasia by anesthetic overdose (see details above).

2.7.1. *Collection and treatment of samples*

Whole intestine was dissected out and the posterior segment was extracted. Distal intestine (DI) was taken from the first diffused sphincter to rectum sphincter as previously described by Torrecillas *et al.*, 2013.

For these studies, DI segment and liver, were fixed in 4% neutral-buffered formalin (formaldehyde [CH_2O], sodium dihydrogen phosphate monohydrate [$H_2NaO_4P*H_2O$] and sodium phosphate dibasic [Na_2HPO_4]).

For immunohistochemical analyses or *in situ* hybridization (ISH) studies, samples were stored in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4, 0.1M at 4ºC. Samples for immunohistochemistry (IHQ) were processed after 48 hours.

For processing, six to eight transversal sections of intestine segments and three to four sections of liver were cut and then dehydrated with an increased graded series of ethanol in a tissue processor (Histokinette 2000, Leica, Nussloch, Germany), submerged in xylene and finally embedded in paraffin wax to be included in a block. Sections were cut (4µm) in a manual rotary microtome (Leica Jung 2055, Germany).

2.7.2. *Morphometric studies and morphological observations*

For optical examinations, samples were stained with the hematoxylin and eosin method (Martoja & Martoja-Pierson 1970). Staining with Alcian Blue/Periodic acid–Schiff (PAS) (pH= 2.5) was used in DI sections in order to differentiate mucus-secreting cells. Micrographs were obtained with a Nikon® Microphot- FXA microscope (objective lens 20X plus eyepiece 10X) equipped with an Olympus® DP50 camera and linked to a computer using image capturing software (cellSens®, Olympus, Hamburg, Germany).

Micrographs of DI and liver were analyzed with Image-Pro® Plus v5 software (Media Cybernetics Inc., Rockville, MD, USA).

From DI, different structural measures were taken as previously described by Torrecillas *et al.*, 2011a, during the Experiment I (36 folds per fish * 3 fish * 3 tanks per diet), II (36 folds per fish * 4 fish * 3 tanks per diet) and III (40 folds per fish * 3 fish * 2 tanks per strategy):

- Thickness of submucosa layer (µm).
- Fold area (µm^2), length (µm) and width (µm).

For number of goblet cells in DI, cell counting (in arbitrary units) was realized in Experiment I (40 folds per fish * 2 fish per tank * 3 tanks per diet), Experiment II (40 folds per fish * 2 fish per tank * 3 tanks per diet) and Experiment III (48 folds per fish * 2 fish per tank * 2 tanks per diet).

From liver, structural measures were taken according to Torrecillas *et al.*, 2007 in Experiment II (80 hepatocytes * 3 fish * 3 tanks per diet) and Experiment III (80 hepatocytes * 3 fish * 2 tanks per diet):

- Area of hepatocytes (μm^2).
- Maximum and minimum length (µm), tacking as reference the centre of the cell nucleus.

For hepatic morphology in Experiment I, a qualitative double-blind evaluation was realized based on vacuolization level of hepatocytes and considering the nuclei displacement and the grade of alignment around sinusoidal spaces to estimate the steatosis level (Torrecillas *et al.*, 2007).

All morphometrical measures were analyzed using individual fish weight as a co-variable.

2.8. GENE EXPRESSION ANALYSES

2.8.1. *RNA extraction and cDNA synthesis*

For these analyses, samples of DI and liver were dissected out and conserved in Invitrogen™ RNA*later*™ Stabilization Solution (Thermo Fisher Scientific Inc., USA) at -20°C. Then, from approximately 100mg of pooled tissues from 3 fish/tank, (N=3, for Experiment I and II and N=2 for Experiment III), and TRI-Reagent (Sigma-Aldrich, Saint Louis, MO, USA), and RNeasy® mini Kit (QUIAGEN, Germany), were used to obtain total RNA. RNA was quantified by spectrophotometry using NanoDrop™ 1000 (Thermo Fisher Scientific Inc., USA) and integrity was evaluated on a 1.4% agarose gel with Gel Red™ (Biotium Inc., Hayward, CA).

Synthesis of complementary DNA (cDNA) for quantitative real-time polymerase chain reaction (RT-qPCR) was realized from 1µg RNA with iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California) in a final volume of 20µl.

2.8.2. *Real time-polymerase chain reaction analyses*

During this research the expression level of some immune-related genes (interleukin 10, *il-10*; tumor necrosis factor alpha, *tnfα*; interleukin 1 beta, *il-1ß*; cluster of quadruple differentiation, *cd-4*; cluster of differentiation 8 alpha, *cd-8α*; t-cell receptor alpha, *tcr-ß*; major histocompatibility complex ii beta, *mhcii-ß*; major histocompatibility complex i alpha, *mhci-α*) and another gene related with eicosanoid production (cyclooxygenase 2, *cox-2*) was measured. The reaction program used during these analyses was 1X (95ºC, 10min), 35x (95ºC, 45s/corresponding annealing temperature, 45s/72ºC, 45s) 1X (72ºC, 30s).

Specific primers sequences, conditions, source and GenBank® references used in this research are registered in Table 9. Two genes, elongation factor 1 alpha (*ef-1α*) and beta actin (*ß-actin*), were tested as housekeeping and *ef-1α* was found to be more stable to make calculations.

Reactions were implemented at least by duplicate in 15µl of final volume, containing 2µl of cDNA (diluted 1/10), 7.5µl Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA) and 0.6µl of each primer (10 mM) in an *iCycler* Optical Module (Bio-Rad, USA). A blank sample was included in each assay as a contamination control, with water replacing cDNA. Relative expression level was calculated by using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Table 9. References, annealing temperatures, sequences and sources of primers for RT-qPCR.

Genes	Genbank® reference	Annealing temperature	Primers sequence (5'➔ 3')	From
il-10	AM268529	52ºC	F'ACCCCGTTCGCTTGCCA R'CATCTGGTGACATCACTC	Buonocore *et al.*, 2007
tnfα	DQ070246.1	58ºC	F'ACAGCGGATATGGACGGTG R'GCCAAGCAAACAGCAGGAC	Román *et al.*, 2013
il-1ß	AJ311925	58ºC	F'GGTGGACAAAGCCAGTC R'CCGAGCCTTCAACATCG	Picchietti *et al.*, 2009
cd-4	AM849811	52ºC	F'GTGATAACGCTGAAGATCGAGCC R'GAGGTGTGTCATCTTCCGTTG	Picchietti *et al.*, 2011
cd-8α	AJ846849	52ºC	F'CTAAGATTCGGCAAAATAACTCGAC R'GATGAGGAGTAGAAGAAGAAGGCC	Picchietti *et al.*, 2011
tcr-ß	AJ493441	52ºC	F' GACGGACGAAGCTGCCCA R' TGGCAGCCTGTGTGATCTTCA	Picchietti *et al.*, 2011
mhcii-ß	AM113466	52ºC	F'CAGAGACGGACAGGAAG R'CAAGATCAGACCCAGGA	Picchietti *et al.*, 2011

mhci-α	AM943118	52ºC	F'CAATACCTCACCCAGA R'CTCCATCTTTCCTCCAGAT	Torrecillas *et al.*, 2015a
cox-2	AJ630649	52ºC	F'CATTCTTTGCCCAGCACTTCACC R'AGCTTGCCATCCTTGAAGAGTC	Picchietti *et al.*, 2009
ef-1α	AJ866727	60ºC	F'GCTTCGAGGAAATCACCAAG R'CAACCTTCCATCCCTTGAAC	Geay *et al.*, 2011
ß-actin	AJ493428	52ºC	F'ATGTACGTTGCCATCC R'GAGATGCCACGCTCTC	Picchietti *et al.*, 2011

2.9. *IN SITU* HYBRIDIZATION STUDIES

In situ hybridization studies were realized at the Aquatic Molecular Pathobiology Laboratory of the Spanish National Research Council (CSIC) located in the Instituto de Investigacións Mariñas (IIM), in Vigo, Spain. Samples of DI from 3 fish per diet, obtained and treated as detailed in section 2.6.1, in glass slides previously treated with a 10% poly-L-lysine solution (Sigma-Aldrich co., St. Luis, USA) were used for ISH analyses.

2.9.1. *Probes synthesis*

Total mRNA was extracted from spleen of European seabass juveniles, purified and used to synthesize first-strand cDNA according to the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) protocol. One µg of total cDNA was used to amplify immune-related genes (*mhcii-ß, cd4, cd8α and tcr-ß*) using forward-reverse specific primers as previously designed by Picchietti *et al.* 2011 (sequences and conditions in Table 9). Specific amplicons of selected genes were isolated after PCR from agarose gel using a recovery kit (Zymoclean gel DNA Recovery Kit, Zymo Research Co.) and used in four ligation reactions with the selected vector (pGem T-easy Vector System; PROMEGA Corporation). Plasmids were transfected into competent E-Coli (JM-109 strain; Promega Corporation), which is deficient in ß-galactosidase activity, and plasmid DNA from the four positive colonies was extracted (Qiagen plasmid midi kit, Quiagen) and sequenced on both strands with the corresponding T7 (5´-AATACGACTCACTATAG- 3') and SP6 (5´ ATTTAGGTGACACTATAG 3´) primers. Checked the sequence and direction of the insert, plasmid DNA was linearized using adequate restriction enzymes. Afterward, sense and antisense digoxigenin-labeled riboprobes were synthesized using SP6 RNA polymerase or T7 RNA polymerase and DIG-RNA Labeling Mix 10x (Roche). RNA probes were purified in Centri-Sep^{TM} spin

columns (ThermoFisher Scientific Inc., USA) and finally diluted to a concentration of 10ng/µl into hybridization mix.

2.9.2. *Staining procedures*

ISH procedures followed methods described previously by Cerdà-Reverter *et al.*, 2000. Firstly, tissue was permeabilized with proteinase K (Ambion™, ThermoFisher Scientific Inc., USA) in TRIS-EDTA buffer (pH 8) at a final concentration of 5µg/ml and washed out with PBS. Slides were submerged in sodium chloride [NaCl] 150mM and incubated with probes at a final concentration of 0.5ng/µl in hybridization mix at 60°C, overnight.

Next day, tissue was washed with SSC (NaCl/sodium citrate) (1X, 1.5X and 2X; ThermoFisher Scientific Inc., USA), at the corresponding hybridization temperature (HT). Slides were then incubated in SSC 2X with RNase A DNase-Protease free (ThermoFisher Scientific Inc., USA) and washed with SSC 2X and 0.2X at HT. Samples were then transferred to MBA (maleic acid [$C_4H_4O_4$] and sodium chloride [$Na_3C_6H_5O_7$]) 1X with tween (1µg/ml) at room temperature. Slides were submerged in a blocking reagent (F. Hoffmann-La Roche AG., Switzerland) to decrease background. Detection of hybridization was performed with anti-digoxigenin-AP fab fragments (F. Hoffmann-La Roche AG., Switzerland). Eventually, colour was revealed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (F. Hoffmann-La Roche AG., Switzerland).

Samples were finally washed and mounted with distyrene, a plasticizer, and xylene (DPX; Merck, Germany). Micrographs were taken with a Nikon microphot- FXA microscope with an Olympus DP50 camera coupled.

2.10. STATISTICAL ANALYSES

All statistical analyses were performed using SPSS 21 software package for Windows (IBM™, Chicago, IL, USA) and followed methods outlined by Sokal & Rolf (1995).

2.10.1 *Statistical analyses with IBM™ SPSS®*

All data were tested for normality and homoscedasticity. Only when necessary, the transformation of data was carried out. The significant level for all analyses was set at

5%, and, unless otherwise indicated, all results are presented as means and standard deviations (mean ± SD).

Data were submitted to non-parametric tests (Kruskal-Wallis and U Mann-Whitney) when were not normally distributed. When Levene's test showed $P<0.05$ and One-way analysis of variance (ANOVA) and Wells tests showed $P<0.05$, post hoc test used was Games-Howell.

When data complied with normality and homoscedasticity, One-way or Two-way ANOVA tests, depending on if one or two independent variables were studied at the same time, were used to determine the possible effects in the studied parameters. When ANOVA test showed significance, individual means were compared using Tukey *post hoc* tests for multiple means comparison.

Correlations were made using the Pearson coefficient to determine the strength of the relationship between selected data.

Survival curves were performed and analyzed using the method described by Kaplan-Meier (Kaplan and Meier, 1958) and compared by the log-rank test to define the responses of European seabass to the different treatments.

Chapter 3

SUPPLEMENTATION OF ARACHIDONIC ACID RICH OIL IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) JUVENILES DIETS: EFFECTS ON LEUCOCYTES AND PLASMA FATTY ACID PROFILES, SELECTED IMMUNE PARAMETERS AND CIRCULATING PROTAGLANDINS LEVELS

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ST, MJ, MI & DM conceived and planned the experiments. LR formulated the diets. FR carried out the experiments and contributed to sample preparation and analyses. ST, CP and LR contributed to analyses. ST, CP, FR, MJ & DM contributed to the interpretation of the results and discussion. ST & DM wrote the paper with input from all authors.

3.1. ABSTRACT

The main objective of this study was to assess the effects of graded levels of dietary arachidonic acid (ARA), supplemented from alternative sources, on fatty acid composition of plasma and HK leucocytes of European seabass (*Dicentrarchus labrax*). For that purpose, European seabass juveniles were fed four diets containing graded levels of ARA as follows: 0.5% (ARA0.5), 1% (ARA1), 2% (ARA2) and 4% (ARA4) for 70 days. At the end of the feeding trial, fatty acid profiles of plasma and HK leucocytes were analyzed. Besides, plasma prostaglandins levels, HK leucocytes respiratory burst activity, peroxidase activity and phagocytic index were assayed. Reducing dietary ARA levels below 1% markedly reduced European seabass growth performance. However, fish fed diet ARA0.5 tried to compensate this dietary ARA deficiency by a selective deposition of ARA on plasma and HK leucocytes, reaching similar levels to those fish fed diet ARA1 after 70 days of feeding. Nevertheless, HK phagocytic capacity was reduced as dietary ARA content was reduced in relation not only to variations on membrane composition but also to changes on fish basal prostaglandins levels. Results obtained demonstrated the importance to supply the necessary quantity of n-6 LC-PUFA, and not only of n-3 LC-PUFA levels, in European seabass diets, in relation to not only growth performance but also immune system function.

3.2. INTRODUCTION

Long chain polyunsaturated fatty acids (LC-PUFA) have important roles in the regulation of fish growth performance, lipid metabolism, cell membrane structure and immune function (Higgs & Dong, 2000; Kiron *et al.*, 2011; Tian *et al.*, 2014). During the past decades, most of the studies addressing LC-PUFA optimum marine fish dietary levels in relation to their biological and physiological role have been focused on n-3 LC-PUFA, particularly on docosahexaenoic acid (DHA, 22:6n_3) and eicosapentaenoic acid (EPA, 20:5n_3) (Sargent *et al.*, 1999a and 1999b; Skalli & Robin 2004; Zuo *et al.*, 2012). However, studies focused on arachidonic acid (ARA, 20:4n-6) have been partially overlooked mainly due to its minor content as membrane component compared to DHA and EPA (Xu *et al.*, 2010). Indeed, it has been along the last years when more attention has been paid to ARA as essential fatty acid (EFA) in marine fish nutrition, predominantly due to its role as eicosanoid precursor (Bell *et al.*, 1993b; Ganga *et al.*, 2005). ARA is recognized to be a main precursor of eicosanoids, the 2-series of PGs and thromboxanes

(TXs) and the 4-series of leukotrienes (LTs) and lipoxins thorough the action of cyclooxygenase (COX) and lipoxygenase enzymes (Lall, 2000). Eicosanoids are involved in the regulation of several physiological processes, including reproduction, growth and development, immune system or stress response (Sargent *et al.*, 1995), thus playing a crucial role on the entire fish life cycle (Tocher, 2003). For instance, dietary ARA influences fish growth, survival and tissue fatty acid profiles (Bessonart *et al.*, 1999; Khozing-Golberg *et al.*, 2006; Atalah *et al.*, 2011), being preferentially retained in various fish species during starvation (Castell *et al.*, 1994). Besides, ARA plays an important role in the regulation of reproduction (Sargent *et al.*, 1999a and 1999b; Fernández-Palacios *et al.*, 1995; Harel *et al.*, 2002; Furuita *et al.*, 2003; Norambuena et al., 2012a and 2013a), lipid metabolism (Norambuena *et al.*, 2012a and 2013a; Martins *et al.*, 2012), metamorphosis (Lund *et al.*, 2008; Boglino *et al.*, 2014) pigmentation (Copeman *et al.*, 2002; Villalta *et al.*, 2005; Lund *et al.*, 2007 and 2010) and resistance to several stressors (Koven *et al.*, 2001 and 2003; Van Anholt *et al.*, 2004; Rezek *et al.*, 2010; Montero *et al.*, 2015c). In terms of immune system function, the role of ARA is widely recognized, being preferentially retained in several fish species leucocytes (Tocher and Sargent, 1986; Waagbø *et al.*, 1995; Farndale *et al.*, 1999; Montero *et al.*, 2003; Petropoulos *et al.*, 2009; Montero & Izquierdo, 2010). Indeed, ARA deposition in membrane phospholipids influences cell membrane fluidity and stability by affecting several signaling pathways, ion transport, trafficking and vesicular transport and membrane associated enzymes activities (Tian *et al.*, 2014; Calder, 2008; Waagbø, 2006). Besides, ARA alters the transcription of gene-encoding proteins involved in lipid metabolism that directly affect immune-related transcription factors (i.e. modulating the *nf-kb*) (Morgan & Liu, 2011) and is involved in the assembly and activation of NADPH oxidase activity (Montero & Izquierdo, 2010; Croker *et al.*, 2001). Eicosanoids derived from ARA, such as PGE2 and LTB4, increase vascular permeability and vasodilatation, induce leucocytes chemotaxis and promote generation of reactive oxygen species (ROS) (Calder, 2006b). Indeed, due to its role as precursor in eicosanoid synthesis, ARA has been also associated with regulation of cytokine release (Tian *et al.*, 2014; Calder, 2006b; Furne *et al.*, 2013; Pirante *et al.*, 2002; Roy Baker, 1990). Several studies in fresh water species point to the effectiveness of an appropriate level of dietary ARA in modulation of immune system function and disease resistance (Khozing-Golberg *et al.*, 2006; Van Anholt *et al.*, 2004; Harel *et al.*, 2001). For example, in guppies (*Poecilia reticulate*) dietary enrichment with arachidonic acid (ARA)-rich triacylglycerols fraction improves disease resistance against

Tetrahymena sp. (Khozing-Golberg *et al.*, 2006). Besides, dietary ARA affects leukocyte relative distribution following intraperitoneal injection with formalin-fixed *Staphylococcus aureus* in striped bass (*Morone saxatilis*) (Harel *et al.*, 2001) and an optimum dietary ARA supplementation increases superoxide dismutase (SOD) and lysozyme activities in Japanese eel (*Anguilla japonica*) (Shahkar *et al.*, 2016).

Likewise, dietary ARA levels (0.36-0.56% total FA) in juvenile Japanese sea bass (*Lateolabrax japonicus*) increase serum lysozyme, alternative complement pathway (ACPH) and superoxide dismutase (SOD) activities, although do not affect respiratory burst activity of HK leucocytes and serum catalase (CAT) (Xu *et al.*, 2010). ARA levels in juvenile turbot (*Scophtalamus maximus*) regulate tissue PGE2 and 6-ketoPGF1 levels (Bell *et al.*, 1994). Feeding European seabass with low ARA levels derived from dietary VO reduces plasma PGE2 content (Mourente *et al.*, 2007), though PGEs production has been described to markedly differ among tissues of the same fish species (Tafalla *et al.*, 1999). Besides, fish immune system function is directly affected by dietary n-3/n-6 ratio (Montero & Izquierdo, 2010; Bell *et al.*, 1996b), due to n-3 LC-PUFA and n-6 LC-PUFA interactions. Despite the importance of European seabass on European aquaculture production, the information available concerning its nutritional requirements is still incomplete compared to other cultured fish species such as salmonids or carps (Oliva-Teles, 2000; Kaushik, 2002; Izquierdo *et al.*, 2005), and particularly those addressing n-6 LC-PUFA effects on European seabass immune system. Thus, the aim of the present study is to assess the effects of graded levels of dietary ARA, supplemented from alternative sources, on European seabass juvenile's fatty acid composition of plasma and HK leucocytes in relation to its immune potential.

3.3. MATERIAL AND METHODS

All animal manipulation described in this paper, comply with the guidelines of the European Union Council (86/609/EU) and Spanish legislation (RD 1201/2005) for the use of laboratory animals and have been approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (ULPGC). The experimental tanks used within the present experiments are located at the aquaculture facilities belonging to the ULPGC.

3.3.1. *Experimental diets*

Four isolipidic and isoproteic experimental dry pelleted diets based on a commercial formulation for European seabass juvenile were prepared to contain 0.59%, 1%, 2%, and 4% of ARA, respectively. The desired ARA content was completed with commercially available ARA oil obtained from *Mortierella alpina* (Vevodar®, DSM Food Specialties, Netherlands). Supplementation of DHA and EPA was done using DHA50 and EPA50 (CRODA, East Yorkshire, UK). Diet ingredients, fatty acid profiles and proximate composition are detailed in Tables 4 and 10.

Table 10. Fatty acids composition (% of total identified fatty acids (TFA)) of the experimental diets.

	ARA0.5	ARA1	ARA2	ARA4
14:0	0.19	4.53	4.99	4.76
14:1 n-5	0.01	0.16	0.17	0.16
14:1 n-7	0.00	0.03	0.04	0.04
15:0	0.04	0.53	0.56	0.53
15:1 n-5	0.00	0.02	0.03	0.02
16:0ISO	0.01	0.03	0.03	0.03
16:0	6.15	16.55	18.10	17.92
16:1 n-7	0.28	5.78	6.04	5.73
16:1 n-5	0.01	0.28	0.28	0.28
16:2 n-6	0.00	0.01	0.01	0.01
16:2 n-4	0.01	0.57	0.56	0.53
17:0	0.01	0.55	0.50	0.48
16:3 n-4	0.08	0.30	0.33	0.31
16:3 n-3	0.03	0.17	0.18	0.18
16:3 n-1	0.01	0.08	0.08	0.08
16:4 n-3	0.03	0.80	0.67	0.64
16:4 n-1	0.00	0.01	0.01	0.01
18:0	3.19	3.92	4.57	4.89
18:1 n-9	17.31	17.36	18.31	18.02
18:1 n-7	0.89	3.00	3.15	3.01
18:1 n-5	0.02	0.13	0.16	0.14
18:2 n-9	0.01	0.05	0.08	0.07
18:2 n-6	13.94	5.86	5.98	6.06
18:2 n-4	0.03	0.24	0.24	0.23
18:3 n-6	0.06	0.33	0.42	0.55
18:3 n-4	0.02	0.16	0.14	0.13
18:3 n-3	42.28	1.61	1.59	1.36
18:3 n-1	0.01	0.02	0.02	0.02
18:4 n-3	0.08	1.39	1.11	1.02
18:4 n-1	0.01	0.13	0.11	0.10

20:0	0.25	0.29	0.35	0.38
20:1 n-9	0.11	0.33	0.34	0.31
20:1 n-7	0.73	2.54	2.58	2.43
20:1 n-5	0.08	0.25	0.27	0.26
20:2 n-9	0.02	0.08	0.09	0.08
20:2 n-6	0.15	0.37	0.40	0.41
20:3 n-9	0.01	0.05	0.04	0.05
20:3 n-6	0.05	0.15	0.23	0.42
20:4 n-6	0.59	1.03	2.03	4.03
20:3 n-3	0.24	0.18	0.17	0.16
20:4 n-3	0.14	0.66	0.56	0.53
20:5 n-3	2.06	9.65	7.68	7.32
22:1 n-11	0.19	2.27	2.12	1.98
22:1 n-9	0.28	0.44	0.43	0.41
22:4 n-6	0.14	0.17	0.18	0.19
22:5 n-6	0.52	0.46	0.50	0.49
22:5 n-3	0.57	1.82	1.40	1.33
22:6 n-3	9.14	14.65	12.19	11.89
Σ Saturates	9.83	26.36	29.07	28.97
Σ Monoenes	19.92	32.61	33.91	32.80
Σ n-3	54.57	30.93	25.54	24.42
Σ n-6	15.45	8.39	9.75	12.16
Σ n-9	17.73	18.26	19.25	18.90
Σ n-3HUFA	12.15	26.97	22.00	21.22
n-3/n-6	3.53	3.69	2.62	2.01

3.3.2. *Experimental conditions*

Six hundred and seventy-two European seabass juveniles were randomly stocked in 12 200 L fiber-glass tanks (56 individual per tank) with an average initial weight of 13.4 ± 0.29 g (mean ± SD) (3.7 kg * m^{-3} initial stocking density). Diets were assayed by triplicate. Tanks were supplied in a flow-through system with seawater, at a temperature of 22.8-24.9ºC, and natural photoperiod (12L:12D). Water dissolved oxygen ranged between 5 and 7 ppm. Fish were fed by hand until apparent satiation, three times a day, six days a week, for 70 days. At the end of the experimental period, whole fish population was sampled for determining survival and growth parameters. All animals were fasted for 24 h before sampling. Then, six animals per experimental tank (18 per diet) were euthanized by an anesthetic overdose (clove oil), and samples of blood were obtained by caudal sinus puncture using 1 ml heparinized syringes. Samples of plasma were obtained

after immediate centrifugation of whole blood at 3000 g during 5 min. Then, an aliquot of plasma was kept at -80ºC until analysis of fatty acid profile. A second aliquot was acidified with formic acid (2 M, 50 µl/mL) and frozen in liquid nitrogen (-80ºC) prior to prostaglandins analysis. Afterwards, samples of HK of 8 fish per tank (27 per treatment) were collected for HK leucocytes immune parameters and fatty acid analyses.

3.3.3. *Biochemical analyses*

Feed composition, plasma and HK leucocytes analyses were conducted following standard procedures (AOAC, 1995). Crude protein content (Nx6.25) was determined by Kjeldahl method, crude lipid was extracted with a chloroform methanol (2:1, v/v) mixture as described by Ref. (Folch *et al.*, 1957) moisture was determined by thermal drying to constant weight in an oven at 110ºC and ash content by combustion in a muffle furnace at 600ºC for 12 h. Fatty acid methyl esters were obtained by transmethylation as described by Ref. (Christie, 1982) and separated by gas chromatography under the conditions described by Ref. (Izquierdo *et al.*, 1992) Fatty acid methyl esters were quantified by flame ionizator detector and identified by comparison with external and well-characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan).

3.3.4. *Immune parameters*

3.3.4.1. Head kidney leucocytes isolation

HK leucocytes were isolated from each fish analyzed under sterile conditions following the technique described by Secombes, (1990) with some modifications. Briefly, HK was excised, cut into small fragments and transferred to 8 ml of supplemented cell culture medium (Leibovitz L-15) (Gibco, Gaithersburg, MD, U.S.A). Cell suspensions were obtained by forcing fragments of the organ through a 100 mm nylon mesh. After centrifugation (400 g, 10 min), cells suspension was layered on a Ficoll® gradient suspension (Lymphocyte Separation Medium; Lonza Walkersville Inc., MD, USA) and centrifuged at 1100 g for 30 min at 4ºC. The interface layer was harvested and diluted in 1 ml of supplemented L-15 medium and again centrifuged at 450 g for 10 min at 4ºC to remove residual Ficoll. Suspension obtained was finally resuspended in L-15 medium supplemented with penicillin/streptomycin/gentamicin (P/S/G). Viable cells were stained with trypan blue and cell suspension concentration adjusted to the desired concentration depending on the parameter studied in a Neubawer's camera.

3.3.4.2. Phagocytic activity of head kidney leucocytes

HK phagocytic activity was measured as described by Puangkaew *et al.* (2004). Aliquots of 500 ml containing 10^7 cells * ml^{-1} in L-15 medium supplemented with P/S/G were seed onto 20 mm diameter glass coverslips in 6-well plates (Nunc, Roskilde, Denmark). Then, phagocyte monolayer was incubated with 10 ml of 10^9 CFU * ml^{-1} (MOI 1:1; bacteria/phagocyte cell ratio) of *Vibrio anguillarum* for 1 h at 22ºC. After washing with PBS, cells were stained with Diff Quick solution (Panreac, Spain). One hundred leucocytes with phagocytic ability per slide were counted and the phagocytic capacity was determined as the percentage of cells with phagocytic ability. All samples were analyzed in triplicate.

3.3.4.3. Head kidney leucocytes peroxidase content

The total HK leucocytes peroxidase content present was measured according to (Quade and Roth, 1997). For that purpose, aliquots of 100 ml containing 1 * 10^6 HK leucocytes * ml^{-1} in L-15 medium supplemented with P/S/G were added to 96-well microtitre plates (Nunc, Roskilde, Denmark) and lysed with 75 ml of 0.02% cetyl trimethyl ammonium bromide (Sigma-Aldrich, Germany). Afterwards, 50 ml of 10 mM 3,30,5,5'-tetramethylbenzidine hydrochloride (Sigma-Aldrich, Germany) and 25 ml of 5 mM H2O2 were added and the reaction stopped after 2 min by adding 50 ml of 2 M sulphuric acid (H_2SO_4). Finally, the optical density was read at 450 nm in a multiscan spectrophotometer (MultiskanFc, Thermo, Chicago) reader. All experiments were carried out by triplicate.

3.3.4.4. Respiratory burst activity of head kidney leucocytes

The generation of intracellular superoxide radicals by HK leucocytes was determined by the reduction of nitro-blue tetrazolium (NBT) according to the technique described by Secombes (1990) and Boesen *et al.* (2001).

For that purpose, aliquots of 100 ml containing 1 * 10^6 HK leucocytes * ml^{-1} in L-15 medium supplemented with P/S/G were dispensed onto 96-well microtiter plates (Nunc, Roskilde, Denmark), 100 ml NBT (1 mg * ml^{-1}) added to each well and incubated at 22ºC for 30 min. After incubation, cells were fixed with 70% methanol and reduced formazan solubilized by adding 120 ml potassium hydroxide (KOH) and 140 ml dimethylsulfoxide ($(CH_3)_2SO$) (Sigma-Aldrich, Germany).

Finally, absorbance was read at 620 nm in a multiscan spectrophotometer (MultiskanFc, Thermo, Chicago). All experiments were carried out by triplicate.

3.3.5. *Prostaglandins analyses*

The frozen acidified plasma samples were extracted using prewashed octadecyl silyl 'Sep-Pak' mini-columns (Sep-Pak C18, Waters Co. Milford, USA) (Powell, 1982) and as described in detail by Bell *et al*. (1993b). Once the plasma was applied to the column, it was washed successively with MiliQ water, 15% ethanol (v/v) and hexane/chloroform (C_6H_{14}/ $CHCl_3$) (65:35, v/v) before elution of prostanoids with ethyl acetate ($C_4H_8O_2$). The extracts were dried under nitrogen (N_2), suspended in 100 ml of methanol (CH_3OH) and stored at -80ºC until analysis. Measurement of prostaglandins was performed using an enzyme immunoassay (EIA) kit for PGE2 according to manufacturer's protocol (Cayman Chemical Co., MI, USA). It must be noted that this EIA kit has shown some cross reactivity with PGE3 in fish samples (Ganga *et al.*, 2005). Therefore, a portion of the observed changes may not be fully attributable to PGE2 and the results obtained have been considered as total PGEs (Torrecillas *et al.*, 2013).

3.3.6. *Statistical analysis*

Statistical analyses followed methods outlined by Ref. (Sokal & Rolf, 1995) and means and standard deviations (SD) were calculated for each parameter measured. All data were tested for normality and homogeneity of variance. Data was subject to one-way ANOVA and significant differences were considered when $P<0.05$. When F values showed significance, individual means were compared using Duncan multiple comparison test. If the variances were not normally distributed, the Kruskal-Wallis non-parametric test was applied to the data. When necessary, dependent variables were correlated with dietary ARA percentage and Pearson correlation coefficient and significance are showed along the text. Analyses were performed using SPSS software (SPSS for windows 11.0).

3.4. RESULTS

3.4.1. *Biological parameters*

After 70 days of feeding, fish fed diet ARA0.5 presented lower ($P<0.05$) final weight, total length and SGR than fish fed the rest of the dietary treatments. Survival was high

along the feeding trial for all the dietary treatments assayed, ranging from 93.3 ± 4.6% to 97.6 ± 1.0 (Table 11).

Table 11. Growth performance and survival in European seabass (*Dicentrarchus labrax*) juveniles fed the experimental diets at the end of the feeding trial (70 days).

Diets	ARA0.5	ARA1	ARA2	ARA4
Initial (t= 0 days)				
Weight (g)	13.33± 0.25	13.28±0.30	13.59±0.52	13.42 ± 0.22
Length (cm)	9.84 ± 0.06	9.81 ± 0.05	9.95 ± 0.10	9.91 ± 0.10
Final (t= 70 days)				
Weight (g)	32.97 ± 1.14a	44.44 ± 1.06b	43.77 ± 0.96b	43.93 ± 3.72b
Length (cm)	13.90 ± 0.12a	15.01 ± 0.14b	15.05 ± 0.03b	15.10 ± 0.40b
SGR (%) [1]	1.35 ± 0.03a	1.79 ± 0.01b	1.73 ± 0.08b	1.75 ± 0.11b

Data expressed in mean ± SD (N = 3 tanks/diet). Different letters within a row denote differences at P<0.05 based on Duncan's multiple range test (one way-ANOVA). [1]Specific growth rate (SGR) = [(ln final weight - ln initial weight)/number of days] * 100.

3.4.2. *Plasma and head kidney leucocytes fatty acids profile*

Plasma and HK leucocytes fatty acid profiles mainly reflected those of the dietary treatment fed (Tables 12 and 13) with certain differences between humoral and cellular fractions. On one hand, increased levels of dietary ARA levels did not affect (P>0.05) plasma saturates, monoenes, Σn-3, Σn-3HUFA and Σn-9 fatty acid percentages, whereas altered Σn-6 content (Table 12). Plasma of fish fed diet ARA1 presented the lowest (P<0.05) Σn-6 percentages compared to the rest of the dietary treatments, whereas plasma of fish fed ARA0.5 presented similar Σn-6 than fish fed diet ARA4 (Table 12). Fish fed ARA1 presented the lowest plasma ARA/EPA and the highest (P<0.05) Σn-3/Σn-6 ratio in relation to the rest of the dietary treatments, whereas fish fed diet ARA0.5 presented similar ARA/EPA and Σn-3/Σn-6 ratios to fish fed diet ARA2 (Table 12). As expected, plasma ARA levels were proportional to those fed, with exception of fish fed diet ARA0.5 which presented similar ARA levels to those of fish fed diet ARA1 (r=0.632, N=12, P=0.026) (Table 12). Similarly, for EPA, deposition rates as EPA plasma/EPA diet, were increased by approximately 3 times in fish fed diet ARA0.5 to those presented by fish fed higher dietary levels (Table 12). Indeed, Elovl6 products 20:2n-6 and 20:3n-3 were higher (P<0.05) in plasma of fish fed diet ARA0.5 compared to fish fed other dietary ARA percentages, despite similar dietary levels (Table 12). In agreement, plasma 20:3n-6 and

18:4n-3 levels were similar to those presented in fish fed ARA1 diet, despite the lower dietary content and 20:4n-3 diet/plasma ratio was reduced in a 60% (Table 12).

On the other hand, HK leucocytes of fish fed diets ARA1 and ARA2 presented the highest ($P<0.05$) saturated fatty acids contents, which were lowest in fish fed diet ARA0.5 ($P<0.05$) (Table 13). Fish fed diet ARA2 had the highest content in monoenes ($P<0.05$), without differences to ARA1 and ARA2 fish. HK leucocytes of fish fed diet ARA0.5 presented the highest Σn-3 content, mainly due to a higher 18:3n-3 (Table 13). Similarly, HK leucocytes of fish fed diet ARA4 had the highest ($P<0.05$) Σn-6 content, due to a greater ARA deposition, resulting in the highest ($P<0.05$) ARA/EPA ratio (Table 13). Indeed, as found for the humoral fraction, HK leucocytes ARA levels were relative to those fed, with exception of fish fed diet ARA0.5 which presented similar ARA levels to those of fish fed diet ARA1 (Table 13). HK leucocytes in 0.5ARA fish presented an increased ($P<0.05$) content of 20:2n-6, despite its levels were similar among diets. Further, 18:3n-6 diet/tissue ratio was highly reduced in fish fed ARA0.5 compared to fish fed other dietary treatments and 20:3n-6% was significantly increased despite a lower dietary content (Table 13). EPA deposition rates were also increased by approximately 3 times in fish fed diet ARA0.5 in relation to fish fed higher dietary levels (Table 13). Besides, fish fed ARA0.5 diet presented reduced 18:4n-3 and 20:4n-3 diet/HK leucocytes proportion than fish fed higher ARA levels (Table 13). Consequently, HK leucocytes of fish fed diet ARA1 presented lower ($P<0.05$) ARA/EPA ratio than fish fed the lowest ARA dietary content, regardless of presenting similar EPA content than fish fed higher dietary ARA concentrations. Σn-3/Σn-6 ratio of HK leucocytes decreased with increasing levels of dietary ARA (Table 13). Indeed, plasma ($r=0.998$, $N=12$, $P<0.001$) and HK leucocytes ($r=0.994$, $N=12$, $P<0.001$) ARA tissue/ARA diet ratios, trend to decrease with increasing levels of dietary ARA (Tables 12 and 13). Besides, ARA plasma and ARA HK leucocytes content increased proportionally ($r=0.995$, $N=12$, $P<0.001$). No significant differences were found among dietary treatments when comparing the DHA levels of both, plasma or HK leucocytes, despite the lower DHA dietary intake in fish fed diet 0.5ARA.

Table 12. Plasma fatty acids composition (% of total identified fatty acids) of fish fed the experimental diets at the end of the feeding trial.

	ARA0.5	ARA1	ARA2	ARA4
14:0	0.75 ± 0.16a	1.71 ± 0.18b	1.79 ± 0.30b	1.64 ± 0.06b
14:1n-5	0.04 ± 0.01a	0.07 ± 0.01b	0.07 ± 0.01b	0.08 ± 0.01b
14:1n-7	0.01 ± 0.02	0.00 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
15:0	0.23 ± 0.04a	0.46 ± 0.05b	0.47 ± 0.05b	0.44 ± 0.03b
15:1 n-5	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
16:0ISO	0.02 ± 0.01a	0.07 ± 0.01b	0.07 ± 0.00b	0.06 ± 0.01b
16:0	21.11 ± 2.31	26.33 ± 2.09	26.11 ± 2.27	25.41 ± 1.86
16:1 n-7	0.80 ± 0.14a	2.01 ± 0.20b	2.01 ± 0.17 b	1.96 ± 0.22b
16:1 n-5	0.06 ± 0.02a	0.23 ± 0.01c	0.21 ± 0.01bc	0.19 ± 0.01b
16:2 n-6	0.00 ± 0.00	0.00 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
16:2 n-4	0.03 ± 0.01	0.04 ± 0.03	0.05 ± 0.01	0.05 ± 0.00
17:0	0.04 ± 0.01	0.05 ± 0.00	0.04 ± 0.00	0.04 ± 0.01
16:3 n-4	0.10 ± 0.01a	0.20 ± 0.00b	0.18 ± 0.01bc	0.18 ± 0.02bc
16:3 n-3	0.05 ± 0.00a	0.13 ± 0.01c	0.12 ± 0.01bc	0.11 ± 0.01b
16:3 n-1	0.31 ± 0.03	0.34 ± 0.01	0.36 ± 0.06	0.39 ± 0.06
16:4 n-3	0.71 ± 0.13	0.50 ± 0.07	0.50 ± 0.14	0.58 ± 0.07
16:4 n-1	0.09 ± 0.02	0.16 ± 0.03	0.16 ± 0.05	0.17 ± 0.03
18:0	4.88 ± 0.34	4.13 ± 0.47	4.06 ± 0.36	4.21 ± 0.43
18:1 n-9	9.57 ± 0.41	9.09 ± 0.85	8.78 ± 0.62	8.84 ± 1.32
18:1 n-7	1.16 ± 0.09a	1.80 ± 0.14b	1.71 ± 0.13b	1.64 ± 0.17b
18:1 n-5	0.05 ± 0.00a	0.12 ± 0.00c	0.11 ± 0.00b	0.10 ± 0.00b
18:2 n-9	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.01
18:2 n-6	4.98 ± 0.19b	1.94 ± 0.16a	2.01 ± 0.21a	1.97 ± 0.24a
18:2 n-4	0.20 ± 0.03ab	0.15 ± 0.00b	0.14 ± 0.01ab	0.13 ± 0.00a
18:3 n-6	0.06 ± 0.01a	0.09 ± 0.01b	0.10 ± 0.01bc	0.12 ± 0.00c
18:3 n-4	0.02 ± 0.01	0.04 ± 0.02	0.06 ± 0.05	0.04 ± 0.01
18:3 n-3	12.93 ± 0.15b	0.67 ± 0.09a	0.72 ± 0.14a	0.67 ± 0.06a
18:3 n-1	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
18:4 n-3	0.18 ± 0.02ab	0.19 ± 0.01b	0.20 ± 0.01b	0.18 ± 0.01b
18:4 n-1	0.02 ± 0.00a	0.05 ± 0.00b	0.05 ± 0.01b	0.04 ± 0.00b
20:0	0.15 ± 0.02b	0.08 ± 0.00a	0.09 ± 0.01a	0.09 ± 0.01a
20:1 n-9	0.02 ± 0.00	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
20:1 n-7	0.49 ± 0.05	0.54 ± 0.02	0.50 ± 0.01	0.47 ± 0.02
20:1 n-5	0.07 ± 0.02	0.08 ± 0.01	0.10 ± 0.05	0.08 ± 0.00
20:2 n-9	0.01 ± 0.01	0.02 ± 0.00	0.01 ± 0.01	0.01 ± 0.01
20:2 n-6	0.49 ± 0.06b	0.22 ± 0.02a	0.21 ± 0.01a	0.21 ± 0.02a
20:3 n-9	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.02	0.01 ± 0.01
20:3 n-6	0.05 ± 0.00a	0.06 ± 0.01ab	0.08 ± 0.01b	0.12 ± 0.01c
20:4 n-6	2.32 ± 0.03a	2.19 ± 0.10a	3.80 ± 0.05b	6.04 ± 0.13c
20:3 n-3	0.20 ± 0.02a	0.07 ± 0.01b	0.07 ± 0.00b	0.07 ± 0.00b
20:4 n-3	0.16 ± 0.00a	0.30 ± 0.01b	0.29 ± 0.02b	0.29 ± 0.02b
20:5 n-3	6.03 ± 0.26a	10.35 ± 0.46d	9.33 ± 0.38c	8.17 ± 0.16b

22:1 n-11	0.18 ± 0.10	0.26 ± 0.07	0.31 ± 0.01	0.25 ± 0.03
22:1 n-9	0.11 ± 0.02	0.08 ± 0.02	0.09 ± 0.01	0.07 ± 0.01
22:4 n-6	0.29 ± 0.01	0.50 ± 0.06	0.51 ± 0.07	0.55 ± 0.09
22:5 n-6	0.64 ± 0.02b	0.47 ± 0.12ab	0.45 ± 0.05ab	0.45 ± 0.01ab
22:5 n-3	0.56 ± 0.03a	0.97 ± 0.09b	0.90 ± 0.06b	0.89 ± 0.08b
22:6 n-3	29.74 ± 3.20	33.10 ± 3.59	33.05 ± 3.80	32.85 ± 3.99
Σ Saturates	27.15 ± 2.29	32.76 ± 2.62	32.56 ± 2.96	31.83 ± 2.36
Σ Monoenes	12.55 ± 0.65	14.34 ± 1.24	13.94 ± 0.89	13.76 ± 1.69
Σ n-3	50.55 ± 3.18	46.28 ± 3.98	45.17 ± 3.97	43.80 ± 4.05
Σ n-6	8.83 ± 0.22c	5.48 ± 0.21a	7.17 ± 0.08b	9.47 ± 0.32c
Σ n-9	9.79 ± 0.41	9.30 ± 0.84	8.98 ± 0.61	9.03 ± 1.31
Σ n-3HUFA	36.68 ± 3.21	44.78 ± 4.08	43.64 ± 4.22	42.27 ± 4.08
ARA/EPA	0.39 ± 0.02b	0.21 ± 0.02a	0.41 ± 0.02b	0.74 ± 0.03c
n-3/n-6	5.73 ± 0.48b	8.47 ± 0.93c	6.30 ± 0.63b	4.63 ± 0.46ab
ARA plasma/ARAdiet	3.93 ± 0.42a	2.12 ± 0.18b	1.87 ± 0.51c	1.49 ± 0.59d

Different letters within a row denote significant differences (P<0.05). N=3 pools of 6 fish/tank. Mean ± SD.

Table 13. Head kidney leucocytes fatty acids composition (% of total identified fatty acids) of fish fed the experimental diets at the end of the feeding trial.

	ARA0.5	ARA1	ARA2	ARA4
14:0	1.19 ± 0.02a	2.50 ± 0.04b	1.27 ± 0.03a	1.98 ± 0.24ab
14:1n-5	0.02 ± 0.00b	0.04 ± 0.00c	0.01 ± 0.00a	0.01 ± 0.00b
14:1n-7	0.05 ± 0.00a	0.07 ± 0.00c	0.06 ± 0.00b	0.05 ± 0.00a
15:0	0.00 ± 0.00a	0.00 ± 0.00c	0.00 ± 0.00d	0.00 ± 0.00b
15:1 n-5	0.07 ± 0.00c	0.08 ± 0.00d	0.02 ± 0.00	0.05 ± 0.00b
16:0ISO	0.08 ± 0.00a	0.08 ± 0.00a	0.09 ± 0.00	0.05 ± 0.00c
16:0	15.29 ± 0.07	20.59 ± 0.45	21.15 ± 0.31	18.69 ± 1.10
16:1 n-7	1.93 ± 0.04a	4.47 ± 0.08c	2.07 ± 0.04a	3.28 ± 0.12b
16:1 n-5	0.08 ± 0.00a	0.18 ± 0.01b	0.20 ± 0.03b	0.11 ± 0.01a
16:2 n-6	0.05 ± 0.00c	0.16 ± 0.01d	0.01 ± 0.00a	0.02 ± 0.00b
16:2 n-4	0.10 ± 0.00b	0.15 ± 0.01c	0.04 ± 0.00a	0.13 ± 0.02c
17:0	0.26 ± 0.00a	0.38 ± 0.01b	0.10 ± 0.00c	0.24 ± 0.03a
16:3 n-4	0.66 ± 0.01c	0.10 ± 0.02a	0.46 ± 0.01b	0.10 ± 0.01a
16:3 n-3	0.97 ± 0.00a	0.68 ± 0.03b	0.19 ± 0.00c	1.17 ± 0.07d
16:3 n-1	0.91 ± 0.00b	0.85 ± 0.03b	0.84 ± 0.03b	0.64 ± 0.03a
16:4 n-3	0.16 ± 0.00a	0.28 ± 0.02b	0.20 ± 0.00a	0.33 ± 0.01c
16:4 n-1	9.46 ± 0.03	9.12 ± 0.87	9.98 ± 0.12	8.69 ± 0.38
18:0	17.66 ± 0.01b	14.43 ± 1.75a	18.33 ± 0.47b	17.74 ± 1.42b
18:1 n-9	2.39 ± 0.05a	4.10 ± 0.17c	4.47 ± 0.07	3.07 ± 0.02b
18:1 n-7	0.10 ± 0.00a	0.18 ± 0.02b	0.60 ± 0.01c	0.13 ± 0.02a
18:1 n-5	0.22 ± 0.00b	0.44 ± 0.00c	0.18 ± 0.02ab	0.16 ± 0.01a
18:2 n-9	7.34 ± 0.01b	8.38 ± 0.41a	7.08 ± 0.05b	6.37 ± 0.20b
18:2 n-6	0.15 ± 0.00a	0.15 ± 0.02a	0.82 ± 0.00b	0.13 ± 0.02a
18:2 n-4	0.61 ± 0.00b	1.07 ± 0.18b	0.12 ± 0.00a	0.58 ± 0.05b
18:3 n-6	0.09 ± 0.00a	0.14 ± 0.02b	0.10 ± 0.00a	0.09 ± 0.01a

18:3 n-4	13.62 ± 0.10c	1.65 ± 0.13ab	1.48 ± 0.01a	1.86 ± 0.14b
18:3 n-3	0.00 ± 0.00a	0.01 ± 0.00b	0.37 ± 0.03c	0.00 ± 0.00a
18:3 n-1	0.28 ± 0.00b	0.59 ± 0.02c	0.14 ± 0.00a	0.30 ± 0.01b
18:4 n-3	0.24 ± 0.00a	0.27 ± 0.02a	0.65 ± 0.01c	0.35 ± 0.02b
18:4 n-1	0.25 ± 0.01ab	0.23 ± 0.01a	0.27 ± 0.02b	0.23 ± 0.00a
20:0	0.07 ± 0.00a	0.16 ± 0.01b	0.07 ± 0.00a	0.16 ± 0.02b
20:1 n-9	1.31 ± 0.00a	1.92 ± 0.03c	1.81 ± 0.02b	1.90 ± 0.01c
20:1 n-7	0.16 ± 0.00ab	0.18 ± 0.02b	0.14 ± 0.00a	0.16 ± 0.01a
20:1 n-5	0.23 ± 0.00b	0.57 ± 0.02c	0.04 ± 0.00a	0.22 ± 0.02b
20:2 n-9	0.93 ± 0.00a	0.80 ± 0.02b	0.68 ± 0.02c	0.68 ± 0.08c
20:2 n-6	0.00 ± 0.00a	0.01 ± 0.00b	0.01 ± 0.00c	0.01 ± 0.00b
20:3 n-9	0.78 ± 0.01d	0.61 ± 0.01c	0.42 ± 0.03b	0.32 ± 0.02a
20:3 n-6	3.10 ± 0.03a	3.47 ± 0.18a	5.76 ± 0.21b	10.08 ± 0.46c
20:4 n-6	0.38 ± 0.01c	0.42 ± 0.02d	0.13 ± 0.00a	0.21 ± 0.01b
20:3 n-3	0.21 ± 0.00b	0.56 ± 0.02d	0.13 ± 0.00a	0.31 ± 0.01c
20:4 n-3	2.96 ± 0.04a	4.47 ± 0.20b	4.63 ± 0.08b	4.40 ± 0.12b
20:5 n-3	0.22 ± 0.00a	0.53 ± 0.02b	0.26 ± 0.03a	0.65 ± 0.02c
22:1 n-11	0.38 ± 0.00b	0.40 ± 0.02b	0.24 ± 0.03a	0.42 ± 0.01b
22:1 n-9	0.09 ± 0.00a	0.14 ± 0.02b	0.10 ± 0.00c	0.11 ± 0.01c
22:4 n-6	0.39 ± 0.00c	0.18 ± 0.02a	0.27 ± 0.03b	0.20 ± 0.01a
22:5 n-6	0.91 ± 0.00a	2.11 ± 0.26c	0.98 ± 0.02a	1.69 ± 0.19b
22:5 n-3	13.67 ± 0.03	12.86 ± 2.25	12.88 ± 0.15	11.95 ± 1.08
22:6 n-3	26.30 ± 0.07a	32.59 ± 1.37c	32.71 ± 0.20c	29.72 ± 0.96b
Σ Saturates	24.42 ± 0.08a	26.70 ± 2.13ab	28.28 ± 0.37b	27.72 ± 1.43ab
Σ Monoenes	33.60 ± 0.12b	23.62 ± 2.95a	21.68 ± 0.20a	21.48 ± 1.56a
Σ n-3	13.22 ± 0.03a	14.66 ± 0.83a	14.44 ± 0.30a	18.34 ± 0.79b
Σ n-6	18.55 ± 0.01	16.00 ± 1.81	18.87 ± 0.40	18.70 ± 1.42
Σ n-9	18.13 ± 0.02	20.43 ± 2.75	18.76 ± 0.23	18.57 ± 1.39
Σ n-3HUFA	1.05 ± 0.00b	0.78 ± 0.00a	1.24 ± 0.03c	2.29 ± 0.04d
ARA/EPA	2.54 ± 0.01c	1.61 ± 0.11b	1.50 ± 0.02b	1.17 ± 0.04a
n-3/n-6	5.24 ± 0.05a	3.36 ± 0.17b	2.83 ± 0.10c	2.49 ± 0.11d
ARA plasma/ARAdiet	1.33 ± 0.03a	1.58 ± 0.04b	1.51 ± 0.04b	1.66 ± 0.04c

Different letters within a row denote significant differences (P<0.05). N=3 pools of 6 fish/tank. Mean ± SD.

