UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Instituto Universitario de Sanidad Animal
y Seguridad Alimentaria

Effects of dietary mannan oligosaccharides (MOS) on European sea bass (*Dicentrarchus labrax*) juvenile culture

Silvia Torrecillas

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controlada de animales acuáticos
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Directors:
Prof. Marisol Izquierdo and Dr. Daniel Montero

A mi familia,

Contents

Abbreviations

AChE Actylcholinesterase
ACP Alternative complement pathway
ACTH Adrenocorticotropic hormone
ADC Apparent digestibility coefficient
ANOVA Analysis of variance
APROMAR Asociación Empresarial de Productos Marinos de España
ARA Arachidonic acid (20:4n-6)
ATP Adenosine-5´-triphosphate
AXOS Arabinoxylo oligosaccharides
BAL Non- specific and bile salt dependant lipolytic enzyme
BHI Brain heart infusion
b-LPH Lipotropic hormone
BSA Bovine serum albumin
CCK Cholecystokinin
CCP Classical complement pathway
CE Cholesterol esters
CFU Colony forming units
CR Catecholamine receptor
CRH Corticotropin releasing hormone
CRP C-reactive protein
DGGE Denaturing gradient gel electrophoresis
DHA Docosohexanoic acid (22:6n-3)
EC European Comission
ECGs Eosinophilic granulocytes
EEC Europe council regulation
EFA Essential fatty acids
ELISA Enzyme linked immuno sorbent assay
EPA Eicosopentanoic acid (20:5n-3)
ETS Electron transport system
EU European Union
FABPs Fatty acid binding proteins
FCR Feed conversion ratio
FI Feed intake
FITC Fluorescein isothiocyanate
FOS Fructo oligosaccharides
G6PD Glucose-6-phosphate dehydrogenase
GALT Gut associated lymphoid tissue
GEP Gastroenteropancreatic system
GH Growth hormone
G-HSH α-melanocyte-stimulating hormone
GLP Glucagon like peptide
GLP-2 Glucagon-like peptide type 2
GLUT2 Glucose transporter type 2
GLUT4 Glucose transporter type 4
GOS Galacto oligosaccharides
GR Glucocorticoid receptor
H&E Hematoxylin and eosin
HBSS Hank´s balanced salt solution
HCl Hidrochloric acid
HDL High-density lipoproteins
HPI Hypothalamus-Pituitary-Interrenal cell
HSC Hypothalamus-Sympathetic nerves-Chromaffin
ICCM Canarian Institute of Marine Science
IFNs Interferons
Ig Immunoglobulin
IMO Isomalto oligosaccharides
ip Intraperitoneal injection
K Condition factor
LCAT Lecithin: cholesterol acyl transferase
LDL Low-density lipoproteins
LPL Lipoprotein lipase
LPS Lipopolisaccharides
MASPs MBL-associated serine proteases
MBL Mannose binding lectin
ME Malic enzyme
MEM Minimal essential medium
MGG May-Grünwald/Giemsa
MLRs Maximum Residue Limits
MMT Million metric ton
MOS Mannan oligosaccharides
MPL Mammalian pancreatic lipase
MT Metric Ton
NADPH Reduced Nicotamide-Adenine-Dinucleotide Phosphate
NAs Natural antibodies
NBT Nitroblue tetrazolium
NBT Nitroblue tetrazolium
NCCs Non-specific cytotoxic cells
PACAP Pituitary adenylate cyclase activating peptide
PAMPs Pathogen associated molecular patterns
PBS Phosphate buffered saline

PGE_2 Prostaglandin E_2
PI Propidium iodide
PI3K Phosphatydilinositol 3 kinase
PKC Protein kinase C
PL Phosphoacylglycerides
PLA_2 Phospholipase A_2
PMA Phorbol 12-myristate 13-acetate
PRP Pattern recognition proteins
PRR Pattern recognition receptors
PUFAs Polyunsaturated fatty acids
RBC Red blood cells
RIA Radioimmunoassay
SAP Serum amyloid protein
SCFAs Short chain fatty acids
scFOS Short chain fructo oligosaccharides
SGLT-1D Glucose co-transporter-1
SGR Specific growth rate
SLGT1 Sodium/Glucose co-transporter-1
TG Triacylglicerides
TSA Tryptone soybean agar
UDP-glucose Uridin diphosphate glucose
UTP Uridin triphosphate
VAM *Vibrio anguillarum* media
VDLD Very-low-density lipoprotein
VFAs Volatile fatty acids
VIE Visible implant elastomer
VIP Vasoactive intestinal peptide
VSI Visceralsomatic index
XOS Xylo oligosaccharides