3.4.3. *Immune parameters*

Dietary ARA levels did not affect significantly (P>0.05) HK leucocytes peroxidase content and neither the respiratory burst activity. HK leucocytes of fish fed diet ARA0.5 presented lower (P<0.1) phagocytic index than HK leucocytes of fish fed diets ARA1 and ARA4, whereas did not differ from that obtained for fish fed diet ARA2 (Table 14). Indeed, HK leucocytes phagocytic index was positively correlated with dietary ARA (r=0.645, N=12, P=0.024) and HK leucocytes ARA content (r=0.618, N=12, P=0.032),

however no significant correlation was found with total HK leucocytes Σn-3HUFA content. Plasma prostaglandins levels were positively correlated with the dietary ARA (r=0.330, N=36, P=0.049; y=0.016x-0.932, R^2=0.88) and with ARA concentration in plasma (r=0.342, N=36, P=0.041; y = 0.006x + 0.485, R^2=0.77) and fish fed diet ARA4 presented higher (P<0.05) circulating prostaglandins levels than fish fed ARA0.5 diet (Table 14). Besides plasma prostaglandins levels were positively correlated with plasma ARA/EPA ratio (r=0.319, N=36, P=0.058).

Table 14. Influence of dietary ARA on European seabass (*Dicentrarchus labrax*) head kidney leucocytes immune parameters and circulating prostaglandins levels at the end of the feeding trial (70 days).

	ARA0.5	ARA1	ARA2	ARA4
Peroxidase content	0.95 ± 0.58	0.93 ± 0.38	0.72 ± 0.33	1.05 ± 0.31
Respiratory burst activity	1.24 ± 0.04	1.30 ± 0.04	1.22 ± 0.15	1.32 ± 0.12
Phagocytic index (%)	19.00 ± 0.33a	21.55 ± 2.36b	20.88 ± 1.35ab	23.11 ± 1.35b
Circulating prostaglandins (pg/ml)	197.94 ± 37.68a	452.49 ± 171.36ab	446.06 ± 57.68ab	773.68 ± 337.01b

Different letters within a row denote significant differences (P<0.1). N= 3 pools of 8 fish/tank. Mean ± SD. Prostaglandins levels are expressed as Mean ± standard error of mean (SEM).

3.5. DISCUSSION

In the present study, dietary levels of ARA below 1% of total fatty acids reduced fish growth performance in agreement with previous studies in this and other fish species (Xu *et al.*, 2010; Bessonart *et al.*, 1999; Atalah *et al.*, 2011; Castell *et al.*, 1994; Shahkar *et al.*, 2016), even when dietary EPA and DHA requirements for this species were well covered (Skalli & Robin, 2004). In fish, it has been suggested that growth performance may be affected by variations on the PGE2/PGF2α ratio, in relation to alterations on muscle fiber formation and protein degradation (Bell & Sargent, 2003), as well as hormonal or molecular regulation of growth-related factors (Izquierdo & Koven, 2011). Interestingly, increments of dietary ARA up to 4% did not negatively affect European seabass growth as demonstrated in other fish species (Xu *et al.*, 2010; Zheng *et al.*, 1996; Ishizaki *et al.*, 1998). This negative effect could be related to a potential inhibitory effect of dietary ARA on EPA bioconversion (Furuita *et al.*, 2003) or EPA incorporation in fish tissues phospholipids (Bell *et al.*, 1996b; Izquierdo, 1996) that would affect ARA/EPA and, hence, eicosanoids synthesis. Indeed, this fact was clearly evidenced on plasma and HK leucocytes fatty acid profiles, which in general terms reflected those of the diets with exception of fish fed diet 0.5ARA, which presented similar ARA, EPA and DHA content

than fish fed diet ARA1. This condition supposes an increase on fatty acid content on HK leucocytes in relation to dietary percentages of approximately from 3.4 to 5.2 for ARA, from 0.5 to 1.4 for EPA and from 1 to 1.5 for DHA. Similarly, ARA, EPA and DHA plasma/diet relation increased from 2 to 4, from 1 to 3 and from 2.5 to 3.25, respectively. Indeed, results obtained showed an increase in plasma and HK leucocytes fatty acids of products of elongation of very long chain fatty acids protein-6 (Elovl6) and Δ6-desaturase pointing to an increased activity of these enzymes, despite the low capability of marine fish of synthetizing LC-PUFA from 18:C precursors (Montero & Izquierdo, 2010), when LC-PUFA are supplemented under fish requirements. A selective incorporation of ARA, EPA and DHA denotes the specific physiological role of those fatty acids in both immune system-related cellular and humoral factors irrespective of European seabass reduced capacity of LC-PUFA biosynthesis. Indeed, previous studies have demonstrated a selective deposition of ARA, EPA and DHA into other immune system-related organs and cells (Castell *et al.,* 1994; Farndale *et al.,* 1999; Montero *et al.,* 2003; Fountoulaki *et al.,* 2003; Betancor *et al.,* 2015b), evidencing their important role on immune system functioning in marine fish species (Montero & Izquierdo, 2010). Changes on LC-PUFA composition of cell membranes affect inflammation processes by a variety of mechanisms, including alterations in cell membrane fluidity, lipid raft formation, cell signaling pathways gene expression or in lipid mediator's production (Izquierdo, 1996). Particularly, variations on the contents of ARA, EPA and DHA of cells involved in the inflammatory pathway appear to be especially important in relation to their function (Izquierdo, 1996). Indeed, in the present study, besides HK leucocytes peroxidase activity and respiratory was not significantly affected by dietary treatments in agreement with previous studies in similar fish species fed ARA graded levels (Xu *et al.*, 2010), fish presenting lower Σn-3/Σn-6 and higher ARA/EPA HK leucocytes content resulted in increased phagocytic index. Actually, in higher vertebrates, an anti-chemotactic effect on leucocytes has been attributed to EPA and DHA in relation to fish oil (FO) intake, although the mechanism involved it is not clear and seems to be related to a lower expression or antagonism of receptors for chemo attractants (Izquierdo, 1996). Nevertheless, a simple reduced prostaglandin production, or alteration on the spectrum and efficacy of prostaglandins produced, may be sufficient to alter immune cell composition and function (Bell & Sargent, 2003) in relation to the phagocytic indexes found as reported in other fish species (Montero & Izquierdo, 2010). In this sense, basal plasma prostaglandins levels found were positively correlated with the dietary ARA

content and ARA/EPA ratio in agreement with previous studies in turbot where dietary ARA/EPA reduction resulted in reduced heart, brain and kidney PGE2 content (Fountoulaki *et al.*, 2003). Eicosanoids are produced from non-esterified C:20 PUFA by the action of cyclooxygenase and lipoxygenase enzymes resulting in metabolites including prostaglandins, leukotrienes, lipoxins and resolvins. Particularly, non-esterified ARA and EPA are precursors of 2-series prostanoids and 4-series leukotriene and lipoxins, and to the 3-series prostanoids and 5-series leukotriene and lipoxins, respectively (Betancor *et al.*, 2015b), being the pattern and properties formed influenced directly by the dietary n-3/n-6 ratio (Tocher, 2003). Therefore, besides the lack of significance among dietary treatments on total PGs levels due to a higher variation among replicates, the higher absolute levels found in fish fed increased ARA percentages might be related to a higher production of PGE2 due to a greater deposition of its precursor on polar leucocytes lipids.

In fish, ARA is mainly stored in polar lipids and it is a minor component of cell membranes compared to EPA (Calder, 2010; Bell *et al.*, 1995). This hypothesis could be applied to all dietary treatments with exception of fish fed diet ARA1, which presented high PGs production with a low dietary and HK leucocytes ARA/EPA ratio. In this sense, it is important to underline that the methodology followed measured the fatty acid profile of total lipids and not only that of the polar lipid fraction. Besides results obtained accounts for total PGs, since PGE2 was not isolated from PGE3 formerly to the analysis. Thus, a high dietary or membrane non-esterified EPA content could be also partially contributing the higher PGs levels found in fish fed diets ARA1 to ARA4, since plasma PGE3 levels has been demonstrated to play a key role as PGs precursor in marine fish and accounts for the main fraction of plasma total PGs in gilthead seabream fed FO based diets (Ganga *et al.*, 2005).

In summary, dietary ARA levels below a 1% of total fatty acids reduced growth performance of European seabass juveniles denoting the importance of ARA for a proper fish development. Indeed, fish fed diet ARA0.5 presented similar HK and plasma ARA, EPA and DHA content than fish fed diet ARA1, regardless of the dietary intake. An increased selective deposition of particularly ARA in European seabass juveniles seems to be related with a fish mechanism to compensate the dietary ARA deficiency in order to guarantee the proper HK leukocytes functioning. However, the resulting altered Σn-3/Σn-6 content affected its phagocytic capacity in relation probably to both: (1) variations on membrane fluidity, lipid rafts formation and cell signaling pathways due to changes in

membrane phospholipids composition and (2) to an altered spectrum and amount of the PGs produced, which in turn will affect not only the functionality of the HK leucocytes evaluated but also may be conditioning fish growth performance.

Thus, the results obtained in the present study evidence the importance of an adequate supply of dietary n-6 LC-PUFA in diets for European seabass juveniles in relation to maximize immune system function efficiency.

Chapter 4

SUPPLEMENTATION OF ARACHIDONIC ACID RICH OIL IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) JUVENILES DIETS: EFFECTS ON GROWTH PERFORMANCE, TISSUE FATTY ACID PROFILE AND LIPID METABOLISM

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ST, MJ, MI & DM conceived and planned the experiments. LR formulated the diets. FR carried out the experiments and contributed to sample preparation and analyses. ST, MB, MJ & DM contributed to the analyses, the interpretation of the results and discussion. ST, DM & MB wrote the paper with input from all authors.

4.1. ABSTRACT

The aim of this study was to evaluate the effects of increasing dietary arachidonic acid (ARA) levels (from 1 to 6% of total fatty acids) on European seabass (*Dicentrarchus labrax*) juveniles' growth performance, tissue fatty acid profile, liver morphology as well as long-chain polyunsaturated fatty acids (LC-PUFA) biosynthesis, triglyceride and cholesterol synthesis and lipid transport. A diet with total fish oil (FO) replacement and defatted fish meal (FM) containing a 0.1g ARA * g^{-1} diet was added to the experimental design as a negative control diet. Dietary ARA inclusion levels below 0.2g * ARA g^{-1} diet significantly worsened growth even only 30 days after the start of the feeding trial, whereas dietary ARA had no effect on fish survival. Liver, muscle and whole-body fatty acid profile mainly reflected dietary contents and ARA content increased accordingly with ARA dietary levels. Tissue eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) levels were positively correlated among them. Hepatic lipid vacuolization increased with reduced dietary ARA levels. Expressions of fatty acyl desaturase 2 (*fads2*) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*hmgcr*) genes were upregulated in fish fed the negative control diet compared to the rest of the dietary treatments denoting the influence of ARA on lipid metabolism. Results obtained highlight the need to include adequate n-6 levels and not only n-3 LC-PUFA levels in European seabass diets.

4.2. INTRODUCTION

Dietary long-chain polyunsaturated fatty acids (LC-PUFA) are necessary for fish growth and development, as well as for an efficient functioning of fish lipid metabolism and immune system (Higgs & Dong 2000; Kiron *et al.*, 2011; Tian *et al.*, 2014). However, during the last decades, a plethora of studies has focused on the effects of n-3 LC-PUFA deposition in fish (Izquierdo & Koven 2011; Morais *et al.*, 2011; Betancor *et al.*, 2015a, b, 2016a, b) and limited attention has been paid to the effects that low levels of n-6 LC-PUFA, in particular arachidonic acid (ARA; 20:4 n-6),
can have on teleost fish species (Montero & Izquierdo 2010; Izquierdo & Koven 2011). Furthermore, the optimum ARA inclusion levels have not been estimated for juveniles of marine fish as most of the requirement studies have been performed on larvae (Bessonart *et al.*, 1999; Atalah *et al.*, 2011; Yuan *et al.*, 2015).

ARA is an essential fatty acid for fish as appropriate levels of this FA are required to achieve proper growth performance, development and survival (Carrier *et al.*, 2011; Boglino *et al.*, 2014; Lund *et al.*, 2007, 2010; Rombenso *et al.*, 2016), lipid metabolism (Martins *et al.*, 2012; Norambuena *et al.*, 2012), reproduction (Furuita *et al.*, 2003; Norambuena *et al.*, 2012, 2013), stress resistance (Koven *et al.*, 2001, 2003; Rezek *et al.*, 2010), immune system function (for review see Montero & Izquierdo 2010) as well as resistance to disease (Xu *et al.*, 2010). It also is the main precursor for the synthesis of two-series prostaglandins (Smith & Murphy, 2002) and is known to affect signaling pathways and membrane-associated enzyme activities by modifying the fatty acid profile of cell membranes (Waagbø 2006; Calder, 2008). Furthermore, not only the individual amounts of ARA have an effect on fish, but an interaction also exists between this and the n-3 LC-PUFA eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3). In this sense, a recent study in Atlantic salmon (*Salmo salar*) showed that combined dietary inclusion of ARA and EPA improved fish performance and changed tissue fatty acid composition (Norambuena *et al.*, 2016). On the other hand, a high EPA/ARA ratio caused reduced growth in gilthead seabream (*Sparus aurata*) juveniles fed feeds containing a de novo oil derived from a GM-oilseed crop (Betancor *et al.*, 2016b). This fact is even more obvious in carnivorous fish species given that these have limited availability to biosynthesize n-3 and n-6 LCPUFA from their precursors in the feeds by the action of fatty acid desaturases and elongases (Mourente *et al.*, 2005). Additionally, the marginal deficiency of essential fatty acids can lead to health-related problems in farmed fish including alterations on stress resistance (Montero *et al.*, 2015), gut integrity (Caballero *et al.*, 2002; Torrecillas *et al.*, 2016) or lipid metabolism (Caballero *et al.*, 2006). Indeed, ARA is known to modify metabolic functions in fish by altering the expression of genes belonging to several pathways (Betancor *et al.*, 2014; Salini *et al.*, 2016) and thus is worthy to evaluate the effect that increasing ARA levels can have on genes involved in LC-PUFA biosynthesis, lipid transport and synthesis as well as cholesterol metabolism.

Thus, the present study aimed to examine the effect of graded levels of dietary supplementation of ARA-rich oil on fish growth, muscle, liver and whole-body fatty acid distribution, liver morphology and lipid metabolism in European seabass (*Dicentrarchus labrax*) as well as to evaluate the potential interactions with either EPA or DHA.

4.3. MATERIALS AND METHODS

4.3.1. *Diets*

Five isolipidic and isoproteic experimental dry pelleted diets were prepared to contain graded levels of arachidonic acid (g fatty acid * g^{-1} diet (dry weight)) as follows: 0.1 (ARA0.1; considered as negative control; C-), 0.2 (ARA0.2), 0.4 (ARA0.4), 0.8 (ARA0.8) and 1.4 (ARA1.4). The desired ARA content was completed with commercially available ARA oil obtained from *Mortierella alpina* (Vevodar®, DSM Food Specialties, the Netherlands). Supplementation of DHA and EPA was done using DHA50 and EPA50 (CRODA, East Yorkshire, UK). Diet ingredients, fatty acid profile and proximate composition are detailed in Table 4 and Table 5.

4.3.2. *Experimental conditions*

Eight hundred and forty European seabass juveniles were maintained in stocking tanks and fed a commercial diet for 4 weeks before starting the feeding trial. Afterwards, with an initial weight and length of 13.4 ± 0.3 g and 9.9 ± 0.1 cm (mean ± SD), animals were randomly allocated in 15 indoor cylindroconical 200 L tanks (56 fish/tank; 3.7 kg * m^{-3} initial stocking density). Tanks were supplied with filtered seawater at a temperature of 22.8–24.9°C in a flow-through system and natural photoperiod (12L/12D). Water dissolved oxygen ranged between 7.3 and 8.1 ppm. Fish were manually fed until apparent satiation with one of the five experimental diets for 70 days (three times a day, 6 days a week). Each dietary treatment was assayed in triplicate. Sampling was performed after 30 and 70 days of feeding. The whole fish population was individually sampled for final weight and length after 24 h of starvation. At the end of the feeding trial, eight fish per tank (N= 24 fish/diet) were sampled for somatic indexes calculation and livers and fillets pooled and used for lipid content and fatty acid analyses. Besides, five fish per tank (N= 15 fish/diet) at the beginning of the experiment and at the end of the feeding trial were sampled for whole body nutrient retention calculations. Five fish per tank (N= 15fish/diet) were sampled individually for liver morphological analyses. Additionally, livers of five fish per tank (N= 15fish/diet) were taken and quickly kept in RNA later and frozen at -80°C until gene expression analysis. The animal experiments described comply with the guidelines of the European Union Council (86/609/EU) and Spanish legislation (RD

1201/2005) for the use of laboratory animals and have been approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria.

4.3.3. *Proximate composition and fatty acid analyses*

Feeds, whole body and tissue proximate composition analyses were conducted following standard procedures (AOAC, 2000). Crude protein content (N × 6.25) was determined by Kjeldahl method, and crude lipid was analyzed as described by Folch *et al.,* (1957). Moisture was determined by thermal dehydration to constant weight at 110°C. Ash content was determined by combustion at 600°C for 12 h. Fatty acid methyl ester profiles were prepared by transmethylation (Christie, 2003) and separated by gas chromatography (GC-14A, Shimadzu, Japan) following the conditions described by Izquierdo *et al.*, (1992). Fatty acid methyl ester quantification was performed by a flame ionizator detector and identification by comparison with external and well characterized fish oil standards (EPA 28, Nippai, Ltd. Tokyo, Japan). All analyses were conducted in triplicate.

4.3.4. *Liver morphological studies*

Individual liver samples were fixed in 4% neutral buffered formalin, embedded in paraffin and stained with haematoxylin and eosin for optical examination (Martoja & Martoja-Pearson, 1970). Liver steatosis level was evaluated by two scientists unaware of the dietary treatments based on hepatocyte vacuolization level and nuclei displacement and alignment around sinusoidal spaces grade. Micrographs were taken at a final magnification of × 40 using an Olympus Cx41 microscope and an Olympus XC50 camera.

4.3.5. *RNA extraction and real-time PCR analysis*

Total RNA of fish liver was extracted using TRI reagent (Sigma-Aldrich, Saint Louis, MO, USA), quantified by measuring absorbance at 260 nm in a spectrophotometer (Nanodrop 1000, Thermo Fisher Scientific Inc., USA). The RNA integrity was verified by Gel Red™ staining (Biotium Inc., Hayward, CA) on a 1% agarose gel. The reverse transcription (RT) reactions were performed using the iScript™ complementary DNA (cDNA) Synthesis Kit (Bio-Rad Hercules, CA) in 20 µl final volume containing 1 µg of total RNA. The *fads2*, fatty acid binding protein 7 *(fabp7)*, *hmgcr* and glucokinase (*gck*) gene expressions were analyzed by real-time PCR, using elongation factor-1 alpha (*ef-*

1α) as housekeeping, under the conditions described by Geay *et al.*, (2011). Specific primers used and annealing temperatures (°C) are indicated in Table 15. The PCR program consisted of an initial DNA denaturation of 94°C for 90 s, followed by 45 cycles at 95°C for 30 s and 60°C for 60 s. All PCR reactions were carried out in a I-cycler with optical module (Bio-Rad Hercules, CA, USA) in a final volume of 20 µl, containing 10 µl Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each primer (10 mM) and 6.6 µl of cDNA (1:10 dilution).

Each run was ended with a melting curve analysis resulting in a melting peak profile specific for the amplified target DNA. All reactions were performed in duplicate for each template cDNA. Blank control reactions replaced cDNA with water. Relative gene expression was estimated by the Δ-Δ method (Livak & Schimittgen, 2001) in relation to fish fed ARA0.2 diet.

Table 15. Primers used for expression analyses by real-time PCR in liver of European seabass (*Dicentrarchus labrax*) fed the different dietary treatments (t = 70 days).

Gene	Accession no.	Forward (5′ ➔ 3′)	Reverse (5′ ➔ 3′)
fads2	EU439924	CCTTCACTGCTCTTCATCCCAA	CCCAGGTGGAGGCAGAAGAA
gck	AM986860	GGTGAAGCAAGCCTGAACTC	CTTCCAGCAGTGACTGTCCA
fabp7	FM000669	GAAGGCACTTGGTGTTGGTT	CAGGGTTTTCACCACCACTT
hmgcr	AY424801	CCAGCTTCGTATTCAGCACA	GCTTTGGAGAGGTCGATGAG
ef1α	AJ866727	GCTTCGAGGAAATCACCAAG	CAACCTTCCATCCCTTGAAC

The table shows the gene accession number and the nucleotide sequences for each specific assay: *fads2,* fatty acid desaturase 2; *gck,* glycerol kinase; *fabp7,* fatty acid binding protein 7; *hmgcr,* 3-hydroxy-3-methylglutaryl-coenzyme A reductase; *ef-1α,* elongation factor 1 alpha.

4.3.6. *Statistical analysis*

All data were tested for normality and homogeneity of variance. Statistical analyses followed the methods described by Sokal & Rolf (1995). The significant level for all analyses was set at 5%, and results are presented as means and standard deviations (SD), with exception of gene expression data that is presented as means and standard error (SE). If necessary, transformation of the data was performed.

Data was submitted to a one-way analysis of variance (ANOVA). Two different statistical analyses were performed for all the parameters evaluated, including and excluding the negative control diet (ARA0.1). When F values showed significance, individual means were compared using post hoc tests for multiple means comparison. When variances were

not homogeneous, non-parametric tests were applied. All analyses were performed using SPSS 21 software package for Windows (IBM, Chicago, IL, USA).

4.4. RESULTS

4.4.1. *Biological parameters*

On one side, feeding European seabass with ARA0.1 along the trial (30 and 70 days) clearly reduced ($P < 0.05$) fish total weight and length as well as specific growth rate (SGR) compared to fish fed higher ARA dietary levels (Table 16); however, all fish presented similar condition factor (K) with exception of fish fed ARA0.1 and ARA0.2 after 70 days of feeding (Table 16). Besides, fish fed ARA0.1 presented lower final perivisceral fat index (PFI) and hepatosomatic index (HSI) than fish fed diets ARA0.2 and ARA0.4, although it was similar to that presented by fish fed diets ARA0.8 and ARA1.4. On the other hand, for fish fed ARA dietary levels from 0.2 to 1.4 (g fatty acid * g^{-1} diet), no significant differences ($P>0.05$) were found for final weight, length, K and SGR. For somatic indexes, fish fed ARA0.2 presented higher viscerosomatic index (VSI), HSI and PFI than fish fed higher ARA levels; however, HSI and PFI were not significantly different ($P>0.05$) from fish fed ARA0.4 (Fig. 5). Indeed, VSI ($r=-0.803$, $N=12$, $P=0.002$), HSI ($r=-0.727$, $N=12$, $P=0.024$) and PFI ($r=-0.709$, $N=12$, $P=0.002$) were significantly negatively correlated with dietary ARA content. Fish fed ARA0.2 diet presented the highest ($P<0.05$) final VSI compared to fish fed the rest of the dietary treatments (Table 16).

Survival was high along the feeding trial for all the dietary treatments ranging from 93.3 ± 4.6 to 97.6 ± 1.0%, with no significant differences observed (Table 16).

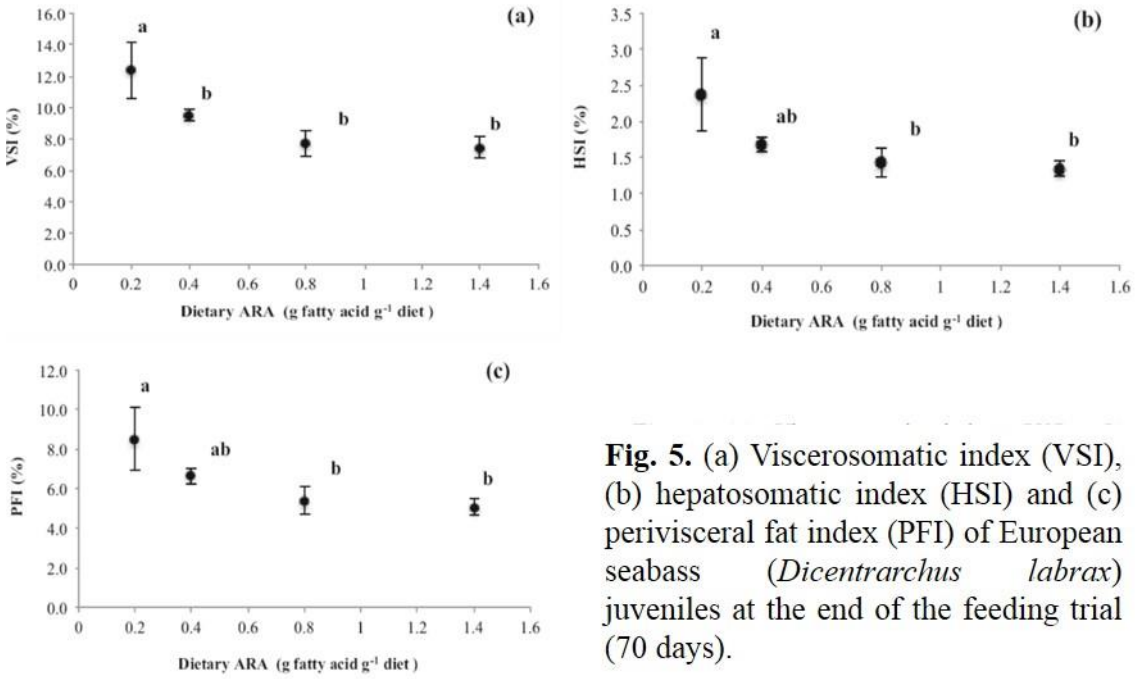

Fig. 5. (a) Viscerosomatic index (VSI), (b) hepatosomatic index (HSI) and (c) perivisceral fat index (PFI) of European seabass (*Dicentrarchus labrax*) juveniles at the end of the feeding trial (70 days).

Table 16. Growth parameters, biometry and feed utilization of fish fed dietary graded level of arachidonic acid (ARA) along the feeding trial.

	Diets				
	ARA0.1 (C-)	ARA0.2	ARA0.4	ARA0.8	ARA1.4
Initial (t= 0 days)					
Weight (g)	13.3 ± 0.2	13.3 ± 0.3	13.6 ± 0.5	13.4 ± 0.2	13.5 ± 0.1
Length (cm)	9.8 ± 0.1	9.8 ± 0.0	9.9 ± 0.1	9.9 ± 0.1	9.9 ± 0.0
K	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0
Mid (t= 30 days)					
Weight (g)	18.1 ± 0.3a	21.9 ± 0.6b	21.9 ± 0.8b	21.9 ± 1.2b	21.0 ± 1.2b
Length (cm)	11.0 ± 0.1a	11.5 ± 0.0b	11.6 ± 0.1b	11.7 ± 0.2b	11.5 ± 0.1b
K	1.3 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0
SGR (%)	0.9 ± 0.1a	1.6 ± 0.1b	1.5 ± 0.1b	1.5 ± 0.1b	1.4 ± 0.2b
Final (t= 70 days)					
Weight (g)	33.0 ± 1.1a	44.4 ± 1.1b	43.8 ± 1.0b	43.9 ± 3.7b	42.8 ± 2.5b
Length (cm)	13.9 ± 0.1a	15.0 ± 0.1b	15.0 ± 0.0b	15.1 ± 0.4b	15.0 ± 0.3b
K	1.2 ± 0.0a	1.3 ± 0.0b	1.3 ± 0.0ab	1.3 ± 0.0ab	1.3 ± 0.0ab
SGR (%)	1.3 ± 0.0a	1.8 ± 0.0b	1.7 ± 0.1b	1.7 ± 0.1b	1.7 ± 0.1b
Total survival (%)	97.0 ± 2.1	93.3 ± 4.6	96.4 ± 3.1	97.0 ± 3.8	97.6 ± 1.0
VSI [(1)]	7.5 ± 0.8a	12.4 ± 1.8b	9.5 ± 0.4a	7.7 ± 0.8a	7.4 ± 0.7a
HSI [(1)]	1.5 ± 0.2a	2.4 ± 0.5c	1.7 ± 0.1bc	1.4 ± 0.2ab	1.3 ± 0.1ab
PFI [(1)]	4.7 ± 0.6a	8.5 ± 1.6c	6.6 ± 0.4bc	5.4 ± 0.7ab	5.1 ± 0.4ab

Values expressed in mean ± SD. N= 3 tanks/diet. Different letters within a line denote significant differences among dietary treatments (P<0.05; one-way ANOVA; Tukey). K condition factor, SGR specific growth rate, FCR feed conversion ratio, PFI perivisceral fat index, HSI hepatosomatic index, VSI viscerosomatic index. [(1)] N= 24 fish/diet.

4.4.2. *Tissue lipid content and fatty acid profiles*

4.4.2.1. Liver fatty acid profiles

Dietary ARA levels did not significantly affect final liver lipid content (P>0.05), and liver fatty acid profile mostly mirrored that of the diet. On one hand, when comparing liver fatty acid profile of fish fed the negative control treatment (ARA0.1) with livers of fish the rest of the dietary treatments, fish fed diet ARA0.1 presented the lowest (P<0.05) saturated and monoenes and the highest (P<0.05) n-3 fatty acid levels (Table 17). No differences (P>0.05) were found for total liver n-3 LC-PUFA or DHA contents among livers of fish fed diets ARA0.1 up to ARA0.8, regardless of the 50–60% lower dietary intake of total n-3 LC-PUFA for fish fed diet ARA0.1 (Tables 5 and 17). Besides, livers of fish fed ARA0.1 showed a relative reduction of a 50–58% EPA in relation to the lower 70–80% EPA dietary percentage when compared with livers of fish fed the rest of the dietary treatments (Table 17).

ARA hepatic content in fish fed ARA0.1 was similar to that presented by fish fed diet ARA0.2, despite that dietary content was relatively lower in a 45%. Certainly, 20:3 n-6 and 18:4 n-3 levels in liver of fish fed ARA0.1 were similar to those presented by fish fed ARA0.2 diet, regardless of the lower dietary content (Tables 5 and 17).

Liver n-3/n-6 ratio tends to decrease as ARA liver content increases, presenting fish fed ARA1.4 diet the lowest (P<0.05). On the other hand, when comparing among profiles of hepatic fatty acid from fish fed ARA0.2 up to ARA1.4, fish fed ARA0.2 presented higher (P<0.05) monoenes and lower (P<0.05) n-6 fatty acids content than fish fed diet ARA0.8 and ARA1.4 diets, but not significantly different (P>0.05) from those presented by fish fed ARA0.4 diet (Table 17). No differences in saturated, total n-3, n-3 LC-PUFA, EPA, docosapentaenoic acid (22:5 n-3, DPA) or DHA fatty acids were detected. However, dietary ARA amount was positively correlated with liver n-3 LC-PUFA (r=0.72, N=12, P=0.009), liver DHA (r=0.77, N=12, P=0.003) and liver DPA (r=0.58, N=12, P=0.047) contents. Moreover, liver EPA content was positively correlated with liver DPA (r=0.97, N=12, P=0.001) and DHA (r=0.88, N=12, P=0.001), as well as liver DPA and DHA amounts found (r=0.95, N=12, P=0.001). Ratio n-3/n-6 tended to decrease as ARA dietary content increased, with fish fed ARA1.4 presenting lower (P<0.05) values than fish fed ARA0.2 diet.

4.4.2.2. Muscle fatty acid profiles

Dietary ARA levels did not significantly affect muscle lipid content (P>0.05) after 70 days of feeding and, in general terms, fatty acid profile mirrored that of the diet (Table 18). On one hand, when comparing muscle fatty acid profile of fish fed the negative control treatment (ARA0.1) with muscle of fish the rest of the dietary treatments, fish fed diet ARA0.1 presented the lowest (P<0.05) saturated, monoenes and n-3 LC-PUFA and highest (P<0.05) n-3 levels. n-6 fatty acid muscle contents of fish fed negative control diet were similar to that presented by fish fed ARA1.4 diet, but higher (P<0.05) than fish fed the rest of the dietary treatments (Table 18). In contrast to the liver, the muscle of fish fed ARA0.1 diet presented an approximately 25% less n-3 LC-PUFA content than the muscle of fish fed higher levels of dietary ARA. Indeed, the muscle of fish fed ARA0.1 presented a relative reduction of a 4–15% in DHA and a 50–55% in EPA content in relation to the lower 25–40% DHA and 72–80% EPA dietary percentage (Tables 5 and 18). Muscle ARA content in fish fed diet ARA0.1 was reduced by a 15, 54, 76, and 84% compared to fish fed diets ARA0.2 up to ARA1.4 respectively, although dietary content was relatively lower in a 45, 71, 85, and 91%, respectively (Tables 5 and 18). Actually, fish fed diet ARA0.1 presented higher (P<0.05) muscle 20:3 n-3 content compared to fish fed other dietary ARA percentages, despite similar dietary levels (Table 18). Muscle n-3/n-6 ratio tended to decrease as ARA dietary content increased, presenting fish fed ARA1.4 diet the lowest (P<0.05). On the other hand, by comparing dietary ARA levels from 0.2 to 1.4 (g fatty acid * g^{-1} diet), no differences (P>0.05) among dietary treatments were found for saturates, n-3 and n-3 LC-PUFA levels. Muscle of fish fed diet ARA0.2 presented the highest (P<0.05) monoenes and lowest (P<0.05) n-6 fatty acid contents (Table 18). Similarly to liver, muscle EPA content was positively correlated with muscle DPA (r=0.99, N=12, P=0.001) and DHA (r=0.95, N=12, P=0.001), as well as liver DPA and DHA amounts found (r=0.97, N=12, P=0.001). Muscle n-3/n-6 tends to decrease as ARA liver content increases; however, fish fed ARA0.2 and ARA0.4 diets presented similar values (P>0.05).

4.4.2.3. Whole body fatty acid profiles

Dietary ARA levels did not significantly affect whole body lipid content (P>0.05) and whole-body fatty acid profile generally reflected that of the diet (Table 19). On one hand, by comparing dietary ARA levels from 0.1 to 1.4 g fatty acid g^{-1} diet, fish fed diet

ARA0.1 presented the lowest (P<0.05) saturated, monoenes and n-3LC-PUFA and the highest (P<0.05) n-3 and n-6 fatty acids levels (Table 19).

Fish fed ARA0.1 and ARA0.2 diets presented similar whole-body ARA and DHA contents, despite of a relative lower 45 and 40% dietary percentage, respectively. Indeed, whole body DHA relative content in fish fed ARA0.1 was only a 5–17% lower than DHA percentages in fish fed higher dietary ARA levels, regardless of the lower 25–40% dietary percentage fed. Similarly, whole body composition of fish fed ARA0.1 diet resulted in an EPA relative reduction of approximately a 50% compared to fish fed the rest of the dietary treatments, despite the lower 70–80% EPA dietary content. Whole body n-3/n-6 ratio tends to decrease as ARA dietary content increases, presenting fish fed ARA1.4 diet the lowest (P<0.05).

On the other hand, excluding negative control treatment, fish fed diet ARA0.2 presented the highest (P<0.05) monoenes and the lowest n-6 levels. Indeed, whole body monoenes and n-3LC-PUFA levels were positively correlated (r=0.77, N=12, P=0.003) among them. No differences (P>0.05) among dietary treatments were found for n-3 levels and fish fed diet ARA0.2 presented lower n-3LC-PUFA level than fish fed diet ARA0.4, but it was not significantly different (P<0.05) from that presented by fish fed diets ARA0.8 and ARA1.4 (Table 19). Whole body ARA content increased accordingly with ARA dietary levels and similarly to the muscle and the liver, whole body EPA content was positively correlated with muscle DPA (r=0.94, N=12, P=0.001) and DHA (r=0.78, n=12, P=0.003), as well as liver DPA and DHA amounts found (r=0.84, N=12, P=0.001). Whole body n-3/n-6 tended to decrease as ARA dietary content increased; however, fish fed ARA0.2 and ARA0.4 diets presented similar values (P>0.05) as detected in muscle.

4.4.3. *Liver morphology*

Qualitative morphological evaluation revealed an effect of dietary ARA level on the liver morphology of European seabass fed the different dietary treatments. Fish fed the negative control diet presented the highest steatosis level when compared to fish fed diets containing higher ARA dietary levels, which presented a moderate to low steatosis level (Fig. 6a). This pattern was more evident in fish fed diets ARA0.4, ARA0.8 and ARA1.4 which, in general terms, presented a more regular-shaped morphology around sinusoidal spaces and a reduction of intracytoplasmic lipid vacuolization resulting in decreased number of hepatocytes with the nuclei displaced to the cellular periphery (Fig. 6b). Fish fed diet ARA0.2 presented an intermediate morphological pattern.

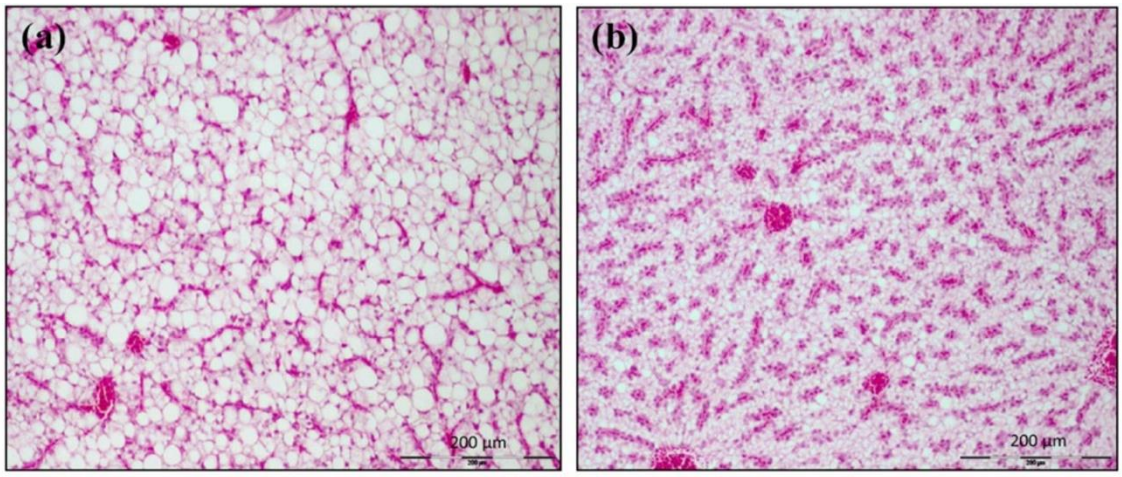

Fig. 6. Hepatocytes (H&E, scale bar 200 µm) morphology of European seabass (*Dicentrarchus labrax*) juveniles at the end of the feeding trial (70 days). (a) Morphological pattern observed in fish diets ARA0.1 and ARA0.2. (b) Morphological pattern observed in fish fed ARA0.4, ARA0.8 and ARA1.4 diets.

4.4.4. *Liver lipid metabolism related gene expression*

In general terms, an upregulation in the expression of the studied genes could be observed in livers of fish fed ARA0.1 in relation to livers of fish fed the rest of the dietary treatments (Fig. 7). Particularly, fish fed diet ARA0.1 presented a significant upregulation ($P<0.05$) of *hmgcr* and *fads2* compared to the rest of the dietary treatments (Fig. 7). When comparing fish fed diets ARA0.2 up to ARA1.4, no significant ($P>0.05$) differences were observed for the genes studied (Fig. 7).

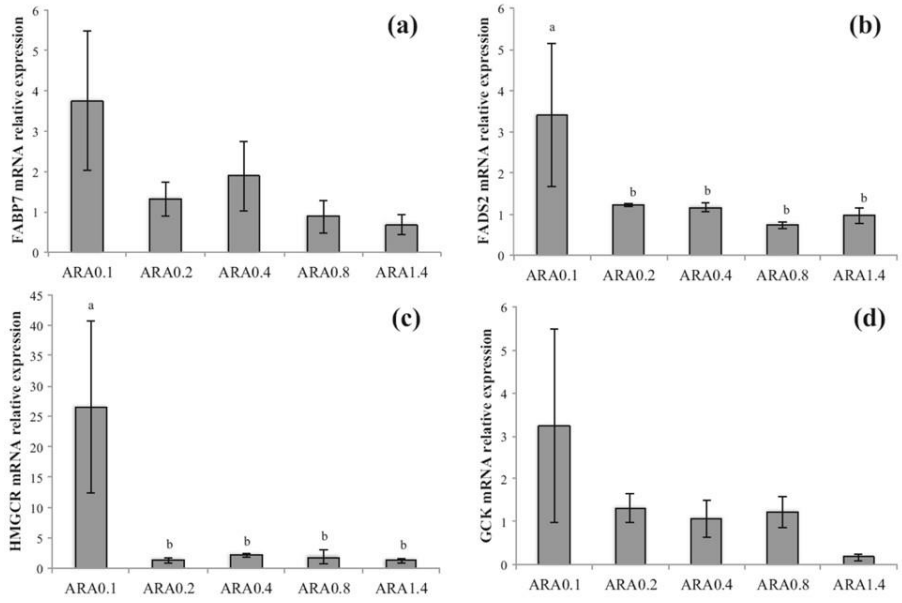

Fig. 7. Liver relative gene expression of European seabass (*Dicentrarchus labrax*) juveniles: (a) *fabp7*, (b) *fads2*, (c) *hmgcr* and (d) *gck* at the end of the feeding trial (70 days). Data expressed (mean ± SE) relatively to fish fed ARA0.2 diet.

Table 17. Liver lipid content (g * 100 g^{-1} dry weight) and fatty acids profile (g fatty acid * g^{-1} tissue) of European seabass (*Dicentrarchus labrax*) fed the experimental diets at the end of the feeding trial (t = 70 days).

	ARA0.1 (C-)	ARA0.2	ARA0.4	ARA0.8	ARA1.4
Lipids (d.w.)	55.05 ± 3.21	50.04 ± 0.86	45.89 ± 1.12	50.12 ± 2.16	51.06 ± 1.41
14:0	0.15 ± 0.02a	0.54 ± 0.07b	0.65 ± 0.07b	0.54 ± 0.08b	0.61 ± 0.04b
16:0	1.68 ± 0.12a	4.01 ± 0.38b	3.57 ± 0.38b	3.29 ± 0.10b	3.25 ± 0.16b
18:0	0.60 ± 0.05a	0.90 ± 0.07b	0.83 ± 0.07b	0.81 ± 0.05b	0.86 ± 0.03b
Total saturates[a]	2.49 ± 0.13a	5.59 ± 0.50b	5.22 ± 0.50b	4.79 ± 0.17b	4.89 ± 0.23b
16:1 n-7	0.25 ± 0.02a	1.14 ± 0.08b	1.14 ± 0.08b	1.02 ± 0.09b	1.06 ± 0.07b
18:1 n-9	4.04 ± 0.39a	6.17 ± 0.26c/B	5.53 ± 0.26bc/AB	5.15 ± 0.55bc/AB	4.80 ± 0.34ab/A
18:1 n-7	0.27 ± 0.01a	0.76 ± 0.04c/B	0.75 ± 0.04c/AB	0.70 ± 0.03bc/AB	0.67 ± 0.03bc/A
20:1 n-7	0.19 ± 0.01a	0.43 ± 0.03c/A	0.46 ± 0.03c/AB	0.39 ± 0.01b/B	0.39 ± 0.02b/AB
22:1 n-11	0.04 ± 0.01a	0.19 ± 0.02bc/AB	0.21 ± 0.02c/B	0.16 ± 0.01b/A	0.17 ± 0.01b/A
Total monoenes[b]	4.88 ± 0.35a	9.02 ± 0.42c/B	8.42 ± 0.42bc/AB	7.70 ± 0.47b/A	7.38 ± 0.42b/A
18:2 n-6	2.54 ± 0.12c	0.94 ± 0.04a/AB	1.13 ± 0.04ab/A	1.09 ± 0.15b/AB	1.28 ± 0.04ab/B
18:3 n-6	0.06 ± 0.01d	0.06 ± 0.00d/A	0.07 ± 0.00c/B	0.10 ± 0.00b/C	0.13 ± 0.00a/D
20:2 n-6	0.13 ± 0.01c	0.09 ± 0.00a/A	0.10 ± 0.00ab/AB	0.11 ± 0.01ab/AB	0.12 ± 0.01bc/B
20:3 n-6	0.02 ± 0.00a	0.02 ± 0.00b/A	0.04 ± 0.00c/B	0.07 ± 0.01d/AB	0.11 ± 0.00e/C
20:4 n-6	0.22 ± 0.01a	0.19 ± 0.02a/A	0.35 ± 0.02b/B	0.74 ± 0.07c/C	1.20 ± 0.04d/D
22:5 n-6	0.13 ± 0.01b	0.08 ± 0.01a/A	0.10 ± 0.01ab/AB	0.12 ± 0.01b/B	0.13 ± 0.01b/B
Total n-6 PUFA[c]	3.12 ± 0.11a	1.43 ± 0.06c/A	1.84 ± 0.06bc/AB	2.29 ± 0.24b/BC	3.03 ± 0.06ab/C
18:3 n-3	6.04 ± 0.11b	0.22 ± 0.01a	0.24 ± 0.01a	0.21 ± 0.02a	0.23 ± 0.01a
18:4 n-3	0.14 ± 0.03	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.00	0.14 ± 0.01
20:3 n-3	0.08 ± 0.01b	0.03 ± 0.00a	0.03 ± 0.00a	0.03 ± 0.00a	0.03 ± 0.00a
20:4 n-3	0.04 ± 0.00a	0.10 ± 0.01b	0.10 ± 0.01b	0.10 ± 0.01b	0.10 ± 0.01b
20:5 n-3	0.57 ± 0.07a	1.22 ± 0.20b	1.18 ± 0.20b	1.29 ± 0.01b	1.34 ± 0.13b
22:5 n-3	0.18 ± 0.02a	0.32 ± 0.06b	0.31 ± 0.06b	0.36 ± 0.01b	0.38 ± 0.04b
22:6 n-3	3.09 ± 0.28a	2.81 ± 0.61a	2.98 ± 0.61a	3.81 ± 0.12ab	4.09 ± 0.43b
Total n-3 PUFA[d]	10.16 ± 0.42b	4.90 ± 0.89a	5.04 ± 0.89a	6.00 ± 0.14a	6.38 ± 0.62a
n-3 LC-PUFA	3.96 ± 0.36a	4.48 ± 0.87ab	4.59 ± 0.87ab	5.59 ± 0.13ab	5.95 ± 0.60b
ARA/EPA	0.39 ± 0.03b	0.15 ± 0.03a/A	0.30 ± 0.03b/B	0.58 ± 0.05c/C	0.90 ± 0.05d/D
EPA/ARA	2.59 ± 0.19c	6.49 ± 0.34e/C	3.35 ± 0.34d/B	1.74 ± 0.15b/A	1.12 ± 0.07a/A
DHA/ARA	14.01 ± 0.60d	14.87 ± 1.14d/C	8.43 ± 1.14c/B	5.16 ± 0.31b/A	3.41 ± 0.24a/A
n-3/n-6	3.26 ± 0.13b	3.40 ± 0.44b/B	2.74 ± 0.44ab/AB	2.64 ± 0.21ab/AB	2.11 ± 0.19a/A

Values expressed in mean ± SD. n = 3 tanks/diet. Different uppercase letters within a line denote significant differences among dietary treatments among fish fed ARA0.2 up to ARA1.4 ($P<0.05$; one-way ANOVA; Tukey). Different lowercase letters within a line denote significant differences among dietary treatments among fish fed ARA0.1 up to ARA1.4 ($P < 0.05$; one-way ANOVA; Tukey).

a 15:0, 17: 0 and 20:0

b 14:1 n-5, 14:1n-7, 15:1 n-5, 16:1 n-5, 18:1 n-5, 20:1 n-5, 20:1 n-9 and 22:1 n-9

c 16:2 n-6 and 22:4 n-6

d 16:3 n-3 and 16:4 n-3.

Table 18. Muscle lipid content (g * 100 g^{-1} dry weight) and fatty acids profile (g fatty acid * g^{-1} tissue) of European seabass (*Dicentrarchus labrax*) fed the experimental diets at the end of the feeding trial (t = 70 days).

	ARA0.1 (C-)	ARA0.2	ARA0.4	ARA0.8	ARA1.4
Lipids (d.w.)	18.87 ± 1.11	16.00 ± 4.90	22.57 ± 4.71	22.32 ± 6.54	14.55 ± 3.08
14:0	0.30 ± 0.02a	0.91 ± 0.06b	0.83 ± 0.06b	0.82 ± 0.05b	0.82 ± 0.04b
16:0	2.82 ± 0.19a	4.50 ± 0.16b	4.24 ± 0.16b	4.14 ± 0.12b	4.40 ± 0.21b
18:0	0.93 ± 0.05a	0.97 ± 0.08a/AB	0.87 ± 0.08a/A	0.98 ± 0.13a/AB	1.14 ± 0.11b/B
Total saturates[a]	4.16 ± 0.20a	6.61 ± 0.17b	6.17 ± 0.17b	6.18 ± 0.19b	6.60 ± 0.35b
16:1 n-7	0.46 ± 0.06a	1.28 ± 0.09b	1.19 ± 0.09b	1.15 ± 0.04b	1.14 ± 0.01b
18:1 n-9	4.58 ± 0.15	4.90 ± 0.10	4.75 ± 0.10	4.65 ± 0.14	4.75 ± 0.19
18:1 n-7	0.35 ± 0.02a	0.70 ± 0.02c/B	0.65 ± 0.02bc/A	0.65 ± 0.01b/A	0.65 ± 0.02bc/A
20:1 n-7	0.28 ± 0.01a	0.53 ± 0.03b	0.50 ± 0.03b	0.50 ± 0.01b	0.49 ± 0.02b
22:1 n-11	0.07 ± 0.01a	0.31 ± 0.03c	0.28 ± 0.03bc	0.29 ± 0.01bc	0.26 ± 0.02b
Total monoenes[b]	5.89 ± 0.24a	8.06 ± 0.18c/B	7.70 ± 0.18b/A	7.56 ± 0.11b/A	7.61 ± 0.15b/A
18:2 n-6	2.33 ± 0.10b	1.29 ± 0.08a	1.31 ± 0.08a	1.34 ± 0.03a	1.42 ± 0.08a
18:3 n-6	0.03 ± 0.00a	0.04 ± 0.00abc/A	0.07 ± 0.00b/AB	0.09 ± 0.00c/B	0.11 ± 0.00d/C
20:2 n-6	0.13 ± 0.00a	0.11 ± 0.00b/AB	0.11 ± 0.00b/A	0.11 ± 0.01ab/AB	0.12 ± 0.00ab/B
20:3 n-6	0.01 ± 0.01a	0.02 ± 0.00b/A	0.04 ± 0.00c/B	0.07 ± 0.00d/C	0.09 ± 0.01e/D
20:4 n-6	0.16 ± 0.01a	0.19 ± 0.04b/A	0.35 ± 0.04c/B	0.69 ± 0.02d/C	1.00 ± 0.06e/D
22:5 n-6	0.10 ± 0.00c	0.08 ± 0.00a/A	0.09 ± 0.00b/B	0.10 ± 0.00bc/B	0.10 ± 0.00bc/B
Total n-6 PUFA[c]	2.79 ± 0.09d	1.77 ± 0.04a/A	2.00 ± 0.04b/B	2.43 ± 0.01c/C	2.89 ± 0.03d/D
18:3 n-3	4.67 ± 0.47b	0.32 ± 0.01a	0.29 ± 0.01a	0.26 ± 0.01a	0.27 ± 0.02a
18:4 n-3	0.08 ± 0.01a	0.16 ± 0.02b	0.15 ± 0.02b	0.14 ± 0.02b	0.14 ± 0.01b
20:3 n-3	0.06 ± 0.01b	0.04 ± 0.00a	0.04 ± 0.00a	0.03 ± 0.00a	0.03 ± 0.01a
20:4 n-3	0.04 ± 0.00a	0.09 ± 0.01b	0.09 ± 0.01b	0.09 ± 0.01b	0.08 ± 0.00b
20:5 n-3	0.58 ± 0.07a	1.28 ± 0.05b	1.24 ± 0.05b	1.19 ± 0.13b	1.17 ± 0.06b
22:5 n-3	0.14 ± 0.00a	0.27 ± 0.01b	0.26 ± 0.01b	0.26 ± 0.02b	0.25 ± 0.02b
22:6 n-3	2.16 ± 0.04a	2.25 ± 0.16ab	2.47 ± 0.16b	2.53 ± 0.12b	2.53 ± 0.17b
Total n-3 PUFA[d]	7.76 ± 0.36b	4.52 ± 0.08a	4.65 ± 0.08a	4.60 ± 0.30a	4.58 ± 0.28a
n-3 LC-PUFA	2.98 ± 0.10a	3.93 ± 0.11b	4.11 ± 0.11b	4.10 ± 0.25b	4.07 ± 0.25b
ARA/EPA	0.28 ± 0.02b	0.15 ± 0.04a/A	0.28 ± 0.04b/B	0.58 ± 0.06c/C	0.85 ± 0.07d/D
EPA/ARA	3.56 ± 0.24b	6.67 ± 0.52c/C	3.59 ± 0.52b/B	1.74 ± 0.18a/A	1.18 ± 0.10a/A
DHA/ARA	13.38 ± 0.91d	11.69 ± 0.30c/D	7.12 ± 0.30b/C	3.69 ± 0.08a/B	2.55 ± 0.28a/A
n-3/n-6	2.78 ± 0.05e	2.56 ± 0.07d/C	2.32 ± 0.07c/C	1.89 ± 0.13b/B	1.59 ± 0.08a/A

Values expressed in mean ± SD. n = 3 tanks/diet. Different uppercase letters within a line denote significant differences among dietary treatments among fish fed ARA0.2 up to ARA1.4 ($P<0.05$; one-way ANOVA; Tukey). Different lowercase letters within a line denote significant differences among dietary treatments among fish fed ARA0.1 up to ARA1.4 ($P < 0.05$; one-way ANOVA; Tukey).

a 15:0, 17: 0 and 20:0

b 14:1 n-5, 14:1 n-7, 15:1 n-5, 16:1 n-5, 18:1 n-5, 20:1 n-5, 20:1 n-9 and 22:1 n-9

c 16:2 n-6 and 22:4 n-6

d 16:3 n-3 and 16:4 n-3

Table 19. Whole body lipid content (g * 100 g^{-1} dry weight) and fatty acids profile (g * fatty acid g^{-1} tissue) of European seabass (*Dicentrarchus labrax*) fed the experimental diets at the end of the feeding trial (t = 70 days).

Lipids (d.w.)	ARA0.1 (C-)	ARA0.2	ARA0.4	ARA0.8	ARA1.4
14:0	0.30 ± 0.02a	0.84 ± 0.03b	0.80 ± 0.03b	0.79 ± 0.00b	0.79 ± 0.04b
16:0	2.14 ± 0.09a	3.66 ± 0.05c/B	3.40 ± 0.05b/A	3.44 ± 0.02b/A	3.56 ± 0.09bc/AB
18:0	0.65 ± 0.01a	0.75 ± 0.02bc/AB	0.72 ± 0.02b/A	0.78 ± 0.03c/B	0.85 ± 0.02d/C
Total saturates[a]	3.19 ± 0.10a	5.47 ± 0.09c/B	5.14 ± 0.09b/A	5.24 ± 0.05bc/AB	5.42 ± 0.15c/B
16:1 n-7	0.48 ± 0.03a	1.24 ± 0.02c/B	1.18 ± 0.02bc/AB	1.14 ± 0.02b/A	1.15 ± 0.04b/A
18:1 n-9	4.20 ± 0.02a	4.75 ± 0.09c/B	4.37 ± 0.09ab/A	4.31 ± 0.02a/A	4.54 ± 0.09b/AB
18:1 n-7	0.34 ± 0.01a	0.66 ± 0.02c	0.62 ± 0.02b	0.60 ± 0.01b	0.60 ± 0.01b
20:1 n-7	0.30 ± 0.01a	0.53 ± 0.01c/B	0.51 ± 0.01c/B	0.47 ± 0.02b/A	0.47 ± 0.02b/A
22:1 n-11	0.10 ± 0.01a	0.34 ± 0.02c/B	0.31 ± 0.02bc/AB	0.27 ± 0.02b/A	0.26 ± 0.03b/A
Total monoenes[b]	5.56 ± 0.07a	7.85 ± 0.10c/B	7.29 ± 0.10b/A	7.09 ± 0.06b/A	7.31 ± 0.12b/A
18:2 n-6	2.34 ± 0.07c	1.39 ± 0.01a/AB	1.33 ± 0.01a/A	1.41 ± 0.04a/B	1.51 ± 0.04b/C
18:3 n-6	0.03 ± 0.00a	0.04 ± 0.00b/A	0.05 ± 0.00c/B	0.08 ± 0.00d/C	0.10 ± 0.00e/D
20:2 n-6	0.13 ± 0.01b	0.11 ± 0.00a	0.11 ± 0.00a	0.11 ± 0.00a	0.11 ± 0.00a
20:3 n-6	0.02 ± 0.00a	0.03 ± 0.00a/A	0.04 ± 0.00b/B	0.07 ± 0.01c/C	0.10 ± 0.01d/D
20:4 n-6	0.17 ± 0.04d	0.19 ± 0.01d/A	0.36 ± 0.01c/B	0.70 ± 0.01b/C	1.02 ± 0.02a/D
22:5 n-6	0.11 ± 0.01a	0.09 ± 0.00b/A	0.11 ± 0.00b/B	0.11 ± 0.00b/B	0.11 ± 0.00b/B
Total n-6 PUFA[c]	2.83 ± 0.02a	1.88 ± 0.01b/A	2.04 ± 0.01c/B	2.52 ± 0.06d/C	3.01 ± 0.02e/D
18:3 n-3	5.11 ± 0.25a	0.41 ± 0.01b	0.32 ± 0.01b	0.31 ± 0.01b	0.32 ± 0.01b
18:4 n-3	0.11 ± 0.01a	0.23 ± 0.01c/B	0.21 ± 0.01bc/AB	0.20 ± 0.01b/A	0.20 ± 0.01b/A
20:3 n-3	0.06 ± 0.00b	0.03 ± 0.00a	0.03 ± 0.00a	0.03 ± 0.00a	0.03 ± 0.00a
20:4 n-3	0.06 ± 0.00a	0.12 ± 0.00c/B	0.12 ± 0.00c/B	0.11 ± 0.00b/A	0.11 ± 0.00b/A
20:5 n-3	0.79 ± 0.04a	1.67 ± 0.07b	1.66 ± 0.07b	1.62 ± 0.07b	1.61 ± 0.07b
22:5 n-3	0.20 ± 0.02a	0.36 ± 0.01b	0.37 ± 0.01b	0.35 ± 0.00b	0.35 ± 0.01b
22:6 n-3	2.65 ± 0.14a	2.80 ± 0.07a	3.20 ± 0.07b	3.18 ± 0.06b	3.18 ± 0.10b
Total n-3 PUFA[d]	9.01 ± 0.17b	5.73 ± 0.14a	6.02 ± 0.14a	5.91 ± 0.14a	5.91 ± 0.18a
n-3 LC-PUFA	3.76 ± 0.20a	4.99 ± 0.13b/A	5.38 ± 0.13c/B	5.28 ± 0.13bc/AB	5.28 ± 0.16bc/AB
ARA/EPA	0.21 ± 0.04b	0.11 ± 0.01a/A	0.22 ± 0.01b/B	0.44 ± 0.03c/C	0.63 ± 0.03d/D
EPA/ARA	4.85 ± 0.86b	8.86 ± 0.20c/D	4.58 ± 0.20b/C	2.30 ± 0.14a/B	1.58 ± 0.07a/A
DHA/ARA	16.42 ± 3.13c	14.82 ± 0.38c/D	8.84 ± 0.38b/C	4.52 ± 0.15a/B	3.11 ± 0.07a/A
n-3/n-6	3.18 ± 0.07d	3.04 ± 0.07cd/C	2.94 ± 0.07c/C	2.34 ± 0.11b/B	1.96 ± 0.05a/A

Values expressed in mean ± SD. n = 3 tanks/diet. Different uppercase letters within a line denote significant differences among dietary treatments among fish fed ARA0.2 up to ARA1.4 ($P<0.05$; one-way ANOVA; Tukey). Different lowercase letters within a line denote significant differences among dietary treatments among fish fed ARA0.1 up to ARA1.4 ($P < 0.05$; one-way ANOVA; Tukey).

a 15:0, 17: 0 and 20:0

b 14:1 n-5, 14:1 n-7, 15:1 n-5, 16:1 n-5, 18:1 n-5, 20:1 n-5, 20:1 n-9 and 22:1 n-9

c 16:2 n-6 and 22:4 n-6

d 16:3 n-3 and 16:4 n-3

4.5. DISCUSSION

Dietary supplementation of ARA from 0.2 up to 1.4 (g fatty acid * g^{-1} diet) did not affect European seabass growth performance and survival after 70 days of feeding when supplemented in a high FM/FO based diet (n-3 LC-PUFA 4.5–5.7 and n-6 LC-PUFA 0.3–1.6 (g fatty acid * g^{-1} diet). This finding is in agreement with previous studies in juvenile Atlantic salmon fed dietary ARA levels from 0.2 to 0.8 (g fatty acid * g^{-1} diet) for 12 weeks supplemented in low FO diets (Dantagnan *et al.,* 2017), in young gilthead seabream (*Sparus aurata*) fed for 54 days increased levels of dietary ARA (0.03 up to 1.68 (g fatty acid g^{-1} diet) in 100% VO diet (Fountoulaki *et al.,* 2003) and in gobid (*Synechogobius hasta*) fed for 8 weeks levels from 0.9 to 6.5 (g fatty acid * g^{-1} diet) in a 48%FM/0%FO based diet (Luo *et al.,* 2012). However, fish fed the negative control diet, which totally replaced FO content and contained defatted FM, presented reduced growth just only 30 days after the start of the feeding trial. Similarly, decreased growth has been observed in European seabass fed total replacement of dietary FM and FO by plant-based ingredients (Geay *et al.,* 2011; Le Boucher *et al.,* 2011, 2013; Torrecillas *et al.,* 2017a, b). Particularly, Torrecillas *et al.,* (2017a) found a clear reduction of weight gain in juvenile European seabass fed for 90 days a diet without FM and FO (n-3 LC-PUFA 0.14% and n-6 LC-PUFA 0.02 g fatty acid * g^{-1} diet). However, supplementation with LC-PUFA (n-3 LC-PUFA 1.1% and n-6 LC-PUFA 0.16 g fatty acid * g^{-1} diet) led to increased growth (Torrecillas *et al.,* 2017a) denoting the important role of LC-PUFA on fish growth (Izquierdo & Koven 2011).

Not only a deficiency but also an excess of dietary LC-PUFA may lead to reduced fish growth performance. For instance, Japanese sea bass (*Lateolabrax japonicus*) fed ARA dietary supplementation greater than 0.7% g fatty acid * g^{-1} diet in zero FO diets for 12 weeks resulted in reduced growth performance despite containing similar n-3 LC-PUFA percentages (Xu *et al.,* 2010).

It is not clear why ARA affects growth in fish, but it has been speculated that ARA can modulate the growth through altering the ratio of prostaglandin E2 (PGE2) and prostaglandin F2 alpha (PGF2α; Palmer 1990) related with alterations on muscle fiber formation and protein degradation (Bell & Sargent 2003). Indeed, it has been suggested that some of the discrepancies reported for dietary ARA effects on fish growth performance may be related to low EPA dietary levels (Norambuena *et al.,* 2016). In this sense, Atlantic salmon fed proportions of ARA/EPA around 1:4.2 significantly increased

weight gain when compared to fish fed low or high ratios of ARA and EPA (1:1.5 and 1:9) after 14 weeks of feeding (Norambuena *et al.*, 2016). However, when supplemented for a total of 20 weeks, fish fed the lowest ARA/EPA ratio were similar in weight gain to fish fed 1:4.2 ARA/EPA ratio. In the present trial, no differences were found in growth performance despite the dietary difference in the ARA/EPA ratio among fish fed diet ARA0.2 (1:8.8) and fish fed higher dietary ARA levels; however, the effect of ARA/EPA ratio on European seabass growth productivity in a long-term feeding study must be evaluated before concluding in relation to this particular subject.

When comparing among ARA-supplemented groups, fish fed ARA0.2 presented higher levels of lipid deposition as denoted by higher PFI, HSI and VSI indexes; meanwhile, there was a reduction in lipid deposition with enhanced levels of dietary ARA. Indeed, there was a strong linear correlation between dietary ARA levels and the indexes (HSI= 0.73, N= 12, P= 0.024; VSI= 0.80, N= 12, P= 0.002; PFI= 0.79, N= 12, P= 0.002) when the feed absent in ARA was exempted from the analysis. Similar results have been observed in other teleost species where increasing levels of dietary ARA levels were negatively correlated with lipid deposition in whole body and individual tissues (Castell *et al.*, 1994; Fountoulaki *et al.*, 2003; Xu *et al.*, 2010; Luo *et al.*, 2012). In fact, it has been suggested that dietary ARA may reduce lipid accumulation and inhibit biosynthesis of fatty acids (Norambuena *et al.*, 2016). In this sense, grass carp (*Ctenopharyngodon idellus*) fed diets containing intermediate levels of ARA (0.3 g fatty acid * g^{-1} diet) showed higher PFI than fish fed lower or higher ARA levels (0.03 and 0.6 g fatty acid * g^{-1} diet; Tian *et al.*, 2014). Additionally, Japanese sea bass showed a significant decrease in HSI and a trend to reduce VSI with increasing dietary levels of ARA (0.36 up to 2.24 g fatty acid * g^{-1} diet; Xu *et al.*, 2010) in FM/VO supplemented with EFA enriched oil diet, similarly to what was observed in the present trial, with the exemption of fish fed negative control diet, where the reduced HSI could be probably attributed to the nutritional status of the fish. Indeed, the trend to higher accumulation of perivisceral fat and increased VSI and HSI, observed in fish fed ARA0.2, is in concordance with the trend to the greater lipid vacuolization of the cytoplasm observed in relation to fish fed higher dietary ARA levels and lower ARA/EPA ratios.

Generally, the fatty acid profiles of fish tissues reflect those of the diets, being this particularly true in marine fish species with a reduced LC-PUFA biosynthesis activity. In this sense, differences in the fatty acid profile among tissues could indicate physiological relevance of that fatty acid for that particular tissue. In the present study, the incorporation

of ARA in tissue total lipids did not vary among the evaluated tissues, with ARA increasing when increasing in diet. Previous studies with differing dietary ARA levels showed a selective incorporation into several organs other than liver and muscle, which are of special importance on immune defense, such as the gills, kidney, HK leucocytes and plasma (Castell *et al.,* 1994; Betancor *et al.,* 2014; Torrecillas *et al.,* 2017b). The role of ARA on immune system function involves effects on cell membrane fluidity and stability, ion transport, trafficking and vesicular transport, membrane-associated enzymes activities, alterations of gene-encoding proteins involved in lipid metabolism or activation of NADPH oxidase activity (Torrecillas *et al.,* 2017b). Besides, eicosanoids derived from ARA increase vascular permeability and vasodilatation, induce leucocytes chemotaxis and promote generation of reactive oxygen species (ROS) (Calder, 2006). Muscle is a storage tissue and as expected, its fatty acid profile reflected dietary contents in agreement with previous studies (Izquierdo *et al.,* 2003; Montero *et al.,* 2005; Torrecillas *et al.,* 2016; Betancor *et al.,* 2016b), although levels of some fatty acids such as EPA, DHA and ARA were found in higher proportion in fish flesh than in the feeds for fish fed the negative control diet.

Indeed, ARA and DHA contents in flesh and whole body for fish fed diets ARA0.1 and ARA0.2 were similar regardless of the different dietary contents, despite that ARA is a minor component of membranes in fish compared to EPA and DHA and is mainly stored in polar lipids (Tocher, 2010; Norambuena *et al.,* 2016).

Besides, EPA content was positively correlated with muscle and whole body DPA and DHA content when negative control was exempted from the analysis. Altogether, it may be indicating not only a selective accumulation of DHA but also pointing to a functional n-3 LC-PUFA biosynthesis towards DHA.

The liver is considered an important site for LC-PUFA synthesis and lipid metabolism in fish (Monroig *et al.,* 2010) and is expected that differences in feed fatty acid profile may elicit changes in this tissue. For fish fed reduced levels of n-3 LC-PUFA in combination with increased levels of C:18 fatty acids (negative control diet), the evident liver morphological pattern alteration observed is in agreement with previous studies in this (Torrecillas *et al.,* 2016, 2017a and 2017b) and other Mediterranean fish species (Caballero *et al.,* 2003). Despite of seeming to indicate that the negative control diet did not fulfill European seabass nutritional requirements for a proper growth performance with consequences in liver health status, signs of a selective accumulation of DHA and an active n-3 LC-PUFA biosynthesis could be observed in their liver as indicated by the

lack of differences in the DHA contents among fish fed diets ARA0.1 up to ARA 0.8 as well as enhanced liver contents of the intermediate product 18:4 n-3, which may indicate an certain activity of elongases and desaturases involved in LC-PUFA biosynthesis. Indeed, when negative control diet was exempted from the analysis, n-3 LC-PUFA, DPA and DHA liver contents were positively correlated with dietary ARA and liver DPA, whereas liver EPA was positively correlated with liver DPA and DHA. Additionally, increased expression of *fads2* was significantly higher in ARA0.1-fed fish particularly when compared to fish fed ARA0.8. In order to achieve isolipidic feeds, high levels of VO were used in the formulation of ARA0.1, which consequently generated high levels of C:18 fatty acids in the feeds and reduced levels of not only ARA but also EPA and DHA. This is the reason why it was expectable to find an upregulation in the expression of fads2 in fish fed the lowest LC-PUFA levels as observed in previous studies substituting FO by VO blends in European seabass (González-Rovira *et al.,* 2009; Geay *et al.,* 2010; Castro *et al.,* 2015b). As *fads2* also participates in the desaturation of 18:2 n-6 to 18:3 n-6 (Monroig *et al.,* 2010), enhanced levels of this intermediate product were also found in the liver of fish fed ARA0.1 compared to dietary content, indicating additional biosynthesis towards n-6 LC-PUFA. Indeed, liver ARA content was positively correlated with liver 18:3 n-6 ($r= 0.98$, $N= 16$, $P<0.001$). The reason why levels of ARA differed greatly in the liver among fish fed the different dietary treatments whereas DHA levels were the same could be due to higher preference of Fads2 protein towards 18:3 n-3 rather than 18:2 n-6 as has been proved *in vitro* (14.5% versus 5.6%; González-Rovira *et al.,* 2009). It must be noted though that the European seabass, as a carnivorous marine fish, has a marine fish pattern in the metabolism of LC-PUFA meaning that it has limited capacity to convert C:18 fatty acids to ARA, EPA and DHA (González-Rovira *et al.,* 2009). Thus, the small rates of LC-PUFA produced are not enough to enhance whole body or flesh levels of DHA, EPA or ARA. Another lipid metabolism related gene that was upregulated in fish fed negative control diet was *hmgcr*, the rate-limiting enzyme in cholesterol biosynthesis (Le Martelot *et al.,* 2009). Upregulation in genes belonging to the cholesterol pathway has been previously described in fish fed more sustainable feeds (Geay *et al.,* 2011; Betancor *et al.,* 2015a), particularly those containing high levels of VO as this is naturally low in cholesterol and can contain cholesterol-lowering phytosterols (Gilman *et al.,* 2003). Thus, the enhancement in the expression of *hmgcr* in fish fed ARA0.1, low in dietary n-3 and n-6 PUFA content and with high percentages 18:2 n-6 and 18:3 n-3 as consequence of not contain FO may correspond to a

compensatory mechanism in order to produce the cholesterol levels necessary to fulfill fish requirements rather than a direct effect of the lack of ARA. These findings are in agreement with previous studies in Atlantic salmon, in which FO replacement by VO elicits not only liver compositional changes but also metabolic alterations in LC-PUFA and cholesterol biosynthetic pathways in order to compensate for deficiencies in the diet, being *fads2*, sterol regulatory element binding transcription factor 1 and 2 *(srebp-1* and *srebp-2*) especially upregulated (Leaver *et al.,* 2008; Morais *et al.,* 2011).

The expression of *fabp7*, a gene related to fatty acid transport and uptake, tended to be upregulated in the liver of European seabass fed ARA0.1, although no differences could be observed among the other dietary treatments. In a similar trial where European seabass were fed diets containing high levels of terrestrial ingredients showed an upregulation in this gene (Geay *et al.,* 2011) and gilthead seabream and cod fed plant-based feed showed upregulation in fabp2 when fed on more sustainable feeds (Lilleeng *et al.,* 2007; Betancor *et al.,* 2016b). Additionally, these fish also showed the lowest performance, what could be suggesting that the upregulation in *fabp7* expression may be associated with the lower growth of these fish, which in turn could elicit compensatory mechanisms such as fatty acid mobilization.

ARA did not show to regulate the hepatic expression of *gck*, a phosphotransferase enzyme involved in triglycerides and glycerophospholipids synthesis, although there was a trend for an upregulation in the liver if fish were fed with the negative control diet. Previous studies in salmonids have also found an upregulation in the expression of *gck* when fish were fed terrestrial ingredient diets (Panserat *et al.,* 2009; Morais *et al.,* 2011), and it was hypothesized that it was related to higher lipid biosynthesis in liver. In the present trial, the higher lipid vacuolization degree in liver was observed in fish fed the lower dietary ARA levels and thus seems the result of activation in triglyceride synthesis although not directly elicited by *gck* as no differences were found in terms of gene expression.

In summary, dietary ARA levels up to a 0.2g fatty acid * g^{-1} diet seem to be necessary to fulfill juvenile European seabass requirements for this fatty acid and guarantee proper growth. Increasing ARA levels between a 0.2g and a 1.4g fatty acid * g^{-1} diet did not enhance fish growth or survival, but levels over a 0.2g fatty acid * g^{-1} diet seemed to inhibit triglyceride biosynthesis as reduced PFI, HSI and VSI were observed. An upregulation in the hepatic expression of *fads2* in fish fed the lowest ARA levels (0.1 g fatty acid * g^{-1} diet) seemed to be related to desaturation not only from 18:2 n-6 to 18:3 n-6 but also from 18:3 n-3 to 18:4 n-3, as also levels of n-3 LC-PUFA were low in this

feed. Additionally, an upregulation in the expression of *hmgcr*, the rate-limiting enzyme in cholesterol biosynthesis, was observed in the liver of ARA0.1-fed fish, what seems to indicate the response of fish to low levels of dietary cholesterol although a direct effect of ARA on the regulation of *hmgcr* cannot be excluded as has been found in mammals. Results from the present study point out the necessity to include adequate levels of n-6 LC-PUFA in diets for European seabass juveniles. Therefore, the new alternatives to FO investigated should aim to contain not only adequate levels of n-3 LC-PUFA but also n-6 LC-PUFA.

Chapter 5

EFFECTS OF DIETARY ARACHIDONIC ACID IN EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) DISTAL INTESTINE LIPID CLASSES AND GUT HEALTH

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ST, MI, MJ & DM conceived and planned the experiments. FRR carried out the experiments and contributed to sample preparation. FR and MB contributed to analyses. FR, ST, MB, MJ & DM contributed to the interpretation of the results and discussion. FR, ST & DM wrote the paper with input from all authors.

5.1. ABSTRACT

The use of low fishmeal/fish oil in marine fish diets affects dietary essential fatty acids (EFAs) composition and concentration and subsequently, may produce a marginal deficiency of those fatty acids with a direct impact on the fish intestinal physiology. Supplementation of essential fatty acids is necessary to cover the requirements of the different EFAs, including the ones belonging to the n-6 series, such as arachidonic acid (ARA). Besides its structural role in the configuration of the lipid classes of intestine, ARA plays an important role on the functionality of the gut associated immune tissue (GALT).

The present study aimed to test five levels of dietary ARA (ARA0.5 (0.5%), ARA1 (1%), ARA2 (2%), ARA4 (4%) and ARA6 (6%) for European seabass (*Dicentrarchus labrax*) juveniles in order to determine: (a) its effect in selected distal intestine (DI) lipid classes composition; and (b) how these changes affected gut bacterial translocation rates and selected GALT-related genes expression pre and post challenge.

No differences were found between DI of fish fed the graded ARA levels in total neutral lipids and total polar lipids. However, DI of fish fed the ARA6 diet presented higher ($P<0.05$) level of phosphatidylethanolamine (PE) and sphingomyelin (SM) than those DI of fish fed the ARA0.5 diet. In general terms, fatty acid profiles of DI lipid classes mirrored those of the diet. Nevertheless, a selective retention of ARA could be observed in glycerophospholipids when dietary levels are low, as reflected in the higher glycerophospholipids-ARA/dietary-ARA ratio for those animals. Increased ARA dietary supplementation was inversely correlated with eicosapentaenoic acid (EPA) content in lipid classes. ARA supplementation did not affect intestinal morphometry, goblet cells number or fish survival, in terms of gut bacterial translocation, along the challenge test. However, after the experimental infection with *Vibrio anguillarum*, the relative expression of *cox-2* and *il-1β* were upregulated ($P<0.05$) in DI of fish fed diets ARA0.5 and ARA2 compared to fish fed the rest of the experimental diets. Although dietary ARA did not affect fish survival, it altered the fatty acid composition of glycerophospholipids and the expression of proinflammatory genes after infection when included at the lowest concentration, which could be compromising the physical and the immune functionality of the DI, denoting the importance of ARA supplementation when low FO diets are used for marine fish.

5.2. INTRODUCTION

Nowadays, due to economic and environmental reasons (Hardy *et al.*, 2010) aquafeeds include important levels of vegetable oils (VO), rich in 18:C polyunsaturated fatty acids (PUFAs). In marine finfish, contrarily to freshwater species, these substitutions are critical in some cases, since they have a limited capacity of elongate and desaturate PUFAs into their long chain families (Tocher, 2003). Thus, marine finfish have dietary requirements of long chain PUFA (LC-PUFAs), in particular for eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3), and arachidonic acid (ARA, 20:4 n-6) (Tocher, 2015), due to their important role into growth performance, nervous system or immune system development and functioning, for what they are recognized as essential fatty acids (EFA) for marine fish (Tocher *et al.*, 2008).

LC-PUFAs are selectively esterified into cell surface glycerophospholipids (GPs) by fatty acyltransferase enzymes, affecting signaling processes as regulation of nuclear receptors and transcription (Crowder *et al.*, 2017), membrane stability and fluidity, and, eventually, cell functions (Tocher, 2003; Fernandez & West, 2005; Yaqoob & Calder, 2007). These functions can be exerted directly by GPs as phosphatidylcholine (PC) and phosphatidylserine (PS) which are activators of protein kinase C (Tocher *et al.*, 2008), or through derivates as phosphoinositides, diacylglycerol, lysophosphatidic acid or oxidized PC, to bind and activate receptors as, for instance, peroxisome proliferator activated receptor (Davies *et al.*, 2001). Similarly, GPs constitute a reservoir of fatty acids that are released by phospholipase A2 (Pla2) to be used by cyclooxygenase (Cox) and lipoxygenase (Lox) enzymes for eicosanoid production (Tocher, 2003) as prostaglandins (PGs), thromboxanes or leukotrienes, among others. Eicosanoids are a group of highly active hormone-like molecules, that exert their biological effects in a paracrine manner in many physiological processes as the inflammatory response (Tocher, 2003; Yaqoob & Calder, 2007).

Given the fact that dietary oils and fats affect fatty acids profile in fish tissues, especially in marine species (Tocher, 2015), the organ function will be also influenced by dietary lipids (Tocher, 2003). For instance, reductions of dietary EFA for gilthead seabream (*Sparus aurata*) together with changes on other fatty acids by the different dietary lipid sources are responsible for alterations in the morphology of intestine

(Caballero *et al.*, 2003 and 2004). The digestive tract of teleosts is one of the main entrances for pathogens (Zapata & Cooper, 1990), and particularly the gut associated immune system (GALT) has a great importance in maintaining its health status (Torrecillas *et al.*, 2012). Fish gut houses a regional immune specialization and it is considered an important place for antigen up-taking, playing a key role achieving oral immune-protection (Rombout *et al.*, 2011). In distal intestine (DI), lymphocytes, granulocytes and leukocytes, are spread on the epithelium and constitute the GALT, a local immune system that reacts to disturbances of homeostasis as those that occur during an infectious process or inclusion of terrestrial sources in diet (Torrecillas *et al.*, 2014; Salinas, 2015). These immune cells can produce eicosanoids to induce immune-cell proliferation, cytokine-release or to chemo-attract other immune cells (Zou & Secombes, 2016). Hence, dietary imbalances of EFAs can lead to modifications on cell membranes composition and, therefore, alter gut morphology, growth performance and fish health (Tocher, 2003; Montero *et al.*, 2001, 2003, 2005, 2008 and 2010).

Increasing research is demonstrating that ARA plays an important role on fish growth performance (Bessonart *et al.*, 1999; Carrier *et al.*, 2011; Koven *et al.*, 2003; Lund *et al.*, 2007; Bae *et al.*, 2010; Luo *et al.*, 2012; Torrecillas *et al.*, 2018a), lipid metabolism (Luo *et al.*, 2012; Martins *et al.*, 2012; Xu *et al.*, 2018), or fish health and disease resistance (Xu *et al.*, 2010; Torrecillas *et al.*, 2017c), among others. Besides, the essential role of ARA and its relative low levels compared to n-3 LC-PUFAs in the marine environment and in fish tissues, have probably led to the strong preference of enzymes involved in eicosanoid synthesis, at the expense of EPA (Liu *et al.*, 2006; Yaqoob & Calder, 2007; Furne *et al.*, 2013). Indeed, the ratio ARA/EPA on the target organ, affects the synthesis of eicosanoids (Ganga *et al.*, 2005 and 2006; Xu *et al.*, 2018). Similarly, ARA-derived eicosanoids compete with those from EPA for the same cell membrane receptors (Sargent *et al.*, 1999a) although those originated from ARA seem to be more biologically active (Leslie, 2004). Beyond eicosanoid production, the ARA role on immunity covers a great number of other mechanisms in cells as the activation of the NADPH oxidase enzyme in leukocytes to trigger the respiratory burst (Brash *et al.*, 2001).

Farmed European seabass presents reduced ARA tissue levels when compared with wild specimens (Lenas *et al.*, 2011; Alasalvar *et al.*, 2002; Bell *et al.*, 2007; Bhouri *et al.*, 2010; Fuentes *et al.*, 2010) indicating a necessary increase of dietary ARA.

Indeed, studies of optimum levels of ARA have been made in larval stages of these species (Koven 2001 and 2003; Atalah *et al.*, 2011; Montero *et al.*, 2015c) but scarce information exists in juveniles regarding ARA content in GPs and its influence in the intestinal immune response (Torrecillas *et al.*, 2017c and 2017d).

Therefore, an experiment was conducted out using graded levels of dietary ARA- for European seabass juveniles to determine the influence and the content of this EFA in lipid classes of DI and the related effects on gut morphology, expression of intestinal immune-related genes, survival and resistance to intestinal infection.

5.3. MATERIAL AND METHODS

5.3.1. *Experimental diets*

Five isolipidic and isoproteic experimental dry pelleted diets based on a commercial formulation were prepared to contain graded levels of ARA (total fatty acids in diet (TFA), %) as follows: ARA0.5 (0.5%), ARA1 (1%), ARA2 (2%), ARA4 (4%) and ARA6 (6%). Diet ingredients, proximate composition, and fatty acid (FA) profiles are reported in Tables 4 and 10. This basal diet was supplemented to achieve desired ARA content in diets ARA2, ARA4, and ARA6 with increasing quantities of Vevodar® (DSM Food Specialties, the Netherlands), a commercial fungal-oil rich in ARA obtained from *Mortierella alpine* (authorized in European Union by Commission Decision 2008/968/CE). Diet ARA0.5, was formulated with defatted fish meal (FM) and without fish oil (FO) to reduce the presence of ARA and supplemented with vegetable oils to reach requirements. When necessary, supplementation of DHA and EPA was done using DHA50 and EPA50 (CRODA, East Yorkshire, UK).

5.3.2. *Fish and experimental conditions*

For this feeding trial, eight hundred and forty European seabass juveniles reared in a commercial farm were maintained in quarantine in the facilities of Marine Science-Technology Park (PCTM) of University of Las Palmas de Gran Canaria (ULPGC), for 4 weeks before the experience, and fed a commercial diet. Tanks were supplied with seawater at a natural temperature of 22.8–24.9 ºC in a flow-through system and kept at a natural photoperiod (12L:12D). Dissolved oxygen ranged between 5-8 ppm. Fish were

fed the experimental diets for 70 days and, at the end of this feeding trial, fish were submitted to a challenge test against *Vibrio anguillarum* via intestinal inoculation.

All animal manipulation in this trial complied European Union Council guidelines (86/609/EU) and Spanish legislation (RD 53/2013) and had been approved by Bioethical Committee of the ULPGC (Ref. 007/2012 CEBA ULPGC).

5.3.3. *Feeding trial*

With an average weight and length of 13.4 ± 0.3 g and 9.9 ± 0.1 cm respectively (mean ± SD), animals were randomly allocated in 15 fiberglass 200 L tanks (55 fish/tank; 4 kg * m^{-3} of stocking density). Diets were assayed in triplicate and animals were fed by hand for 70 days until apparent satiation, three times a day, 6 days a week. After 70 days, samples of DI were taken for biochemical, histological and gene-expression analyses. Survival was recorded during the whole period of the feeding trial.

5.3.4. *Challenge trial*

After 70 days of experiment, fish were transferred to the Biosecurity Facilities of ULPGC in PCTM (Telde, Las Palmas, Canary Island, Spain). After 2 weeks of adaptation to the new experimental conditions, fish were inoculated with a sublethal dose (10^{7} CFU ^{x} ml^{-1} per fish) of *V. anguillarum* using the method of anal cannulation assayed previously in similar experimental conditions (Torrecillas *et al.*, 2007). Fish were fed their corresponding experimental diets for 7 days, as frequent than before. At 2 days after the infection, samples of DI were taken for immune-related genes analyses. Survival was recorded along this trial.

5.3.5. *Lipid class and fatty acid content of selected glycerophospholipids of distal intestine*

At day 70, eight fish per tank (N= 24 fish/diet), were used for biochemical analysis. The intestine was extracted out for analysis and distal section was separated as previously described by Torrecillas *et al.,* (2013). Fish tissues were kept at -80º C until the analysis. Biochemical composition of distal intestine and diets were conducted following standard procedures from Association of Official Analytical Chemists (AOAC, 2016). The analysis of lipid class and fatty acid composition of selected

glycerophospholipids (GPs) was conducted in the Institute of Aquaculture, Stirling University (UK). Separation of main lipid classes was realized in 10 × 10 cm plates (VWR, Lutterworth, UK) by double development high-performance thin-layer chromatography (HPTLC), using the technics described by Tocher & Harvie (1988), and Olsen & Henderson (1989). Firstly, plates were pre-run in diethyl ether and then activated at 120º C for 1 hour. The lipid classes were visualized after spraying with 3% (w/v) copper acetate, containing 8% (v/v) phosphoric acid by charring at 160º C for 20 min. Quantification was made by densitometry using a CAMAG-3 thin layer chromatography scanner (Version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS Planar Chromatography Manager. Samples and authentic standards run alongside, in the same conditions, on HPTLC plates, as the way to determine the identities of individual lipid classes by contrasting Rf values. Total GPs, including PC, PS, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were isolated from HPTLC plates and subjected to acid-catalyzed transesterification according to the method of Tocher & Harvie (1988). Afterwards, extraction and purification were performed as described by Christie (2003). To separate and quantify fatty acid methyl esters (FAMEs) of selected GPs, a gas-liquid chromatography was executed using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) with the conditions determined previously (Izquierdo *et al.*, 1992).

5.3.6. *Histological studies*

Samples from DI (N= 6 fish/diet) obtained after 70 days of feeding and taken as described elsewhere (Torrecillas *et al.*, 2013) were fixed in neutral-buffered formalin (4%). After 48 hours, tissues were dehydrated with an increased graded series of ethanol, submerged in xylene and embedded in paraffin blocks. Sections of 4µm were cut and stained with hematoxylin and eosin (H&E) and Alcian Blue-PAS (pH= 2.5) (Martoja & Martoja-Pierson, 1970), for optical examinations and to differentiate mucus-secreting cells, respectively. Micrographs analyzed were obtained with a Nikon Microphot- FXA microscope (objective lens 20X plus eyepiece 10X) equipped with an Olympus DP50 camera. Cell count and measures of DI were made according to Torrecillas *et al.* (2007), using Image-Pro Plus v5 software (Media Cybernetics Inc., Rockville, MD, USA). Structural measures of DI were studied with a light microscope (N= 72; 12 sections per fish × 6 fish per tank × 3 tanks per diet) and using individual

fish weight as co-variable. Following measures were calculated: fold area, FA; fold perimeter, FP; fold length, FL; fold width, FW; submucosa width, SW. To estimate mucus production, the number of mucus-secreting cells by unit of area was counted (N= 288; 48 folds per fish × 2 fish per tank × 3 tanks per diet).

5.3.7. *RNA extraction, cDNA synthesis and Quantitative Real-Time PCR analysis*

After 70 days of feeding and during challenge trial (2 days), DI (N= 9 fish/diet) samples were collected in order to realize real time (RT) qPCR analyses. Tissues were submerged into Invitrogen™ RNAlater™ Stabilization Solution (Thermo Fisher Scientific Inc., USA) and conserved at -20°C. Then, using TRI-Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and RNeasy® mini Kit (QUIAGEN, Germany), total RNA was extracted from 100 mg of pooled tissues, (N= 3 fish/tank). RNA was quantified by spectrophotometry using Nanodrop 1000 (Thermo Fisher Scientific Inc., USA) and integrity was evaluated on a 1.4% agarose gel with Gel Red™ (Biotium Inc., Hayward, CA). The synthesis of cDNA was realized from 1µg RNA with iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California) in 20µl final volume. Selected genes related to GALT functioning and eicosanoid production were as follows and respectively: interleukin 10 (*il-10*), interleukin-1beta (*il-1ß*), tumor necrosis factor alpha (*tnfα*), and cyclooxygenase 2 (*cox-2*). RT-qPCR reactions were performed by triplicate and conditions were 1X (95°C, 10min), 35x (95°C, 45s/corresponding annealing temperature, 45s/72°C, 45s) 1X (72°C, 30s). Conditions, sequences and references are registered in Table 9. Two genes, elongation factor 1 (*ef-1*) and *β-actin*, were tested as housekeeping but *ef-1* was found to be more stable to make calculations. Reactions were performed in an iCycler Optical Module (Bio-Rad, USA), the final volume used was 15µl, containing 2µl of cDNA (diluted 1/10), 0.6µl of each primer (10 mM) and 7.5µl of Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA). Blank samples, with 2µl of water replacing cDNA, were included in each assay as a contamination control. The Livak & Schmittgen (2001) method was used to calculate relative expression of each gene.

5.3.8. *Statistical analysis*

All statistical analyses were performed using SPSS 21 software package for Windows (IBM, Chicago, IL, USA). All data, presented as mean ± SD, were tested for normality and homoscedasticity. Statistical analyses followed methods outlined by Sokal & Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA). When F values showed significance, individual means were compared using post hoc tests for multiple means comparison. When data were not normally distributed, data analysis was made by non-parametric test (Kruskal-Wallis and U Mann-Whitney). When Levene's test showed $P<0.05$, but ANOVA and Wells test showed $P<0.05$, post hoc test used was Games-Howell. Pearson coefficient was used for correlations and statistical significance was set at $P<0.05$. Survival curves were performed and analyzed using the method described by Kaplan-Meier (Kaplan & Meier, 1958).

5.4. RESULTS

5.4.1. *Growth parameters*

Fish growth presented differences at the end of feeding trial (Torrecillas *et al.*, 2018a). Fish fed the lowest dietary ARA levels showed lower ($P<0.05$) weight (g) (ARA0.5 =33.0 ± 1.1) than those from the other diets (ARA1= 44.4 ± 1.1; ARA2= 43.8 ± 1.0; ARA4= 43.9 ± 3.7; ARA6= 42.8 ± 2.5) (mean ± SD). Dietary ARA levels did not affect ($P>0.05$) cumulative survival percentages between for European seabass fed the experimental diets for 70 days (over 95% for all diets).

5.4.2. *Lipid class composition of distal intestine*

No differences were found between diets in the Σ neutral lipids or the Σ polar lipids of DI (Table 20). Regarding polar lipids, PC, followed by PE, were in higher proportion than the rest of lipid class (Table 20). Lysophosphatidylcholine (LPC) presented the lowest proportion (Table 20). Among polar lipids, SM and PE were the only lipid class affected by dietary ARA ($P=0.041$ and $P=0.049$; respectively) (Table 20). Fish fed diet ARA6 had significant ($P<0.05$) higher level of PE than control diet (ARA0.5) (Table 20). Similarly, SM was more abundant in ARA6 than in ARA0.5, ARA1 and ARA2 (Table 20). Besides, significant correlations between dietary ARA

and lipid classes in DI were found for PE (0.743/P=0.001), PC (0.640/P=0.010) and SM (0.700/P=0.004), (Pearson coefficient/P value).

Table 20. Lipid class composition in distal intestine of *D. labrax* juveniles at the end of feeding trial (70 days).

	Lipid class in distal intestine				
	ARA0.5	ARA1	ARA2	ARA4	ARA6
TAG (1)	55.20±9.36	55.77±11.30	56.40±9.06	51.27±10.47	53.87±2.81
FFA (2)	10.20±4.79	7.50±5.20	7.97±4.10	9.07±4.04	6.57±0.74
Cholesterol/sterols	8.97±1.03	9.83±0.65	9.13±0.31	9.47±0.32	10.37±0.83
Unknown neutral lipid	4.40±2.43	3.87±2.11	4.03±1.97	4.03±1.83	3.07±0.23
Σ neutral lipids	78.77±1.56	76.97±5.31	77.53±3.54	73.83±4.42	73.87±2.05
PA/PG/CL (3)	1.20±0.82	1.13±0.55	1.03±0.51	1.13±0.67	0.97±0.15
PC	6.03±0.42	6.63±1.50	5.87±0.23	7.07±0.59	7.70±0.17
PS	2.07±0.31	3.27±2.80	3.63±2.50	4.60±2.78	2.70±0.95
PE	3.90±0.46a	4.57±0.98ab	4.47±0.47ab	5.10±0.40ab	5.77±0.67b
PI	1.83±1.07	1.80±0.75	1.83±0.74	2.17±0.76	1.97±0.38
SM (4)	2.23±0.32a	2.03±0.51a	1.97±0.64a	2.60±0.40ab	3.17±0.32b
LPC (5)	0.43±0.15	0.73±0.40	0.57±0.21	0.40±0.17	0.47±0.31
Pigmented material	3.53±0.64	2.87±0.45	3.10±0.61	3.10±0.85	3.40±0.53
Σ polar lipids	21.23±1.56	23.03±5.31	22.47±3.54	26.17±4.42	26.13±2.05

Triacylglycerols, (2) free fatty acids, (3) phosphatidic acid/phosphatidylglycerol/cardiolipin, (4) sphingomyelin (5) lysophosphatidylcholine. Letters denote significant differences after ANOVA analysis. All results are expressed as mean ± SD.

5.4.3. *Fatty acid composition of selected glycerophospholipids in distal intestine*

The FA composition of four main GPs (PC, PE, PS and PI) was analyzed in DI (Tables 21 a, b, c & d). Increasing dietary ARA levels mirrored the content of ARA in GPs (GPsARA). However, the lowest dietary ARA level (ARA0.5) induced a selective incorporation of ARA in all the GPs, reflected in the content of ARA ($P<0.05$; Tables 21a to 21d). Equally, the higher GPsARA/dietary ARA ratio ($P<0.05$) in ARA0.5 than in the rest of diets for PC, PE and PS, reflected the selective incorporation of ARA (Tables 21a to 21c). In PI, no differences ($P>0.05$) were found in the GPsARA/dietary ARA ratio between fish fed ARA0.5 and ARA1 diets (Table 21d). The GPsARA/dietary ARA ratio in all GPs analyzed in DI, reflected that content of ARA

was higher than dietary ARA. Significant ($P<0.05$) correlations were found in DI between dietary ARA levels and the GPsARA in all analyzed polar lipids: PC (0.992/$P<0.001$), PS (0.872/$P<0.001$), PE (0.969/$P<0.001$), PI (0.750/$P=0.001$) (Pearson coefficient/P value) (Tables 21a to 21d).

For all GPs analyzed in DI, increasing dietary ARA induced an accumulation of Σn-6 PUFA ($P<0.05$), mainly due to the increased GPsARA in the different GPs, (Tables 21a to 21d). Moreover, in PC, PE and PS, dietary ARA induced a significant ($P<0.05$) reduction of Σn-3 PUFA (Tables 21a to 21c). The increment of dietary levels of ARA was inversely correlated with the EPA content in GPs, although negative correlations were not significant ($P>0.05$), except for PE (data not shown), due to reduced dietary EPA level in diet ARA0.5 compared to the other diets (Table 10). Negative and significant ($P<0.05$) correlations between dietary ARA level and EPA content were found for all GPs when ARA0.5 diet was excluded from the statistical analysis: PC (-0.904/$P<0.001$), PS (-0.777/$P=0.003$), PE (-0.941/$P<0.001$), and PI (-0.807/$P=0.002$) (Pearson coefficient/P value) (Tables 21a to 21d). Besides, differences of Σ saturated and Σ PUFA were found in PC, with the higher ($P< 0.05$) Σ PUFA level and the lower ($P<0.05$) level of Σ saturated in those fish fed ARA0.5 diet, due to significant increases of oleic, linoleic and alpha-linolenic acids, (Table 21a). Differences in DHA content were found in PS and PE among fish fed the different dietary treatments (Tables 21b and 21c). In PS, lower ($P<0.05$) level of DHA was found in fish fed ARA0.5 diet than ARA1, ARA2, and ARA4 (Table 21b). In PE, lower ($P<0.05$) level of DHA was found in fish fed ARA0.5 and ARA6 diets when compared with the rest of experimental diets (Table 21c).