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Chapter 1

Introduction

1.1. EUROPEAN SEA BASS (*Dicentrarchus labrax*) AQUACULTURE PRODUCTION

Actually, 50% of the total aquatic food consumed worldwide comes from aquaculture (APROMAR, 2010). In 2008, total aquaculture production for human consumption reached 68.4 MMT in contrast with the 67.0 MMT from fisheries sector, and it is expected to reach the 100 MMT in 2030. Consequently, aquaculture represents the animal production sector with higher growth in the last decades, increasing its production from 0.6 MMT in the 50´s to 68.4 MMT in 2008, and achieving a commercial market value of 84791 million €. This production raise has derived also in an increase of fish consumption from 11kg/cap in 1970 to over 20 kg/cap nowadays. In 2008, total fish aquaculture production in the European Union (EU) reached 626650 MT, which signified approximately 2562 million €. Spanish fish aquaculture production represented the 10.2% of the total EU production, achieving a commercial market value of 279 million €.

Regarding marine fish production in the south of Europe and Mediterranean regions, gilthead sea bream (*Sparus aurata*), European sea bass (*D. labrax*) and turbot (*Psetta maxima*) are the main cultivated species. Total sea bass world production in 2009 reached 66738 tonnes increasing its production by a 6.9% from 2008 and it is predicted to continue increasing in the next years.

The main European sea bass producer countries are located in the Mediterranean basin and include Greece, Turkey, Spain and Italy. Spanish sea bass production during 2009 was 13840 tons, increasing a 40.7% from 2008 and representing the 96% of the total sea bass used for human consumption. Canary Islands are the main sea bass producer in Spain. It produces the 32% of the total national production; the rest of the

production is divided between Murcia, Andalucía, València and Catalunya (Fig.1.1).

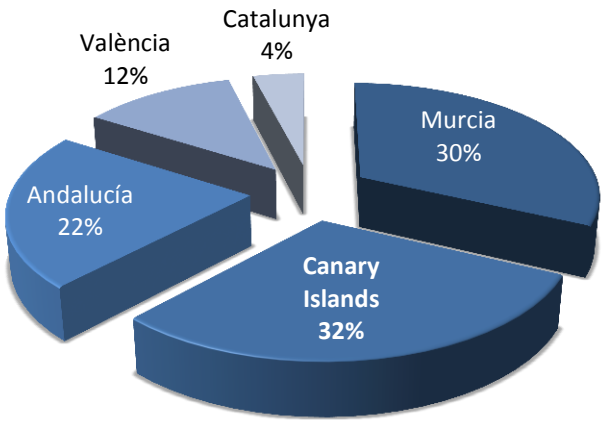


Figure 1.1. Percentage sea bass production in Spain divided by regions (APROMAR, 2010)

Amazingly, and despite its importance in Mediterranean aquaculture (including Canary Islands), European sea bass is a carnivorous species particularly sensitive to handling and disease under culture conditions. This sensitivity imposes two main limitations to the optimization of European sea bass production:

1. **Fragile health and welfare**. One of the main problems for European sea bass production development is its high susceptibility to disease and stress. Main economic losses in aquaculture are derived from disease outbreaks; hence, maintain fish health is determinant in order to achieve optimal production success. Bacterial diseases are considered a very important limitation in juvenile European sea bass culture. In intensive production conditions the relationships between host, environment and pathogen can be altered easily leading in a pathology outbreak that causes more or less serious mortalities and reduced growth rates. In this sense, the reinforcement of the innate immune system in this species through the use of alternative in feed antibiotics could markedly prevent general pathology outbreaks, avoiding the excessive use of therapeutics.
2. **Limited nutrient utilization efficiency.** Marine aquaculture is a relatively new activity and great efforts are actually being done in order to achieve fish better growth or feed conversion ratio (FCR, Ingested food/generated biomass), and consequently, reduce production expenses since the feed suppose approximately the 40-60% of total production costs. In most of the cases,