Table 21a FAMES in phosphatidylcholine in distal intestine.

Fatty acid	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	19.86±1.09a	26.98±0.54b	27.37±0.80b	27.91±1.56b	25.93±0.59b
18:0	9.25±0.40	9.26±0.73	8.95±0.91	8.85±0.55	9.27±0.88
Σ saturated	30.88±1.17a	39.55±0.41b	40.32±1.17b	40.38±1.68b	38.90±1.49b
18:1 n-9	13.36±0.36b	10.51±1.04a	11.08±0.37ab	10.84±1.72ab	10.60±1.13ab
Σ monoenes	18.95±0.78	18.12±1.08	19.13±0.52	17.95±2.02	18.04±1.09
18:2 n-6	6.71±0.42c	2.51±0.18b	2.01±0.07a	1.87±0.04a	1.67±0.13a
20:4 n-6	3.10±0.08a	3.08±0.19a	5.26±0.13b	8.56±0.39c	11.28±0.12d
Σ n-6 PUFA	13.09±0.57d	7.10±0.16a	8.90±0.20b	12.05±0.38c	14.62±0.28e
18:3 n-3	11.82±1.06c	0.34±0.05b	0.29±0.04ab	0.26±0.02ab	0.23±0.05a
20:5 n-3	5.25±0.37a	8.20±0.79c	7.21±0.19bc	6.09±0.09ab	5.47±0.74a
22:6 n-3	18.89±2.23	25.17±1.70	22.82±0.73	22.13±3.47	21.65±1.29
Σ n-3 PUFA	37.09±1.35c	35.22±1.33bc	31.65±1.07abc	29.60±3.58ab	28.41±2.23a
Σ PUFA	50.17±0.81b	42.32±1.45a	40.55±1.26a	41.67±3.45a	43.06±2.56a
∑ n-3/n-6	2.84±0.22a	4.96±0.13c	3.56±0.05a	2.46±0.34ab	1.94±0.12b
∑ n-3 LC-PUFA	24.15±2.44a	33.37±1.21b	30.02±0.91ab	28.22±3.56ab	27.12±2.03ab
GPsARA/ARA diet	5.26±0.14d	2.99±0.19c	2.60±0.07b	2.12±0.10a	1.78±0.02a

Table 21b FAMES in phosphatidylserine in distal intestine.

Fatty acid	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	7.72±1.63	7.74±1.40	6.54±0.37	6.08±0.81	7.82±2.57
18:0	34.04±1.43	32.64±1.14	34.29±0.84	34.74±2.41	32.57±2.95
Σ saturated	44.90±0.73	43.54±2.78	43.89±1.12	43.89±1.26	43.87±2.17
18:1 n-9	7.39±1.61	5.38±0.49	4.60±0.65	4.69±0.44	7.22±2.35
Σ monoenes	12.90±1.58	10.19±0.84	9.45±1.06	9.36±1.06	12.33±2.91
18:2 n-6	2.11±0.39b	1.05±0.24a	0.71±0.15a	0.62±0.04a	0.77±0.11a
20:4 n-6	1.31±0.22a	1.82±0.50a	2.44±0.25ab	4.03±1.07bc	4.27±0.75c
Σ n-6 PUFA	8.10±0.22b	5.48±0.55a	6.16±0.33ab	7.72±1.33b	8.03±0.87b
18:3 n-3	2.69±0.30a	0.25±0.20b	0.16±0.03b	0.13±0.03b	0.16±0.04b
20:5 n-3	1.06±0.13a	1.92±0.35b	1.39±0.09ab	1.06±0.11a	1.00±0.27a
22:6 n-3	29.15±2.33a	36.39±2.65b	37.06±0.61b	35.87±1.99b	32.93±3.46ab
Σ n-3 PUFA	34.05±1.87a	40.73±3.01b	40.47±0.72ab	38.97±1.64ab	35.75±3.88ab
Σ PUFA	42.20±1.59	46.27±3.47	46.66±1.01	46.75±2.26	43.80±4.80
∑ n-3/n-6	4.21±0.35a	7.44±0.25b	6.58±0.24b	5.15±0.96ab	4.45±0.02a
∑ n-3 LC-PUFA	30.21±2.21a	38.31±3.01b	38.44±0.53b	36.94±1.90b	33.93±3.60ab
GPsARA/ARA diet	2.22±0.38d	1.77±0.49c	1.21±0.13bc	1.00±0.27ab	0.67±0.12a

Table 21c	FAMES in phosphatidylethanolamine in distal intestine.				
Fatty acid	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	6.97±0.68	10.02±1.86	9.73±0.99	9.26±1.02	8.27±1.00
18:0	16.69±0.96	17.30±1.66	17.47±0.79	17.38±2.13	16.88±1.38
Σ saturated	25.78±1.68	30.12±1.87	29.84±2.01	29.15±3.11	27.34±0.36
18:1 n-9	6.66±0.13a	3.92±0.60b	4.05±0.11b	4.14±0.84ab	3.53±0.42b
Σ monoenes	14.87±0.45	12.72±0.80	11.79±1.52	12.22±1.17	12.77±1.44
18:2 n-6	2.72±0.33a	0.98±0.14b	0.80±0.14b	0.84±0.19b	0.96±0.68ab
20:4 n-6	6.43±0.78a	5.96±0.48a	9.27±0.57b	13.13±1.29c	16.13±1.13d
Σ n-6 PUFA	14.17±1.20bc	8.71±0.60a	12.24±0.85b	16.18±1.37c	19.50±0.54d
18:3 n-3	3.70±0.41a	0.18±0.01b	0.16±0.01b	0.19±0.03b	0.22±0.11b
20:5 n-3	5.01±0.27b	7.46±0.34d	6.00±0.41c	4.46±0.47ab	3.68±0.17a
22:6 n-3	35.10±0.86a	38.98±2.33b	38.40±2.09b	36.29±0.26ab	35.07±1.61a
Σ n-3 PUFA	45.14±0.52bc	48.41±2.10c	46.02±2.59bc	42.38±0.70ab	40.39±1.76a
Σ PUFA	59.34±1.51	57.16±2.48	58.36±3.53	58.64±1.95	59.89±1.33
∑ n-3/n-6	3.20±0.25bc	5.57±0.32d	3.76±0.05c	2.63±0.19ab	2.07±0.14a
∑ n-3 LC-PUFA	40.11±0.76a	46.44±2.13b	44.41±2.50b	40.75±0.64a	38.75±1.75a
GPsARA/ARA diet	10.91±1.32d	5.79±0.47c	4.58±0.28c	3.26±0.32ab	2.54±0.18a

Table 21d	FAMES in phosphatidylinositol in distal intestine.				
Fatty acid	ARA0.5	ARA1	ARA2	ARA4	ARA6
16:0	10.52±5.29	10.11±2.06	10.89±2.05	9.43±1.15	9.29±1.70
18:0	22.42±2.40	26.35±1.22	24.50±1.51	24.39±2.09	25.01±1.18
Σ saturated	37.00±6.13	39.52±1.59	42.44±3.96	41.05±4.24	40.22±3.94
18:1 n-9	10.29±1.47b	6.06±0.65a	5.69±0.41a	7.00±2.63a	6.13±1.00a
Σ monoenes	16.13±3.18	11.14±1.29	11.42±1.19	12.27±4.26	11.48±2.20
18:2 n-6	2.20±0.38b	1.12±0.15a	0.85±0.14a	0.93±0.22a	0.87±0.15a
20:4 n-6	14.48±1.86a	17.90±1.66ab	17.39±3.05ab	21.50±0.51b	21.13±0.62b
Σ n-6 PUFA	19.16±2.22a	20.51±1.77ab	19.75±3.07a	23.69±0.63b	23.50±0.54b
18:3 n-3	3.49±0.90b	0.22±0.05a	0.21±0.03a	0.21±0.14a	0.32±0.31a
20:5 n-3	2.37±0.49a	3.86±0.82b	2.87±0.21ab	2.00±0.22a	1.92±0.26a
22:6 n-3	20.71±4.14	22.90±3.23	22.04±3.45	19.57±1.96	21.13±2.70
Σ n-3 PUFA	27.52±4.88	28.73±2.64	26.37±3.61	22.94±1.68	24.60±3.02
Σ PUFA	46.86±7.10	49.34±2.59	46.14±4.98	46.68±1.40	48.30±3.50
∑ n-3/n-6	1.43±0.12c	1.41±0.20c	1.35±0.27bc	0.97±0.09a	1.05±0.11ab
∑ n-3 LC-PUFA	23.08±4.56	26.76±2.62	24.91±3.58	21.57±1.81	23.06±2.90
GPsARA/ARA diet	24.57±3.16e	17.39±1.61d	8.59±1.50c	5.33±0.13b	3.33±0.10a

5.4.4. *Histological studies*

Morphometry of DI revealed no significant (P>0.05) differences in any intestinal measure (Table 22) when related to fish real weight. Similarly, no effect of dietary ARA was observed in the density of goblet cells by unit of area in relation to the real fish weight (Table 22).

Table 22. Measures of morphometry and number of goblet cells in distal intestine.

Diet/ parameters	ARA0.5	ARA1	ARA2	ARA4	ARA6
FA	252.99±21.12	198.76±22.27	215.94±36.01	222.36±26.11	225.45±32.28
FP	12.26±2.90	14.17±3.36	13.67±2.08	13.29±1.36	12.84±1.40
FL	3.90±0.85	3.74±0.51	4.04±0.51	4.09±0.20	4.08±0.46
FW	1.38±0.43	1.25±0.16	1.29±0.11	1.38±0.12	1.35±0.07
SW	68.24±7.56	50.38±4.55	60.04±13.58	50.54±6.18	50.85±6.04
GC	34.84±9.22	29.69±1.16	33.24±2.09	32.21±1.54	29.53±0.86

All measures considering individual fish weight (g) as co-variable. FA =fold area ($\mu m^2/g$), FP=fold perimeter (µm/g), FL= fold length (µm/g), FW= fold width (µm/g), SW= submucosa width ([µm/g] * 100). GC= goblet cells/area (arbitrary units * 10^4).

5.4.5. *Relative expression of selected genes after feeding trial and challenge test against Vibrio anguillarum*

The dietary levels of ARA affect, but not significantly (P>0.05), the cumulative mortality after challenge test against *V. anguillarum*. Despite the differences in the survival percentages were not significant, there was a trend to lower mortality in fish fed diet ARA6, which did not present mortality along the experimental intestinal infection, whereas the survival percentage of fish fed diets ARA0.5, ARA1, ARA2 and ARA4 ranged between 76.5 and 88.2%.

The relative expression of immune related genes, including *il-1β, tnfα, il-10* and *cox-2*, were analyzed in DI at both basal and 2 days post infection (Fig.1).

After the feeding period (basal level), increased expression of proinflammatory *il-1β* (P=0.030) was found in fish fed ARA0.5 diet in comparison to fish fed ARA1 and ARA2 (Fig.1). After 2 days post infection, there was an upregulation of *il-1β* relative

gene expression in fish fed ARA0.5 and ARA2 diets when compared with those fish fed the rest of the diets (P<0.001) (Fig.1). No differences (P>0.05) were found in expression of *tnfα* at any sampling point (Fig.1). No differences (P>0.05) were found at basal level for *cox-2* relative expression (Fig.1). At 2 DPI, *cox-2* gene expression was upregulated (P<0.05) in fish fed ARA2 (Fig.1) compared to fish fed the rest of the dietary treatments. For *il-10* expression, an increment of *il-10* relative expression was found in fish fed ARA1 and ARA6 (P=0.002) at basal level compared to fish fed the other diets, whereas after infection a reduction was found in fish fed ARA2 compared to those fed the rest of the diets (P<0.001) (Fig.1).

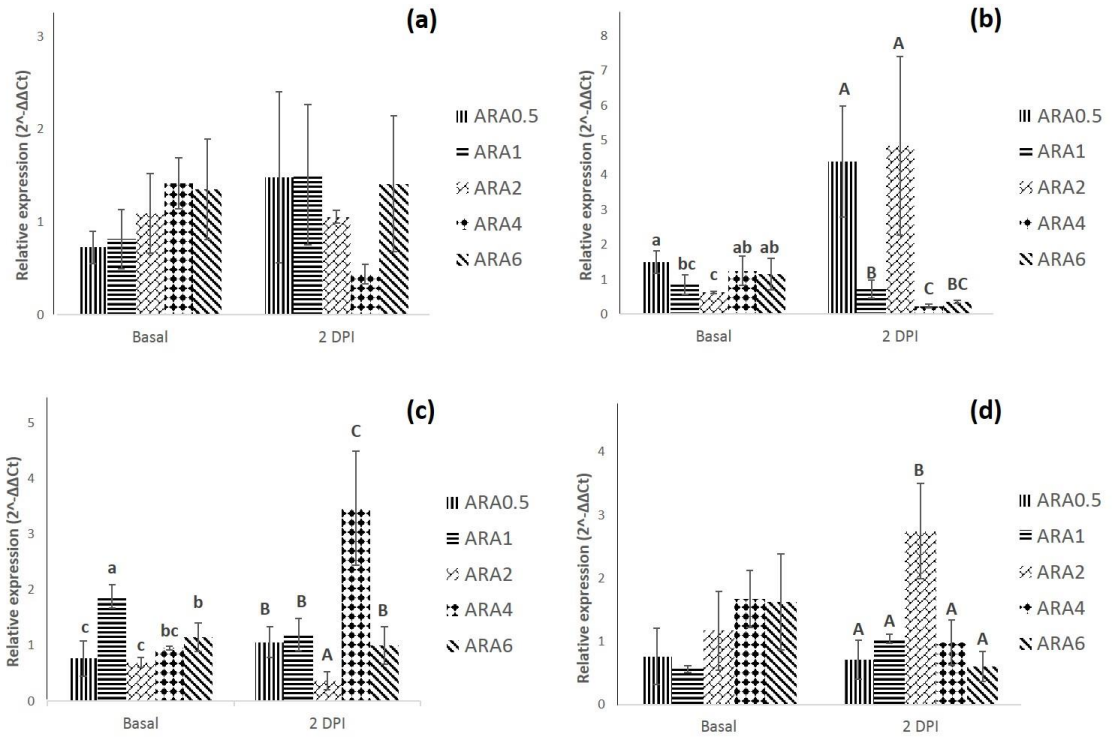

Fig.8. RT-qPCR of immune-related genes in distal intestine of European seabass (*Dicentrarchus labrax)* juveniles at basal time and at 2 days post infection (DPI): (a) *tnfα*; (b) *il-1β*; (c) *il-10*; (d) *cox-2*. N= 9 fish/diet. All values of relative expression are represented as mean ± SD. Differences were significant when P<0.05, after One-way ANOVA. Differences between diets within same sampling point indicated with letters: lowercase for Basal and uppercase for 2DPI.

5.5. DISCUSSION

Fish have dietary requirements of GPs for normal growth, maintain homeostasis, survival, or immune system correct function (Tocher *et al.,* 2008). Among other functions, GPs are related with lipid transport and plasticity of the cell-membranes (Tocher *et al.,* 2008). Besides, GPs, act as precursors of metabolism mediators as diacylglycerol or phosphoinositides, these last related with cell polarity to keep cytoarchitecture, which is determinant in epithelial barrier and transport functions (Shewan *et al.,* 2011). GPs has been described to be affected by the dietary fatty acid profile, both the amount of each GP and also de fatty acid composition of each lipid class (Olsen *et al.,* 2003).

In this study, levels of dietary ARA were correlated with the concentration of the different lipid class levels in DI of European seabass. Although increased dietary ARA significantly increased the concentration of PE and SM in DI, it was also correlated to PC level, a lipid class that is required for SM synthesis (Patel & Witt, 2017) and is related to PE through remodeling pathways (Tocher *et al.,* 2008). Previous studies have demonstrated the importance of SM in epithelial barriers of fish and other vertebrates, despite the structural differences between marine and terrestrial epithelia (Feingold, 2007; Pullmannová *et al.,* 2014; Cheng *et al.,* 2018). In fact, this polar lipid, disposed in the outer leaflet of the cell membrane with another choline-container lipid as PC (Tocher *et al.,* 2008), is more abundant in membranes of temperate-water fish suggesting its role in the membrane fluidity (Storelli *et al.,* 1998; Palmerini *et al.,* 2009). In Atlantic salmon, reductions in dietary EPA and DHA increased skin SM levels, denoting alterations of the barrier function of the skin with reductions of these EFAs (Cheng *et al.,* 2018). Besides, SM has been shown to be linked with the regulation of the release of ARA, by the inhibition of the c-Pla2α bound to the GPs (Nakamura & Murayama, 2014). In the present experiment, SM in DI increased when ARA increased in diet, with the subsequent decrease of the n-3 LC-PUFA/ARA ratio. The increase of SM in the gut of fish fed high dietary ARA could be ameliorating a possible increase of cPla2 activity induced by the high amount of ARA in the GPs of those fish fed the higher levels of ARA in diet.

It is known that high LC-PUFA content induces the decarboxylation of PS to PE at membrane level of different organelles as mitochondria or Golgi (Kainu *et al.,* 2013).

In the present study, PE levels in DI were increased by dietary ARA, with the highest level corresponding to those fish fed the highest dietary ARA level. This could be related to the fact that the generation of PE through the PS decarboxylation pathway generated preferentially PE species with a PUFA at the sn-2 position (Bleijerveld *et al.*, 2007). However, the synthesis of PE through decarboxylation of PS has been shown to be promoted by DHA and not by ARA (Ikemoto *et al.,* 1999), and thus, other metabolic pathways different than PS decarboxylation cannot be rejected to explain the increases of PE in the DI of the fish fed high ARA in diet.

Dietary ARA also influenced fatty acid profiles of lipid classes in the distal section of the intestine. Olsen *et al.*, (2003) showed that the effect of the type of dietary lipid is reflected in the fatty acid profile of the intestine and it is dependent of the section of intestine studied. In this study, correlations were found between dietary ARA and content of ARA for the four GPs studied in DI.

As described for other species, PI was the lipid class with the highest content of ARA (Bell & Sargent, 2003). Moreover, due to the abundance of PC and PE in the tissue studied, higher ARA content was found in those GPs in agreement with previous research (Bell *et al.,* 1995). Besides, the increased content of ARA in studied GPs with respect to the dietary level occurred in all diets and GPs analyzed, although with more intensity in fish fed the lowest ARA level as reflected in the higher ratio GPs-ARA/dietary for those animals. This selective retention can be considered as a way to keep functionality during EFA deficiencies (Skalli *et al.,* 2006) as negative effects of EFA deficiencies can be magnified at chronic stressful situations. Indeed, ARA reductions were found in liver polar lipids when gilthead seabream were subjected to high stocking densities probably due to its preferential utilization in that stressful situation (Montero *et al.,* 2001). Moreover, DHA concentration was also higher than dietary DHA levels in all studied GPs, particularly in PE and PS. The relatively high levels of ARA and/or DHA despite their dietary inclusion were in agreement to their preferential incorporation previously found by other authors in European seabass tissues (Farndale *et al.,* 1999; Eroldoğan *et al.,* 2013; Torrecillas *et al.,* 2015a) including in polar lipids (Torrecillas *et al.,* 2013) and in other species (Bell *et al.,* 2001; Montero *et al.,* 2001 and 2003; Fountoulaki *et al.,* 2003; Dantagnan *et al.,* 2017). Furthermore, results from the present study indicate that inclusion of EPA in GPs was negatively correlated by the supplementation of ARA in diet, suggesting competition between EPA

and ARA during phospholipid esterification, in agreement with previous studies (Bell *et al.*, 1991, Bessonart *et al.*, 1999; Fountoulaki *et al.*, 2003; Atalah *et al.*, 2011). Competition between both fatty acids as substrate for different enzymes is of especial relevance during eicosanoid synthesis, as both fatty acids are substrates for eicosanoid production, affecting different fish functions, including immune system (Bell *et al.*, 1996b).

The graded dietary levels of ARA used in the present study did not affect survival, in agreement with previous studies using graded dietary ARA levels in European seabass larvae (Atalah *et al.*, 2011) or in other marine species such as gilthead seabream, Senegal sole (*Solea senegalensis*) or Japanese sea bass (*Lateolabrax japonicus*) (Fountoulaki *et al.*, 2003; Villalta *et al.*, 2005; Xu *et al.*, 2010). Other studies in gilthead seabream have found positive effects (Bessonart *et al.*, 1999) related to stress resistance (Koven *et al.*, 2001; Willey *et al.*, 2003). Besides, low or too high dietary ARA levels has been described to induce a reduction of fish survival during a bacterial challenge in Atlantic salmon (*Salmo salar*) (Dantagnan *et al.*, 2017). In the present experiment, the graded levels of dietary ARA did not affect survival after challenge test, but had a direct effect on fish health, as described for other species such as Atlantic salmon (Dantagnan *et al.*, 2017) or guppy (*Poecilia reticulata*) (Khozing-Goldberg *et al.*, 2006). Indeed, a previous study has related dietary ARA with mechanisms of protection against damage in the intestine (Tarnawski *et al.*, 1989). In this sense, intestine is an organ subjected to injury, intestinal barrier being highly compromised and subsequently acting as one of the main entrances for pathogens (Ellis, 2001; Campos-Pérez *et al.*, 2000).

The relation between intestine and eicosanoid synthesis has been widely studied in different fish species (Sargent *et al.*, 1999a; Tocher, 2003; Calduch-Giner *et al.*, 2016). Although ARA and EPA are substrates for Cox and Lox enzymes to produce eicosanoids (Bell & Sargent, 2003, Tocher *et al.*, 2008), these enzymes seem to have stronger preference for released-ARA than for EPA at least in freshwater fish and salmonids (Bell & Sargent, 2003; Tocher *et al.*, 2008; Furne *et al.*, 2013). In this trial, the supplementation of dietary ARA did not influence directly basal levels of *cox-2* relative expression in gut, suggesting no effect on PGE2 production in intestine as described for other vertebrates (Tateishi *et al.*, 2014) which is also supported by the absence of significant differences in PI levels, the main pool of ARA for eicosanoids

production (Yaqoob & Calder, 2007). However, after infection with *V. anguillarum*, in the present study, European seabass juveniles fed 2% of ARA in diet increased *cox-2* relative expression, which has been related with protection to gastric mucosal defenses including stimulation of mucus secretion and maintenance of mucosal blood flow (Wallace & Devchan, 2005). The gastro-protective properties of Cox-2-derived PGs have been demonstrated in eel (*Anguilla anguilla*) gastric mucosa (Faggio *et al.*, 2000), and *cox-2* expression in the intestine has been also associated to a response of Atlantic salmon to acute stress, mainly in DI (Oxley *et al.*, 2010).

The upregulation of *cox-2* levels found in the present study after bacterial infection was coincident with the increased *il-1β* gene relative expression. The Cox-2 enzyme and proinflammatory cytokines such as Il-1β seems to be linked through the p38 mitogen-activated protein kinase (P38 mapk) (Camacho-Barquero *et al.*, 2007), which is known to be present in fish (Ribeiro *et al.*, 2010; Yang *et al.*, 2014b). The Mapk can be activated by ARA metabolites in a dose-dependent manner (Alexander *et al.*, 2001) which in turn can activate *cox-2* expression (Sui *et al.*, 2014). Besides, Mapk constitutes a signaling pathway involved in regulation of multiple cell functions including autophagy, a cell process of self-degradation to maintain homeostasis in which proinflammatory cytokines are implicated (Sui *et al.*, 2014). PE plays an important role in autophagy because it is utilized by proteins required for the formation of autophagosomes to attach to cell membranes (Ichimura *et al.*, 2000; Iula *et al.*, 2018), and, besides, these autophagic vesicles are utilized for secretion of cytosolic Il-1β (Iula *et al.*, 2018). At the same time, Il-1β has been suggested to be involved in the PE synthesis via Mapk (Sluzalska *et al.*, 2017). In this way, the modification of PE levels in DI can be related with the secretion of Il-1β. In the present study, increased *il-1β* relative expression at basal time in diet ARA0.5 could be related to PE reductions in that diet. Besides, other authors have showed that increased levels of Il-1β can reduce SM synthesis without affecting other choline-GPs as PC (Kronqvist *et al.*, 1999). In this experiment, reductions in SM levels could be related to increments in ARA release in those fish fed the lowest ARA level or to regulation of its synthesis, both mechanisms affected by Il-1β release.

In conclusion, ARA is selectively retained in the GPs of DI of European seabass, supporting its important physiological role in this tissue. This ARA selective retention is especially evident when low dietary ARA levels are fed, as reflected in the higher

glycerophospholipids-ARA/dietary-ARA ratio found. However, these variations were not enough to alter DI morphology or/and bacterial translocation rates, regardless of the ARA-deficiency related upregulation of DI proinflammatory genes. Altogether pointing to a long-term compromised physical barrier integrity and immune functionality of the DI, denoting the importance of ARA supplementation when low FO diets are used for marine fish.

Chapter 6

FEEDING EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*) JUVENILES WITH A FUNCTIONAL SYNBIOTIC ADDITIVE (MANNAN OLIGOSACCHARIDES AND *PEDIOCOCCUS ACIDILACTICI*): AN EFFECTIVE TOOL TO REDUCE LOW FISH MEAN AND FISH OIL GUT HEALTH EFFECTS?

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ST, MJ, MI & DM conceived and planned the experiments. AM formulated the diets. FR carried out the experiments and contributed to sample preparation and analyses. ST, MJ & P-SB contributed to analyses. ST, FR, MJC, JR & DM contributed to the interpretation of the results and discussion. AF-M contributed to the statistical analysis. ST & DM wrote the paper with input from all authors.

6.1. ABSTRACT

The aim of this study was to assess the effects of dietary mannan oligosaccharides (MOS), *Pediococcus acidilactici* or their conjunction as a synbiotic in low fish meal (FM) and fish oil (FO) based diets on European seabass (*Dicentrarchus labrax*) disease resistance and gut health.

For that purpose, European seabass juveniles were fed one of 6 diets containing different combinations of MOS (Biomos® and Actigen©; Alltech, Inc., Kentucky, USA) and *Pediococcus acidilactici* (BAC, Bactocell®; Lallemand Inc., Cardiff, UK) replacing standard carbohydrates as follows (MOS (%)/BAC (commercial recommendation): high prebiotic level (HP) = 0.6/0, low prebiotic level (LP) = 0.3/0, only probiotic (B) = 0/+, high prebiotic level plus probiotic (HPB) = 0.6/+, low prebiotic level plus probiotic (LPB) = 0.3/+, control (C) = 0/0 for 90 days. After 90 days of feeding trial, fish were subjected to an experimental infection against *Vibrio anguillarum*. Additionally, inducible nitric oxide synthase (Inos) protein and tumor necrosis factor α (Tnfα) protein gut patterns of immunopositivity and major histocompatibility complex class ii (*mhcii*), T-cell receptor beta (*tcr-β*), regulatory T-cell subset (*cd-4*$^+$ T lymphocytes) and effector T cell (*cd-8α*$^+$ T lymphocytes) gene expression patterns in gut by *in situ* hybridization were evaluated after 90 days of feeding. The effects of both additives on distal gut through gut associated lymphoid tissue (GALT) gene expression was also studied. Fish fed the prebiotic and its combination with *P. acidilactici* presented increased weight regardless of the dose supplemented after 90 days of feeding, however no effect was detected on somatic indexes. For distal gut, morphometric patterns and goblet cells density was not affected by MOS, *P. acidilactici* or its combination. Anti-Inos and anti-Tnfα gut immunopositivity patterns were mainly influenced by MOS supplementation and not by its combination with *P. acidilactici*. The *mhcii-β*, *tcr-β*, *cd-4* and *cd-8α* positive cells distribution and incidence was not affected by diet. Fish fed HP dose presented a clear upregulation of *tnfα*, cyclooxygenase-2 (*cox-2*), *cd-4* and *il10*, whereas *P. acidilactici* dietary supplementation increased the number of interleukin-1 beta (*il-1β*) and *cox-2* gene transcripts. Synbiotic supplementation resulted in a reduction of MOS-induced gut humoral proinflammatory response by increasing the expression of some cellular-immune system related genes. Fish mortality after *V. anguillarum* infection was reduced in fish fed LPB and LP diets compared to fish fed the non-supplemented diet after 90 days of feeding.

Thus, overall pointing to the combination of a low dose of MOS and *P. acidilactici* as synbiont (LPB) as a viable tool to potentiate European seabass juvenile's growth and disease resistance when supplemented in low FM and FO diets.

6.2. INTRODUCTION

A successful replacement of fish meal (FM) and fish oil (FO) by terrestrial meals (TM) and vegetable oils (VO) in feeds for marine fish species is determinant to achieve a sustainable aquaculture sector development (Tacon & Metian, 2015a; FAO, 2016). However, in some cases, high levels of dietary TM and VO are associated with variable side-effects on fish growth performance and health (Sitjà-Bobadilla *et al.*, 2005; Montero & Izquierdo, 2010; Calduch-Giner *et al.*, 2012; Kiron, 2012; Oliva-Teles, 2012; Torrecillas *et al.*, 2017d). These effects depend mainly on the fish species studied, the level of replacement and the source of terrestrial meals and oils used. Particularly, a high replacement of marine raw materials in European seabass (*Dicentrarchus labrax*) has been associated with gut morphological alterations such as engrossment of the lamina propria and submucosa, increased mucus production, intestinal upregulation of several inflammation related genes and altered microbiota populations (Torrecillas *et al.*, 2017a).

In this sense, it is necessary to develop complementary tools that will help to reduce the negative-side effects of high FM and FO dietary replacement for more sustainable fish feed raw materials (Sitjà-Bobadilla *et al.*, 2005; Montero & Izquierdo, 2010; Calduch-Giner *et al.*, 2012; Kiron, 2012; Oliva-Teles, 2012; Torrecillas *et al.*, 2017a and 2017d). During the last decade, prebiotics and probiotics among other compounds, have been proposed as potential candidates to enhance fish immune status and growth performance when diets are formulated with high levels of dietary plant raw materials (Song *et al.*, 2014; Torrecillas *et al.*, 2016; Rimoldi *et al.*, 2016; Azeredo *et al.*, 2017), by counteracting some of those associated side effects. Among them, mannan oligosaccharides (MOS) increased bacterial disease resistance, stimulated local and systemic immune system response and protected fish gut integrity (Torrecillas *et al.*, 2014; Guerreiro *et al.*, 2017).

Besides, MOS promoted fish growth, feed utilization and reduced hepatocyte vacuolization of several Mediterranean fish species such as European seabass or gilthead seabream (*Sparus aurata*) (Torrecillas *et al.*, 2007, 2011a, 2013, 2015a; Dimitroglou *et al.*, 2010; Gültepe *et al.*, 2012). Specifically, for European seabass, MOS supplementation

in total FO replaced diets by soybean oil (SBO) moderates the downregulation of several GALT-related genes involved in the functioning of gut mucous barrier, such as major histocompatibility complex class i alpha (*mhci-α*), cyclooxygenase 2 (*cox-2*) or transforming growth factor β (*tgf-β*), and helps to preserve gut immune homeostasis by increasing distal gut mucous cell diffusion rates (Torrecillas *et al.*, 2015b). Similarly, lactic acid bacteria (LAB) dietary supplementation is related with beneficial effects of fish growth performance and health (Ringø *et al.*, 2010; Cruz *et al.*, 2012; Carnevali *et al.*, 2006). LAB can avoid gut colonization by pathogenic bacteria through the production of inhibitory substances as bacteriocins, lactic and acetic acids (Balcazar *et al.*, 2006a). Specifically, *Pediococcus acidilactici* dietary supplementation promoted growth performance, optimized feed efficacy, improved survival rates, triggered immune response and increased mucus production in several species of fish and shrimp (Castex *et al.*, 2008; Standen *et al.*, 2013; Ferguson *et al.*, 2010; Harper *et al.*, 2011; Neissi *et al.*, 2013). Unfortunately, to date, few studies studied the combined use of dietary prebiotics and probiotics as synbiotics in intensive reared fish species, which potentially could enhance the beneficial effects of each functional additive (Cerezuela *et al.*, 2011; Azimirad *et al.*, 2016), particularly favoring probiotic ability settlement as part of the fish gut microbiota (Cerezuela *et al.*, 2011). However, the mechanisms implied in most cases remain unclear, being dependent on fish species, dose supplemented, time of supplementation and the combination of prebiotic and probiotic tested (Cerezuela *et al.*, 2011).

Thus, the aim of the present experiment is to study the effects of MOS, *P. acidilactici* and their combination, on growth performance, gut morphology, gut associated lymphoid tissue function and disease resistance against *V. anguillarum* for European seabass juveniles fed a diet based on a high substitution of marine raw materials by TM and VO.

6.3. MATERIALS AND METHODS

6.3.1. *Diets*

Six experimental diets were formulated based in a 5% FM and 6% FO content containing different combinations of mannan oligosaccharides (MOS, Biomos® and Actigen© (second generation of MOS; Alltech, Inc., Kentucky, USA) and *Pediococcus acidilactici* (BAC, Bactocell®; Lallemand Inc., Cardiff, UK) replacing standard carbohydrates as

follows (MOS (%)/BAC (commercial recommendation): high prebiotic level (HP) = 0.6/0, low prebiotic level (LP) = 0.3/0, only probiotic (B) = 0/+, high prebiotic level plus probiotic (HPB) = 0.6/+, low prebiotic level plus probiotic (LPB) = 0.3/+, control (C) = 0/0. Diets were isoenergetic and isonitrogenous, covered all known nutritional requirements for this species and were manufactured by extrusion process in BioMar Tech-Centre (Brande, Denmark). Diets raw materials and proximate composition are detailed in Table 6.

6.3.2. *Experimental conditions*

6.3.2.1. Experiment I: feeding trial

One thousand and eight hundred European seabass juveniles reared in a local farm (Tinamenor S.L., Castillo del Romeral, Gran Canaria, Canary Islands, Spain) were transferred to the facilities of the Parque Científico-Tecnológico Marino (PCTM) at University of Las Palmas de Gran Canaria (ULPGC; Telde, Canary Islands, Spain) and adapted for 4 weeks to facility water conditions (7.5–7.9 ppm dissolved O_2, 22.0–23.1 °C).

After that, fish were randomly distributed in 18 fiberglass tanks of 500 L (100 fish/tank) at an initial density of 4 kg * m^{-3} (mean weight ± SD: 19.7 ± 0.11 g; mean length ± SD: 11.32 ± 0.04 cm) in a flow through system with natural photoperiod (12L/12D). All groups were fed until apparent satiation 3 times a day, 6 days a week for 90 days.

After 60 and 90 days all fish were individually weighted and measured to calculate growth performance and feed utilization parameters. After 90 days, fish were sampled for gut (N=4 fish/tank) and liver morphology (N=3 fish/tank), gut immunohistochemistry (N=4 fish/tank) gut in situ hybridization (N=4 fish/tank), gut gene expression (N=4 fish/tank), and to calculate viscerosomatic indexes (N=5 fish/tank).

6.3.2.2. Experiment II: challenge test

After 90 days of feeding, 20 fish/diet from LP, LPB and C diets, were moved to the Marine Biosecurity (MBS) facilities of the PCTM-ULPGC (Telde, Canary Islands, Spain), adapted to the new rearing conditions for 2 weeks and challenged against *V. anguillarum* (strain 507, isolated from a clinical outbreak in Canary Islands; 10^6 CFU * ml^{-1} per fish; Torrecillas *et al.*, 2017) by intraperitoneal injection. Fish were fed their respective diets two times a day. Fish survival was recorded daily along the challenge

experiment. The observed day-to-day pattern of changes was described by Kaplan-Meier curves for each dietary treatment.

Animal manipulation complied guidelines of the European Union Council (86/609/EU) and Spanish legislation (RD 53/2013) and was approved by Bioethical Committee of the ULPGC.

6.3.3. *Diets proximate analyses*

Feed biochemical composition was analyzed according to standard procedures (AOAC. 1995). Dry matter content was determined after drying in an oven (110°C) to constant weight and ash content by combustion in a muffle furnace (600°C, 12 h). Crude lipid was extracted as described in Ref. (Folch *et al.*, 1957) and crude protein content (Nx6.25) by Kjeldahl method (AOAC, 1995).

6.3.4. *Morphometric studies*

Distal intestine (DI), from the first diffused sphincter to rectum sphincter and liver were dissected out and fixed at 4°C in 4% paraformaldehyde.

After 48 h, samples were dehydrated and embedded in paraffin. Sections of 4 µm were stained with haematoxylin and eosin (H&E) (Martoja & Martoja-Pierson 1970) for optical examination. DI sections were also stained with Alcian Blue-PAS (pH=2.5) to differentiate goblet cells secreting acid mucins. H&E stained liver (N=3 fish/tank) and DI (N=4 fish/tank) slides were observed under light microscope. Micrographs of each individual section were taken at a final magnification of 20X and 40X using an Olympus Cx41 microscope and an Olympus XC50 camera. Cell count (N=4 fish/tank) and morphometric characteristics evaluated with Image Pro Plus v5 software (Media Cybernetics Inc., Rockville, MD, USA), according to (Torrecillas *et al.*, 2011a) for DI and (Torrecillas *et al.*, 2007) for liver.

6.3.5. *Immunohistochemical analyses*

Immunohistochemical studies of DI sections (N=4 fish/tank) were performed for anti-Inos and anti-Tnf*α* as described before in Ref. (Torrecillas *et al.*, 2017d) for the same fish species. Briefly, after antigen retrieval (High pH, Dako, Denmark), endogenous peroxidase activity was blocked, and sections were incubated overnight at 4°C with primary rabbit polyclonal anti-Tnf*α* and anti-Inos-2. Immunohistochemical staining was

carried out using the horseradish peroxidase (HRP) anti-rabbit EnVision + System (Dako, Denmark), diaminobenzidine (Dako, Denmark) was used as chromogen. Negative controls were processed simultaneously and obtained by replacing primary antibodies with primary antibody diluent.

The pattern of immunoreactivity was evaluated by 3 scientists, unaware of the experimental treatments and accordingly to a scoring scale established by Ref. [8] for the same fish species and antibodies as follows: weak and strong for each area/cell evaluated. None of the negative controls showed Inos or Tnf*α* immunoreactivity.

6.3.6. *In situ hybridization and staining procedures*

In situ hybridization (ISH) for *mhcii-β, tcr-β, cd-4*, and *cd-8α* immune-related genes was performed in DI sections (N=4 fish/tank).

DI samples were fixed in 4% PFA, dehydrated, included in paraffin and cut into 4 µm transverse sections. Sections were laid and stored at 37°C overnight in poly-L slides. For probes synthesis, total RNA was extracted from spleen of juvenile European seabass. First-strand cDNA was synthesized according to the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) protocol. Genes *mhcii-β, tcr-β, cd-4* and *cd-8α* were amplified by using forward-reverse specific primers described in Table 9. Specific amplicons were isolated from agarose gel (Zymoclean gel DNA Recovery Kit, Zymo Research Co.), cloned into pGEM T-easy vector (Promega, Madison, WI, USA) and sequenced on both strands.

DNA vectors were linearized using adequate restriction enzymes and then sense and antisense digoxigenin-labelled riboprobes were synthesized by using T7 or SP6 RNA polymerase. ISH procedures followed methods described previously by Ref. (Suárez-Bregua *et al.*, 2017).

6.3.7. *Gene expression studies*

RNA was extracted from 100 mg of DI (samples were pooled, 4 fish/tank) with TRI-Reagent (Sigma-Aldrich, Saint Louis, MO, USA) and RNeasy® mini Kit (QIAGEN, Germany). Quantified by spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific Inc., USA) and integrity evaluated on a 1.4% agarose gel with Gel Red™ (Biotium Inc., Hayward, CA).

cDNA was synthesized in 20 µl final volume with 1 µg RNA and iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California). For gene expression analyses, RT-PCR conditions were 1× (95°C, 10min), 35× (95°C, 45s/corresponding annealing temperature, 45s/72°C, 45s), 1× (72°C, 30s), using primers previously described by different authors (Buonocore *et al.*, 2007; Picchietti *et al.*, 2009 and 2011; Geay *et al.*, 2011; Román *et al.*, 2013). The *ef-1α* and *β-actin* genes were tested as housekeeping, and calculations made using the most stable one, *ef-1α*. Specific primers sequences, source and Genbank® references are detailed in Table 9.

Reactions were done by duplicate in a 15 µl final volume, containing 2 µl of cDNA (1/10), 7.5 µl Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA) and 0.6 µl of each primer (10 mM) in a iCycler Optical Module (Bio-Rad, USA). A blank sample was included in each assay as a negative control. Relative expression level was calculated by using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen (2001)) in relation to house-keeping reference genes and normalized using the corresponding control diet data.

6.3.8. *Statistical analyses*

Statistical analyses followed methods described in Sokal & Rolf, (1995). Means and standard deviations (SD) were calculated for each parameter measured. All data were tested for normality and homoscedasticity of variance. When required, data arcsine square root transformation was performed, particularly when data were expressed as %. Based on the main objective of the study, all results presented focusing on the effects of prebiotic and probiotic and their combination were analyzed by two way-ANOVA analyses with MOS and *P. acidilactici* established as fixed factors. The effect of MOS and *P. acidilactici* on individually measured parameters was evaluated considering the effect of tank as a random factor in a nested ANOVA analysis, however no effect of tank was detected in any dependent variable analyzed. Significant differences were considered when $P<0.05$. The effect of MOS dietary levels was compared using *post hoc* GT2 Hochberg for multiple means comparison.

To study the variability on DI relative gene expression by diet, a principal component analysis (PCA) was performed and statistical differences evaluated using PERMANOVA analyses. The number of permutations was established in 999, considering statistical significances under permutational p (P perm) <0.05. Survival data from challenge was analyzed using Kaplan-Meier survival analysis and compared by the log-rank test to

define the responses of European seabass to the different treatments. Analyses were performed using the SPSS Statistical Software System v21.0 (SPSS, Chicago, IL, USA) and PRIMER 7 with PERMANOVA complement (Auckland, New Zealand).

6.4. RESULTS

6.4.1. *Experiment I: growth performance*

Table 23. Growth parameters of European seabass (*Dicentrarchus labrax*) juveniles 90 days of feeding.

Diets	C	HP	LP	B	HPB	LPB
60 days						
Weight (g)	45.42±2.54	46.18±0.76	45.81±3.12	45.00±0.91	44.93±0.63	45.17±1.44
Length (cm)	14.66±0.28	14.52±0.19	14.55±0.21	14.57±0.26	14.62±0.18	14.54±0.02
Condition factor (K)[a]	1.44±0.02	1.51±0.03	1.49±0.05	1.46±0.05	1.44±0.07	1.47±0.05
SGR[b]	1.57±0.12	1.6±0.03	1.59±0.12	1.57±0.02	1.57±0.04	1.55±0.05
90 days						
Weight (g)	63.70±4.04	68.24±0.81[A]	69.24±1.89[A]	66.74±1.89	66.11±1.89[A]	68.16±6.07[A]
Length (cm)	16.37±0.33	16.54±0.12[A]	16.63±0.26[A]	16.58±0.16	16.55±0.16[A]	16.69±0.38[A]
Condition factor (K)[a]	1.45±0.02	1.51±0.04	1.51±0.04	1.46±0.01	1.46±0.03	1.46±0.03
SGR[b]	1.40±0.08	1.47±0.01	1.50±0.03	1.46±0.03	1.45±0.04	1.47±0.10
ISI[c]	1.16 ± 0.09	1.17 ± 0.05	1.11 ± 0.03	1.05 ± 0.13	1.01 ± 0.11	1.07 ± 0.05
VSI[d]	9.37±0.76	9.97±0.69	9.90±0.81	8.95±0.47	8.98±0.44	9.12±0.33
HSI[e]	1.76±0.19	1.62±0.07	1.75±0.08	1.65±0.10	1.63±0.05	1.65±0.08
PFI[f]	6.53±0.59	7.18±0.53	6.98±0.70	6.19±0.40	6.29±0.42	6.47±0.30

Diets: C (control diet), HP (high prebiotic level), LP (low prebiotic level), B (only probiotic), HPB (high prebiotic level plus probiotic) and LPB (low prebiotic level plus probiotic). (a) Condition factor (K)=fish weight/fish length3, (b) Specific growth rate (SGR) = [Ln (final weight)-Ln (initial weight)]/days of feeding), (c) Intestinal-somatic index (ISI) = 100 * (gut weight/fish weight), (d) Viscerosomatic index (VSI) = 100 * (viscera weight/fish weight), (e) Hepatosomatic index (HSI) = 100 * (liver weight/fish weight), (f) Perivisceral fat index (PFI) = 100 * (perivisceral fat/fish weight). Data expressed as mean ± SD. Two-way ANOVA analyses (P<0.05) MOS *post hoc* analyses were calculated by GT2 Hochberg test and did not revealed significant differences between MOS dietary levels, denoted by A letter. NS= No significant.

No differences were found in growth performance parameters after 60 days of feeding. At the end of the feeding period (90 days), two-way ANOVA analyses revealed individual effect of dietary MOS on final weight (F=9.013, N=222 fish/diet, P=0.001) and length (F=3.536, N=222 fish/diet, P=0.029), whereas no effect was detected on specific growth rate (SGR) (P>0.05, N=3 tank/diet) (Table 23). *Post hoc* analyses did not reveal significant differences for final weight or length between prebiotic levels (P>0.05). No individual effect was detected for probiotic dietary supplementation on evaluated parameters (P>0.05, N=222 fish/diet). MOS and *P. acidilactici* presented a significant

interaction for final weight (F=7.938, N=222 fish/diet, P=0.001), however no differences were observed between HPB and LPB treatments (Table 23). No differences were found for somatic indexes (P>0.05, N=15 fish/diet) and no effect of tank as random factor was detected on the parameters studied.

6.4.2. *Experiment I: liver morphometry*

After 90 days of feeding, probiotic supplementation reduced hepatocyte maximum length (F=4.547; N=9 fish, P=0.038), however no effect was detected in hepatocyte area or minimum cellular length (Table 24). No significant effect of prebiotic supplementation, interaction between both factors or tank as random factor was detected on the parameters studied.

Table 24. Liver morphology of European seabass (*Dicentrarchus labrax*) juveniles after 90 days of feeding.

Diet	C	HP	LP	B	HPB	LPB
Area (μm^2)	70.88±5.29	61.36±8.66	72.28±2.51	62.66±1.64	63.53±3.42	63.82±0.01
Maximum length (μm)	10.13±0.33	9.90±0.79	10.58±0.23	9.65±0.25	9.63±0.38	9.70±0.28
Minimumm lenght (μm)	6.75±0.62	6.33±0.68	6.88±0.25	6.57±0.29	6.59±0.23	6.70±0.02

C (control diet), HP (high prebiotic level), LP (low prebiotic level), B (only probiotic), HPB (high prebiotic level plus probiotic) and LPB (low prebiotic level plus probiotic). All data are represented as mean ± SD. N= 3 (fish/tank). Two-way ANOVA analyses (P<0.05). MOS *post hoc* analyses were calculated by GT2 Hochberg test and did not revealed significant differences between MOS dietary levels. NS= No significant.

6.4.3. *Experiment I: gut morphological and functional studies*

6.4.3.1. Distal gut morphometry and mucus production

At the end of the feeding trial, histopathological evaluation of fish distal intestines showed an intact epithelial barrier and well-organized fold pattern in all treatments. No differences were found for distal gut fold length, fold width, fold area, submucosa thickness or density of goblet cells by unit of area (N=12 fish) among fish fed the different dietary treatments when related to fish real weight (Table 25). No effect of tank as random factor was detected on the parameters studied.

Table 25. Distal intestine morphometric parameters for European seabass (*Dicentrarchus labrax*) juveniles normalized by real fish weight at the end of the feeding trial (90 days).

Diets	C	HP	LP	B	HPB	LPB
Fold area ($\mu m^2/g$)	381.45±41.78	312.44±56.57	382.26±30.11	371.33±150.37	308.45±52.46	333.57±0.92
Fold length (µm/g)	4.16±0.25	3.53±0.51	3.72±0.25	4.01±1.25	3.56±0.14	3.48±0.08
Fold width (µm/g)	1.44±0.08	1.31±0.10	1.47±0.07	1.73±0.41	1.39±0.17	1.43±0.17
Submucosa thickness (µm/g)	0.34±0.03	0.34±0.01	0.32±0.03	0.42±0.08	0.36±0.04	0.30±0.09
Goblet cells acid mucins/μm^2*10^4	18.28 ± 4.10	19.66 ± 2.95	18.67 ± 1.37	17.72 ± 0.83	18.76 ± 0.53	20.50 ± 2.29

C (control diet), HP (high prebiotic level), LP (low prebiotic level), B (only probiotic), HPB (high prebiotic level plus probiotic) and LPB (low prebiotic level plus probiotic). All data are represented as mean ± SD. N= 4 (fish/tank). Two-way ANOVA analyses ($P<0.05$). MOS *post hoc* analyses were calculated by GT2 Hochberg test. NS= No significant.

6.4.3.2. Gut immunohistochemistry

Anti-Inos and anti-Tnf*α* immunostaining was defined by the presence of a brown reaction in the basal zone and apical region of gut folds and was detected in all the intestinal sections studied with exception of negatives controls (Fig. 9A and B). The staining intensity was classified as strong versus weak, and in general terms, it was stronger in the basal zone than in the apical region of the fold, regardless of the antibody studied (Fig. 9C and D). For both antibodies, two clear patterns of immunostaining were detected. The first one to fish fed MOS which presented the stronger immunostaining pattern on both, the apical and basal regions (Fig. 9C and D). The second one to fish fed diets including *P. acidilactici*, which only presented strong immunoreactivity on the basal region compared to the apical zone (Fig. 9E and F) and was similar to the pattern observed in fish fed control diet. Distal gut submucosa and lamina propria infiltrated leucocytes presented immunoreactivity against anti-Inos (Fig. 9G) and to anti-Tnf*α* (Fig. 9H), whereas a slight trend to higher lamina propria and submucosa immunoreactivity to anti-Inos was observed in fish fed supplemented diets. Mucus contained in goblet cells presented immunopositivity to anti-Inos (Fig. 9G) but not to anti-Tnf*α* regardless of the dietary treatment (Fig. 9H).

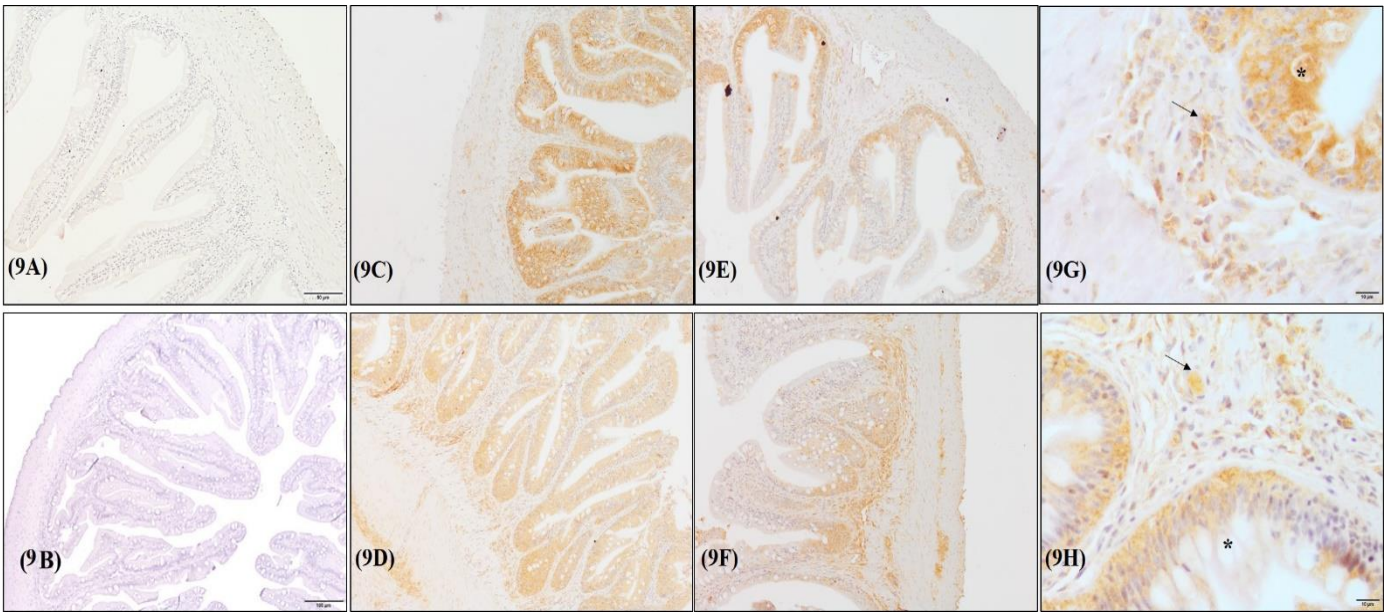

Fig.9. Photomicrographs of the distal intestine immunoreactivity with the anti-Inos and anti-Tnfα antibodies at the end of the feeding trial (90 days). Immunoreactivity to anti-Inos and anti-Tnfα was detected in all the fish distal intestinal sections studied with exception of negatives controls (Fig.9A and B). The higher distal gut immunoreactivity patterns (strong) to anti-Inos and anti-Tnfα are represented in Fig.9C and D respectively and correspond to fish fed MOS based diets. Fig.9E (anti-Inos) and Fig.9F (anti-Tnfα) represent the lower reactivity patterns observed and correspond to fish fed control and *P. acidilactici* diet. Bar 50µm. Observe the stronger immunoreactivity detected for both antibodies on the basal fold region compared to the apical fold region, regardless of the treatment fed. Distal gut submucosa and lamina propria infiltrated leucocytes presented immunoreactivity against anti-Inos (arrows; Fig.9G) and to anti-Tnfα (arrows; Fig.9H). Bar 10µm. Positive immunoreactivity of goblet cells against anti-Inos antibody (asterisk; Fig.9G) and negative to anti-Tnfα (asterisk; Fig.9H).

6.4.3.3. *In situ* hybridization

In general terms, *mhcii-β* cells were higher number than the rest, followed by *tcr-β*, *cd-8α* and *cd-4* expressing cells, being *cd-4* cells the less frequent (Fig. 10). No differences were found in the distribution of stained cells in relation to diet. *mhcii-β* positive cells were mainly infiltrated between or immediately below enterocytes and goblet cells and, less frequently, located in lamina propria-submucosa (Fig. 10A and B). Similarly, *tcr-β* positive cells were found in all sections studied and principally located below enterocytes and less frequently in lamina propria-submucosa (Fig. 10C and D). The *cd-8α* and *cd-4* expressing cells were detected primarily in lamina propria and close to enterocytes basis, respectively (Fig. 10E and H). The *mhcii-β, tcr-β, cd-4* and *cd-8α* sense-probes assay did not result in any staining.

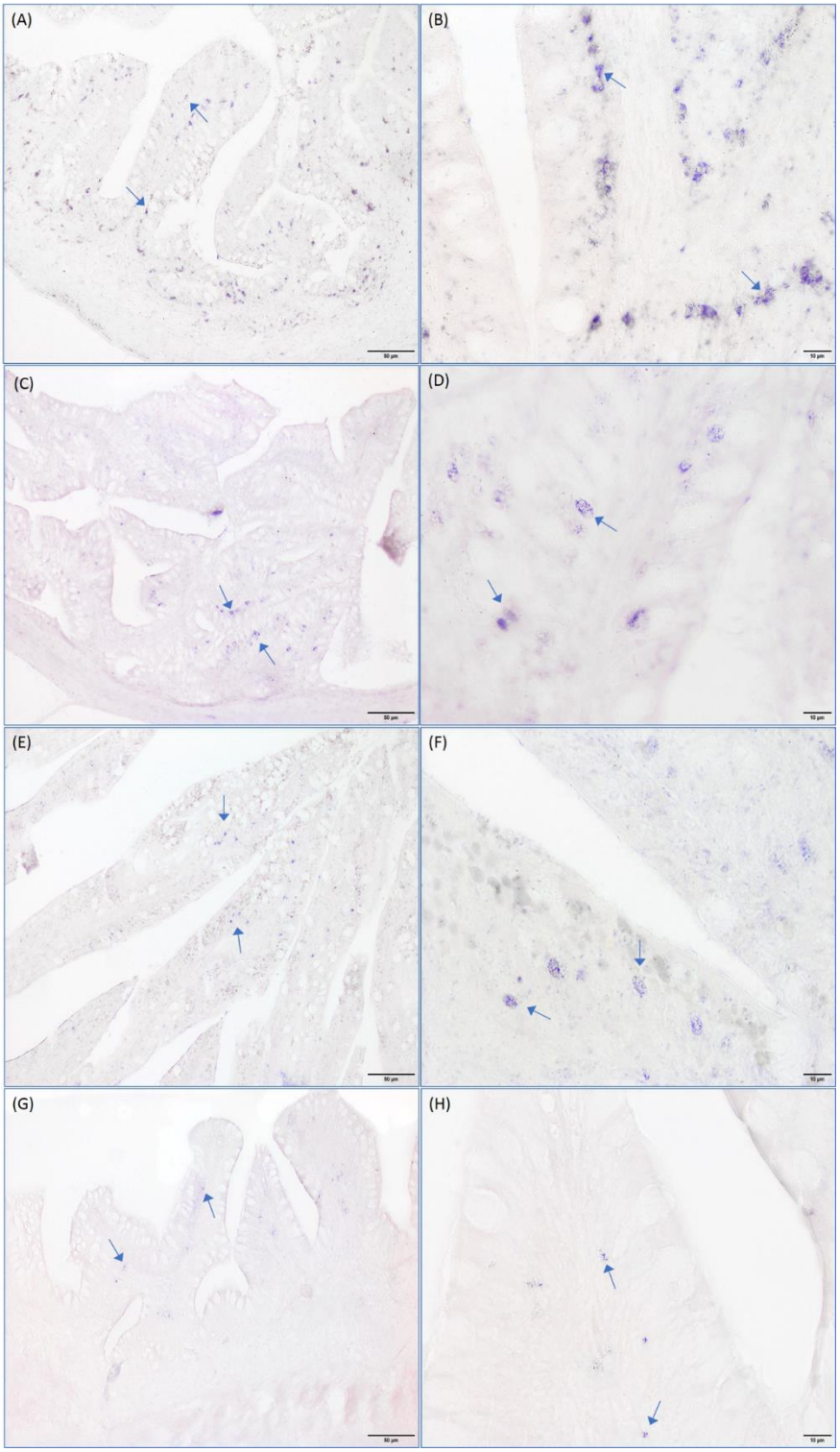

Fig. 10. Photomicrographs of *in situ* hybridization patterns of European seabass (*Dicentrarchus labrax)* juveniles distal intestine observed for (A,B) *mhcii-β,* (C,D) *tcr-β*, (E,F) *cd-8α*, and (G,H) *cd-4* immune-related genes after 90 days of feeding. No differences were found in the distribution of stained cells in relation to the diet supplemented. The *mhcii-β* positive cells were found in higher number than *tcr-β*, *cd-8α*, and *cd-4* expressing cells. (A) *mhcii-β* positive cells were detected mainly infiltrated between or located below basal membrane of enterocytes and goblet cells (arrows). Bar 50µm. (B) Detailed micrograph of *mhcii-β* positive cells location (arrows). Bar 10µm. (C) *tcr-β* positive cells distribution was mainly located below enterocytes nuclei (arrows). Bar 20µm. (D) Detailed micrograph of *tcr-β* positive cells distribution (arrows). Bar 10µm. (E,F) *cd-8α* expressing cells were detected primarily within lamina propria or as mucosal infiltrates (arrows). Bar 50-10µm. (G,H) *cd-4* positive cells located principally within or as mucosal infiltrates in lamina propria (arrows). Bar 50-10µm.

6.4.3.4. Gut gene expression

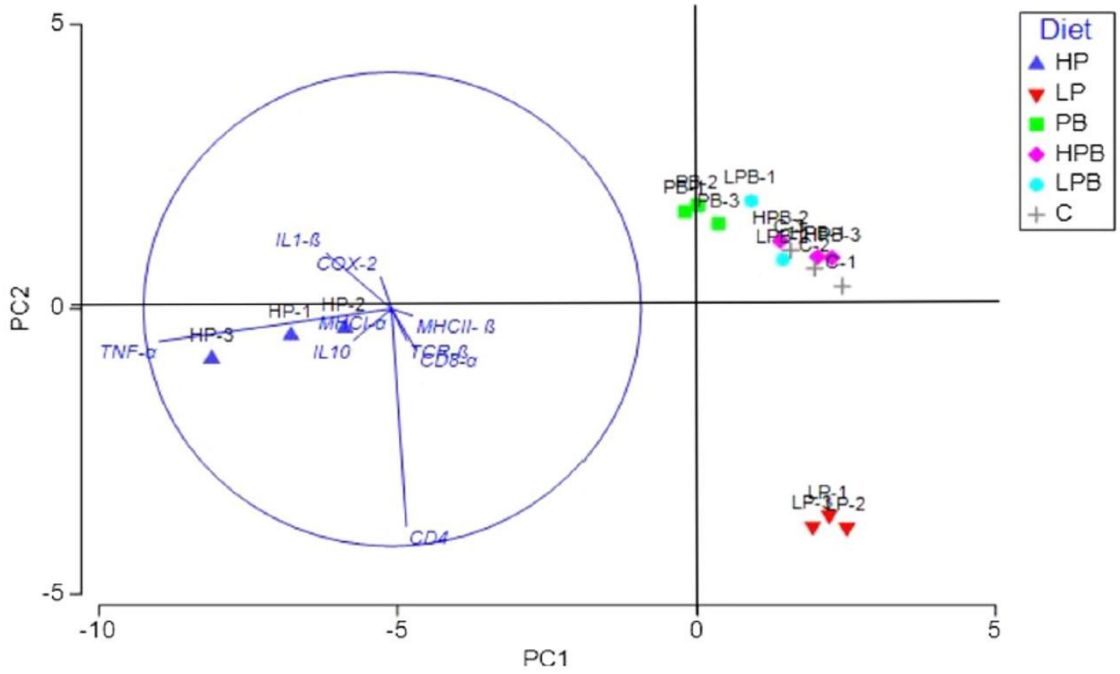

Fig. 11. Principal component analysis (PCA) to study the variability of European seabass (*Dicentrarchus labrax*) juveniles' distal gut in relation to dietary treatment. Diets: C (control diet), HP (high prebiotic level), LP (low prebiotic level), B (only probiotic), HPB (high prebiotic level plus probiotic) and LPB (low prebiotic level plus probiotic).

PERMANOVA analyses indicated the significance of MOS (P=0.01), *P. acidilactici* (P=0.02) and their interaction (P=0.01). PCA attributed a 78.7% of the total variability on the DI relative gene expression results to the dietary treatment (Fig. 11). The first principal component (PC1) accounted for a 59.4% of the total DI GALT-gene expression variability, and clearly detached HP diet from the rest of the dietary treatments to the left side of the axis. PC1 linear combination of variables attributed the main negative values to inflammation process humoral factors (*tnfα, il-1β, il-10* and *cox-2*), whereas the positive values were determined by the cellular-immune system related genes and demonstrated synergistic effects of supplementing both products together on DI functionality (Fig. 11). PC2 accounted for a 19.3% of the total variability observed and seceded LP treatment from the rest, being *cox-2* and *il-1β* the main positive variables and *cd-4* the main negative, indicating the importance of MOS dose of supplementation when fed alone. Synbiotic, control and *P. acidilactici* diets are all clustered on the up-right PCA graph, indicating low variability among them in total DI GALT-gene expression, however particular effects as described above have been detected.

After 90 days of feeding, two-way-ANOVA analysis revealed individual effect of dietary MOS on *tnfα* (F=82.76, P=0.001), *il-10* (F=9.87, P=0.004), *mhcii-β* (F=22.81, P=0.001), *cd-4* (F=114.24, P=0.001), *cd-8α* (F=10.86, P=0.002), *tcr-β* (F=5.56, P=0.021) and *cox-2* (F=22.28, P=0.001) (Table 26). Comparing dietary MOS doses, the lowest MOS dietary level upregulated *cd-8α*, *cd-4*, *tcr-β* and *mhcii-β* gene expression, whilst downregulated *tnfα, cox-2* and *il-10* compared to the highest MOS level.

On the other hand, probiotic dietary supplementation downregulated the gene expression of *tnfα* (F=64.41, P=0.001), *il-10* (F=75.93, P=0.001), *mhci-α* (F=30.07, P=0.001), *cd-4* (F=208.90, P=0.001) and *tcr-β* (F=16.29, P=0.002) (Table 26), and increased the number of transcripts of *il1β* (F=6.55, P=0.027) and *cox-2* (F=9.70, P=0.010) genes compared to non-supplemented diets (Table 26). Synbiotic supplementation resulted in an upregulation of *il-1β* (F=77.51, P=0.001) and *mhcii-β* (F=22.78, P=0.001), and in downregulation of *tnfα* (F=120.16, P=0.001), *il-10* (F=36.78, P=0.001), *mhci-α* (F=10.98, P=0.002), *cd-4* (F=90.74, P=0.001), *cd-8α* (F=26.39, P=0.001), *tcr-β* (F=7.28, P=0.001) gene expression (Table 26). In relation to MOS dietary levels, the highest dose of MOS presented an upregulation of *cd-8α, il-10*, *cox-2* and *tnfα* gene expression in relation to lowest MOS dietary levels, whereas low doses of MOS presented higher number of transcripts for *mhcii-β*, *cd-4* and *tcr-β* (Table 26).

Table 26. RT-PCR of immune-related genes in distal intestine of European seabass (*Dicentrarchus labrax*) juveniles fed different dietary treatments at the end of the feeding trial (90 days).

diets	C	HP	LP	B	HPB	LPB
tnfα	1.05 ± 0.38	9.47 ± 1.21	1.24 ± 0.20	2.52 ± 0.28	1.16 ± 0.38	1.19 ± 0.02
Il-1β	1.01 ± 0.13	3.41 ± 0.17	0.54 ± 0.25	2.08 ± 0.25	0.57 ± 0.12	3.87 ± 1.23
cox-2	1.04 ± 0.35	3.00 ± 1.11	2.30 ± 0.02	4.07 ± 0.86	5.08 ± 0.64	0.29 ± 0.05
Il-10	1.03 ± 0.29	2.34 ± 0.26	1.40 ± 0.00	1.14 ± 0.09	0.60 ± 0.18	0.64 ± 0.01
mhci-α	1.00 ± 0.01	0.40 ± 0.17	0.35 ± 0.08	0.07 ± 0.05	0.17 ± 0.07	0.36 ± 0.12
mhcii-β	1.01 ± 0.20	0.09 ± 0.03	0.91 ± 0.29	0.36 ± 0.07	0.61 ± 0.05	1.05 ± 0.17
cd-4	1.07 ± 0.46	2.06 ± 0.06	5.69 ± 0.13	0.64 ± 0.19	1.28 ± 0.26	1.08 ± 0.40
cd-8α	1.00 ± 0.09	0.63 ± 0.19	2.09 ± 0.18	0.94 ± 0.08	1.51 ± 0.41	1.03 ± 0.25
tcr-β	1.01 ± 0.14	0.35 ± 0.24	1.25 ± 0.31	0.33 ± 0.08	0.47 ± 0.32	0.46 ± 0.20

Diets: C (control diet), HP (high prebiotic level), LP (low prebiotic level), B (only probiotic), HPB (high prebiotic level plus probiotic) and LPB (low prebiotic level plus probiotic). Data presented as mean ± SD. N = 3 tanks/diet. Two-way ANOVA analyses (P<0.05). MOS *post-hoc* analyses were calculated by GT2 Hochberg test. NS = No significant.

6.4.4. *Experiment II: challenge test against Vibrio anguillarum*

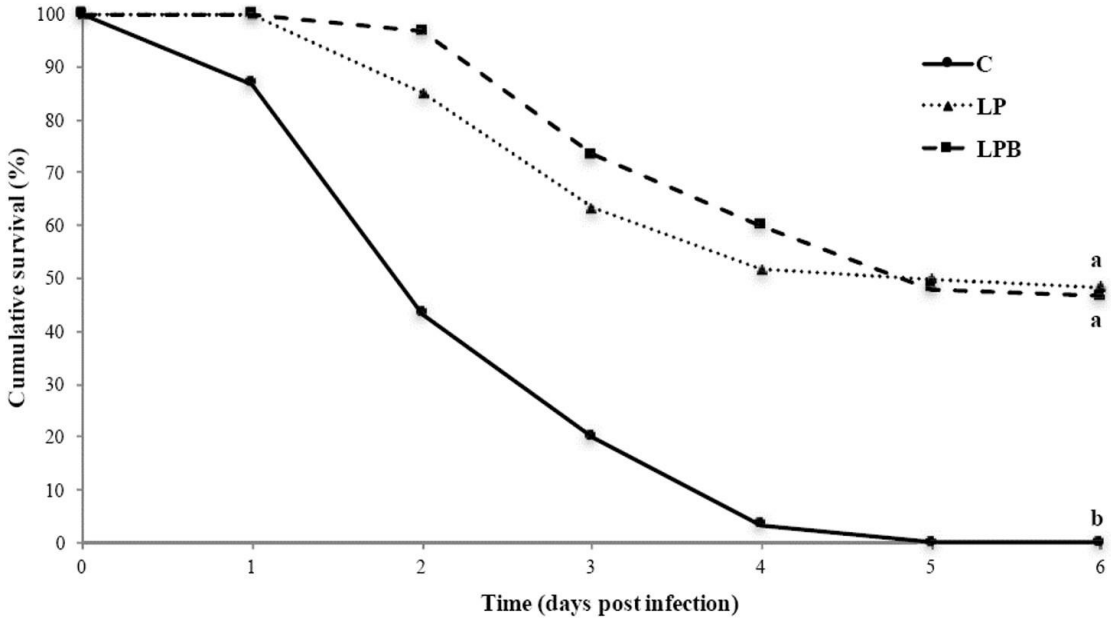

Fig. 12. Cumulative survival (%) of European seabass (*Dicentrarchus labrax)* juveniles along the challenge test against *V. anguillarum.* Different letters denote statistical differences (P<0.05; Kaplan-Meier survival; Mantel-Cox, Generalized Wilcoxon, Tarone-Ware analyses). Diets: C (control diet), LP (low prebiotic level), and LPB (low prebiotic level plus probiotic).

After 90 days of feeding, fish fed C diet presented lower (P<0.05) survival against *V. anguillarum* infection than fish fed diets supplemented with LP and LPB (Fig. 12), No differences (P>0.05) in final survival percentages were found between LP and LPB groups at the end of the challenge test period. As can be observed in Fig. 12, the pattern of mortality also differs from fish control diet to fish fed supplemented diets (LP and LPB), presenting fish fed control diet a higher incidence of mortality in the first 48 h of challenge (Fig. 12).

6.5. DISCUSSION

The use of sustainable raw materials in diets for marine fish is essential to achieve a sustainable aquaculture sector development. However, marine raw materials replacement by terrestrial protein and oil sources has been associated, in some cases, with depleted fish growth, reduced immune response or increased disease incidence.

Particularly for European seabass juveniles, high dietary substitution levels of FM and FO (i.e. down to 5%FM/6%FO in % of total ingredients) by terrestrial sources is related to lower weight gain and feed intake, altered gut associated lymphoid tissue function and increased gut bacterial translocation rates when compared to fish fed higher levels of FM and FO (58%-20%FM and 15%FO-6%FO in % of total ingredients) (Torrecillas *et al.*, 2017a). In this context functional additives may play a key role as an effective tool to buffer some of the growth and health associated side-effects mentioned. In the present study, dietary MOS supplemented alone or combined with *P. acidilactici* in 5%FM and 6%FO based diets for 90 days improved European seabass juveniles weight gain when compared to non-supplemented diets regardless the prebiotic dietary level. The positive effect of dietary MOS at similar doses on European seabass growth performance is well documented, particularly when included in high FM and FO based diets (50%FM/15%FO) (Torrecillas *et al.*, 2007, 2011a, 2013, 2015a), and it is related to its gut mucosa protective function and to a better nutrient absorption (Torrecillas *et al.*, 2014). Nevertheless, this is the first study which combine a low dietary inclusion of FM/FO and the use of MOS, and further studies must be conducted to elucidate MOS mode of action in a nutritionally forced situation. In agreement with results obtained, *P. acidilactici* supplementation by itself neither enhanced growth performance in fresh water species (Standen *et al.*, 2013; Aubin *et al.*, 2005; Merrifield *et al.*, 2011b), however it clearly delayed intestinal inflammatory response in Atlantic Salmon (Vasanth *et al.*, 2015). However, *P. acidilactici* supplementation cooperated positively with other immune modulators such as β-glucans or FOS by promoting fish and shellfish growth performance (Azimirad *et al.*, 2016; Boonanuntanasarn *et al.*, 2016), despite it did not differ from prebiotic supplementation alone as found in present study, focusing its role as immunomodulator and not as a growth promoter. In fact, dietary administration of MOS and/or *P. acidilactici* did not affect European seabass somatic indexes, however hepatocyte maximum length was reduced in fish fed diets with *P. acidilactici*, pointing

to a potential positive role of this product in European seabass lipid metabolism when fed in low FM/FO based diets.

Fish gut morphological patterns may be directly affected by reductions in dietary FM and FO (Torrecillas *et al.*, 2017a; Caballero *et al.,* 2003; Krogdahl *et al.,* 2003; Aslaksen *et al.*, 2007; Bakke-McKellep *et al.*, 2007; Olsen *et al.*, 2007; Bonaldo *et al.*, 2008; Øverland *et al.,* 2009) as well as after dietary functional additives supplementation (Torrecillas *et al.*, 2014, 2015b; Standen *et al.*, 2013; Ferguson *et al.,* 2010; Harper *et al.*, 2011). In this sense, fish fed MOS and *P. acidilactici* supplemented diets presented a trend to greater lamina propria and submucosa immunoreactivity to anti-Inos compared to fish fed control diet, which may be an indicative of variations in leucocytes populations related to microbiota composition changes in agreement with earlier studies in other fish species (Torrecillas *et al.*, 2013; Ferguson *et al.*, 2010).

However, gut morphometric analyses or ISH studies did not reveal a clear functional additive-derived effect on any parameter evaluated. Nevertheless, it is worthy to highlight the relative high number of DI *mhcii-β* labelled cells found, despite of dietary functional additives supplementation, and probably associated to adaptations of gut microbiota to a 5%FM/6%FO dietary content (Torrecillas *et al.*, 2007). Previous studies reported differences in intestine *mhcii-β* cells location in Atlantic salmon (*Salmo salar*) fed high levels of soybean meal as an attempt of fish to maintain homeostasis or improve tolerance when resident microbiota population is altered (Romarheim *et al.*, 2013) and based on *mhcii-β* antigen expression by several phagocytic-cell populations (Li *et al.*, 2006; Cuesta *et al.*, 2006). Altogether denoting, again, the importance of diet formulation when testing functional ingredients and how it could be limiting or/and modifying functional additives specific modes of action (*i.e.*, triggering of gut mucus production, GALT cellular populations distribution).

In terms of gene expression, fish fed the highest prebiotic dosage presented an upregulation of several immune response-related genes, such as for example *tnfα* or *cox-2*, compared to fish fed LP or non-supplemented diet in agreement with previous studies in the same fish species fed higher levels of marine origin ingredients (Torrecillas *et al.*, 2015b). Although as observed on the PCA analysis, and despite individual and combined effect of both dietary supplements, when MOS is combined with *P. acidilactici* total DI GALT-gene expression is really similar to fish fed non-supplemented diets. This fact may indicate a "tone-down like effect" in relation to fish fed the highest prebiotic diet (HP) by

upregulating the expression of some cellular-immune system related genes in agreement with the gut immunohistochemical patterns observed. Actually, *P. acidilactici* supplementation by itself did not trigger any clear response in terms of DI GALT-gene expression in relation to the control treatment, despite some variations have been detected depending on the MOS dosage combined with. For example, feeding European seabass with the highest dose of dietary MOS resulted in a clear upregulation of *tnfα*, *cd-4* and *cox-2* genes without causing a negative effect on fish growth performance. This result is in agreement with similar studies in the same fish species when MOS was included in diets with higher dietary levels of marine raw materials (Torrecillas *et al.*, 2015b). However, when MOS was combined with dietary *P. acidilactici*, *tnfα* gene expression was downregulated to almost basal levels, whereas *cox-2* gene expression was upregulated. Besides, as indicates the PCA analysis, LP distal gut GALT-gene expression is clearly detached from HP and the rest of the dietary treatments, showed the importance of dietary MOS dosage when supplemented in low fish meal and fish oil diets. Certainly *tnfα*, which was the main variable affecting PC1 variations, is released in response to pathogen-associated molecular patterns (PAMPs) by macrophages or activated T-cells presenting cells, playing a key role on migration of leucocytes to the inflammation site (Ming *et al.*, 1987; Young *et al.*, 2001). On one hand, the role of dietary MOS on the stimulation of leucocyte migration in the distal gut of European seabass has been clearly described by genetic and morphologic studies in relation to reduced gut bacterial translocation (Torrecillas *et al.*, 2014 and 2015b). This fact has been related to the activation of *nf-kβ* (Ming *et al.*, 1987; Young *et al.*, 2001) via mannose receptor or dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SING), to the modulation of the toll like receptor 4 (TLR4) via alterations in microbiota populations or even as a continuous response to the indigestible MOS itself (Torrecillas *et al.*, 2015b). On the other hand, probiotics stimulate gut epithelial homeostasis by controlling alterations in microbiota populations and helping to stabilize the gut mucosal immunological barrier (Madsen *et al.*, 2001). Particularly, *P. acidilactici*-feeding combined with an experimentally induced gut inflammation condition in Atlantic salmon resulted in not only milder and delayed inflammatory responses compared to non-supplemented fish but also to a faster recovery (Vasanth *et al.*, 2015). Actually, when HP was supplemented with *P. acidilactici*, *il-10* expression was downregulated in response to lower proinflammatory levels. Similarly, *cd-4*$^{+}$ lymphocytes and *tcr-β* gene expression seems to be reduced in fish fed diets LPB and HPB when compared to fish fed only MOS,

particularly when combined with the lowest dose of MOS. Altogether pointing to a synergistic effect of both products in terms of balancing MOS-derived distal gut inflammatory like status. On the contrary, synbiotic-feeding increases *cox-2* gene expression, especially when combined with LP dose and in absence of inflamed gut symptomatology evidence. Inducible *cox-2* upregulation may correspond to a different microbiota pattern after probiotic supplementation, but the homeostatic function of constitutively expressed *cox-2* in absence of explicit inflammation (Kirkby *et al.*, 2013) may be considered.

Unfortunately, despite in the present study European seabass disease resistance against *V. anguillarum* was not evaluated in HP diet, our findings show that feeding European seabass with low prebiotic or low synbiotic diets reduces mortality against *V. anguillarum* infection after 90 days of feeding, though no differences were found between both treatments. In this sense, it is well documented how dietary MOS triggers European seabass cellular and humoral innate immune response and enhances its disease resistance in a high FM and FO diet basis (Torrecillas *et al.*, 2014; Guerreiro *et al.*, 2017). However, almost all former studies were based in a shorter period of supplementation pre-challenge, indicating also the importance of diet formulation in relation to dose-time-effect studies with functional additives (Torrecillas *et al.*, 2014).

Overall our results show that a dietary level of 5%FM/6%FO for 90 days did not trigger an evident intestinal inflammatory like status in terms distal gut morphology. However, when MOS was combined with a low content of FM and FO, the dosage supplemented is a key factor in terms of the gut gene expression proinflammatory pattern,
being greater in fish fed higher doses of MOS. Results obtained indicate that MOS-derived distal gut humoral proinflammatory genes up regulation could be partially counteracted by *P. acidilactici* by reducing in particular *tnfα*, *il-1β* and Inos when supplemented as a synbiont, helping European seabass to accomplish a faster intestinal homeostasis status with no detrimental effect on fish growth performance.
Furthermore, dietary MOS at low doses, as well as its combination with *P. acidilactici*, reduced European seabass mortality when challenged against *V. anguillarum* by intraperitoneal injection. Altogether pointing to the combination of a low dose of MOS and *P. acidilactici* as synbiont (LPB) as a viable tool to potentiate growth and disease resistance of European seabass juveniles when supplemented in low FM and FO diets.

Chapter 7

SYNBIOTICS IN DIETS FOR EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*): FEEDING STRATEGIES TO IMPROVE THE BENEFICIAL ACTION OF FUNCTIONAL INGREDIENTS IN DIETS LOW IN FISHMEAL AND FISH OIL

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ST, RG, MJC, MI & DM conceived and planned the experiments. AM formulated the diets. FR carried out the experiments and contributed to sample preparation and analyses. AF-M contributed to the statistical analysis. MC-SR contributed to analyses. FR, ST, MJC & DM contributed to the interpretation of the results and discussion. FR, ST & DM wrote the paper with input from all authors.

7.1. ABSTRACT

This study aimed to evaluate different feeding strategies based on the supplementation with mannan oligosaccharides (MOS) and *Pediococcus acidilactici* in singular or synbiotic administration on 5%FM/6%FO based diets for European seabass in order to assess their effects on survival, growth performance, liver and intestine morphology, systemic immune parameters, relative expression of genes related to gut associated lymphoid tissue (GALT) and disease resistance against *Vibrio anguillarum*. For that purpose, 4 diets were assayed for 39 weeks (% MOS/*P. acidilactici* (commercial recommendations)): only prebiotic (P) = 0.3/0; only probiotic (B) = 0/+; synbiont: prebiotic plus probiotic (PB) = 0.3/+; and control (C) = 0/0. Diets were assayed in three sequential periods: period 1 (P1), 0-25 weeks; period 2 (P2), 25-31 weeks; period 3 (P3), 31-39 weeks. In P1, fish were fed P, B, PB and C diets. At the end of P1, fish fed P and PB were divided in three groups and fed along P2 and P3 with different feeding strategies. Thus, in P2 strategies assayed were P, P/PB, B, PB, PB/P and C and in P3, strategies were P, P/PB/P, P/PB/PB, B, PB, PB/P/PB, PB/PB/P and C. After 39 weeks fish were experimentally infected with *Vibrio anguillarum*. Additionally, at the end of each feeding period (P1, P2 and P3) growth parameters and somatic indexes were calculated. After 39 weeks of feeding, morphology of distal intestine and liver was evaluated. At the end of P3 and after bacterial challenge, samples of distal intestine were taken for gut associated immune tissue (GALT)-related gene analysis, and samples of blood was taken to evaluate serum lysozyme and bactericidal activity and total peroxidase content.

Growth performance of fish was enhanced after 25 weeks (P1) by P diet whereas strategies P/PB/P and PB influenced positively growth at P3 (39 weeks). In distal intestine, fold length was increased by *P. acidilactici* supplementation whereas synbiotic (PB) reduced hepatocytes size and vacuolization, after 39 weeks of feeding. Synbiotic and P/PB/P strategies enhanced basal lysozyme and peroxidase compared to C, after 39 weeks of feeding whereas P, P/PB/P, PB, PB/P/PB and B strategies enhanced peroxidase activity compared to control diet after challenge. At the end of feeding trial, functional ingredients reduced the upregulation of proinflammatory genes in distal intestine. Similarly, the capacity of immune response after bacterial challenge seemed to be enhanced by P and PB/P/PB strategies. Results of the present Experiment reflect that different feeding strategies, using the same functional additives, induce different effects in growth performance, intestine and liver morphology, immune parameters and the

expression of GALT-related genes, denoting the importance of mode of use of these ingredients.

7.2. INTRODUCTION

During the last decades, the increasing growth of aquaculture together with the increased prices and reductions in the availability of fish oil (FO) and fish meal (FM) have forced to reduce the use of those ingredients by the inclusion of alternative sources in feedstuffs for farmed fish (Tacon & Metian, 2017). However, the use of alternative ingredients, such as those from vegetable origin, implies several issues to reach a good performance and/or to maintain the fish health status. Particularly for European seabass (*Dicentrarchus labrax*), total substitution of dietary FM/FO has been shown to impact negatively in fish growth (Geay *et al.,* 2011; Le Boucher *et al.,* 2011 and 2013; Torrecillas *et al.,* 2017a), while high levels of substitution alter intestine morphology (Torrecillas *et al.,* 2017a). In this fish species, alterations in distal intestine cytoarchitecture have been correlated with both, poor fish growth, through digestibility disturbances (Torrecillas *et al.,* 2017a), and modifications in gut microbiota environment with the subsequent alteration of the gut extrinsic, physical, and immune barriers against pathogens (Torrecillas *et al.,* 2011a and 2013; Miao *et al.,* 2017). Moreover, European seabass feeding free FM/FO diets present alterations in other tissues, such as liver, altering its lipid metabolism which may produce negative effects on fish health (Geay *et al.,* 2011). Those morphological alterations in liver have been associated, among others, to dietary FO reductions and to the subsequent depletion of essential fatty acids (EFAs) (Torrecillas *et al.,* 2017b).

Besides, the reduction of FO down to 3% (of total ingredients) have been related to increased bacteria translocation through intestinal mucosa epithelium whereas FM reductions down to 5% induced an intestinal inflammatory-like response, reducing fish capacity to face bacterial infection (Torrecillas *et al.,* 2017d).

The use of functional dietary ingredients as prebiotics and probiotics has been demonstrated to be a reliable tool to counteract those potential negative effects on health when using low FM/FO diets in marine fish (Torrecillas *et al.,* 2015b, 2016 and 2018b; Rimoldi *et al.,* 2016; Terova *et al.,* 2016). Among the different prebiotics, mannan oligosaccharides (MOS) has shown to potentiate fish growth performance, innate immune system, disease resistance, intestinal morphology, and mucus secretion of European seabass (*Dicentrarchus labrax*) (Torrecillas *et al.,* 2014). The capacity of prebiotics to increase fish disease resistance relies, in part, in their ability to avoid fish colonization by

pathogenic bacteria (Huynh *et al.*, 2017). However, the effectiveness of these functional additives will depend on form of presentation, dosage, time of administration, fish species, fish age, culture conditions, and feed production process (Dimitroglou *et al.*, 2010 and 2011; Torrecillas *et al.*, 2014).

Probiotics have been shown to induce beneficial modulations of fish microbiota and to prevent the attachment of pathogenic bacteria in intestine (Balcázar *et al.*, 2006a; Gatesoupe, 2008; Sun *et al.*, 2011). Besides, they have been described to improve fish growth performance and health (Hai, 2015) and particularly for European seabass, the dietary supplementation with certain probiotics enhanced growth performance (Carnevali *et al.*, 2006; Frouël *et al.*, 2008). However, any beneficial effect on fish species will depend on the probiotic strain characteristics, such as being gram +/-, indigenous/exogenous, spore/non-spore former, mode of presentation, use, viability, or time, among other factors (Nayak, 2010; Ramos *et al.*, 2013), altogether influencing its relationship with the host as demonstrated in freshwater and marine fish species (Nayak, 2010; Newaj-Fyzul *et al.*, 2014). In particular, the lactic acid bacteria (LAB) *Pediococcus acidilactici*, is one of the most used probiotics in marine aquaculture (Cruz *et al.*, 2012; Newaj-Fyzul *et al.*, 2014; Hai, 2015) and has been related to improvements in growth, survival, disease resistance and immunomodulation in fish (Merrifield *et al.*, 2010 and 2011b; Ferguson *et al.*, 2010; Neissi *et al.*, 2013; Ramos *et al.*, 2013; Standen *et al.*, 2013; Vasanth *et al.*, 2015; Mondaloo *et al.*, 2017; Hoseinifar *et al.*, 2015a and 2017).

Beyond their individual effects, the combined use of both, prebiotics and probiotics, gives rise to an alternative functional additive, also known as synbiotic (Gibson & Roberfroid, 1995), which can be used to face the undesired effects of high dietary levels of FM/FO substitutions on fish intestinal health. The mentioned considerations for prebiotics and probiotics make the effectiveness of the synbiotic dependent on the species in which is used (Huynh *et al.*, 2017). This synbiotic approach has been tested in European seabass juveniles, using MOS and *Pediococcus acidilactici*, improving growth and disease resistance when supplemented for 90 days in fish fed highly substituted diets (Torrecillas *et al.*, 2018b).

Therefore, based on previous results (Torrecillas *et al.*, 2018b), the aim of this study was to evaluate different feeding strategies for European seabass fed 5%FM-6%FO based diets supplemented with prebiotic MOS and probiotic *Pediococcus acidilactici* in singular or synbiotic administration. The strategies consisted on administration of synbiont or

either single prebiotic or probiotic, in a series of alternate periods. This study focused on the effects in fish survival, growth performance, liver and intestine morphology, systemic immune parameters, relative expression of genes related to gut associated lymphoid tissue (GALT) and disease resistance against *Vibrio anguillarum*.

7.3. MATERIAL AND METHODS

7.3.1. *Experimental diets*

Four extruded isolipidic and isoproteic diets manufactured by BioMar (Tech-Centre, Brande, Denmark) were formulated to contain low fish oil (6%) and fish meal (5%) levels. A combination of mannan oligosaccharides (MOS, Biomos® and Actigen©; Alltech, Inc., Kentucky, USA) and a commercial presentation of the probiotic *Pediococcus acidilactici* (BAC, Bactocell®; Lallemand Inc., Cardiff, UK) were included in diets replacing standard carbohydrates as follows (MOS (%) / BAC (commercial recommendation)): only prebiotic (P) = 0.3/-, only probiotic (B) = 0/+, prebiotic plus probiotic (PB) = 0.3/+. Another diet, without supplementation, was included as a control (C) = 0/-. Ingredients and proximal composition of diets are detailed in Table 6.

7.3.2. *Experimental conditions*

One thousand and two hundred European seabass juveniles reared in a local farm (Aquanaria, S.L., Castillo del Romeral, Gran Canaria, Canary Islands, Spain) were maintained in stocking tanks and fed the control diet of this experiment during 4 weeks in the facilities of the Parque Científico-Tecnológico Marino (PCTM) at the University of Las Palmas de Gran Canaria (ULPGC) (Telde, Gran Canaria, Canary Islands, Spain) until achieving the experimental size.

7.3.3. Feeding trial

Fish were fed the experimental diets until apparent satiation (3 times / day, 6 days / week) for a total period of 39 weeks (Total period; TP), distributed in 3 sequential periods: Period 1 consisting of 25 weeks of feeding (P1; 0-25 week), followed by Period 2 consisting of 6 weeks (P2; 25-31 week) and ending with a Period 3 consisting of 8 weeks (P3; 31-39 week) (detailed in Table 27). In order to cope with the strategies designed for sequential periods 2 and 3 (P2 and P3), both functional product supplementation was

alternated as follows: P/PB/P, P/PB/PB, PB/P/PB and PB/PB/P, corresponding P to only MOS, and PB to MOS + *P. acidilactici* supplementation (Table 27). Altogether a control diet, non-supplemented, for the whole period (C), a total period strategy of continuous dietary supplementation of MOS (P), *P. acidilactici* (B) or their combination (PB) were also evaluated (Table 27).

Along the whole experimental period of feeding, tanks were in a flow-through system supplied with aeration, and under a natural photoperiod (~12L / 12D). Dissolved oxygen and temperature were measured weekly and ranged between 7.5-8.0 ppm and 21-22º C, respectively.

For P1 (first 25 weeks of feeding), fish were randomly distributed in 500l fiberglass circular tanks at an initial density of 4 kg * m^{-3} (100 fish/tank) and experimental treatments were assayed in triplicate. Fish initial weight and length was 19.7 ± 0.1 g and 11.3 ± 0.1 cm (mean ± SD) respectively.

Table 27. Diets and corresponding strategy acronym during Periods 1 (0-25 week), 2 (25-31 week) and 3 (31-39 week).

PERIOD 1 (0-25 week)		PERIOD 2 (25-31 week)		PERIOD 3 (31-39 week)	
DIET	**STRATEGY ACRONYM**	**DIET**	**STRATEGY ACRONYM**	**DIET**	**STRATEGY ACRONYM**
P	P	**P**	P	**P**	P
		PB	P/PB	**P**	P/PB/P
		PB	P/PB	**PB**	P/PB/PB
PB	PB	**PB**	PB	**PB**	PB
		P	PB/P	**PB**	PB/P/PB
		PB	PB	**P**	PB/PB/P
B	B	**B**	B	**B**	B
C	C	**C**	C	**C**	C

At the end of P1, fish of each treatment, in order to follow the feeding strategies described above, were pooled and distributed in duplicate in 500l fiberglass circular tanks (16 fish/tank). For P2 and P3, fish were subjected to a change of diet, if necessary, for 6 and 8 weeks respectively, as described in Table 27, until the end of the feeding trial. At the end of the total period of feeding.

7.3.4. *Challenge test against Vibrio anguillarum*

At the end of the total period (39 weeks of feeding), a total of 18 fish from each dietary treatment (P, P/PB/P, P/PB/PB, PB, PB/P/PB, PB/PB/P, B, and C; N=18), were experimentally infected against *Vibrio anguillarum*.

Fish were transferred to the marine biosecurity station (MBS) facility at the PCTM at ULPGC, fed the same strategy used along the P3 and challenged against *V. anguillarum* (strain 507; adjusted to 10^3 CFU ml^{-1} for each fish) diluted in phosphate buffered saline (PBS) by intestinal inoculation as described previously (Torrecillas *et al.*, 2011). Samples of blood and distal intestine were recollected at 2- and 7- days after challenge from 3 fish/tank (N=6) for the different analyses. Fish mortality was registered daily during the trial.

7.3.5. *Sampling procedures*

At P1 (25 weeks), P2 (31 weeks) and P3 (39 weeks) all fish were individually weighed (W (g); whole body weight) and measured (L (cm); length from the tip of the head to the end of the caudal fin), and growth parameters and somatic indexes calculated as follows: relative growth (RG; [[Wfinal – Winitial] * $Winitial^{-1}$] * 100); (condition factor (K; W/L^3); specific growth rate (SGR; [(Ln(Wfinal)-Ln(Winitial))/days of feeding] * 100,); viscerosomatic index (VSI; [viscera weight * W^{-1}] * 100); hepatosomatic index (HSI; [liver weight * W^{-1}] * 100); perivisceral fat index (PFI; [perivisceral fat weight * W^{-1}] * 100). At P2, growth parameters and somatic indexes were calculated for groups fed the same diet at that moment: P, P/PB, PB, PB/P, B, and C (Table 27).

At the end of P3 and bacterial infection trial, blood and tissue samples were collected for immunological (N = 6 fish/treatment), morphological (N = 6 fish/treatment) and gene expression analyses (N = 6 fish/treatment).

Before all samplings, animals were starved for 24 hours. Fish were anaesthetized with clove oil (5 ml/100 l) prior any handling. Animal manipulation protocols were made under guidelines of European Union Council (86/609/EU) and Spanish (RD 53/2013) legislations and approved by Bioethical Committee of the ULPGC (University of Las Palmas de Gran Canaria) (REF: 007/2012 CEBA ULPGC).

7.3.5.1. Histological studies

Intestine samples were obtained at the end of the total feeding period (39 weeks). Distal intestine (DI), as previously described by Torrecillas *et al.*, (2013), and liver were dissected out, stored in 4% paraformaldehyde (pH = 7.4, 0.1M) and conserved at 4ºC. After a minimum of 48 hours, samples were included in paraffin. Sections of 4µm were cut with a manual rotary microtome (Leica Jung 2055, Germany) and stained with hematoxylin and eosin (H&E) (Martoja & Martoja-Pierson 1970). Besides, new DI sections were also stained using Alcian Blue-Periodic Acid Schiff (pH= 2.5) staining with the purpose of marking acid mucus secreting cells (Martoja & Martoja-Pierson 1970). Micrographs were taken using the image capturing software (Cell B®, Olympus, Hamburg, Germany) in a computer connected to a Nikon Microphot- FXA microscope with an Olympus DP50 camera coupled. The software Image Pro Plus v5 (Media Cybernetics Inc., Rockville, MD, USA), was used to treat acquired images. The density of goblet cells per fold area and morphometrical variables (fold length, FL; fold width, FW; submucosa width, SW) were evaluated from DI samples according to Torrecillas *et al.*, (2011a). From liver, hepatocytes morphometrical measures were taken at the end of the total feeding period (39 weeks) as described by Torrecillas *et al.*, (2007): hepatocytes area and hepatocytes maximum and minimum length (HA, HMx and HMn, respectively), tacking as reference the center of the cell nucleus. Both, distal intestine and liver morphometrical measures were weighted using individual fish weight.

7.3.5.2. Immune-parameters analyses

Blood samples were obtained by caudal vein of individual fish using non-heparinized syringes from three animals from each tank at the end of basal time (39 weeks) and after 2 days of pathogen inoculation. Serum was obtained by clotting overnight at 4ºC and stored at -80ºC until analyses. Serum bactericidal activity capacity was determined following the method described by Wiklund & Dalsgaard (2002). Serum lysozyme activity was determined according to the method described by Anderson & Siwicki (1994). Total peroxidase content was estimated from serum following the methodology described by Quade and Roth (1997).

7.3.5.3. Relative expression of GALT-related genes

At 0h and 2 and 7 days after pathogen inoculation, distal gut were dissected out and conserved in Invitrogen™ RNA*later*™ Stabilization Solution (Thermo Fisher Scientific Inc., USA) according to manufacturer's guidelines. Afterwards, total RNA was extracted from 100 mg of tissue (N=3 pool/diet, 2 fish/pool) with TRI-Reagent (Sigma-Aldrich, Germany) and RNeasy® mini Kit (QUIAGEN, Germany), quantified by spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific Inc, USA) and the integrity was evaluated on a 1.4% agarose gel with Gel Red™ (Biotium Inc., Hayward, CA).

cDNA was synthesized in 20µl final volume with 1µg RNA and iScript™ cDNA Synthesis Kit (Bio-Rad Hercules, California). Both, elongation factor one alpha (*ef-1α*) and β-actin genes were tested as housekeeping, and *ef-1α* demonstrated to be the more stable to make calculations. Gut associated lymphoid tissue (GALT)-related genes analyzed were: major histocompatibility complexes one-alpha and two-beta (*mhci-α* and *mhcii-ß*), interleukins 10 and 1 beta (*il-10* and *il-1β*), clusters of quadruple differentiation 4 and 8 alpha (*cd-4* and *cd-8α*), cyclooxygenase 2 (*cox-2*), tumor necrosis factor alpha (*tnfα*) and t-cell receptor-beta (*tcr-ß*). Specific primer sequences, references, and conditions are registered in Table 9, described in Chapter 2.

For gene expression analysis in DI, conditions of RT-qPCR were 1x (95ºC, 10min), 35x (95ºC, 45s/annealing temperature, 45s/72ºC, 45s) 1x (72ºC, 30s).

Reactions were implemented by duplicated in 15µl of final volume, containing 2µl of cDNA (diluted 1/10), 7.5µl Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA) and 0.6µl of each primer (10 mM) in an *iCycler* Optical Module (Bio-Rad, USA). Blank reactions, with water replacing cDNA, were included in each analysis as a contamination control. Calculations of relative expression were realized by using $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

7.3.6. *Statistical analyses*

Statistical analyses followed methods described by Sokal & Rolf (1995). Statistical calculations were executed with SPSS® v21 software package for Windows (IBM™, Chicago, IL, USA). The significant level for all analyses was set at 5%, and, unless otherwise indicated, all results are presented as means and standard deviations (mean ± SD). Previously, data were tested for normality and homoscedasticity. When data met the aforementioned conditions, One-way ANOVA was used to compare the dependent

variable mean values. Tukey *post hoc* test was used for determining differences between groups. Data of relative gene expression were also analyzed using the permutational multivariate analysis of variance (PERMANOVA) complement of PRIMER 7® (Auckland, New Zealand). A principal component analysis (PCA) was performed and statistical differences were evaluated using PERMANOVA analyses with the number of permutations set in 999. Statistical significances were considered with a *P*-value after permutation procedure (P(perm)) <0.05. Survival data were analyzed using the method described by Kaplan and Meier (1958) and compared by the log-rank test to define the responses of European seabass to the different strategies.