European sea bass share commercial feed formulation with gilthead sea bream however they are two different species having in the wild different feeding habits and possibly nutritional requirements (Izquierdo, 2005). This fact together with the extreme sensibility of this species to stressors, leads to an average higher FCR than gilthead sea bream, possibly related to nutritional unbalance on the former, since studies on nutritional requirements are much more common in gilthead sea bream than in European sea bass (Oliva-Teles, 2000; Izquierdo, 2005). It has been widely reported that stressful situations affect fish metabolism, usually demanding transitory energy and altering some metabolic routes as gluconeogenesis. Consequently, fish species especially sensitive to handling, as European sea bass, use part of the energy supplied through feed to fight against those stressful situations reducing growth and then, increasing production costs.

1.2 FISH HEALTH AND WELFARE

1.2.1. Innate immune system

The immune system of fish, as in higher vertebrates, is usually divided in two integral components: (1) the innate, natural or non-specific defense system formed by a series of cellular and humoral components and (2) the adaptative, acquired (specific) immune system characterized by the humoral immune response through the production of antibodies and by the cellular immune response mediated by T-lymphocytes, capable of reacting specifically with antigens (Gómez and Balcázar, 2008). However, there is evidence from mammalian and fish immunology, that these are combinational systems (Magnadóttir, 2006). Innate immunity generally will precede specific immunity by activating and determining the nature of the adaptive response and co-operating in homeostasis maintenance (Fearon and Locksley, 1996; Fearon, 1997; Magnadóttir, 2006) by direct cell-to-cell contact involving adhesion molecules and by the production of chemicals agents as cytokines, which can induce a broad range of activities via multiple target cell types (Engelsma *et al.*, 2002; Gómez and Balcázar, 2008). Cytokines that regulate innate immune response by mediating in the inflammatory response are produced by macrophages, nonspecific cytotoxic cells (NCCs) and lymphocytes as response to microbial antigens or compounds released from damaged cells producing migration of leucocytes to the site of infection (Gómez and Balcázar, 2008).

So, when a pathogen gets in touch with the host, innate immune response is activated and it can lead to three situations: (1) success and no disease outbreak or infection; (2) activation of specific immune system or (3) failure, death or infection as indicated in Fig. 1.2.