7.4. RESULTS

7.4.1. *Feeding trial*

7.4.1.1. Survival and growth parameters

No differences (P>0.05) on fish survival were found among the different feeding strategies in any of the experimental periods, being survival percentage almost 100% (P1: diets P, B, C a 97% and diet PB a 96%; P2 and P3: 100%). At the end of the first period (P1: 25 weeks), significant (P>0.05) effects of diet were found in final weight, length, relative growth, SGR, condition factor (K), PFI and VSI. Fish fed P diet presented higher (P<0.05) weight and fish fed P and PB diets presented higher (P<0.05) length than those fed C diet (Table 28). Fish fed P diet presented higher (P<0.05) relative growth and SGR than the other fish whereas fish fed PB diet showed lower (P<0.05) condition factor than fish fed the other diets (Table 28). Besides, fish fed P and PB diets presented higher (P<0.05) PFI than fish fed C diet whereas those fed PB diet showed higher (P<0.05) VSI than fish fed C diet (Table 28). No differences (P>0.05) were found in HSI at P1.

Table 28. Growth parameters and somatic indexes of European seabass (*Dicentrarchus labrax*) juveniles at the end of Period 1 (0-25 week).

	C	P	PB	B
P1 (0-25 week)				
W (g)	129.8±1.2a	140.7±5.0b	131.8±4.0a	134.7±3.6a
L (cm)	20.4±0.2a	20.9±0.3b	20.8±0.4b	20.6±0.2ab
RG (%)	558.6±6.2a	614.8±25.2c	565.4±20.4ab	586.3±18.6b
SGR (%)	1.08±0.01a	1.12±0.01c	1.08±0.02ab	1.10±0.02b
K (%)	1.52±0.02b	1.55±0.04b	1.47±0.02a	1.54±0.01b
HSI (%)	2.1±0.0	2.0±0.2	2.6±0.0	2.2±0.2
PFI (%)	3.9±1.1a	5.2±1.0b	6.4±0.3b	4.8±0.0ab

VSI (%)	7.1±1.3a	8.8±0.8bc	10.4±0.2c	8.1±0.3ab

Growth parameters and somatic indexes were calculated as follows: condition factor (K): (W/L^3), where W is body weight and L is body length from the tip of the head to the end of the caudal fin; SGR: [((Ln (Wf)-Ln (Wi))/days of feeding] x 100, where Wf is final body weight and Wi is initial body weight; viscerosomatic index (VSI): [(viscera weight/body weight)] x100; hepatosomatic index (HSI): [(liver weight/body weight)] x100; perivisceral fat index (PFI): [(perivisceral fat weight/body weight)] x100. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

Growth parameters at P2 are summarized in Table 29. At P2 (31 weeks), no differences (P>0.05) were found in weight, length or condition factor. Significative differences (P<0.05) were found for relative growth, SGR, HSI, PFI and VSI at P2 (Table 29). Fish fed PB and C strategies up to this period, presented higher RG and SGR than those fed P, P/PB and B strategies (Table 29). Fish from P/PB and PB strategies presented higher (P<0.05) HSI than those from P diet (Table 29). For PFI, fish fed P/PB, PB/P and B strategies had higher (P<0.05) percentage than those fed P (Table 29). Finally, fish fed B diet had higher (P<0.05) VSI than those fed P diet (Table 29).

Table 29. Growth parameters and somatic indexes of European seabass (*Dicentrarchus labrax*) juveniles from Period 2 (P2, 25-31 week).

	C	P	P/PB(1)	PB(2)	PB/P	B
P2 (25-31 week)						
W (g)	157.6±0.5	165.5±0.5	163.9±8.6	160.9±4.0	156.4±6.8	157.7±0.9
L (cm)	22.0±0.0	22.3±0.2	22.3±0.4	22.1±0.3	21.9±0.3	22.0±0.0
RG (%)	21.4±0.4b	15.9±0.2a	17.3±2.1a	21.6±0.8b	19.4±1.7ab	17.2±2.9a
SGR (%)(3)	0.46±0.01b	0.35±0.00a	0.38±0.04a	0.47±0.02b	0.42±0.03ab	0.38±0.06a
K (%)	1.48±0.00	1.49±0.04	1.47±0.04	1.50±0.03	1.49±0.00	1.48±0.00
HSI (%)	1.9±0.1ab	1.7±0.2a	2.4±0.4b	2.4±0.3b	2.0±0.1ab	2.0±0.4ab
PFI (%)	4.2±0.3ab	3.4±0.2a	5.3±1.1b	4.9±1.2ab	5.4±0.2b	5.1±0.7b
VSI (%)	8.2±0.9ab	6.2±0.0a	8.8±1.6ab	8.7±1.1ab	8.8±0.2ab	8.9±0.4b

(1) Group of fish fed P at P1 and PB at P2 (strategies P/PB/P and P/PB/PB at P3, according to Table 27).
(2) Group of fish fed PB at P1 and P2 (strategies PB and PB/PB/P at P3, according to Table 27).
(3) Calculated from week 25 to 31. Growth parameters and somatic indexes were calculated as follows: condition factor (K): (W/L^3), where W is body weight and L is body length from the tip of the head to the end of the caudal fin; SGR: [((Ln (Wf)-Ln (Wi))/days of feeding] x 100, where Wf is final body weight and Wi is initial body weight; viscerosomatic index (VSI): [(viscera weight/body weight)] x100; hepatosomatic index (HSI): [(liver weight/body weight)] x100; perivisceral fat index (PFI): [(perivisceral fat weight/body weight)] x100. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

Results of growth parameters and indexes calculated after 39 weeks (P3: 31 to 39 weeks and TP: 0-39 weeks) are summarized in Table 30. At P3 (39 weeks), differences (P<0.05) were found for weight, length, relative growth, SGR, condition factor, VSI and PFI (Table

30). Fish fed P/PB/P and PB diets presented higher (P<0.05) weight than those fed B, C and PB/P/PB diets (Table 30). Fish fed P/PB/P, PB/PB/P and PB, presented higher (P<0.05) length than those fed P/PB/PB (Table 30). Fish fed P/PB/P and PB had higher (P<0.05) relative growth and SGR than fish fed B (Table 30). Fish from P/PB/PB diet had higher condition factor than those from C, P/PB/P, PB/P/PB, PB/PB/P and B (Table 30). Fish from P, PB and PB/PB/P had higher (P<0.05) PFI than those from C diet (Table 30). Fish from P, PB presented higher (P<0.05) VSI than fish from C diet (Table 30). No differences (P>0.05) were found for HSI in any diet at P3 (39 weeks) (Table 30).

Table 30. Growth parameters and somatic indexes of European seabass (*Dicentrarchus labrax*) juveniles from Period 3 (P3, 31-39 week) and Total Period (TP, 0-39 week).

	C	P	P/PB/P	P/PB/PB	PB	PB/P/PB	PB/PB/P	B
P3 (31-39 week)								
W (g)	188.5±12.8a	211.4±15.0ab	227.7±15.3b	207.3±14.5ab	231.7±17.1b	192.1±4.2a	207.9±6.0ab	184.1±3.3a
L (cm)	23.2±0.1ab	23.2±0.1ab	24.5±0.2c	22.4±0.9a	24.2±0.8c	22.9±0.2ab	23.8±0.7bc	22.8±0.1ab
RG (%)	19.6±8.7ab	27.7±8.5ab	33.6±12.0bc	31.8±1.3abc	46.0±6.3c	23.0±4.8ab	27.4±0.0ab	16.7±3.0a
SGR (%)(1)	0.32±0.13ab	0.43±0.12ab	0.51±0.16bc	0.49±0.02abc	0.67±0.08c	0.37±0.07ab	0.43±0.00ab	0.28±0.05a
K (%)	1.51±0.12a	1.69±0.15ab	1.55±0.06a	1.86±0.10b	1.64±0.04ab	1.60±0.00a	1.55±0.10a	1.55±0.01a
HSI (%)	1.9±0.3	2.0±0.1	1.9±0.2	1.9±0.3	1.8±0.0	2.0±0.2	1.8±0.1	2.0±0.1
PFI (%)	4.7±1.1a	6.5±0.1b	6.0±1.3ab	5.5±1.2ab	7.1±0.4b	5.8±0.4ab	6.4±0.2b	5.6±0.6ab
VSI (%)	8.0±0.8a	10.3±0.7b	9.4±1.7ab	8.7±1.9ab	10.4±0.4b	9.3±0.3ab	9.6±0.2ab	9.1±0.5ab
TP (0-39 week)								
RG (%)	856.6±64.9a	973.8±76.2ab	1056.5±77.8b	953.3±73.5ab	1069.5±86.4b	869.4±21.3a	949.4±30.2ab	838.2±16.7a
SGR (%)(2)	0.84±0.03a	0.88±0.03ab	0.91±0.02b	0.87±0.03ab	0.91±0.03b	0.84±0.01a	0.87±0.01ab	0.83±0.01a

(1) Calculated from 31 to 39 weeks. (2) Calculated from 0 to 39 weeks. Growth parameters and somatic indexes were calculated as follows: condition factor (K): (W/L^3), where W is body weight and L is body length from the tip of the head to the end of the caudal fin; SGR: [((Ln (Wf)-Ln (Wi))/days of feeding] x 100, where Wf is final body weight and Wi is initial body weight; viscerosomatic index (VSI): [(viscera weight/body weight)] x100; hepatosomatic index (HSI): [(liver weight/body weight)] x100; perivisceral fat index (PFI): [(perivisceral fat weight/body weight)] x100. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

At the end of TP (0-39 week), relative growth and SGR were calculated (Table 30). Fish fed P/PB/P and PB presented higher (P<0.05) relative growth and SGR than fish from C, PB/P/PB and B diets (Table 30).

7.4.1.2. Histological studies

7.4.1.2.a. Distal intestine morphometry

Morphological observations revealed an intact epithelial barrier and organized folds along the distal intestine (Fig.13). After One-way ANOVA analyses, the different strategies of use of synbionts did not induce effects on DI morphology, except in fold length (Table 31), where fish from B strategy showed a significant ($P<0.05$) longer fold than those fed P, P/PB/P and P/PB/PB diets (Table 31).

No significant differences ($P>0.05$) were found between feeding strategies in the density of goblet cells in DI by unit of area (Table 31).

Table 31. Intestinal morphometry and density of goblet cells in distal intestine of European seabass (*Dicentrarchus labrax*) juveniles at the end of Period 3 (31-39 week).

	C	P	P/PB/P	P/PB/PB	PB	PB/P/PB	PB/PB/P	B
FL[1]	18.4±0.1ab	16.4±2.2a	15.5±0.4a	15.3±0.6a	15.6±0.2ab	18.1±0.9ab	17.9±1.6ab	20.2±0.7b
FW[2]	5.8±0.8	5.2±0.1	5.3±0.1	5.0±0.1	5.0±0.3	5.3±0.6	5.3±0.1	6.0±0.2
SW[3]	1.5±0.1	1.7±0.1	1.6±0.0	1.4±0.1	1.5±0.1	1.7±0.2	1.5±0.1	1.5±0.1
GC[4]	2.18±0.07	2.67±0.05	2.32±0.09	2.46±0.20	2.60±0.59	2.46±0.38	2.42±0.49	2.22±0.15

All values are represented as measure ± SD. Letters denote significant differences ($P<0.05$) after One-way ANOVA. All distal intestine measures were weighted with individual fish weight and ([1]) fold length, expressed as µm * 10; ([2]) fold width, expressed as µm * 10; ([3]) submucosa width, expressed as µm * 10; ([4]) goblet cells, expressed as cells/fold area * 10000 in arbitrary units. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

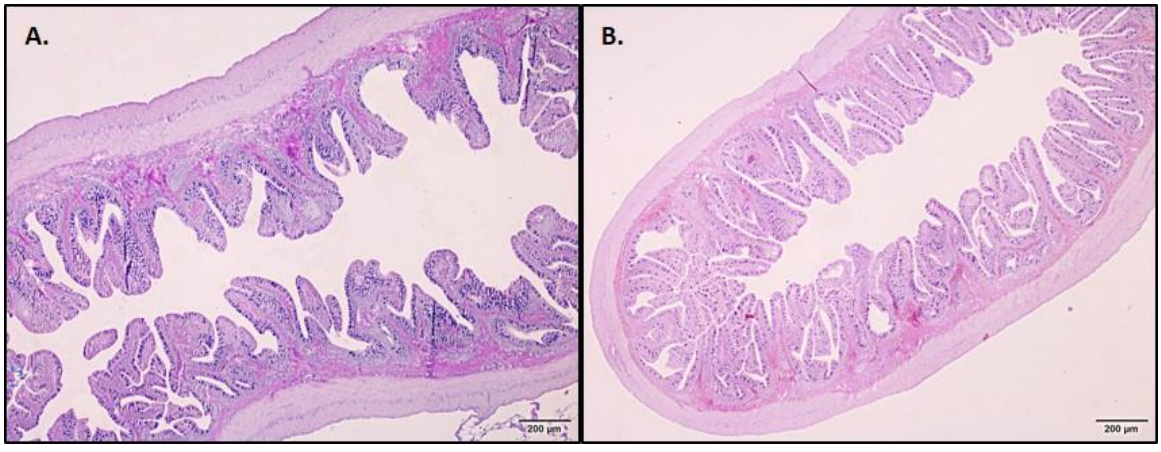

Fig.13. Distal intestine of European seabass (*Dicentrarchus labrax)* juveniles at the end of feeding period (39 weeks). Image A: P diet. Image B: B diet. Bar 200µm.

7.4.1.2.b. Liver morphometry

At the end of P3 (31-39 week), significant (P<0.05) effects by supplementation were found in liver morphometry (Table 32). Fish from PB treatment presented the smaller (P<0.05) hepatocytes, measured as area, minimum and maximum length (Table 32). In particular, the strategy PB induced hepatocytes with smaller (P<0.05) area than those from C, shorter (P<0.05) maximum length than those from fish fed B and C, and finally reduced (P<0.05) minimum length than those from fish fed C, P and B (Table 32). Despite these, in comparation with hepatocytes from control diet, a lower vacuolization level, with more regular-shaped morphology and better organization around sinusoidal spaces of hepatocytes were found in fish from PB, P, P/PB/P, P/PB/PB and PB/PB/P strategies (Fig.14).

Table 32. Liver morphometry of European seabass (*Dicentrarchus labrax)* juveniles at the end of Period 3 (31-39 week).

	C	P	P/PB/P	P/PB/PB	PB	PB/P/PB	PB/PB/P	B
HA[1]	4.5±0.1b	4.2±0.2ab	3.9±0.1ab	4.3±0.1ab	2.8±0.2a	3.8±0.6ab	4.2±0.1ab	4.4±0.6ab
HMx[2]	6.2±0.3c	5.4±0.1bc	5.1±0.0ab	5.6±0.1bc	4.4±0.1a	5.5±0.3bc	5.7±0.0bc	6.1±0.5c
HMn[3]	3.9±0.1b	3.7±0.1b	3.3±0.1ab	3.6±0.1ab	2.8±0.1a	3.6±0.0ab	3.6±0.1ab	4.0±0.4b

([1]) Hepatocytes area (HA), expressed as μm^2 * 10; ([2]) Hepatocytes maximum length (HMx), expressed as µm * 10; ([3]) Hepatocytes minimum length (HMn), expressed as µm * 10. All values are represented as mean ± SD. Letters denote significant differences (P<0.05) after One-way ANOVA. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

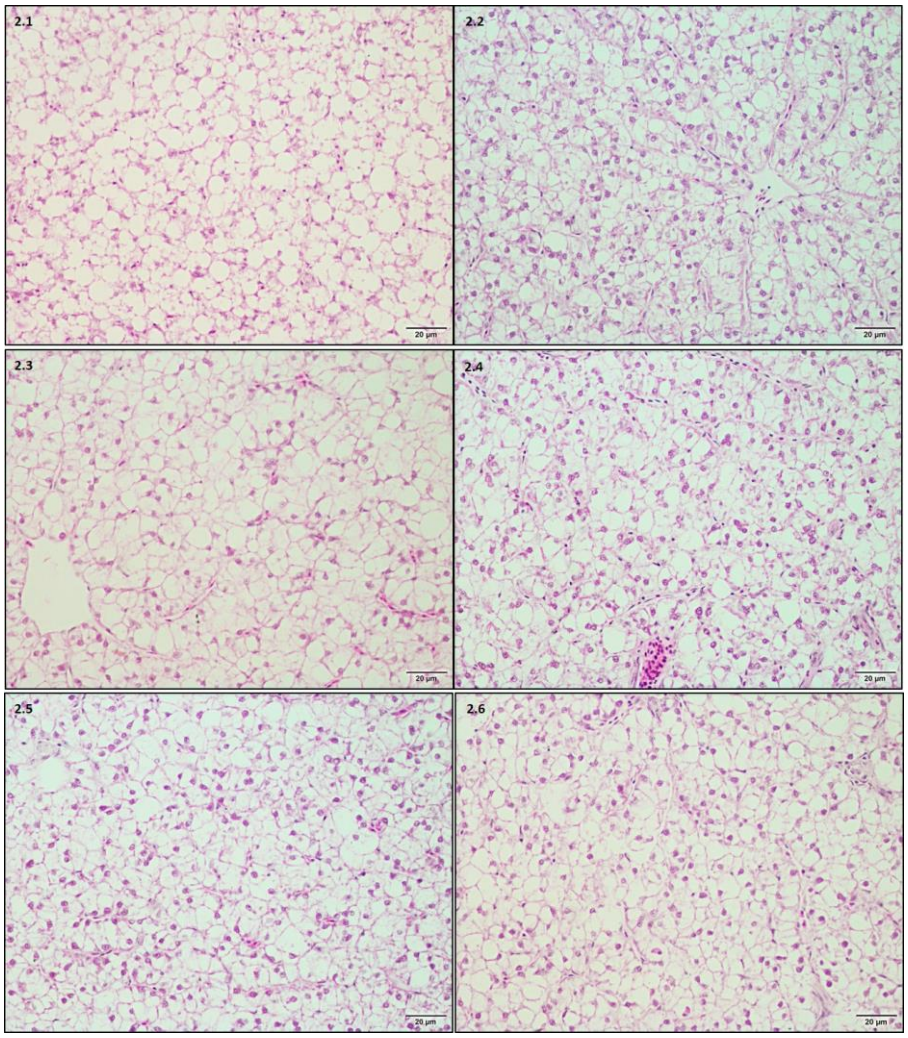

Fig.14. Hepatocytes from European seabass (*Dicentrarchus labrax*) juveniles at the end of feeding period (39 weeks). Bar 20µm. 2.1: C diet; 2.2: PB; 2.3: P; 2.4: P/PB/P; 2.5: P/PB/PB and 2.6: PB/PB/P. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici,* (PB) synbiotic supplementation with MOS + *P. acidilactici*.

7.4.1.3. Relative expression of GALT-related genes after feeding trial

At the end of feeding experience, immune related genes were analyzed in DI by One-way ANOVA analyses (Fig.15). These analyses found different effects of functional ingredients in the expression of all genes, except for *tcr-ß*, and *cd-4* expressions (Fig.15). One-way ANOVA indicated that expression of *mhcii-ß* was upregulated ($P<0.05$) by PB and PB/P/PB when compared to fish from P, P/PB/P, PB/PB/P, B, and C strategies (Fig.15). One-way ANOVA analysis of the expression of *mhci-α* showed that was downregulated ($P<0.05$) in P diet than the rest of diets (Fig.15). According to One-way ANOVA, expression of *cd-8α* was downregulated ($P<0.05$) in fish from PB/PB/P strategy

when compared to C strategy (Fig.15). One-way ANOVA showed an upregulation of *cox-2* in P/PB/P, P/PB/PB, PB, PB/P/PB, and B strategies compared to C strategy (Fig.15). A downregulation ($P<0.05$) of *il-10* expression was found by One-way ANOVA in all strategies with respect to C fish (Fig.15). Expression of proinflammatory *il-1β* was found downregulated ($P<0.05$), by One-way ANOVA, in fish from P, P/PB/P, P/PB/PB, PB, and PB/P/PB strategies when compared to PB/PB/P and C (Fig.15). The *tnfα* expression was also found downregulated ($P<0.05$) by One-way ANOVA in fish from P, PB, and PB/P/PB with respect to C (Fig.15).

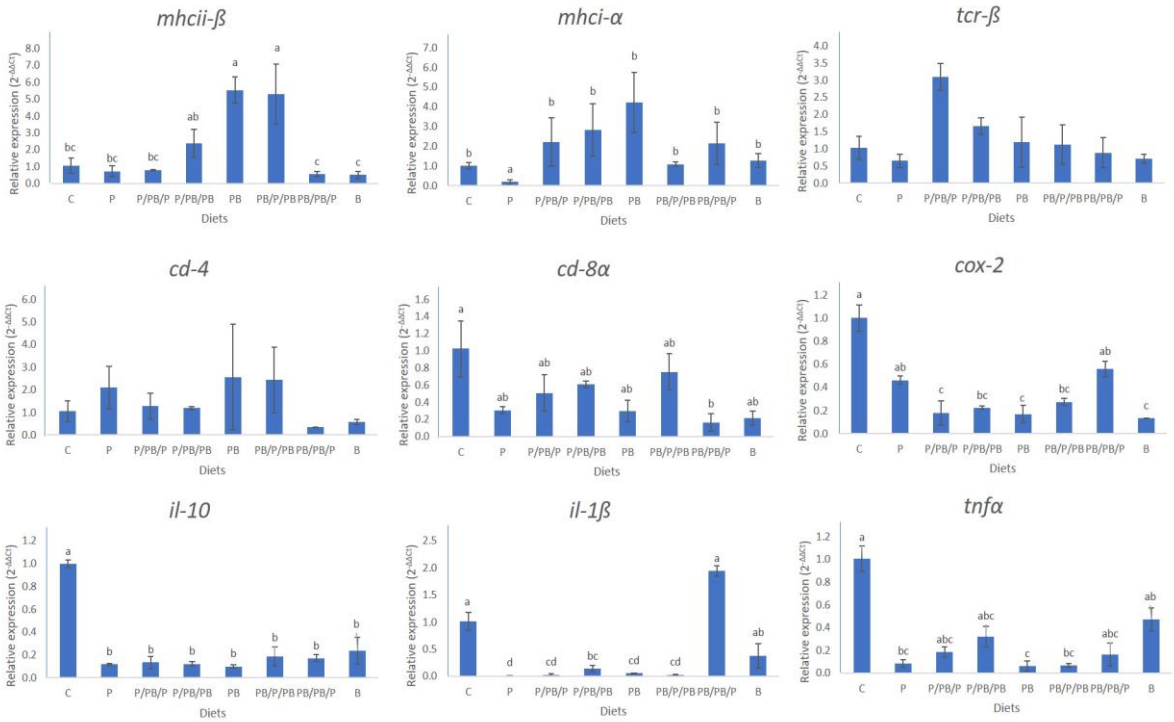

Fig.15. Relative basal (non-infected) expression of GALT-Related genes in distal intestine of European seabass (*Dicentrarchus labrax*) juveniles at the end of feeding trial (39 weeks). All values are represented as measure ± SD. Letters denote significant differences between diets after One-way Anova analyses. Analyzed genes: major histocompatibility complex ii-beta, *mhcii-ß*; major histocompatibility complex i alpha, *mhci-α;* t-cell receptor alpha, *tcr-ß;* cluster of quadruple differentiation, *cd-4*; cluster of differentiation 8 alpha, *cd-8α;* cyclooxygenase 2, *cox-2;* interleukin 10, *il-10;* interleukin 1- beta, *il-1ß;* tumor necrosis factor alpha, *tnfα.* Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici.*

The significances found by PERMANOVA analyses were P(perm) =0.001 for MOS, P(perm)=0.030 for *P. acidilactici*, and P(perm)=0.020 for synbiotic. The analysis of PCA assigned a 70.8% on the total GALT-gene expression variability in DI to the sum of the first and second principal components (PC1 and PC2). The PC1 accounted for the 52.6% of variation and detached the PB/PB/P, B and C from the rest of dietary strategies to the left side of the axis (Fig.16). To this model, components of acquired immunity were responsible for the negative values on the PC1 (*cd-4*, *cd-8α, tcr-β,* and *mhcii-β*) whereas factors related to inflammation (*il-1β, tnfα, il10,* and *cox-2*) were distributed to the positive side of this axis (Fig.16). The PC2 accounted for a 18.3% of the total variability and detached P from the rest of strategies with *mhci-α* and *mhcii-β* as the main positive components of analysed genes whereas P/PB/PB and PB strategies were separated to the negative side of this axis (Fig.16).

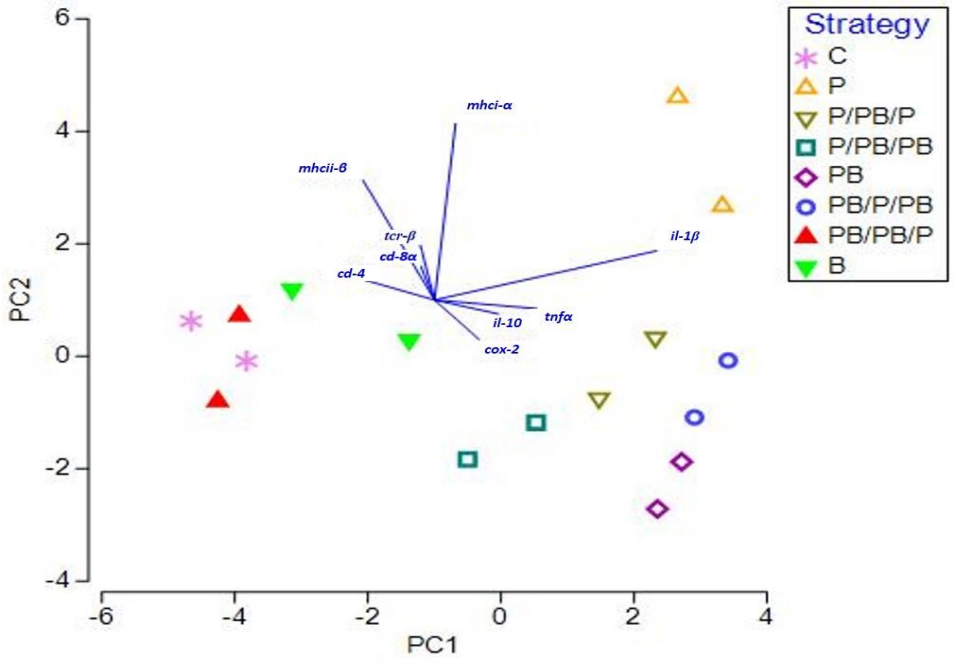

Fig.16. Principal component analysis (PCA) of distal intestine GALT-gene expression variability of European seabass (*Dicentrarchus labrax*) juveniles in relation to feeding strategies at the end of feeding trial (39 weeks). Analyzed genes: major histocompatibility complex ii beta, *mhcii-ß*; major histocompatibility complex i alpha, *mhci-α;* t-cell receptor alpha, *tcr-ß;* cluster of quadruple differentiation, *cd-4*; cluster of differentiation 8 alpha, *cd-8α;* cyclooxygenase 2, *cox-2;* interleukin 10, *il-10;* interleukin 1 beta, *il-1ß;* tumor necrosis factor alpha, *tnfα.* Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici.*

7.4.1.4. Serum immune parameters assayed after feeding trial (39 weeks)

Prior to challenge test, lysozyme and peroxidase activities were measured in fish serum and values are reflected in Fig.17. Higher value of lysozyme level ($P<0.05$) was found after One-way ANOVA in fish from PB/P/PB diet when compared to those fed P/PB/P (Fig.17). On the other hand, One-way ANOVA analysis showed that peroxidase level was increased ($P<0.05$) in fish from P/PB/P diet when compared to those from P, PB/P/PB, PB/PB/P, B, and C diets (Fig.17).

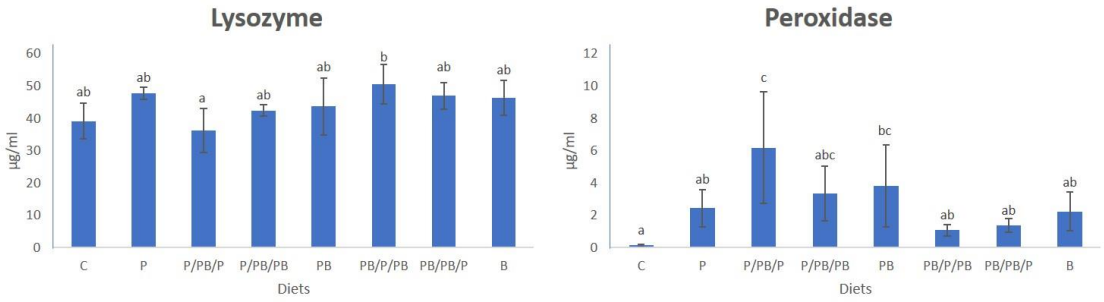

Fig.17. Serum immune-parameters of non-infected European seabass (*Dicentrarchus* labrax) juveniles at the end of feeding period (39 weeks). All values are represented as measure ± SD. Letters denote significant differences ($P<0.05$) after One-way Anova analyses. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

7.4.2. *Challenge test against Vibrio anguillarum*

7.4.2.1. Survival and relative expression of immune-related genes after challenge test

No differences ($P>0.05$) were found in cumulative survival between the different strategies along this infection assay against *V. anguillarum* (Fig. 18). Inoculated bacteria were recovered from internal organs of all sampled fish.

At 2- and 7-days post infection, gene expression of immune-related genes was measured in DI of infected fish. One-way ANOVA analyses are represented in Fig.19. After 2 days post infection, according to One-way ANOVA, fish from P, P/PB/P, PB/P/PB, and PB/PB/P presented lower ($P<0.05$) *mhcii-ß* expression than the rest of treatments (Fig.19). No differences ($P>0.05$) were found after 7 days post infection for *mhcii-ß* (Fig.19).

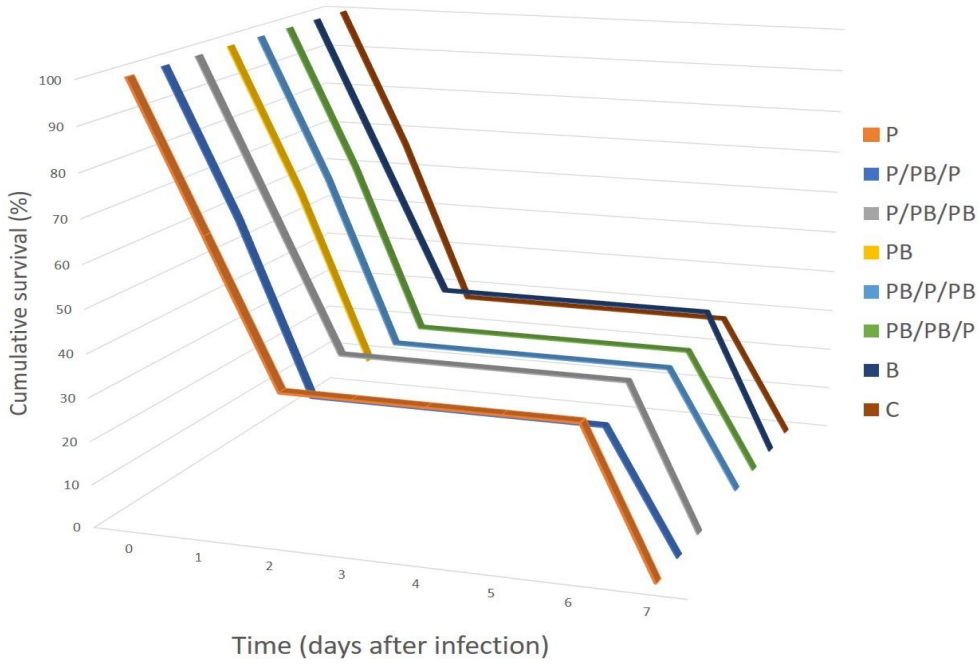

Fig.18. Cumulative survival of European seabass (*Dicentrarchus labrax*) juveniles along intestinal bacterial infection challenge. No differences ($P>0.05$) were detected after Kaplan-Meier analysis.

At 2 days post infection, analyses indicated that *mhci-α* was downregulated ($P<0.05$) in fish fed PB/P/PB and PB/PB/P than fish from C, P/PB/P, PB and B strategies (Fig.19). At 7 days post infection, *mhci-α*, expression was higher ($P<0.05$) in P/PB/PB fish than PB, PB/PB/P and B, according to One-way ANOVA analyses (Fig.19).

At 2 days post infection, *il-10* expression was found higher ($P<0.05$) for fish from B strategy with respect to those from P/PB/P (Fig.19). At 7 days post infection, analyses demonstrated that the expression of *il-10* was significantly increased ($P<0.05$) in P/PB/PB, PB/P/PB and PB than the rest of diets, but there were differences ($P<0.05$) between PB/P/PB, with the lowest ($P<0.05$) level, and the other two (Fig.19). Besides, at

7 days post infection, expression of *il-10* in PB/PB/P was the lowest (P<0.05) among all diets, according to One-way ANOVA analyses (Fig.19).

At 2 days post infection, One-way ANOVA showed that fish from B diet presented higher (P<0.05) *cd-4* expression than those from PB/P/PB (Fig.19). At 7 days post infection, *cd-4* expression was lower (P<0.05) in fish fed P, P/PB/P and B diets than fish fed C, according to One-way ANOVA (Fig.19).

At 2 days post infection, One-way ANOVA indicated that *cd-8α* expression was higher (P<0.05) in fish from PB strategy than those from P (Fig.19). At 7 days post infection, *cd-8α* expression was reduced (P<0.05) in P, PB and B strategies, with differences (P<0.05) between P, lower than the other two, according to One-way ANOVA (Fig.19).

At 2 days post infection, *cox-2* was upregulated in C than P according to One-way ANOVA (Fig.19). At 7 days post infection, One-way ANOVA showed that *cox-2* expression was upregulated (P<0.05) in P/PB/PB than the rest of diets except PB (Fig.19).

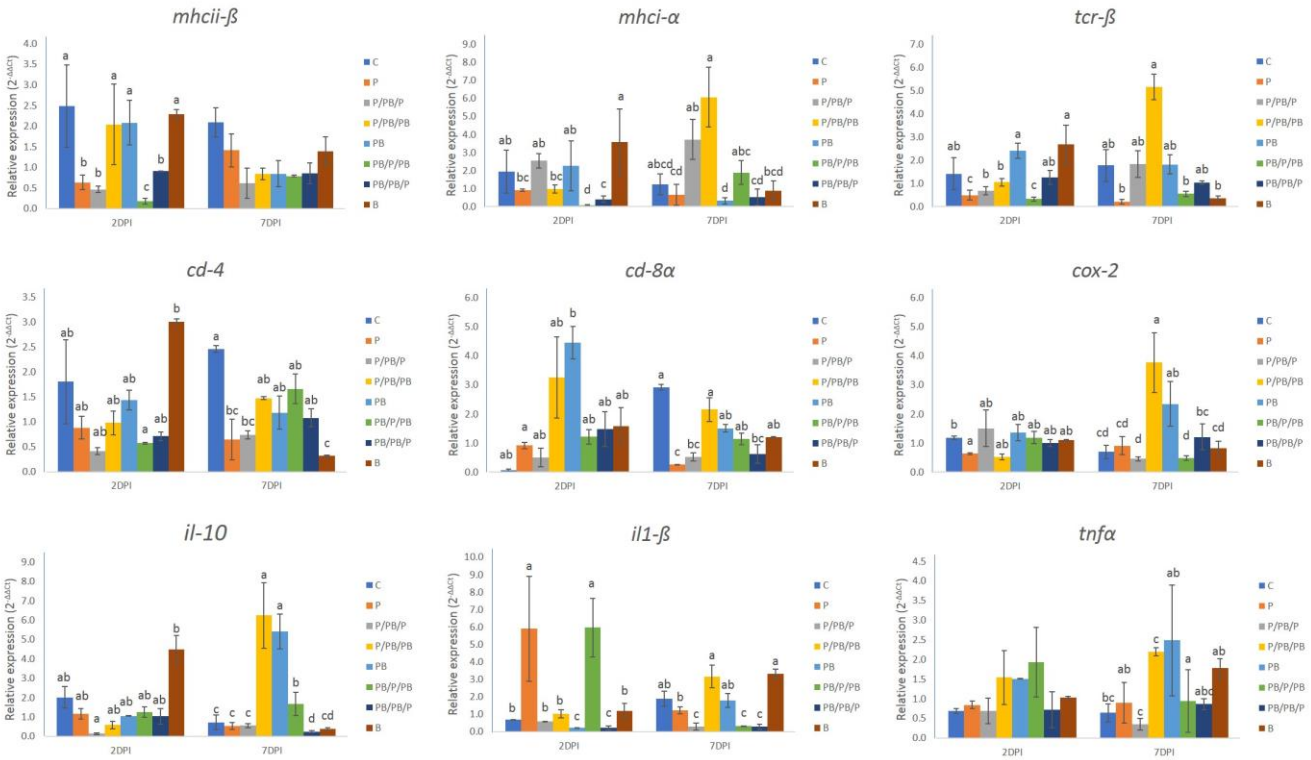

Fig.19. Data of immune-related expression in distal intestine of European seabass (*Dicentrarchus labrax*) juveniles after 2- and 7-days post infection (DPI). All values are represented as measure ± SD. Letters denote significant (P<0.05) differences between diets after One-way Anova analyses. Analyzed genes: major histocompatibility complex ii beta, *mhcii-ß*; major histocompatibility complex i alpha, *mhci-α;* t-cell receptor alpha, *tcr-ß;* cluster of quadruple differentiation, *cd-4*; cluster of differentiation 8 alpha, *cd-8α;* cyclooxygenase 2, *cox-2;* interleukin 10, *il-10;* interleukin 1- beta, *il-1ß;* tumor necrosis factor alpha, *tnfα.* Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici,* (PB) synbiotic supplementation with MOS + *P. acidilactici.*

At 2 days post infection, One-way ANOVA showed that the expression of *il-1β* was higher (P<0.05) for P and PB/P/PB than the rest of diets, but PB and PB/PB/P presented the lower (P<0.05) expression for this gene (Fig.19). At 7 days post infection, *il-1β*, expression was lower (P<0.05) in P/PB/P, PB/P/PB, and PB/PB/P than P, P/PB/P, and B strategy, according to One-way ANOVA (Fig.19).

At 2 days post inoculation, results of PERMANOVA analysis showed significative influence of MOS (P(perm)=0.013) and not for *P. acidilactici* or the synbiotic (P(perm)>0.05) (Fig.20). The PCA analyses displayed a 75.0% of total variability for the sum of PC1 and PC2. The PC1 scored the 42.2% of total variability and PB/P/PB was clearly detached from the other diets (Fig.20). In this axis, *mhcii-ß*, *mhci-α*, *tcr-ß*, *cd-4*, *il-10*, and *cox-2* were the main responsible for the negative values (Fig.20). The PC2 accounted for the 32.8% of total variability. In this axis, C diet was clearly separated from the rest and *cd-8α* expression was the main responsible of positive results (Fig.20).

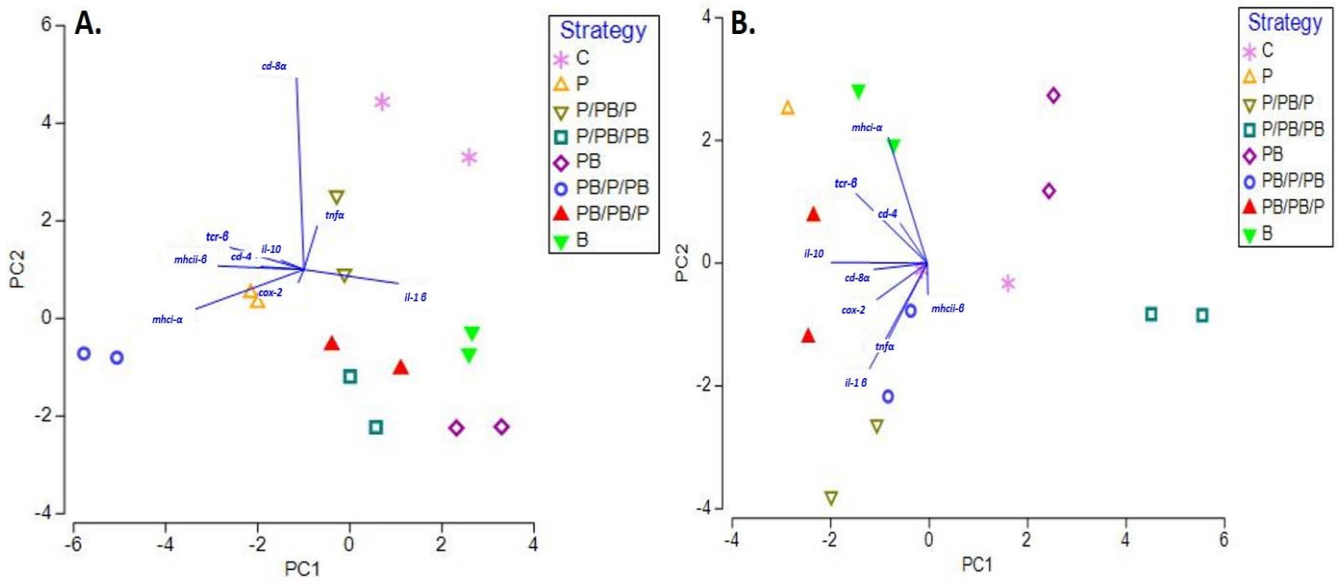

Fig.20. Principal component analysis (PCA) of distal intestine GALT-gene expression variability of European seabass (*Dicentrarchus labrax*) juveniles in relation to feeding strategies after infection with *Vibrio anguillarum*. A: PCA after 2 days post infection. B: PCA after 7 days post infection. Analyzed genes: major histocompatibility complex ii-beta, *mhcii-ß*; major histocompatibility complex i alpha, *mhci-α;* t-cell receptor alpha, *tcr-ß;* cluster of quadruple differentiation, *cd-4*; cluster of differentiation 8 alpha, *cd-8α;* cyclooxygenase 2, *cox-2;* interleukin 10, *il-10;* interleukin 1- beta, *il-1ß;* tumor necrosis factor alpha, *tnfα*. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

At 7 days post inoculation, PERMANOVA analysis showed significance for probiotic and synbiotic (P(perm)=0.043 and P(perm)=0.009 respectively. The analysis of PCA attributed a 68.3% of total variability to the sum of PC1 and PC2. The PC1 presented the

43.3% of total variability. For PC1 axis, the PB and P/PB/PB diets, were attached from the rest and the expression of *mhcii-ß* was the only responsible for positive values (Fig.20). The PC2 showed a 25% of total variability, and control diet was attached from the rest, being proinflammation-related genes (*cox-2*, *il-1β*, and *tnfα*) the main responsible for negative values in this axis (Fig.20).

7.4.2.2. Serum immune parameters after challenge test

At 2 days post infection, lysozyme, peroxidase and bactericidal activities were measured in serum. One-way ANOVA analyses are showed in Fig.21.

Results of One-way ANOVA analysis showed that lysozyme level was significantly improved ($P<0.05$) in fish from P/PB/P and P/PB/PB diets with respect to fish from PB/P/PB strategy (Fig.21). No differences were found in bactericidal activity after 2 DPI by One-way (Fig.21). On the other hand, One-way ANOVA showed that peroxidase level was enhanced in fish from all diets, particularly in P/PB/P, PB, PB/P/PB and B fish with respect to those from C diet (Fig.21).

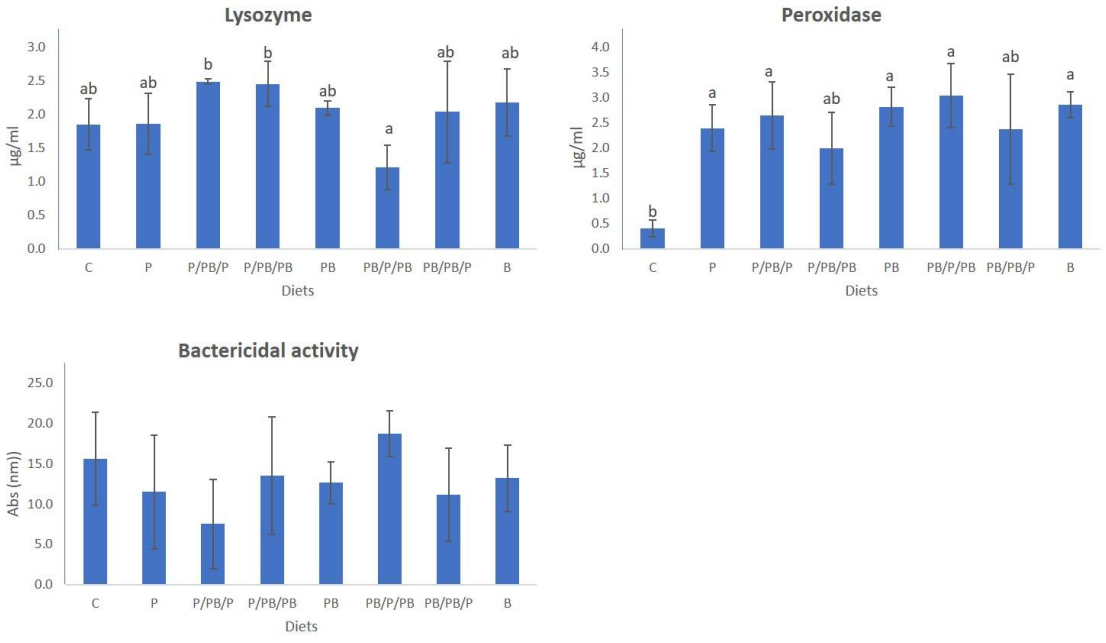

Fig.21. Serum immune-parameters of infected European seabass (*Dicentrarchus labrax*) juveniles at day 2 post-infection. All values are represented as measure ± SD. Letters denote significant ($P<0.05$) differences between diets. Acronyms of diets: (C) control diet non-supplemented, (P) dietary supplementation with MOS, (B) dietary supplementation with *P. acidilactici*, (PB) synbiotic supplementation with MOS + *P. acidilactici*.

7.5. DISCUSSION

The successful supplementation of functional ingredients, including synbiotics, in diets with high substitutions of FM and FO has been already demonstrated on performance of European seabass juveniles (Torrecillas *et al.*, 2015b, 2017a, and 2018b). In this line, results of the present study indicate that fish growth performance, in terms of weight, length, condition factor, relative growth, SGR, and VSI, was affected by dietary treatments in periods 1 and 3 (0 to 25 and 31 to 39 weeks of feeding, respectively). Besides, at period 2 (25 to 31 weeks) differences were found in relative growth, SGR and VSI. Those results were related to the strategy of administration of functional ingredients, indicating the importance of feeding design. Previous studies have demonstrated that the use of diets with 5%FM/6%FO, as the basal diet used in this study, can affect growth of European seabass (Torrecillas *et al.*, 2017a and 2018b), but the singular use of MOS or in synbiotic combination with *P. acidilactici*, for 13 weeks, improved growth performance (Torrecillas *et al.*, 2018b). Similarly, results of the present study demonstrated that MOS (P) and synbiotic (PB) increased fish growth performance after 25 weeks of feeding, compared to non-supplemented diet whereas after a longer period of 39 weeks, synbiotic (PB) and P/PB/P strategy increased weight and length, compared to those fish fed control diet. The role of MOS as a growth promoter for European seabass, has been described in European seabass previously (Torrecillas *et al.*, 2007, 2011a, 2013) including when fed highly substituted diets (Torrecillas *et al.*, 2015a, 2018b) related to its capacity to induce better use of dietary energy and feed utilization , altogether induced by a better intestinal health status (Torrecillas et al., 2011a, 2015a). The supplementation of *P. acidilactici* has also shown the capacity to promote fish growth previously, as demonstrated in different fish species (Shelby *et al.*, 2006; Neissi *et al.*, 2013; Azimirad *et al.*, 2016). This capacity could be related, among others, to the proteolytic ability of LAB, in general, and *P. acidilactici*, in particular, (Toe *et al.*, 2019), which could improve fish digestion. Other studies have shown that some LAB can induce an increment of insulin growth factor I expression and a reduction of myostatin expression which, in conjunction, may contribute to improve European seabass growth after 25 or 59 days of supplementation (Carnevali *et al.*, 2006). However, a growth beneficial effect of *P. acidilactici* was not found when using a single dietary supplementation for European seabass (Torrecillas *et al.*, 2018b). Hence, effects of MOS and *P. acidilactici* on growth parameters found in the present experiment are in agreement with those showing that

MOS and synbiotics are a valuable strategy to enhance European seabass growth fed highly substituted diets with terrestrial raw materials. However, the reasons that alternate feeding with MOS or synbiotic, in P/PB/PB, PB/P/PB and PB/PB/P strategies, did not improve fish growth could be related to negative effects of intermittent feeding with functional ingredients itself. In this line, the cessation of functional additives supplementation causes the rapid loss of positive effects as reduction of dietary inhibitory substances or nutrient/enzymatic contributions to digestion (Balcázar *et al.,* 2006a) and, particularly, the abrupt suspension of probiotic supplementation could even induce a gut dysbiosis status (Liu *et al.,* 2016). However, P/PB/P increased weight and length at taking into account the whole period (39 weeks), denoting the importance of the selection of the strategy with functional ingredient has great importance to improve growth performance of European seabass fed 5%FM/6%FO based diets. Moreover, at the end of P1, P2 and P3, prebiotic P (P1 and P3), synbiotic PB (P1 and P3), as well as strategies P/PB and PB/P (P2) and PB/PB/P (P3) presented an increment of PFI, which could be in part responsible for the increased condition factor found for P (P1).

High level of vegetable oils in diets for European seabass juveniles has been described to increase hepatocytes size (Torrecillas *et al.,* 2016). In this sense, MOS has been described to reduce hepatocytes area in juveniles of European seabass (Torrecillas *et al.,* 2011a). Similarly, the potential effect of *P. acidilactici* on hepatic lipid metabolism has been pointed out previously, reducing hepatocytes size and indicating a positive role in lipid metabolism on juveniles of this fish species when fed 5%FM/6%FO diets for 90 days (Torrecillas *et al.,* 2018b). In agreement with this, in the present study, a synbiotic supplementation (PB diet) was related to a reduction in hepatocyte size at P3 (39 weeks of feeding). The effect of the dietary synbiont on hepatocytes could be pointing to a better lipid absorption and a subsequent mobilization of hepatic lipids in European seabass. Again, the alternate use of P and PB in the selected strategies (P/PB/P, P/PB/PB, PB/P/PB, PB/PB/P) of this study failed to reproduce the beneficial effect of continuous feeding with synbiotic in fish liver, maybe related with readjustment of gut homeostasis duet to dietary changes (Balcázar *et al.,* 2006a; Liu *et al.,* 2016). Consistently with this, it has been shown that low number of probiotic bacteria in the intestine is related to its benefits (Liu *et al.,* 2013) and stopping feeding a probiotic reduces rapidly the presence of probionts in fish gut (Ferguson *et al.,* 2010), affecting the equilibrium of different microbiota populations previously induced by the functional ingredient.

Previous studies with European seabass fed vegetable diets, showed a reduction in lysozyme activity (Geay *et al.,* 2011). Results in this study show that functional ingredients can counteract the negative effects of terrestrial raw materials in diets for European seabass. The improvement in the immune parameters of European seabass by functional ingredients has been demonstrated previously after 90 days of supplementation for those fish fed highly substituted diets with terrestrial meals and oils (Torrecillas *et al.*, 2018b). At the present study, systemic immunity was influenced by MOS and *P. acidilactici*, which induced higher serum peroxidase level at P3. The beneficial effect in local and systemic immune parameters of European seabass by dietary MOS supplementation is in agreement with previous research (Torrecillas *et al.,* 2007, 2011a and 2015b). Different studies have also shown that *P. acidilactici* has the potential to increase serum lysozyme activity (Neissi *et al.,* 2013; Hoseinifar *et al.,* 2015a) in agreement with the results obtained in the present study, which seems to be a frequent characteristic of probiotics (Balcázar *et al.,* 2007; Ai *et al.,* 2011; Ye *et al.*, 2011). Nevertheless, some studies failed to find effects on lysozyme by probiotic supplementation (Geng *et al.,* 2011; Kazuń *et al.,* 2018) and some authors have related this absence to low attachment capacity of probionts to fish epithelia (Balcázar *et al.*, 2007), length of the experiment, selected strain, or administration dose (Geng *et al.*, 2011). Other authors have suggested that even when lysozyme can be induced by probiotics, its levels cannot be kept high for long periods, which at the same time may depend on the capacity of probiotic to remain in the intestine (Panigrahi *et al.,* 2005).

The capacity of functional ingredients to influence fish health has been related to the presence of a highly reactive GALT and its capacity to develop tolerance against non-damaging and frequent antigen-like substances (Nutsch & Hsieh, 2010; Rombout *et al.*, 2011; Kim *et al.*, 2012; Tafalla *et al.,* 2016). In this sense, it is reasonable to link the existing relationship between mechanisms that regulate homeostasis and the consequent communication between intestinal microbiota and host immune system to keep immune-tolerance as many authors have pointed out before (Gómez & Balcázar, 2008; Ashida *et al.*, 2011; Gómez *et al.*, 2013; Viera *et al.,* 2013; Hamilton *et al.,* 2015; Salinas, 2015). Indeed, it has been described that microbiota can ferment some fibers to produce short-chain fatty acids (SCFA) which can diffuse into the blood and hence, through G protein coupled receptor, can modulate innate parameters of systemic immunity as serum lysozyme activity (Maslowsky & Mackay, 2011). However, in this study, the alternate

administration of same functional ingredients seemed to produce different effects, indicating the importance of administration strategies to obtain a systemic health enhancement in European seabass juveniles fed low FO/FM diets. On the other hand, previous research found that continuous feeding for a long period with immunostimulants can affect host health negatively, impeding and adequate response when disease appears (Bai *et al.,* 2010; Chang *et al.,* 2000). Besides, not only continuous but also discontinuous supplementation can change the effects. For instance, the positive effects of probiotics will not last long time after finishing the treatment due to the competitions with indigenous microflora (Balcázar *et al.,* 2006a). In relation to this, it has been measured that after suspending a 32 days supplementation period, *P. acidilactici* strains will be present in the intestine of Nile tilapia (*Oreochromis niloticus*) modulating microbial populations only up to 17 days (Ferguson *et al.,* 2010). Therefore, it could be expected that different changes in feeding strategies for a long period could be affecting differently fish systemic immune parameters as serum lysozyme and peroxidase. The results of the present study indicate that PB and P/PB/P strategies increased the basal level of the analyzed systemic immune parameters of European seabass fed low FO/FM diets for a long-term experiment (39 weeks).

Moreover, MOS have been described to improve fish intestinal morphology in European seabass (Torrecillas *et al.,* 2013; Salem *et al.,* 2015) as well as other fish species (Dimitroglou *et al.,* 2009). Similarly, probiotics have been related to improvements in intestinal morphology in European seabass (Frouël *et al.,* 2008). In agreement with this, results in the present study have shown that an increased fold length was found in fish fed B diet compared to those fed PB, PB/P/PB and PB/PB/P. In Nile tilapia, intestinal surface was increased after eight weeks of supplementation with a multi-species probiotic (Standen *et al.,* 2015 and 2016) or six weeks with *Bacillus subtilis* (Vieira de Azevedo *et al.,* 2016). The presence of beneficial bacteria by probiotic supplementation has been described to have positive effects on intestinal morphology (Standen et al., 2015 and 2016) suggesting that it could enhance cellular renewal in the intestinal tissue, affecting positively to fold structure (Vieira de Azevedo *et al.,* 2016).

Different authors have pointed out the importance of selected feeding strategy to maximize the potential benefit of using functional ingredients (Sajeevan *et al.,* 2009; Bai *et al.,* 2010). Thus, at the present study, PERMANOVA analysis showed the influence of MOS, *P. acidilactici* and synbiotic in the basal results of expression of GALT-related

genes. The capacity of *P. acidilactici* to regulate genes of innate immunity has been found in previous research in common carp (*Cyprinus carpio*) (Mondaloo *et al.*, 2017) and European seabass (Lamari *et al.*, 2016; Torrecillas *et al.*, 2018b) whereas the role of MOS in regulation of expression of GALT-related genes has been pointed out before in European seabass fed highly substituted diets (Torrecillas *et al.*, 2015b). Similarly, synbiotics have shown to be a reliable strategy to enhance health of European seabass fed low FM/FO based diets (Torrecillas *et al.*, 2018b). At basal point, the strategy based on single use of prebiotic (P) influenced a downregulation of *mhci-α* expression, which could indicate the reduction in the migration of cd-8α^+ lymphocytes, usually related to their recruitment for keeping gut homeostasis through regulating tolerance of antigens from vegetable origin after a long period of feeding with these based diets (Rombout *et al.*, 2014), as it has been suggested before for European seabass (Torrecillas *et al.*, 2015b). Despite no significant differences, *cd-4* gene expression was increased by two-folds in P, PB and B diets when compared to fish from C, whereas *cd-8α* expression presented a trend to be reduced in all supplemented diets other than the control diet, particularly for P, PB, B and PB/PB/P although only significantly for this last strategy, which might be an effect of functional ingredients to reduce proinflammatory-induced effects of vegetable compounds. The expression of *cd-8α* is usually related to expression of *mhcii-ß* in a proinflammatory-like context when fish are fed terrestrial ingredients (Romarheim *et al.*, 2013). Besides, the upregulation of *mhcii-ß* expression, as this found in PB and PB/P/PB, has been found previously related to the expression of proinflammatory cytokines and probably linked to the influence of *il-1β* in different immune cells or in the functionality of enterocytes according to findings by other authors (Torrecillas *et al.*, 2017d; Romarheim *et al.*, 2013). Hence, the increased expression of *mhcii-ß* has been also previously connected to an attempt to keep homeostasis through the development of immune-tolerance between resident microbiota and host immune cells in the intestinal environment created by the high levels of terrestrial components of the based diet by induced the development or reactivity of T-cells (Rombout *et al.*, 2011; Romarheim *et al.*, 2013; Tafalla *et al.*, 2016). Indeed, *il-1β* and, to a lesser extent, all the other analyzed genes related with the proinflammatory response, influenced greatly the distribution of P, PB, P/PB/P, P/PB/PB, and PB/P/PB strategies in the PCA analysis, pointing out to the positive influence of MOS, in contrast to results of C diet. These results highlight the positive influence of functional ingredients when supplemented in highly substituted diets but also the importance of changes in mode of supplementation of functional ingredients

on European seabass. In this line, it is known that the abrupt suspension of probiotic supplementation can provoke a reduction of immune performance, maybe related to the development of an immune-dependence (Liu *et al.*, 2016) and results of PB/PB/P are in agreement with this idea. Moreover, the reduction of *il-10* in fish fed all supplemented diets could be related to its proposed role in the necessary immune-tolerance processes (Tafalla *et al.*, 2016), but it could be also related to the low level of proinflammatory-related gene expression in fish fed these diets. In both cases, low relative expression of proinflammatory genes level and low *il-10* expression levels are pointing out to a situation of non-inflammatory status in the intestine of fish fed functional ingredients compared to those fed non-supplemented diets.

As expected, the most marked differences between the different strategies of supplementation, were observed after fish infection with *V. anguillarum*. At 2 days post infection, PCA analysis revealed that MOS supplementation influenced significantly the results observed by gene expression in distal intestine of European seabass. At this point, according to PCA, mobilization of cd-8α^+ lymphocytes was important in supplemented diets but the fact that C diet was detached from the rest in the PCA analysis may implies that those lymphocytes were already present at the site of infection, due to their role in inflammation associated to vegetable-based diets (Romarheim *et al.*, 2013). In diets P and PB/P/PB, *il-1β* expression was upregulated to face the infection, although in PB and PB/PB/P the effect was the contrary. Again, downregulation of the expression of this important cytokine to face bacterial infections could be related with the already mentioned low capacity of response against a pathogen threat that the fish immune system can present after a long feeding period with immunostimulants (Bai *et al.*, 2010; Chang *et al.*, 2000).

However, at 7 days post infection, the differentiated distribution of strategies, primary influenced by MOS and synbiotic, according to PCA, was responsibility of two groups of genes: one composed by genes related to inflammation (*cox-2, il-1β, tnfα)* and the other with genes related to acquired immunity (*mhci-α, cd4, tcr-ß)*. Again, the same functional ingredients, administered in different strategies for this species, induced different immune performance. Besides, PCA analysis showed the influence of *mhcii-ß* for developing an acquired response at that moment, detaching PB and P/PB/PB diets from the rest, which could imply the recruitment of cd-8α^+ lymphocytes to this function. At this point, *il-10* expression was increased to control the proinflammatory response in PB strategy,

probably to avoid damage of self-recognition or to microbiota, in the context of an intestinal inflammation (Chaudry *et al.*, 2011).

Therefore, after 39 weeks of dietary supplementation with different strategies of functional ingredients for European seabass fed 5%FM/6%FO based-diets, influenced differently growth performance, liver and intestine morphology and modulated fish systemic and local immunity, pointing out to the importance of feeding strategies for this species. Affectation of systemic immune parameters could be related to the development of a relationship between dietary functional ingredients and the resident intestinal microbiota. Besides, local immune parameters in fish distal intestine, indicate that MOS, *P. acidilactici* and their synbiotic combination can modulate GALT-related genes after feeding to reduce negative effects of vegetable diets in the intestine, even after long feeding period. After bacterial infection, continuous feeding with MOS and synbiotic, showed to be adequate for developing a proinflammatory response.

In summary, the results of the present study show that the long-term use of synbionts in diets for European seabass fed 5%FM/6%FO based-diets, is a good strategy to enhance growth, lipid metabolism and fish health. The supplementation of single prebiotics or synbiont improved fish growth performance after 25 weeks of feeding, whereas after 39 weeks of feeding the best strategy int terms of growth performance points to be PB or alternation of prebiotic/synbiont/prebiotic (P/PB/P), and being the beneficial effects of the single supplementation of each of them masked by the other alternate strategies. Indeed, particularly PB reduced the level of hepatocyte vacuolization after 39 weeks of feeding but also fish fed P/PB/P presented a reduced maximum hepatocyte length. Besides, fish fed P and PB strategies presented lower levels of proinflammatory cytokines basal gene expression at the end of the feeding trial, indicating probably the reaching of a homeostasis status in terms of microbiota establishment and stability. In terms of capacity of response after infection, a global vision of the data seems to point to a better capacity of response of fish intestinally inoculated when fed P and PB/P/PB strategies, which was not evident in fish fed PB and PB/PB/P. However, it cannot be determinant in order to choose a feeding strategy since, the grade of infection of the sampled fish was not determined. More studies are necessary to reveal the precise mechanisms underlying the benefits of the functional additive supplementation with alternation strategies during long feeding periods.

Chapter 8

8. GENERAL DISCUSSION

8.1. What are the main effects of high fishmeal and fish oil substitutions with terrestrial meals and oils on European seabass health status?

Some of the most important effects of FM/FO substitutions on fish health are related with the gut local immunity due to the direct influence of dietary modifications on the GALT (Ellis, 2001; Dimitroglou *et al.*, 2011; Salinas, 2015; Torrecillas *et al.*, 2015b). For instance, European seabass fed for 90 days a diet based on a 36% to a 39% of terrestrial meal sources (g * kg^{-1} in diet) in combination with defatted FM or, alternatively, non-defatted FM, combined with a 100% down to a 31% (g * kg^{-1} in diet) of vegetal fats and oils, modified profiles of FA in phosphoglycerides from cell membranes, but also affected the relative abundance of different polar lipids, in agreement with our findings (Chapter 5) and with other similar studies (Castro *et al.*, 2015b and 2016a). Those effects on phosphoglycerides affect fluidity and functionality of membranes (Olsen *et al.*, 2004; Venou *et al.*, 2006; Kaushik *et al.*, 1995, 1998a and 2004; Richard *et al.*, 2006) and modify the activity of cells, including immune cells (Montero & Izquierdo, 2010). Thus, depending on dietary vegetal sources, one pathway of synthesis of eicosanoids could dominate over the rest which could activate a chronic proinflammatory response (Calder 2006b). For instance, LO, rich in n-3 PUFAs, precursors of EPA and DHA, included in diets up to 60% for 281 days, leads to an increment of 3-series prostaglandins, whereas SBO, rich in n-6 PUFAs, precursors of ARA, increases PGE2 in plasma of gilthead seabream (Ganga *et al.*, 2005). Besides, dietary inclusion of a vegetal blend of oils (RO, LO, PO) up to 60% for 64 weeks alters eicosanoid production in plasma of European seabass where PGE2 levels have been found to be reduced in relation to a reduction in respiratory burst activity (Mourente *et al.*, 2007). Moreover, total depletion of dietary FO using SBO for European seabass during 8 weeks of feeding can affect intestinal mucus production, a defensive tool that contains humoral components of immunity and helps fish to avoid microbial attachment (Magnadottir, 2005), by altering goblet cells size in

skin, proximal and distal gut, but also by decreasing cell density in proximal intestine (Torrecillas *et al.*, 2015b). Besides, different combinations of low %FM/%FO levels (58/15, 20/6, 20 /3, 10/6, 10/3, 5/6, 5/3, and 0/0) and terrestrial sources for 90 days were correlated with changes in density of goblet cells and increased GALT activity in DI (Torrecillas *et al.*, 2017a). Similarly, 18:C PUFAs as ALA or LA, abundant in vegetal sources as LO or SBO respectively, can influence the activity of Pparγ, present in different cells, including immune cells, and through it they can alter the pattern of expression of different genes including immune-related genes such as *il-1β, il-6* and *tnfα*, among others (Rosen *et al.*, 2000; Marion-Letellier *et al.*, 2016). Thus, high substitutions of marine raw materials can increase immune-cellular activity in DI, with upregulation of different immune-related genes (Torrecillas *et al.*, 2017a; chapters 5, 6 and 7), particularly proinflammatory genes as *il-1β, tnfα,* and *cox-2*, usually related to tissue damage in villi and microvilli, and can activate a consequent engrossment of submucosa due to the infiltrate of different immune-cell populations (Torrecillas *et al.*, 2017a and 2017d). These changes can affect intestinal homeostasis.

High substitutions of marine meals and oils, have also seen to alter microbiota environment. For instance, SBM levels up to 75% in feedstuffs for northern snakehead (*Channa argus*), for 63 days, produced a reduction in abundance and diversity of intestinal bacteria (Miao *et al.*, 2017). In brown trout (*Salmo trutta fario*) feeding 60 days with a commercial diet containing 25%FM/20%FO diets supplemented with terrestrial meals and oils reduced diversity and richness in microbiota species, respect to those fish fed the same diets supplemented with 0.1% (w/v) of EFAs (Manzano *et al.*, 2012). In European seabass, substituting dietary FM/FO levels with terrestrial meals and oils (58/15; 20/6; 10/6; 5/6; 20/3; 10/3; 5/3; 0/0 and 0/0+LC-PUFA) for 90 days, also affected richness and diversity of intestinal microbiota in European seabass (Torrecillas *et al.*, 2017a). The importance of microbiota lays in the fact that they are, together to the local immune system, responsible of keeping homeostasis in this organ as a way to keep favorable conditions for their growth, maintaining a state of immunotolerance with host defenses (Gómez & Balcázar, 2008; Ashida *et al.*, 2011; Gómez *et al.*, 2013; Viera *et al.*, 2013; Hamilton *et al.*, 2015; Salinas, 2015).

Then, in European seabass fed diets with 100%FO substitutions with SBO, or 20%FM/3%FO, substituted with different terrestrial meals and oils, the immune system

presented a low capacity to respond to alterations of homeostasis with the consequent decreasing resistance against intestinal pathogens (Torrecillas *et al.*, 2015b and 2017d).

Beyond local immunity, FM/FO substitutions on European seabass have demonstrated to affect different parameters of systemic immunity. For instance, 60% inclusions of different VO as (RO, LO or OO) for 34 weeks reduced the number of circulating leucocytes and the burst respiratory activity of head kidney leucocytes (Mourente *et al.*, 2005a), whereas for the same fish species, FM/FO free diets, using different terrestrial sources of protein (SBM, wheat gluten, whole wheat, white sweet lupin, and soy lecithin) and LO, for 9 months, reduced plasma lysozyme activity and increased plasma complement activity (Geay *et al.*, 2011). However, it has been pointed out in gilthead seabream that a dietary blend of vegetable meal ingredients, in levels from 50% up to 100%, for 6 months, can increase complement activity, however this effect uses to be counteracted by the frequent liver steatosis produced by high level of substitutions (Sitjà-Bobadilla *et al.*, 2005).

Therefore, high levels of substitutions can produce alterations in local and/or systemic components of immunity, but also changes in the equilibrium of microbiota, and can predispose fish to future infections.

8.2. What are the main causes and implications of fishmeal and fish oil substitutions effects on fish resistance against bacterial infections?

High FM and FO replacement may be considered as a nutritional stress for fish, compromising its local and systemic health status and increasing its susceptibility to infection as described previously for European seabass (Torrecillas *et al.*, 2017d) and as supported by our data (Chapters 6 and 7).

In fish, the main ways of entry for pathogens are the skin, gills and digestive tract (Muiswinkel & Nakao, 2013). These organs must act as a barrier to impede the entrance of pathogens (Magnadottir, 2005) and it has been demonstrated that better intestinal barrier integrity in European seabass can reduce pathogen translocation into fish tissues (Torrecillas *et al.*, 2013). However, the gut integrity can be compromised in European seabass by high reductions of dietary marine raw materials (Torrecillas *et al.*, 2017d). Besides, in gilthead seabream fed low FM/FO diets (5%/16% or 5%/42%), supplemented

with a blend of vegetal sources for 20 months, proteome showed altered pattern of proteins that indicate lack of intestinal integrity and functionality, as the downregulation of mucins (*muc*), related to increased inflammation symptoms, or angiotensin-converting enzymes (*ace*), involved in intestinal inflammation and innate immunity, in comparation to those fish fed a FM25%FO100% diet (Piazzon *et al.*, 2017). Similarly, reductions of 50%FM (%DM) using SBM for 15 weeks in Japanese seabass led to increased symptoms of enteritis, reductions of height in folds and microvillus, and in muscularis thickness, of distal gut (Wang *et al.*, 2016b). Moreover, high inclusion of terrestrial meals and oils can modify unions between epithelial cells (Kano-Sueoka *et al.*, 2001) altering tissue morphological pattern. Indeed, in Chapter 5, dietary ARA level was found to affect the esterification of ARA in all the studied polar lipids (PC, PE, PS and PI) of cell membranes in distal gut of European seabass and, besides, it affected PE and SM levels. Particularly, the functions of SM are related to normal functioning of epithelia in vertebrates, including fish (Storelli *et al.*, 1998; Feingold, 2007; Palmerini *et al.*, 2009; Pullmannová *et al.*, 2014; Cheng *et al.*, 2018). Additionally, the possible affectation of intestinal integrity can lead to the release of damage-associated molecular patterns (DAMPs) that will affect the pattern of immune-genes expression on defensive cells (Secombes & Wang, 2012) conditioning a subsequent local immune response during infection, as described in different studies with European seabass (Torrecillas *et al.*, 2015b, 2017a, 2017d). The damage of epithelial barriers is a factor that leads to increased probability of bacterial pathogen success in translocation and infection (Torrecillas *et al.*, 2013 and 2017d). The release of DAMPs is followed by the increment in the expression of proinflammatory cytokines (Secombes & Wang, 2012) as demonstrated in European seabass fed 5%FM diets for 90 days (Torrecillas *et al.*, 2017). In this sense, studies with 50% and 75% substitutions of FM by SBM for eight weeks in Japanese seabass induced increased circulating levels in serum of damage-key indicators of intestinal mucosal barrier integrity, D-lactate and diamine oxidase, in relation to increased release of proinflammatory cytokines such as Tnfα, Il-1β, Il-8, and Il-2 (Zhang *et al.*, 2018).

Moreover, high levels of terrestrial oils and meals for 90 days in European seabass, are related to the increasing presence of some bacterial species, as *Clostridium spp.*, that can produce tissue damage by production of proteases, inducing the increasing production of proinflammatory cytokines by fish tissues (Secades & Guijarro, 1999; Torrecillas *et al.*, 2017a). The intestinal morphological alterations are related to reductions in the efficacy

of epithelial barriers as first line of defense (Torrecillas *et al.*, 2011a and 2013; Miao *et al.*, 2017) and consequently influencing bacterial translocation rates in fish, as demonstrated in European seabass juveniles (Torrecillas *et al.*, 2012).

Once the bacteria have reached the MALT of intestine, must face fish immune responses. Fish defenses have evolved not only to reduce the possibilities of bacterial translocation through epithelia but to diminish bacterial survival capacity after their entry by the action of humoral and cellular immune mechanisms (Secombes & Wang, 2012). Nevertheless, even though GALT protects physical barrier of intestinal epithelia, its components and mechanisms can be altered by types and levels of dietary oil sources. Thus, feeding European seabass juveniles with FM/FO free diets for 90 days, induced a higher infiltration of leucocytes in distal gut submucosa and a greater accumulation of lipids on proximal gut lamina propria, each effect attributed to low dietary FM and FO contents respectively (Torrecillas *et al.*, 2017a). In that experiment, reductions of FM down to 5%, irrespective of FO level, reduced the functionality of immune-cells as leucocytes, denoted by reductions of *mhcii-β* expression levels and, at the same time, induced the contribution of all nucleated cells in immunity through the increment of *mhci-α* expression levels (Torrecillas *et al.*, 2017d).

In Chapter 6 of the present thesis, dietary substitutions of FM/FO affected cellular immunity in DI of European seabass, with Mhcii-β-positive cells, studied by ISH technics, being present in a higher density and wider distribution respect to those reported by other authors in the same species when fed a commercial diet with 100%FM/100%FO (Picchietti *et al.*, 2011). As discussed in Chapter 6, the increased number of Mhcii-β-positive cells was related to the attempt of keeping homeostasis through the immune-tolerance developed between intestinal microbiota and host immune-cells in this new environment created by dietary high levels of terrestrial compounds. The increment in *mhcii-β* gene expression in antigen presenting cells has been also found previously in Atlantic salmon fed a SBM-based diet for 47 days and related to a role in maintaining intestinal homeostasis when intestinal microbiota was altered by terrestrial sources (Romarheim *et al.*, 2013). Thus, high substitutions of marine meals and oils affect local cellular and humoral immune responses and the state of immunotolerance that allows microbiota to growth and collaborate with host defenses (Gómez & Balcázar, 2008; Dimitroglou *et al.*, 2011). In fact, levels of FM/FO (%\%) 20/6, 10/6, 5/6, 20/3, 10/3, 5/3, 0/0 and 0/0+LC-PUFA in combination with vegetal sources for 90 days have also found

to affect microbiota of European seabass juveniles and this was related to an altered gene expression of proinflammatory cytokines *il-1β* and *tnfα* (Torrecillas *et al.*, 2017a). Feeding low %FM/%FO levels of 20/6, 10/6, 5/6, 20/3, 10/3, 5/3 and 0/0 supplemented with a mix of terrestrial sources ((blood meal (spray-dried), soya protein concentrate, corn gluten meal, wheat gluten, rapeseed meal, wheat; RO, LO and PO) for 90 days (Torrecillas *et al.*, 2017d) or even total FO substitution with SBO for 60 days (Torrecillas *et al.*, 2015b) with a 61% of FM altered the expression of several immune-related genes in DI of European seabass juveniles (Torrecillas *et al.*, 2015b and 2017d). In this regard, when European seabass juveniles were fed 3%FO, irrespective of FM level, presented increased intestinal bacterial translocation rates (Torrecillas *et al.*, 2017d). In those experiments, *ex vivo* studies pointed out to increased bacterial translocation as the main reason for the high mortalities found *in vivo* (Torrecillas *et al.*, 2017d). The same explanation can be attributed to the experimental infection results presented in Chapter 6 of this work where fish fed the non-supplemented diet (5%FM/6%FO) presented lower survival than fish fed functional diets supplemented with *P. acidilactici* and/or MOS for 90 days, prior to pathogen exposition. This finding was accompanied of a lower capacity to mount an efficient intestinal immune response against the experimental intestinal bacterial infection when fish were fed the non-supplemented diet with functional additives. Indeed, in Chapters 5, 6 and 7, the patterns of immune-related genes in European seabass were found affected before and after infection with *V. anguillarum*. Particularly, in Chapter 5, changes in the immune-related genes in the DI of European seabass juveniles were suggested to be related to dietary ARA levels through the mitogen-activated protein kinase (Mapk) which can be activated by ARA derivatives in relation to ARA level in tissues (Alexander *et al.*, 2001). The activation of Mapk leads to the increment of *cox-2* expression and it is also related to an important cellular process as autophagy, that involves PE (Sui *et al.*, 2014), also altered by dietary ARA levels (Chapter 5). Autophagy is connected to increased *il-1β* release (Iula *et al.*, 2018) and reductions of SM synthesis (Kronqvist *et al.*, 1999), both effects found in that experiment for fish fed dietary ARA levels under 4% (TFA in diet, %) for 90 days (Chapter 5).

Apart from local immunity, systemic immunity can be also affected by high levels of terrestrial oil sources. Thus, as described in Chapters 3, 4, and 5 of the present thesis, FA profiles were affected by dietary graded levels of ARA in different tissues (muscle, liver, intestine, plasma and HK) of European seabass, as well as described for same fish species

fed VO in previous studies (Izquierdo *et al.*, 2003, Montero *et al.*, 2005; Mourente *et al.*, 2005a and 2005b; Mourente & Bell, 2006; Mourente *et al.*, 2007; Geay *et al.*, 2011; Castro *et al.*, 2015a and 2016a; Torrecillas *et al.*, 2015b, 2016 and 2017b). In this sense, gilthead seabream fed a diet with a 60% of FO replacement by SBO for 204 days presented reduced alternative complement activity and phagocytic activity of HK macrophages in relation to altered FA profile of immune cell membranes (Montero *et al.*, 2003). Particularly, the reduction in dietary LC-PUFAs for fish by the inclusion of vegetal sources could reduce the availability of nicotinamide adenine dinucleotide phosphate, affecting the innate mechanism of respiratory burst of peripheral leukocytes (Montero & Izquierdo, 2010) which is also a common pathway to be affected by bacteria to evade the fish immune response (Sepulcre *et al.*, 2007). In this regard, European seabass fed a diet with a 60% of FO replacement by VO sources, for 64 weeks, presented reduced respiratory burst activity of HK macrophages (Mourente *et al.*, 2007), whereas the same percentage of FO replacement by VO was found to reduce the number of circulating leukocytes and HK macrophages respiratory burst after only 34 weeks in other study (Mourente *et al.*, 2005a). Similarly, total FM/FO replacement by terrestrial sources for European seabass also reduced serum lysozyme activity after 39 weeks of feeding (Geay *et al.*, 2011). In Chapter 3, a selective deposition of ARA, DHA and EPA in HK leucocytes and plasma of European seabass juveniles fed a diet without FO and with defatted FM for 90 days, reflected the importance of EFAs in the immunological functions and the importance of including appropriate dietary levels during FO substitutions. In that Chapter 3, modifications in FA profiles in plasma and HK were reflected in the systemic immunity function, with changes in HK leucocytes phagocytic index and plasma prostaglandins levels. In previous studies, dietary FA have been found to alter the incorporation of EFAs in tissues which led to altered PGs serum levels in gilthead seabream (Ganga *et al.*, 2005). In Chapter 3, PGs plasma levels in European seabass juveniles were affected by dietary FA levels and they were correlated to ARA/EPA ratios, probably related to dietary ARA level and its influence on EPA bioconversion and/or EPA incorporation into GPs, as suggested previously by Ganga *et al.*, (2005).

Therefore, high dietary substitutions of FM/FO for European seabass affected no only tissue integrity and functionality, favoring the entrance of the potential bacterial

pathogen, and, in the end, increases higher mortalities rates in those fish fed the lower FO levels (Torrecillas *et al.*, 2015b, 2017a, 2017d).

8.3. Is it necessary the supplementation of arachidonic acid in diets with low fishmeal and fish oil content for European seabass juveniles?

Most of the marine species, as European seabass, have very low ability, if at all, to elongate and desaturate FA chains even when they have the complete group of genes to perform this function (Mourente & Dick, 2002; Sargent *et al.*, 2002; Mourente *et al.*, 2005b; Kabeya *et al.*, 2018). In consequence, LC-PUFAs, as ARA, cannot be synthetized from their precursors 18:C PUFAs, as LA, and ALA, abundant in vegetable oils, and then, marine species have specific requirements (Higgs & Dong, 2000; Oliva-Teles, 2000). Besides, not only reductions of FO can lead to ARA deficiencies, but also reductions of FM due to the high percentage of LC-PUFA contained in non-defatted FM (Turchini *et al.*, 2009).

Arachidonic acid plays a crucial role in fish survival (Bessonart *et al.*, 1999; Koven *et al.*, 2001; Atalah *et al.*, 2011; Montero *et al.*, 2015c), growth (Bessonart *et al.*, 1999; Koven *et al.*, 2003; Lund *et al.*, 2007; Bae *et al.*, 2010; Carrier *et al.*, 2011; Luo *et al.*, 2012; Montero *et al.*, 2015c; Torrecillas *et al.*, 2018a; Ding *et al.*, 2018; Ma *et al.*, 2018), lipid metabolism (Martins *et al.*, 2012; Ma *et al.*, 2018; Xu *et al.*, 2018), stress resistance (Koven *et al.*, 2001; Van Anholt *et al.*, 2004; Ganga *et al.*, 2006, 2011a and 2011b; Carrier *et al.*, 2011; Atalah *et al.*, 2011; Montero *et al.*, 2015c), smoltification and osmoregulation (Bell & Sargent, 2003; Oxley *et al.*, 2010; Van Anholt *et al.*, 2012), pigmentation (Estevez *et al.*, 1997; Villalta *et al.*, 2005; Lund *et al.*, 2007 and 2008), egg quality (Furuita *et al.*, 2003) and immune system (Hughes *et al.*, 1996, Sanderson *et al.*, 1997; Huang *et al.*, 1992; Xu *et al.*, 2010; Dantagnan *et al.*, 2017; Ding *et al.*, 2018), among others. In this line, and according to our data at Chapter 3, reductions of dietary ARA below 1% (% TFA) can reduce growth performance in European seabass juveniles, in terms of weight, length and SGR. Besides, results at the Chapter 4 of the present thesis indicate that liver morphology was affected by low ARA level, as suggested by other authors in other marine fish species (Castell *et al.*, 1994; Fountoulaki *et al.*, 2003; Luo *et al.*, 2012; Xu *et al.*, 2010 and 2018). The liver of fish fed the lowest dietary ARA level (g

TFA/g of diet) presented higher steatosis level and irregular-shaped morphology around sinusoidal spaces with increased intracytoplasmic lipid vacuolization and higher number of hepatocytes with displaced nuclei to the cellular periphery, when compared to livers from those fish fed higher dietary ARA levels (Chapter 4). Those fish fed the lowest dietary ARA percentage, also presented an upregulation of the hepatic expression of *hmgcr* gene, related to lipid metabolism, and it was suggested to exist a direct effect by dietary ARA levels under 1% (% TFA) (Chapter 4).

Dietary ARA also influenced lipid contents and FA profiles in DI. For instance (Chapter 5) dietary ARA influenced some gut phosphoglycerides (PE and SM) and the FA composition of all studied GPs, with a selective retention of this EFA for the lowest dietary ARA levels, indicanting its importance in the intestinal functioning. Moreover, the inclussion of ARA in diets was inversely correlated to EPA content in the intestinal lipid classes (Chapter 5).

Important immune functions of ARA have been demonstrated for marine fish previously (Castell *et al.,* 1994; Xu *et al.,* 2010; Torrecillas *et al.,* 2017b) and our data in Chapters 3 and 5 are in agreement with those results of ARA regulation of defensive responses. Thus, the expression of several proinflammatory cytokines was altered in fish fed the ARA0.5 and ARA2 (% of TFA) diets, when challenged against an intestinal pathogen, indicating the effect of dietary ARA on immune response to pathogens.

Systemic immunity also can be affected due to dietary ARA levels. In this line, European seabass fed below 1% of dietary ARA (as TFA) presented a selective deposition of this EFA on HK leucocytes although the resulting increment of ARA was not enough to increase phagocytic activity levels up to levels of those fish fed higher dietary ARA levels and this reduction was related to FA profiles variations on immune cell membranes and to altered PGs plasma levels (Chapter 3), demonstrating the effects of dietary ARA far from the digestive tract where it was administrated.

Altogether, these data demonstrate the importance of dietary supplementation with ARA for European seabass to keep adequate growth performance, lipid metabolism and local and systemic immune functioning.

8.4. Is it possible to minimize deleterious effects of high inclusion of terrestrial meals and oils on diets for European seabass with the addition of functional ingredients?

Different studies have demonstrated the beneficial effects of MOS (Torrecillas *et al.*, 2007, 2011a, 2011b, 2012, 2013, 2015a, 2015b, 2016; Salem *et al.*, 2016; Terova *et al.*, 2009), and probiotics (Carnevalli *et al.*, 2006; Frouël *et al.*, 2008) on European seabass physiology.

Some of these studies have been made using diets with total replacement of FO (Torrecillas *et al.*, 2015b and 2016). For instance, MOS inclusion in totally FO replaced diets by SBO combined with a 51.5% of non-defatted FM resulted in higher SGR, reduced hepatocyte area and increased the gut mucous cell size and density which, as an overall outcome, resulted in a partial compensation of negative effects of total FO replacement (Torrecillas *et al.*, 2015b and 2016). Our results in Chapter 6 and 7 corroborate the benefits of including functional ingredients. The inclusion of MOS and *P. acidilactici* and their synbiotic combination on highly substituted diets (5%FM/6%FO) improved fish growth. However, it seems that the time of supplementation is determinant in order to choose the functional additive to be feed. For example, single supplementation of MOS or probiotic *P. acidilactici* for 25 weeks increased fish SGR, however it was the combination of P/PB/P and PB (synbiont) which resulted in a better fish performance at the end of the experiment (39 weeks). Besides, MOS has been found to affect positively to liver morphology of European seabass before (Torrecillas *et al.*, 2011a). In Chapter 6, *P. acidilactici* was related with the same effect in European seabass fed a 5%FM/6%FO based diet after 90 days of feeding. In Chapter 7, European seabass fed a 5%FM/6%FO based diet for 39 weeks with continuous supplementation of a synbiotic composed by MOS and *P. acidilactici* (PB treatment), resulted in a reduction of hepatocytes area compared to those from fish fed control diet.

Furthermore, functional ingredients can enhance local immunity response in gut of European seabass. For instance, MOS inclusion in totally FO replaced diets by SBO combined with a 51.5% of non-defatted FM, counteracted the effects of the inclusion of terrestrial sources on GALT-related genes of European seabass such as *cox-2* and *il-1β* expression and particularly contributed to homeostasis through regulation of *tgf-β* expression levels (Torrecillas *et al.*, 2015b). In this sense, the inclusion of 0.4 g * kg^{-1} of

MOS in diets with FO or SBO as unique lipid source, combined with a 51.5% of non-defatted FM, for European seabass for 90 days, modulated *tnfα* gene expression in DI, probably in relation to leucocyte mobilization which has been described before for the same species (Torrecillas *et al.*, 2015b). In that regard, MOS effects on the recruitment of leucocytes and on the proinflammatory cytokines levels has been proposed to be related to changes in microbiota and/or to the activation of NF-*κβ* in macrophages (Torrecillas *et al.*, 2015b).

Besides, MOS, at levels of 4 g * kg^{-1} for 90, and probiotic, *Lactobacillus spp.* at a concentration of 3 to 8 * 10^8 CFU * g/diet for 103 days, have been described before to improve fish intestinal morphology in European seabass (Torrecillas *et al.*, 2013; Salem *et al.*, 2015; Frouël *et al.*, 2008;). However, such results were not found in this species when fed a 5%FM/6%FO based diet for 90 or 270 days and supplemented with functional additives when compared to non-supplemented fish (Chapter 6 and 7, respectively) denoting the importance of the dietary formula when supplementing functionals additives, and suggesting that functional additives dosage and time of supplementation must be adapted to the fish species, size and diet formula.

Supplementation of MOS at levels of 4 g * kg^{-1} for 90 days and LAB probiotics, using different species of *Lactobacillus spp.* at concentrations from 3 to 8 * 10^8 CFU * g/diet for 103 days, have been related to a better gut barrier functionality in European seabass (Torrecillas *et al.*, 2013; Frouël *et al.*, 2008). Results discussed on Chapter 6 showed that singular or synbiotic administration of both functional ingredients for 90 days were related to reduced fish mortality after bacterial infection, then demonstrating the increased fish resistance to bacterial infection even with a low inclusion of FM/FO (%5FM/6%FO in diet) on basal diet when supplemented in a relative short-time period (90 days).

Besides, the influence of systemic immune parameters by MOS in European seabass have been demonstrated (Torrecillas *et al.*, 2007 and 2011a). In Chapter 7 of the present thesis, the continuous administration of MOS in synbiotic administration with *Pediococcus acidilactici* (PB treatment) or alternate with singular administration of MOS, in P/PB/P and PB/P/PB strategies, in European seabass fed 5%FM/6%FO based diets for 39 weeks, resulted in increased systemic immune parameters level as lysozyme and peroxidase when compared with fish from non-supplemented diets in non-infected fish. On this Chapter 7 and after a bacterial infection, continuous and singular administration of MOS,

P. acidilactici or synbiotic, or alternate use of those MOS and synbiotic in P/PB/P and PB/P/PB strategies, increased peroxidase levels. This affectation of systemic immune parameters by orally-administered functional ingredients could be related to the capacity of microbiota to ferment dietary fibers to produce short-chain fatty acids (SCFA) which can diffuse into the blood modulating innate parameters of systemic immunity through G protein coupled receptor (Maslowsky & Mackay, 2011).

Therefore, these data demonstrate that local/systemic immune response and resistance to bacterial infection can be increased when European seabass is fed highly FM/FO substituted diets supplemented with functional ingredients as MOS and *P. acidilactici*.

8.5. Which characteristics must be considered for dietary supplementation with functional ingredients for European seabass?

Different authors have described the importance of controlling different parameters as dose, time, mode of use, *etc*., during the supplementation of functional ingredients (Torrecillas *et al.*, 2014; Dimitroglou *et al.*, 2011; Nayak, 2010). In particular, our data reinforce this idea. Indeed, as described in Chapter 6, it is also important the diet formulation that could alter number or distribution of intestinal immune cellular populations and, in general, the intestinal homeostasis, and, consequently, altering or limiting the modes of action of functional ingredients, as described before (Torrecillas *et al.*, 2015b). For this reason, the next recommendations are restricted to European seabass juveniles fed a 5%FM/6%FO based diet supplemented with terrestrial meals and VO as described in Chapters 6 and 7.

Data from Experiment II (Chapter 6) showed that supplementing MOS, *P. acidilactici* or their combination in 5%FM/6%FO based diets did not improve growth performance after a feeding period under 90 days. However, single MOS supplementation for a period of 90 days potentiated fish growth in terms of final weight and length and, in combination to *P. acidilactici* as synbiont, regardless of the dose fed.

In this sense, and despite the fact that feeding with a 5%FM/6%FO based diet for 90 days (Chapter 6) did not cause a pathological intestinal inflammatory-like status in DI morphology, the addition of both functional ingredients altered the GALT-related genes pattern of expression. On one hand, the use of a particularly high (0.6%) level of MOS

led to a higher level of expression of several proinflammatory genes in the distal gut, such as *tnf-α*, *il10*, and *cox-2*, whereas *P. acidilactici*, downregulated *tnf-α* and *il10* and upregulated *il-1β* and *cox*-2. Besides, fish fed diets including *P. acidilactici,* presented lesser immunoreactivity to anti-Inos compared to fish fed MOS diets, presenting a more similar pattern to those fish fed control diet. Altogether indicating that distal gut humoral proinflammatory genes upregulation could be partially counteracted by *P. acidilactici*, helping to European seabass to reach faster an intestinal homeostatic status. Indeed, these results mentioned together with the lower mortalities found in fish fed the lower dose of MOS combined with *P. acidilactici* as synbiont (LPB), point to this diet as a good candidate to potentiate European seabass health and disease resistance when fed low FM/FO levels.

The strategy of administration is important too. In Experiment III (Chapter 7), MOS (P) and synbiotic (PB) after 25 weeks, altogether synbiotic and P/PB/P strategy, after 39 weeks, enhanced growth of European seabass fed the experimental diets. Indeed, particularly PB reduced the level of hepatocyte vacuolization after 39 days of feeding but also fish fed P/PB/P presented a reduced maximum hepatocyte length. Besides, feeding strategies also affected GALT-related genes when analyzed after 39 weeks of feeding. Thus, P, P/PB/P, P/PB/PB, PB and PB/P/PB reduced *il-1β* expression and P, PB and PB/P/PB reduced *tnfα* expression, two important proinflammatory cytokines. In this line, low levels of *il*-10 expression in fish fed supplemented diets could be related to the low levels of expression proinflammatory-related genes but also to its role in achieving immunotolerance (Tafalla *et al.,* 2016). This cytokine is expressed by different immune cells to control proinflammatory cytokines and it affects specially the function of regulatory T-cells (Treg) (Chaudry *et al.,* 2011). A high level of *il-10* expression is necessary to control Tregs, particularly in the context of intestinal inflammation, avoiding producing self-damage or to microbiota (Chaudry *et al.,* 2011). Besides, MOS reduced *mhci-α* expression whereas PB and PB/P/PB increased *mhcii-β* expression. The *cd-8α* expression has been found related to proinflammatory effects of vegetal diets (Romarheim *et al.,* 2013) and all functional ingredients presented a trend to reduce its level at this point. In terms of capacity of response after infection, a global vision of the data seems to point to a better capacity of response of fish intestinally inoculated when fed P and PB/P/PB strategies, which was not evident in fish fed PB and PB/PB/P. In this context, different authors have pointed out that long feeding periods with functional ingredients can make the immune system less capable to react when facing a pathogen

(Bai *et al.*, 2010; Chang *et al.*, 2000). However, it cannot be determinant in order to choose a strategy since, the grade of infection of the sampled fish was not determined.

Thus, not only innate but also acquired local immune responses in the intestine were affected by using different combinations of MOS and synbiotic. Similarly, results of systemic immune parameters were differently affected by feeding strategies. After 39 weeks, PB/P/PB enhanced plasma lysozyme level whereas PB and P/PB/P enhanced peroxidase level. Therefore, local and systemic parameters were differently affected by the same functional ingredients, MOS and synbiotic, when administered in different feeding strategies along 39 weeks for European seabass fed 5%FM/6%FO based-diets.

Therefore, the dietary dose of 0.3% of MOS in synbiotic combination with *P. acidilactici* at a commercial level for a period of 90 days was an effective tool to reduce the deleterious effects of high FM/FO substitutions on European seabass growth performance and enhanced intestinal homeostasis, increasing fish resistance to bacterial infections (Chapter 6). However, in a mid-term basis (25 weeks) it seems that feeding P or B, without combining, is the best option for European seabass, in terms of promoting growth performance, indicating the importance of time of supplementation when feeding functional diets (Chapter 7). Indeed, on a long-term basis (39 weeks) fish fed PB and P/PB/P strategies presented higher weight and PB strategy reduced also steatosis level in liver, whereas fish fed P and PB/P/PB strategies point to have a better capacity of response after being intestinally inoculated with *V. anguillarum* in terms of gene expression results obtained, however no differences were found in survival percentages (Chapter 7).

8.6. Is it profitable for the aquaculture sector to invest in research and development in the field of functional ingredients based on the revenues from the production outputs?

High marine raw materials substitutions by more sustainable ingredients affects fish health and disease resistance, which could have detrimental economic consequences for the aquaculture sector. Therefore, every improvement that leads to higher fish welfare, production performance and disease resistance will be a guarantee for the future of the growing sector of aquaculture. The study of functional ingredients used in the present thesis focused in fish health, in terms of infection resistance, and presented results of growth performance. Obtained results indicate a net improvement in both parameters.

However, optimization of profit must be considered in aquaculture research. In this sense, European seabass is a species with high economic and social value in Mediterranean aquaculture. Its global production in Spain in 2017 reached more than 21 MT with an economic value over one hundred million euros (APROMAR, 2018). Besides, feedstuffs for European seabass, in conjunction with those for breeding of gilthead seabream, accounted for 2% of global supplies in 2017 (APROMAR, 2018). As an approach to the study of profitability, an analysis was made for the growth improvements produced in European seabass fed the different strategies collected in Chapters 7, for 270 days of feeding with 5%FM/6%FO based diets. According to that study, for current mean market sale-prices of this species, the value-added of these strategies, enriched with functional ingredients, can account for 1.6 euros for each kilogram of feedstuff (Torrecillas & Montero, personal communication from PROINMUNOIL project), ensuring an economic return for the present R&D action. This demonstrate that the use of functional ingredient is not only an acceptable and reasonable strategy to reduce the occurrence of disease outbreaks and their devastating effects, but also a tool that can increase the benefits from the production outputs when high levels of terrestrial raw materials are used in feedstuffs.

Therefore, this expected benefit justifies the investment in R&D actions to extend the use of these products to other species and breeding conditions.

Chapter 9

9. GENERAL CONCLUSIONS

1. Dietary ARA levels below 0.2g fatty acid * g^{-1} diet reduced growth performance of European seabass juveniles denoting the importance of ARA for a proper fish development.

2. When dietary ARA levels did not cover the requirements for growth, there was a selective deposition of this essential fatty acid in head kidney leukocytes, although functionality of those immune cells was reduced in terms of phagocytic activity.

3. The supplementation of dietary ARA was reflected in the fatty acid profile of different tissues, including head kidney, liver and muscle, with ARA incorporation increasing in tissue when increasing in diet.

4. Supplementation of dietary ARA levels over 0.2 g fatty acid * g^{-1} diet up to 1.4 g fatty acid * g^{-1} diet did not improve growth of European seabass juveniles, although a reduction in lipid deposition was found with higher levels of dietary ARA, reflected in a reduction of hepatosomatic index and perivisceral fat content.

5. Dietary ARA levels were incorporated in the lipid classes and particularly in the glycerophospholipids of distal intestine (phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol) of European seabass juveniles, with a selective retention of this fatty acid particularly when dietary ARA did not cover requirements for growth (below 0.2 g fatty acid * g^{-1} diet). ARA content was higher than dietary ARA levels in all the analyzed glycerophospholipids of distal intestine.

6. Dietary ARA levels below 0.2 g fatty acid * g^{-1} diet were related to an increased basal expression of proinflammatory cytokines in distal intestine of European seabass juveniles.

7. A dietary single supplementation of mannan oligosaccharides (MOS; 0.3 or 0.6% in diet) or 0.3%MOS plus probiotic *P. acidilactici*, in a low fish meal/fish oil based diet, increased growth performance of European seabass juveniles after 90 days of feeding.

8. Synbiotic supplementation (0.3% MOS plus probiotic *P. acidilactici*) in low fish meal and fish oil based diets counteracted the MOS-derived upregulation of humoral proinflammatory genes of distal intestine by reducing in particular *tnfα*, *il-1β* gene expression and Inos production after 90 days of feeding (short-term strategy).

9. Dietary MOS at low doses, as well as its synbiont with *P. acidilactici*, reduced European seabass mortality when challenged against *V. anguillarum* by intraperitoneal injection after 90 days of feeding (short term strategy).

10. On a mid-term basis (25weeks) feeding P or B without combining among them was the best option for European seabass, in terms of promoting growth performance, indicating the importance of time of supplementation when feeding functional diets.

11. On a long-term basis (39 weeks) feeding European seabass with synbiont (PB) and the alternate strategy of P/PB/P increased final fish weight. The beneficial effects of the single supplementation of each of them (P or PB) seemed to be masked by the other alternate strategies. Synbiont (PB) dietary supplementation reduced liver steatosis and alone or within the combined strategy with prebiotic (P/PB/P and PB/P/PB) increased serum immune potential.

12. On a long-term basis (39 weeks) and based on the gene expression values found, feeding P and PB or their combination in P/PB/P or PB/P/PB strategies seemed to reduce some of the typical distal gut innate and acquired inflammatory-like response caused by low FM/FO dietary levels in European seabass, indicating probably a restore and maintenance of the homeostasis status. This effect was not evident when prolonged and continued use of *P. acidilactici* was included in the alternate strategies (PB/PB/P or P/PB/PB) or in singular and continuous supplementation (B).

13. In terms of capacity of response after infection, a global vision of the data seems to point to a better capacity of response of fish intestinally inoculated when fed P and PB/P/PB strategies, which was not evident in fish fed PB and PB/PB/P. However, it cannot be determinant in order to choose an strategy since, the grade of infection of the sampled fish was not determined.

Chapter 10 / Capítulo 10

10. RESUMEN EN ESPAÑOL

10.1. INTRODUCCIÓN

10.1.1. EL CRECIENTE SECTOR DE LA ACUICULTURA: CIFRAS DE LA PRODUCCIÓN DE LUBINA EUROPEA

Desde 2014, la acuicultura ha sido responsable de proveer más del 50% del pescado para consumo humano y actualmente es la responsable del incremento de las capturas (FAO, 2016 y 2018). En el caso particular de la lubina europea (*Dicentrarchus labrax*), en 2016 el 97.1% de su consumo en la región mediterránea provino de la acuicultura (APROMAR, 2018). Esta especie, la número 73 de la producción acuícola mundial, supuso un mercado de 720 millones de euros en 2018, siendo en España, el tercer productor mundial, la segunda especie más cultivada y la primera en relación al retorno económico (APROMAR, 2017 y 2018).

10.1.2. MATERIAS PRIMAS PARA PIENSOS EN ACUICULTURA

10.1.2.1. *Situación actual y alternativas sostenibles*

En 2017, el cultivo de lubinas y doradas requirió el 2% de la producción mundial de piensos y ese mismo año, la acuicultura española requirió 129 toneladas métricas de piensos (APROMAR, 2018). Para esta producción de piensos, la acuicultura dependió durante largo tiempo de ingredientes provenientes de peces capturados del medio marino y transformados en aceites y harinas de pescado (FO y FM, respectivamente) (Naylor *et al.*, 2009; Tacon y Metian, 2015; SEAFISH, 2018). Esta fuente de nutrientes es adecuada para alimentar a las especies de cultivo debido a las apropiadas cantidades y calidades de los aminoácidos, ácidos grasos esenciales (EFAs), vitaminas y minerales, además de poseer un sabor y textura que los hacen más aceptables para los peces (Sargent y Tacon, 1999; Hasan *et al.*, 2007; NRC, 2011).

Sin embargo, la producción de FM y FO ha tenido diferentes altibajos en las últimas décadas debido a diversos factores como la sobrepesca y malas prácticas, la contaminación del medio natural y el cambio climático (FAO, 2016 y 2018; Barange *et*

al., 2018). Todo esto está llevando a la escasez y encarecimiento de los FM y FO, cuestionando su sostenibilidad como principal alimento en acuicultura (Tacon y Foster, 2003; FAO, 2018). Por ello, se hace necesario incluir nuevos ingredientes que sean sostenibles a nivel económico y medioambiental, como los de origen terrestre, con una buena relación coste-efectividad, una producción sostenible y un gran margen de crecimiento (Gatlin *et al.*, 2007; Turchini *et al.*, 2009; Hardy *et al.*, 2010; Tacon *et al.*, 2011).

10.1.2.2. *Consideraciones para la inclusión de ingredientes terrestres en piensos para acuicultura*

El uso de ingredientes vegetales en dietas para peces se ha encontrado con diferentes problemas de formulación que se han ido solventando en los últimos años (Francis *et al.*, 2001; Hardy *et al.*, 2010; Krogdahl *et al.*, 2010; Li *et al.*, 2014; Betancor *et al.*, 2015a; Domínguez *et al.*, 2017). Esto ha llevado a que puedan incluirse en las dietas para especies como la lubina europea, importantes niveles de sustitución aunque posean bajos niveles de ácidos grasos poliinsaturados de cadena larga (LC-PUFAs), entre otros nutrientes. (Berge *et al.*, 2002; Kaushik *et al.*, 2004; Bonaldo *et al.*, 2011; Torrecillas *et al.*, 2017a, 2017b y 2017d) En consecuencia, prescindir totalmente de los ingredientes marinos en piensos para acuicultura hace necesario el estudio de los requerimientos de EFAs en las especies a cultivar (Tocher, 2010 y 2015).

10.1.3. UN RÁPIDO VISTAZO AL SISTEMA INMUNE EN PECES

En los peces, el tracto gastrointestinal constituye una barrera defensiva cubierta por mucus y con un tejido linfoide con función defensiva (Uribe *et al.*, 2011; Van Muiswinkel y Nakao, 2013), altamente expuesta a los cambios dietéticos y a interacciones entre microorganismos y sistema inmune (Bernard *et al.*, 2006, Dimitroglou *et al.*, 2011; Van Muiswinkel y Nakao, 2013). El mucus, provee un medio para que los microorganismos se anclen, alimenten y crezcan (Gomez *et al.*, 2013), aunque, paradójicamente, es también reconocido como una parte de la barrera defensiva debido a sus elementos antiadhesivos y antibacterianos (Ellis, 2001; Dimitroglou *et al.*, 2011; Salinas, 2015), evitando así que la superficie intestinal sea colonizada por la mayor parte de los patógenos. De esta forma, un incremento en la producción de mucus puede reducir el anclaje de patógenos, ayudando a que sean eliminados con las heces, al tiempo que reduce los daños por la fricción que genera el movimiento de la comida durante la digestión, ayudando al

mantenimiento de la integridad del tejido (Ellis, 2001; Torrecillas *et al.*, 2011a). De hecho, el incremento de la producción de mucus en lubina europea ha sido relacionado con un incremento de la resistencia a infección (Torrecillas *et al.*, 2011a).

En relación con la salud, la integridad intestinal, es crucial. Dieta con altos niveles de substitución de FM/FO, pueden llevar a disrupciones de las uniones entre células epiteliales (Kano-Sueoka *et al.*, 2001), incrementando las probabilidades de translocación bacteriana (Torrecillas *et al.*, 2013). En parte por estas razones, la salud intestinal de los peces es frecuentemente afectada por dietas con alto nivel de sustitución de ingredientes marinos (Khroghdal *et al.*, 2010; Torrecillas *et al.*, 2017a).

Pero el tracto digestivo debe no solo actuar como una barrera pasiva que reduzca la capacidad de translocación, sino también disminuir la capacidad de supervivencia de los patógenos, de lo cual se encargará el sistema inmune (Secombes y Wang, 2012). En este sentido, las respuestas inmunes de los peces se dividen entre las innatas, con una rápida e inespecífica reacción contra las amenazas, y la adquirida, que requiere más tiempo para desarrollar formas específicas de enfrentar las amenazas (Tort *et al.*, 2003). A pesar de estas diferencias, ambas vías no son independientes puesto que interactúan y pueden estar activas al mismo tiempo (Kum y Sekkin, 2011). Así, para reducir la supervivencia bacteriana tras su entrada en los tejidos, el intestino aloja un tejido reactivo, llamado tejido linfoide asociado al intestino (GALT) que estará a cargo de la defensa reaccionando ante variaciones de la homeostasis como las que ocurren durante procesos infecciosos, situaciones estresantes o sustituciones de ingredientes marinos en las dietas (Torrecillas *et al.*, 2014; Salinas, 2015).

Para que el GALT cumpla su misión, los antígenos deben ser detectados y clasificados entre los potencialmente dañinos y los no dañinos y frecuentes, y eso requiere su comunicación con la microbiota y células epiteliales (Pelaseyed *et al.*, 2014).

La detección de antígenos implica la conexión entre la inmunidad innata y adquirida para detectar y presentar los antígenos a las células especialistas (Rombout *et al.*, 2014). Además, deberá existir una comunicación entre células T y la microbiota residente, la cual ayudará a educar el sistema inmune en un proceso que se denomina inmunotolerancia (Nutsch y Hsieh, 2010). En el intestino de los vertebrados más evolucionados, como parte del proceso de detección y procesado de antígenos, hay pequeñas células secretoras de mucus que actúan captando pequeñas partículas desde el lumen y las presentan a las células inmunes de la lámina propia (Pelaseyed *et al.*, 2014). Se sabe que esta capacidad también existe en el intestino de los teleósteos a través de otras células, similares a células

M, y su interacción con linfocitos intraepiteliales (Ellis, 1995; Fuglem *et al.*, 2010; Torrecillas *et al.*, 2017d). Esta estimulación de la inmunidad local influirá en el desarrollo de la inmunidad sistémica lo que convierte al intestino en un elemento fundamental para lograr la inmuno-protección oral (Le Breton, 2009; Dimitroglou *et al.*, 2011; Rombout *et al.*, 2011).

10.1.4. IMPORTANCIA DE LOS ÁCIDOS GRASOS ESENCIALES EN LOS PECES

10.1.4.1. *Las series n-3 y n-6*

Dependiendo del patrón de alimentación o, de forma más general, del medio en que vivan, podemos definir dos grupos de peces, los de agua dulce y los marinos (Monroig *et al.*, 2013). En la mayor parte de las especies dulceacuícolas las necesidades de LC-PUFAs pueden ser cubiertas con los de cadena corta como el ácido linoleico (18:2 n-6) y ácido α-linolénico (18:3 n-3), abundantes en los ingredientes de origen vegetal (Sargent *et al.*, 1995; Mourente *et al.*, 2005b). En contraste, las especies marinas, como la lubina europea, viven y han evolucionado en un medio en el que abundan los LC-PUFAs de la serie n-3 y, en menor medida, de la n-6 (Monroig *et al.*, 2013). Por ello, estas especies tienen escasa o nula capacidad de elongar y desaturar cadenas cortas de ácidos grasos (FA), incluso cuando poseen todos los genes necesarios para hacerlo (Mourente y Dick, 2002; Sargent *et al.*, 2002; Mourente *et al.*, 2005b; Kabeya *et al.*, 2018). Por ello, los LC-PUFA son esenciales para las especies marinas que, además, poseen requerimientos muy específicos (Higgs y Dong, 2000; Oliva-Teles, 2000).

Entre los de la serie n-3 LC-PUFAs destacan el ácido eicosapentaenoico (20:5 n-3; EPA) y el ácido docosahexaenoico (22:6 n-3; DHA), ambos abundantes en las membranas celulares, lo que lleva a ser requeridos en mayor cantidad respecto a los del grupo n-6 (Bell y Sargent, 2003; Izquierdo, 1996; Yaqoob y Calder, 2007). Niveles insuficientes de EPA y DHA conducen a un bajo crecimiento, altas mortalidades y problemas de funcionamiento del sistema inmune y nervioso, además de alteraciones morfológicas en órganos (Izquierdo, 2005; Montero *et al.*, 2001 y 2005).

10.1.4.2. *El ácido araquidónico*

El ácido graso más importante de la serie n-6 LC-PUFAs es el ácido araquidónico (20:4 n-6; ARA). El hecho de que los tejidos de los peces posean bajos niveles ARA

comparados con los de la serie n-3 ha inducido a prestar menos atención a sus requerimientos dietéticos, que se estiman cubiertos a bajos niveles. Sin embargo, se ha visto que en los tejidos de lubinas cultivadas los niveles de ARA son menores que en las salvajes. Además, diferentes investigaciones han demostrado los importantes roles del ARA en el metabolismo lipídico (Martins *et al.*, 2012; Ma *et al.*, 2018; Xu *et al.*, 2018), crecimiento (Bessonart *et al.*, 1999; Koven *et al.*, 2003; Lund *et al.*, 2007; Bae *et al.*, 2010; Carrier *et al.*, 2011; Luo *et al.*, 2012; Montero *et al.*, 2015c; Torrecillas *et al.*, 2018a; Ding *et al.*, 2018; Ma *et al.*, 2018), supervivencia (Bessonart *et al.*, 1999; Koven *et al.*, 2001; Atalah *et al.*, 2011; Montero *et al.*, 2015c) o sistema inmune (Hughes *et al.*, 1996; Sanderson *et al.*, 1997; Huang *et al.*, 1992; Xu *et al.*, 2010; Dantagnan *et al.*, 2017; Ding *et al.*, 2018), entre otros.

Como para el grupo de los n-3, las deficiencias dietéticas de ARA se compensan con su retención selectiva, sobre todo en tejidos donde parece influir en su correcto funcionamiento (Castell *et al.*, 1994; Farndale *et al.*, 1999; Montero *et al.*, 2003; Fountoulaki *et al.*, 2003; Ganga *et al.*, 2005). Así, deficiencias dietéticas de ARA pueden conducir a diversos problemas que pueden acrecentarse en condiciones comerciales, llevando a la afección del bienestar animal amén de importantes pérdidas económicas y (Farndale *et al.*, 1999). En la actualidad existe escasa información sobre los requerimientos mínimos de ARA en lubinas juveniles, pero también de los niveles máximos, los cuáles, de ser superados, pueden ser tóxicos (Watanabe, 1982; Furuita *et al.*, 2003; Blanchard *et al.*, 2008; Xu *et al.*, 2010; Luo *et al.*, 2012).

Así, la falta de información sobre los niveles mínimos y óptimos de ARA para lubinas incrementa los riesgos al hacer sustituciones.

10.1.4.3. *Combinaciones críticas de sustituciones dietéticas de harina y aceite de pescado en lubina europea*

La FM contiene un importante porcentaje de LC-PUFA por lo que sus reducciones, además de las de FO, pueden magnificar las deficiencias nutricionales en peces (Turchini *et al.*, 2009). Además, la suplementación de LC-PUFA en dietas con bajos niveles de FM y FO ha sido asociado con un mejor ambiente de desarrollo para la microbiota de estos peces y aunque el crecimiento no se vea afectado, se ha demostrado en lubinas que la morfología del intestino anterior sí puede verse afectada en relación a la absorción lipídica (Torrecillas *et al.*, 2017a). Además, se ha demostrado que cambios en la morfología de

los órganos puede producir efectos negativos en la salud del animal y, por ejemplo, alteraciones del intestino distal (DI) se han correlacionado con cambios en la respuesta defensiva y en la microbiota, señalando la sensibilidad de este órgano a la sustitución de ingredientes marinos en la dieta y su gran influencia en el sistema inmune del animal (Van den Ingh *et al.,* 1991; Miao *et al.,* 2017; Vigneulle y Laurencin, 1991; Torrecillas *et al.*, 2011a y 2013). También en lubina, la sustitución total de FM y FO produjo modificaciones del metabolismo hepático (Torrecillas *et al.,* 2017b) y efectos negativos en la salud y, en lubinas alimentadas con niveles de FO bajo el 3% y de FM bajo el 5%, redujeron, así mismo, la capacidad de los peces para enfrentarse a una infección intestinal (Geay *et al.,* 2011).

10.1.5. AÑADIENDO PREBIÓTICOS Y PROBIÓTICOS COMO INGREDIENTES FUNCIONALES EN DIETAS PARA ACUICULTURA

La sustitución de ingredientes marinos en dietas para lubinas pueden reducir la resistencia de los animales frente a brotes infecciosos en explotaciones comerciales, los cuales suelen ser tratados con antibióticos y otros productos que, a la larga, pueden inducir resistencia bacteriana y modificar la microbiota natural, predisponiendo a los peces a futuras complicaciones, incluyendo nuevas infecciones (Becattini *et al.,* 2016; Falony *et al.,* 2016; Zhernakova *et al.,* 2016). Además, en lubina, desarrollar nuevas formas de luchar contra las *vibriosis* ha sido una preocupación creciente en los últimos años, como complemento al uso de vacunas (Mosca *et al.,* 2014). En este campo, los ingredientes funcionales, con capacidad para influir diferentes funciones al mismo tiempo (Roberfroid, 2000), pueden ser usados para contrarrestar o reducir algunas de las consecuencias indeseables debidas al uso de dietas con bajos niveles de FM/FO (Panigrahi y Azad, 2007; Kiron, 2012).

10.1.5.1. *Prebióticos: manan-oligosacáridos en dietas para peces marinos*

Los prebióticos pueden ser definidos como ingredientes no digeribles por el animal que los ingiere, pero con efectos positivos en su fisiología además de para la microbiota residente (Gibson y Roberfroid, 1995; Burr *et al.,* 2005; Dimitroglou *et al.,* 2011; Song *et al.,* 2014) y, dado que algunos de esos efectos positivos tienen que ver con la mejora de parámetros del sistema inmune, suelen ser incluidos en el grupo de inmunoestimulantes. Para cumplir su misión, los prebióticos deben alcanzar el tracto intestinal intactos donde serán fermentados por la microbiota pudiendo, además, modular

estas comunidades lo que es especialmente interesante en sustituciones de ingredientes marinos (Guerreiro *et al.*, 2017; Dimitroglou *et al.*, 2011; Torrecillas *et al.*, 2014).

Los prebióticos son principalmente carbohidratos que actúan directa o indirectamente en la mejora de la inmunidad pasiva y, probablemente, de la activa (Dimitroglou *et al.*, 2011; Song *et al.*, 2014). Particularmente en el caso de los manan-oligosacáridos (MOS), estudiados en esta Tesis Doctoral, parece que sus efectos positivos están parcialmente derivados de la respuesta no específica del sistema inmune contra su presencia. Así, los prebióticos emulan el primer paso del encuentro entre un posible patógeno y las defensas del hospedador (Huang *et al.*, 2009; Areschoug y Gordon, 2008). Además, el MOS puede ser reconocido como lugar de unión por los patógenos, que acabarán siendo expulsados con las heces al no haberse anclado eficazmente, interrumpiendo la colonización de los tejidos (Arason, 1996; Redondo y Álvarez-Pellitero, 2010; Torrecillas *et al.*, 2014).

Más allá de la inmunidad, la administración de MOS ha sido extensamente estudiada en diferentes especies de peces, incluyendo la lubina, demostrando efectos positivos en el crecimiento, supervivencia, respuesta al estrés, ingesta de alimento, utilización de nutrientes, metabolismo lipídico, mejora de la morfología e integridad de la pared intestinal y el incremento de la secreción de mucus, tanto en condiciones experimentales como comerciales (Salze *et al.*, 2008; Dimitroglou *et al.*, 2009 y 2010; Gültepe *et al.*, 2011 y 2012; Torrecillas *et al.*, 2007, 2011a, 2012, 2013, 2015a y 2015b, 2016; Salem *et al.*, 2015; Eryalçyn *et al.*, 2017; Gelibolu *et al.*, 2018a y 2018b).

Sin embargo, los efectos de los prebióticos dependen de la forma de presentación, dosis, tiempo de administración, especie, edad y condiciones de cultivo (Dimitroglou *et al.*, 2010 y 2011; Torrecillas *et al.*, 2014), lo que ha llevado a que se obtengan resultados experimentales contradictorios (Azeredo *et al.*, 2017), al variar uno o varios de esos factores.

En lubina, el MOS han demostrado ser una herramienta eficaz para hacer frente a la inclusión de ingredientes vegetales en las dietas, compensando los frecuentes efectos negativos derivados de la sustitución de FM/FO. Esos efectos del MOS no se restringen al sistema inmune local en el tracto digestivo, donde son administrados, sino que también se ven en la inmunidad sistémica (Torrecillas *et al.*, 2007, 2011a, 2013, 2015a, 2015b). La respuesta positiva en lubinas se ha relacionado con niveles de entre el 0.2 y 0.6% de MOS en dieta, encontrándose que niveles del 0.4% resultaron mejorar notablemente el crecimiento y la inmunoestimulación (Torrecillas *et al.*, 2007, 2011a, 2011b, 2012, 2013, 2015b, 2016).

10.1.5.2. *Probióticos*

La microbiota es altamente variable entre individuos de la misma especie criados en diferentes localizaciones, dado que las larvas son colonizadas no solo por transmisión materna sino también desde el medio circundante (Balcazar *et al.,* 2006a, Gatesoupe *et al.*, 2007, Dimitroglou *et al.,* 2011). Desde el instante de la colonización, la microbiota intestinal va a ser un factor clave que conduzca al desarrollo y maduración del sistema inmune y además será parte de las barreras defensivas, contribuyendo a la homeostasis, al mantenimiento de la integridad de la barrera intestinal y al correcto funcionamiento de los mecanismos antibacterianos (Gómez y Balcázar, 2008; Ashida *et al.,* 2011; Dimitroglou *et al.,* 2011). La microbiota intestinal constituye un ecosistema que será configurado y modulado a través de mecanismos de competencia y exclusión entre las diferentes especies presentes, durante la vida del pez (Gómez y Balcázar, 2008). Esas interacciones deben ocurrir sin alertar al sistema inmune por lo que la microbiota regulará la expresión de genes del hospedador para transformar el tracto intestinal en un ambiente favorable (Gómez y Balcázar, 2008). En este proceso, los mecanismos de inmunotolerancia sirven para evitar que el sistema inmune reaccione contra antígenos frecuentes y no dañinos que provienen de la propia microbiota o desde la comida ingerida (Kim *et al.*, 2012).

El término probiótico se puede definir *grosso modo* como microorganismos que son ingeridos vivos y son beneficiosos para el hospedador actuando como un nuevo componente de la microbiota durante el período de administración (Parker, 1974). Hay que subrayar que la microbiota indígena es muy competitiva y la presencia y dominancia de un probiótico durará solo durante su administración en altas dosis (Balcázar *et al.*, 2006a). La administración continua de un probiótico termina por inducir cambios favorables en las poblaciones de la microbiota del hospedador, como la reducción de especies potencialmente dañinas (Balcázar *et al.*, 2006a, Dimitroglou *et al.*, 2011) o la mejora en el crecimiento del animal (Ringø *et al.*, 2016 y 2018). También se ha demostrado que los probióticos pueden servir para modular el estrés en lubinas mediante la reducción de los niveles corporales de cortisol (Carnevali *et al.*, 2006), lo que es de gran interés en rutinas de manejo diarias de la acuicultura que pueden comprometer la respuesta inmune del animal. Sin embargo, aunque temporal, la estrecha relación que se crea entre el probiótico y el resto de especies que componen la microbiota puede crear

una suerte de dependencia (Liu *et al.*, 2016) que puede afectar a la homeostasis e inducir una mayor susceptibilidad a la infección.

Igual que para los prebióticos, el uso de probióticos requiere de la consideración de diferentes condiciones como la cepa a usar, la viabilidad, la dosis, modo y periodo de suplementación, etc (Nayak, 2010). El uso de la cepa láctica *P. acidilactici* ha demostrado inducir efectos beneficios en el crecimiento, supervivencia, desarrollo corporal, modulación de microbiota, respuesta inmune humoral y celular, resistencia a infección, etc., en diferentes especies acuáticas, incluyendo la lubina europea (Tabla 3) (Aubin *et al.*, 2005; Merrifield *et al.*, 2010 y 2011b; Ferguson *et al.*, 2010; Neissi *et al.*, 2013; Ramos *et al.*, 2013, Standen *et al.*, 2013; Vasanth *et al.*, 2015; Mondaloo *et al.*, 2017; Hoseinifar *et al.*, 2015 y 2017).

10.1.5.3. *Sinbióticos*

El término sinbiótico, hace referencia al uso sinérgico de diferentes tipos de ingredientes funcionales, con la potencialidad de mejorar las funciones individuales de sus componentes (Gibson y Roberfroid, 1995). No todos los sinbióticos pueden inducir efectos beneficiosos, pudiendo incluso aumentar la susceptibilidad a infección del hospedador. Por tanto, los efectos de este aditivo funcional dependerán de la especie, dosis, tiempo de administración, etc., siendo importante que se evalúen en primer lugar los resultados individuales de los productos a combinar (Huynh *et al.*, 2017). Así, aunque el uso de prebióticos como el MOS y probióticos como *P. acidilactici* han demostrado ser seguros y valiosos como estrategia para mejorar el crecimiento, salud y bienestar de los peces, su combinación sinérgica puede incrementar los efectos individuales. Un uso eficaz de esta herramienta en una especie tan relevante como la lubina europea permitirá afrentar retos presentes y futuros de la acuicultura como es la reducción dietética del FO y FM.

10.2. OBJETIVOS

Esta tesis doctoral tiene por objetivos definir herramientas nutricionales y estrategias para mejorar el crecimiento y salud de lubinas europeas al ser alimentadas con dietas con bajos niveles de FM/FO. Con ese propósito se han evaluado: **(1)** los efectos de diferentes niveles dietéticos de ácido araquidónico (20:4 n-6), incluyendo aquellos que pueden estar presentes en dietas con reducciones de aceite de pescado, en el crecimiento, contenido y

composición de ácidos grasos de los glicerofosfolípidos del intestino posterior y en el perfil de ácidos grasos de diversos tejidos, además de los efectos en parámetros inmunes celulares y humorales, y en la resistencia a infección; **(2)** la efectividad del prebiótico manan-oligosacárido, del probiótico *Pediococcus acidilactici*, y de su combinación sinbiótica como complementos dietéticos; y **(3)** la evaluación de diferentes estrategias de suplementación con los ingredientes funcionales, administrados individualmente o como sinbiótico, de manera alterna o continua, para maximizar el potencial de resistencia frente a patógenos.

Para cubrir estos objetivos principales, una serie de objetivos específicos fueron diseñados y llevados a cabo en los diferentes experimentos:

1.- Evaluar la necesidad de suplementar con ácido araquidónico las dietas de lubinas europeas juveniles. Esta evaluación se realize no solo en términos de crecimentos sino también de salud (Capítulo 3), metabolismo lipídico (Capítulo 4) e integridad intestinal y composición de las clases lipídicas (Capítulo 5).
2.- Balancear la salud intestinal de lubinas europeas juveniles a través del uso de ingredientes funcionales (prebiótico -MOS- y probiótico -*P. acidilactici*-) en dietas con bajo contenido en harina y aceite de pescado (Capítulo 6).
3.- Determinar las mejores estrategias de alimentación para mejorar la acción beneficiosa de los ingredientes funcionales en lubinas europeas juveniles alimentadas con dietas con bajo contenido en harina y aceite de pescado (Capítulo 7).

Los objetivos específicos se repartieron en los siguientes capítulos:

- ***Capítulo 3:*** suplementación de un aceite rico en ácido araquidónico en dietas para lubina europea (*Dicentrarchus labrax*): efectos en leucocitos y perfiles plasmáticos de ácidos grasos, parámetros inmunes y niveles de prostaglandinas circulantes.

- ***Capítulo 4:*** suplementación de un aceite rico en ácido araquidónico en dietas para juveniles de lubina europea (*Dicentrarchus labrax*): efectos en el crecimiento, perfiles de ácidos grasos en tejidos y metabolismo lipídico.

- ***Capítulo 5:*** efectos del ácido araquidónico dietético en las clases lipídicas del intestino distal y la salud intestinal de lubina europea (*Dicentrarchus labrax)*.

- ***Capítulo 6:*** alimentación de juveniles de lubina europea (*Dicentrarchus labrax*) con un aditivo funcional sinbiótico (manan-oligosacáridos y *Pediococcus acidilactici*): ¿una herramienta efectiva para reducir los efectos en la salud intestinal de los bajos niveles de harina y aceite de pescado?

- ***Capítulo 7:*** sinbióticos en dietas para lubina europea (*Dicentrarchus labrax*): estrategias de alimentación para mejorar la acción beneficiosa de los ingredientes funcionales en dietas con niveles bajos de harina y aceite de pescado.

10.3. MATERIAL Y MÉTODOS

Para lograr la consecución de los objetivos de esta Tesis Doctoral en el contexto del Proyecto PROINMUNOIL, se llevaron a cabo los siguientes experimentos:

- **Experimento I:** ensayo de suplementación de ARA (Capítulos 3, 4 y 5)
- **Experimento II:** ensayo de suplementación con ingredientes funcionales (Capítulo 6)
- **Experimento III:** ensayo de estrategias de alimentación con ingredientes funcionales (Capítulo 7)

10.3.1. INSTALACIONES Y PECES PARA LOS ENSAYOS EXPERIMENTALES

Los experimentos fueron llevados a cabo en las instalaciones del Parque Científico-Tecnológico Marino (PCTM), localizado en la ciudad de Telde (Gran Canaria, Islas Canarias, España), gestionado por el Instituto Universitario de Acuicultura Sostenible y Ecosistemas Marinos (IU-EcoAqua) en la Universidad de Las Palmas de Gran Canaria (ULPGC). Los animales utilizados fueron manipulados de acuerdo con las directrices del Consejo de la Unión Europea (86/609/EU) y la legislación española (RD 53/2013). Los protocolos desarrollados fueron previamente aprobados por el Comité de Bioética de la ULPGC (REF: 007/2012 CEBA ULPGC).

Los juveniles de lubina europea fueron criados en granjas comerciales y transportados a las instalaciones del PCTM. Durante las 4 semanas previas los peces fueron aclimatados en tanques de 2000l y alimentados con dietas comerciales diseñadas para esta especie. Los peces fueron alimentados 3 veces al día, 6 días a la semana. Antes de los muestreos, los animales ayunaron durante 24 horas.

Los tanques fueron provistos de agua de mar filtrada en un sistema de aguas abiertas y bajo un fotoperiodo natural (12L / 12O). La temperatura del agua fluctuó diariamente de

acuerdo con las medias estacionales (21-25ºC) y las concentraciones de oxígeno se encontraron entre las 7.0 y 8.0ppm durante los períodos experimentales

10.3.2. PROCEDIMIENTOS Y DIETAS DEL EXPERIMENTO I

Los peces fueron distribuidos al azar en 15 tanques con una densidad final de 4 kg * m^{-3} y las dietas se ensayaron por triplicado. Se establecieron dos puntos de muestreo, a los 30 y 70 días, en los que se calcularon los parámetros de crecimiento (Figura 1). La mortalidad fue registrada diariamente. Tras los 70 días de alimentación se tomaron muestras para análisis bioquímicos, morfológicos y de expresión génica. Al final del período de alimentación, los peces fueron sometidos a un ensayo de infección bacteriana contra un patógeno intestinal y se tomaron muestras para análisis de expresión génica.

Cinco dietas experimentales isolipídicas e isoproteicas secas y peletizadas fueron formuladas y producidas en la planta de producción de dietas experimentales del IU-EcoAqua para contener una gradación de ARA (% en dieta): 0.5%, 1%, 2%, 4% y 6%. El contenido deseado de ARA fue logrado usando un aceite obtenido del hongo *Mortierella alpina* (Vevodar®, DSM Food Specialties, Holanda). Para suplementar los niveles de DHA y EPA se usaron DHA50 y EPA50 (CRODA, East Yorkshire, RU), respectivamente. Los ingredientes de la dieta, la composición bioquímica y su perfil de FA están detallados en las Tablas 4 y 5.

10.3.3. PROCEDIMIENTOS Y DIETAS DE LOS EXPERIMENTOS II Y III

Para el Experimento II, los peces fueron distribuidos al azar en 18 tanques a una densidad final de, aproximadamente, 4 kg * m^{-3}, para ensayar las dietas por triplicado en cada tratamiento. El procedimiento del Experimento II está representado en la Figura 2. Se establecieron dos puntos de muestreo, a los 60 y 90 días, y en ambos se calcularon parámetros de crecimiento. Además, al final del período de alimentación con las dietas experimentales, se tomaron muestras para realizar estudios histológicos y moleculares (incluyendo de hibridación *in situ* y expresión génica). A lo largo del ensayo, la supervivencia fue registrada diariamente.

Tras los 90 días de alimentación con las dietas experimentales, los peces fueron sometidos a un desafío de infección con un patógeno intestinal. Al final de este período, se tomaron muestras para análisis de expresión génica.

El Experimento III fue desarrollado como una extensión del Experimento II, realizado durante tres períodos consecutivos: período 1 (P1, 0-25 semanas), período 2 (P2, 25-31 semanas) y período 3 (P3, 31-39 semanas). Para el P1, los peces fueron distribuidos al azar en 12 tanques con una densidad final de aproximadamente 4 kg * m^{-3}. Para el P2 y P3, los peces fueron redistribuidos en 16 tanques de acuerdo a la representación detallada en la Figura 3, para ensayar por duplicado ocho nuevos tratamientos, durante 6 y 8 semanas, respectivamente. Las diferentes estrategias y los días de alimentación están resumidos en la Tabla 7.

El procedimiento del Experimento III está representado en la Figura 4. En los puntos de muestreo (semanas 25, 31 y 39), se calcularon parámetros e índices de crecimiento. Además, al final de la semana 39, se tomaron muestras de sangre y tejidos para análisis inmunes, morfológicos y de expresión génica. Tras esto, los peces fueron sometidos a un desafío por infección bacteriana mediante la inoculación intestinal de *V. anguillarum* y se tomaron muestras para análisis de expresión génica.

Para el Experimento II, seis dietas experimentales y extruidas, isoenergéticas e isonitrogenadas, fueron formuladas para contener 5% FM y 6% FO y los carbohidratos estándares fueron reemplazados usando manan-oligosacáridos (MOS, Bio-Mos® y Actigen©; Alltech, Inc., Kentucky, EEUU) y *Pediococcus acidilactici* (BAC, Bactocell®; Lallemand Inc., Cardiff, RU) de la siguiente manera (%MOS/BAC): HP=0.6/0, LP=0.3/0, B=0/BAC, HPB=0.6/BAC, LPB=0.3/BAC, C=0/0. Los niveles de BAC en las dietas fueron determinados atendiendo a recomendaciones comerciales. Las dietas fueron producidas en el Centro Tecnológico de BioMar (Brande, Dinamarca). Los ingredientes de cada dieta están recogidos en la Tabla 6, mientras que la composición proximal se encuentra en las Tablas 6 (EII) y 8 (EIII).

Para el Experimento III, cuatro dietas fueron seleccionadas en base a los datos obtenidos en el Experimento II y renombradas de la siguiente manera: (MOS (%) / BAC (presencia +/ ausencia -)): solo prebiótico (P) = 0.3/-; solo probiótico (B) = 0/+; prebiótico más probiótico (PB) = 0.3/+; y dieta control (C) = 0/-.

10.3.4. ENSAYOS DE DESAFÍO FRENTE A PATÓGENO

Los peces fueron transferidos a las instalaciones de bioensayo del PCTM. Durante las dos semanas de adaptación, los peces fueron alimentados con sus correspondientes dietas. Tras esto, fueron infectados con *V. anguillarum* (cepa 507, aislada en cultivo tras

un brote clínico en las Islas Canarias) con una dosis subletal (EI: 10^7 UFC * ml^{-1}; EII: 10^6 UFC * ml^{-1}; EII: 10^3 UFC * ml^{-1}). La inoculación fue realizada por vía anal en los Capítulos EI y EIII y mediante inyección intraperitoneal en el EII.

10.4. CAPÍTULO 3

El objetivo principal de este estudio fue medir los efectos de diferentes niveles dietéticos de ARA, suplementado desde fuentes alternativas, en la composición de FA del plasma y leucocitos del riñón anterior de lubina europea. Para ello, juveniles de esta especie fueron alimentados durante 70 días con cuatro dietas que contenían una gradación de ARA de la siguiente manera: 0.5% (ARA0.5), 1% (ARA1), 2% (ARA2) y 4% (ARA4).

Al final del ensayo de alimentación, los perfiles de FA del plasma y de los leucocitos del riñón anterior, además de los niveles de prostaglandinas del plasma, el estallido respiratorio de los leucocitos del riñón anterior, la actividad peroxidasa y el índice fagocítico, fueron analizados. La reducción de niveles dietéticos de ARA bajo el 1% redujo el crecimiento de manera marcada. Sin embargo, los peces alimentados con la dieta ARA0.5 intentaron compensar esta deficiencia mediante una deposición selectiva de ARA en el plasma y leucocitos del riñón anterior, alcanzando niveles similares a los de los animales alimentados con ARA1, tras 70 días. Sin embargo, la capacidad fagocítica de los leucocitos del riñón anterior fue reducida con la reducción de ARA en la dieta en relación no solo a variaciones de la composición de la membrana, sino a cambios en los niveles basales de prostaglandinas. Los resultados obtenidos demuestran la importancia de añadir la cantidad necesaria de n-6, y no solo de n-3 LC-PUFA, en dietas para lubinas europeas en relación con el crecimiento, pero también con la inmune.

10.5. CAPÍTULO 4

El objetivo de este estudio fue el de evaluar los efectos del aumento de los niveles de ARA en la dieta (desde el 1 al 6% del total de FA) en el crecimiento, perfil de FA en los tejidos, además de la morfología, biosíntesis de LC-PUFA, síntesis de triglicéridos y colesterol y transporte lipídico en hígados de juveniles de lubina europea. Una dieta con total sustitución de FO y FM-desgrasada, conteniendo un 0.1g de ARA * g^{-1} en dieta, fue añadida al diseño experimental como control negativo. La inclusiones de niveles dietéticos de ARA por debajo de los 0.2g * ARA g^{-1} en dieta, empeoraron el crecimiento significativamente, incluso tras solo 30 días de ensayo, mientras que los diferentes niveles

de ARA en dieta no afectaron a la supervivencia de los peces. Los perfiles de FA del hígado, músculo y cuerpo entero reflejaron principalmente los de la dieta, mientras que los niveles de ARA en tejidos lo hicieron de acuerdo a sus niveles en dieta. Los niveles de EPA, DPA y DHA se correlacionaron positivamente entre ellos. La vacuolización lipídica en el hígado se incrementó con los niveles más bajos de ARA en dieta. Las expresiones génicas de la desaturasa de ácidos grasos 2 (*fads2*) y de la 3-hidroxi-3-metilglutaril coenzima A reductasa (*hmgcr*) en hígado aumentaron en los peces alimentados con el control negativo cuando se compararon con los de los peces alimentados con el resto de tratamientos, denotando la influencia del ARA en el metabolismo lipídico. Los resultados obtenidos subrayan la necesidad de incluir los niveles adecuados de n-6, y no solo los de n-3 LC-PUFA, en dietas para lubinas europeas.

10.6. CAPÍTULO 5

El uso de niveles bajos de FM y FO, en dietas para peces marinos, afecta a la composición y concentración de los niveles de EFAs y, consecuentemente, puede producir una deficiencia marginal de esos FA con un impacto directo en la fisiología intestinal. La suplementación de distintos EFAs para cubrir requerimientos es necesaria incluyendo la de aquellos pertenecientes a la serie n-6, como el ARA. Además de su rol en la configuración de las clases lipídicas del intestino, el ARA juega un rol importante en la funcionalidad del GALT.

El presente estudio estuvo dirigido a probar cinco niveles de ARA en dieta (ARA0.5 (0.5%), ARA1 (1%), ARA2 (2%), ARA4 (4%) y ARA6 (6%) para juveniles de lubina europea en orden a determinar (1) sus efectos en la composición de determinadas clases lipídicas y (2) como esos cambios afectaron los ratios de translocación bacteriana y la expresión de determinados genes del GALT antes y después de un ensayo de infección.

En los peces, cualquiera que fuera la dieta suministrada, no se encontraron diferencias en el porcentaje final de los lípidos totales polares ni neutros del DI. Sin embargo, en el DI de los peces alimentados con ARA6, se presentaron niveles más altos de fosfatidiletanolamina y esfingomielina que en el de los peces alimentos con la ARA0.5. En términos generales, los perfiles de FA de las clases lipídicas del DI reflejaron los de las dietas. Sin embargo, se observó una retención selectiva de ARA en los glicerofosfolípidos (GPs) cuando los niveles dietéticos eran bajos, como se refleja en el

alto ratio de ARA en GPs/ARA en dieta, para esos animales. El incremento del nivel de ARA en dieta fue inversamente correlacionado con el contenido de EPA de las clases lipídicas. La suplementación de ARA no afectó a la morfología del intestino, al número de células mucosas, ni a la supervivencia a lo largo del desafío de infección. Sin embargo, tras la infección experimental con *V. anguillarum*, la expresión relativa de ciclooxigenasa 2 (*cox-2*) e interleukina-1 β (*il-1β*) aumentó en el DI de los peces alimentados con las dietas ARA0.5 y ARA2, comparado con los peces alimentados con el resto de las dietas. Aunque el ARA de la dieta no afectó a la supervivencia, los niveles más bajos alteraron la composición en FA de los GPs y, tras la infección, la expresión de genes proinflamatorios, lo que podría estar comprometiendo la funcionalidad física e inmune del DI, denotando la importancia de la suplementación de ARA cuando se usan dietas con bajos niveles de aceites de pescado en peces marinos.

10.7. CAPÍTULO 6

El objetivo de este estudio fue ensayar los efectos dietéticos del MOS, *Pediococcus acidilactici* o su combinación como sinbiótico en dietas con niveles bajos de FM y FO, en la salud intestinal y resistencia a la enfermedad de lubinas europeas. Para ese propósito, juveniles de lubinas europeas fueron alimentados con alguna de las 6 dietas que contenían diferentes combinaciones de MOS y *P. acidilactici*, como reemplazo de los carbohidratos, de la siguiente manera: (MOS (%)/BAC (recomendación comercial): alto nivel de prebiótico (HP) = 0.6/0, bajo nivel de prebiótico (LP) = 0.3/0, solo probiótico (B) = 0/+, alto nivel de prebiótico más probiótico (HPB) = 0.6/+, bajo nivel de prebiótico más probiótico (LPB) = 0.3/+, control (C) = 0/0, durante 90 días.

Tras 90 días de alimentación, los peces fueron sometidos a un desafío frente a *Vibrio anguillarum*. Adicionalmente, se midieron los patrones de inmunopositividad de la enzima óxido nítrico sintetasa inducible (Inos) y del factor de necrosis tumoral alfa tumor necrótico α (Tnfα) y los patrones de expresión génica mediante hibridación *in situ* del complejo mayor de histocompatibilidad de clase ii (*mhcii*), el receptor de células T beta (*tcr-β*), el subgrupo de células T reguladoras (linfocitos T *cd-4*$^{+}$) y las células T efectoras (linfocitos T *cd-8α*$^{+}$), fueron medidos en el intestino tras 90 días de alimentación.

Los efectos de ambos aditivos en el DI, a través de la expresión génica del GALT, fueron también estudiados. Los peces alimentados con prebióticos y su combinación con *P. acidilactici* presentaron un incremento de peso de manera independiente a la dosis suplementada tras 90 días de alimentación, aunque no se detectaron efectos en los índices

somáticos. En DI, la morfología y la densidad de células mucosas no se vieron afectadas por el MOS, *P. acidilactici*, o su combinación. Los patrones de inmunopositividad de anti-Inos y anti-Tnfα en el intestino fueron influenciados principalmente por la suplementación de MOS y no por su combinación con *P. acidilactici*. La distribución e incidencias de células positivas *mhcii-β*, *tcr-β*, *cd-4* y *cd-8α*, no fueron afectadas por la dieta. Los peces alimentados con la dosis HP presentación una clara sobreexpresión de *tnfα*, *cox-2*, *cd-4* e *il10*, mientras que la suplementación con *P. acidilactici* incrementó el número de transcritos genéticos de *il-1β* y *cox-2*. La suplementación con sinbiótico resultó en una reducción de la respuesta proinflamatoria inducida por MOS, mediante el incremento de algunos genes relacionados con la inmunidad celular. La mortalidad de los peces tras la infección con *V. anguillarum* fue reducida en los peces alimentados con las dietas LPB y LP, comparadas con la de los peces alimentados con dietas no suplementadas tras 90 días de alimentación.

Por tanto, de forma general la combinación de dosis bajas de MOS y su combinación sinbiótica con *P. acidilactici* (LPB) apunta a ser una herramienta viable para potenciar el crecimiento y la resistencia a enfermedad de las lubinas europeas juveniles cuando son alimentadas con dietas bajas en FM y FO.

10.8. CAPÍTULO 7

Este estudio tuvo como objetivo la evaluación de diferentes estrategias de alimentación basadas en la suplementación alterna o continua con MOS y *P. acidilactici* en administración individual o sinbiótica en dietas 5%FM/6%FO para lubina europea, para ensayar los efectos en la supervivencia, crecimiento, morfología de hígado e intestino, parámetros de la inmunidad sistémica, expresión de genes relacionados con el GALT y resistencia a infección con *V. anguillarum*. Para ese propósito, 4 dietas fueron ensayadas (% MOS/*P. acidilactici* (recomendación comercial)): solo prebiótico (P) = 0.3/0, solo probiótico (B) = 0/+, prebiótico más probiótico (PB) = 0.3/+ y control (C) = 0/0, por 39 semanas.

Las dietas se ensayaron en tres periodos consecutivos: periodo 1 (P1), 0-25 semanas; periodo 2 (P2), 25-31 semanas; periodo 3 (P3), 31-39 semanas. En P1, los peces fueron alimentados con las dietas P, B, PB y C. Al final de P1, los peces alimentados con P y PB fueron divididos en tres grupos para ser alimentados con diferentes estrategias durante P2 y P3. Así, en P2 las estrategias ensayadas fueron P, P/PB, B, PB, PB/P y C, mientras que

en P3 fueron P, P/PB/P, P/PB/PB, B, PB, PB/P/PB, PB/PB/P y C. Tras 39 semanas, los peces fueron infectados con *V. anguillarum*. Además, al final de cada período, se calcularon parámetros e índices de crecimiento. Tras 39 semanas de alimentación, el DI y el hígado fueron recogidos para análisis histológicos. Al final del P3 y tras la infección experimental, se recogieron muestras de DI fueron para el análisis de expresión génica del GALT, mientras que se recogieron muestras de sangre y riñón anterior para evaluar diferentes parámetros humorales inmunes.

El crecimiento de los peces alimentados con P mejoró tras el P1 mientras que las estrategias P/PB/P y PB mejoraron el crecimiento en P3. En el DI, la longitud de las vellosidades fue incrementada por el probiótico, mientras que el sinbiótico redujo la vacuolización y tamaño de los hepatocitos tras 39 semanas. Las estrategias de sinbiótico y P/PB/P mejoraron la lisozima basal y la peroxidasa comparadas con la dieta C, tras 39 semanas, mientras que las estrategias P, P/PB/P, PB, PB/P/PB y B, mejoraron la actividad peroxidasa en comparación con la C tras la infección. Al final del período de alimentación, los ingredientes funcionales redujeron la sobreexpresión de genes proinflamatorios en el DI. De forma similar, la capacidad de la respuesta inmune durante el desafío por infección pareció mejorar para las estrategias P y PB/P/PB. Los resultados del presente experimento reflejan que las diferentes estrategias de alimentación, usando los mismos ingredientes funcionales, inducen diferentes efectos en el crecimiento, morfología intestinal y hepática, expresión génica del GALT y parámetros inmunes, señalando la importancia del modo de uso de estos aditivos.

10.9. CONCLUSIONES

1. Los niveles dietéticos de ARA bajo el 0.2g de ácidos grasos por gramo de dieta, redujeron el crecimiento de las lubinas europeas juveniles, demostrando la importancia del ARA para un desarrollo apropiado en esta especie.

2. Cuando los niveles de dietéticos de ARA no cubren los requerimientos para el crecimiento, se produce una deposición selectiva de este ácido graso esencial en leucocitos de riñón anterior, aunque la funcionalidad de esas células inmunes se ve reducida en términos de actividad fagocítica.

3. La suplementación dietética de ARA se reflejó en el perfil de ácidos grasos de diferentes tejidos, incluyendo el riñón anterior, hígado y músculo, con un incremento en la incorporación de ARA al incrementar su nivel en dieta.

4. La suplementación de niveles dietéticos de ARA sobre los 0.2g y hasta los 1.4g de ácidos grasos por gramo de dieta no mejoraron el crecimiento de las lubinas europeas juveniles, aunque se halló una reducción en la deposición lipídica con el incremento de los niveles dietéticos de ARA, lo que se reflejó en una reducción del índice hepatosomático y el contenido de grasa perivisceral.

5. Los niveles dietéticos de ARA son incorporados en las clases lipídicas y, particularmente, en los glicerofosfolípidos del intestino distal (fosfatidilcolina, fosfatidilserina, fosfatidiletanolamina y fosfatidilinositol) de las lubinas europeas juveniles, con una retención selectiva de ARA particularmente cuando sus nivel dietético no cubre los requerimientos para el crecimiento (bajo los 2g de ácidos grasos por gramo de dieta). El contenido de ARA fue mayor que el contenido dietético en todos los glicerofosfolípidos analizados en el intestino distal.

6. Niveles dietéticos de ARA bajo los 0.2g de ácidos grasos por gramo de dieta, se relacionaron con un incremento en la expresión basal de citoquinas proinflamatorias en el intestino distal de lubinas europeas juveniles.

7. La suplementación individual de manan-oligosacáridos (MOS; 0.3 ó 0.6% en dieta) o la combinación sinbiótica de 0.3%MOS y el probiótico *P. acidilactici* en una dieta base baja en harina y aceite de pescado, incrementó el crecimiento de las lubinas europeas juveniles tras 90 días de alimentación.

8. La suplementación sinbiótica de un 0.3% de MOS en dieta y *P. acidilactici* en dietas con bajo contenido de harinas y aceite de pescado, contrarresta la sobreexpresión de genes proinflamatorios, en particular *tnfα, il-1β*, y la producción de Inos, debida al MOS, en el intestino distal, tras 90 días de alimentación (estrategia a corto plazo).

9. La suplementación dietética individual de MOS en bajas dosis, o en un sinbiótico combinado con el probiótico *P. acidilactici*, redujo la mortalidad de las lubinas europeas juveniles, durante el desafío de infección mediante inyección peritoneal con *V. anguillarum*, tras 90 días de alimentación (estrategia de corto plazo).

10. Un período de alimentación de medio plazo (25 semanas) con P o B, individualmente, es la mejor opción para promover el crecimiento lubinas europeas juveniles, indicando la importancia del tiempo de suplementación con dietas funcionales.

11. A largo plazo (39 semanas) la alimentación de lubinas europeas con sinbióticos (PB), o una estrategia alterna de P/PB/P, incrementó el peso final de los peces. Los efectos beneficiosos de la suplementación individual de cada dieta (P o PB) parecen haber sido enmascarados en las estrategias donde se alternaron. La suplementación con el sinbiótico (PB) redujo los niveles de esteatosis hepática y solo o alternado en las estrategias con prebióticos (P/PB/P y /PB/P/PB), incrementó el potencial inmune del suero.

12. A largo plazo (39 semanas) y en base a los valores de expresión génica encontrados, la alimentación con P y PB o su combinación en las estrategias P/PB/P o PB/P/PB pareció reducir parte de la típica respuesta inflamatoria de la inmunidad innata y adquirida en el intestino distal, causada por los bajos niveles dietéticos de harina y aceite de pescado para lubinas europeas, indicando una probable restauración y mantenimiento del estatus homeostático. Este efecto no es evidente con el uso prolongado y continuo de *P. acidilactici* incluido en las estrategias alternas (PB/PB/P o P/PB/PB) o en suplementación continua e individual (B).

13. En términos de capacidad de respuesta tras la infección, una visión global de los datos parece señalar hacia una mejor capacidad de respuesta de los peces inoculados intestinalmente cuando fueron alimentados con las estrategias P y PB/P/PB, la cual no fue evidente en los peces alimentados con las estrategias PB y PB/PB/P. Sin embargo, esto no puede determinar la elección de una estrategia, dado que no se determinó el grado de infección en los peces usados en los análisis.

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