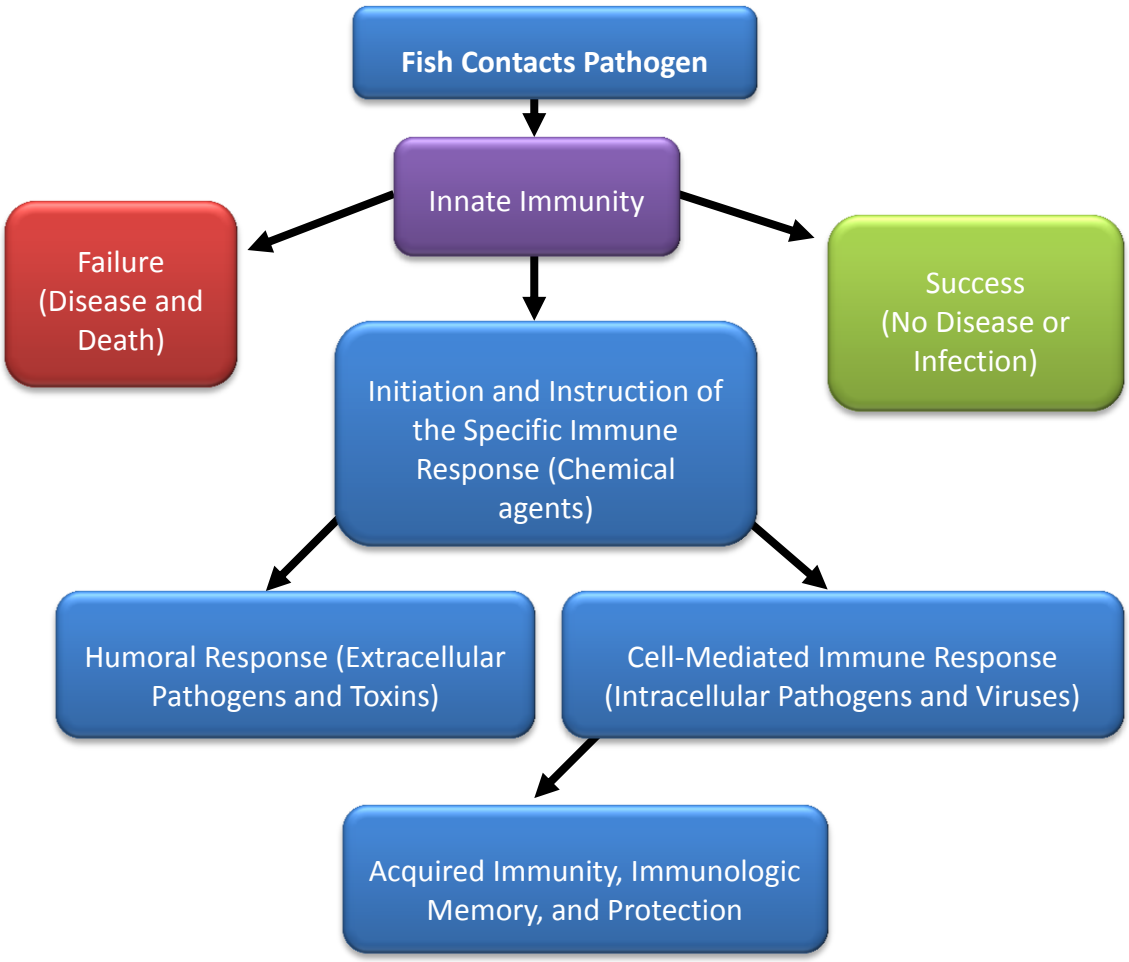


Figure 1.2. Schematic representation of fish response against a potential pathogen.

1.2.1.1. The innate immune system recognition of non-self

The identification of characteristic molecular patterns of microbes, such as lipopolysaccharides (LPS), peptidoglycans, bacterial DNA, double stranded viral RNA and pathogen associated molecular patterns (PAMPs), in the innate immune system of multicellular organisms is carried out through a variety of germline-encoded pattern recognition receptors (PRR) or pattern recognition proteins (PRP) (Janeway, 1989; Kaisho and Akira, 2001; Elward and Gasque, 2003; Magnadóttir, 2006). They are mainly divided into two categories, the first one regarding to recognition of foreign or pathogen associated molecular patterns, and the second regarding to molecular patterns exposed through damage of the host's own tissues due to

infection, necrotic changes and natural cell death (Matzinger, 1998; Magnadóttir, 2006). The PRR can be soluble components like the complement protein C3, lectins and various other humoral innate components or they can be expressed as receptors on phagocytes and other cells of the immune system (Magnadóttir, 2006). When a molecular pattern is recognized by a protective ligand they bound protecting cells from, for example, some humoral innate components, but if these patterns change as for example in apoptosis, the danger signal will induce a PRR response (Elward and Gasque, 2003; Magnadóttir, 2006). Then, when recognition molecules are activated, opsonization and phagocytosis of the pathogen can be induced by stimulation of natural cytotoxic cells or activation of different signalling/executive processes like the complement system and the lytic pathway or an acute phase response (Magnadóttir, 2006).

1.2.1.2. Main parameters of the innate immune system

The components of the innate immune system are divided in physical parameters or ephitelial barriers, cellular and humoral factors.

1.2.1.2.1. Ephitelial barriers

The first barrier against infection includes dermis, epidermis, scales and mucus (Ellis, 2001; Magnadóttir, 2006; Gómez and Balcázar, 2008). The mucosal immune system in fish encompasses the above mentioned tissues and represents the primary barrier to repel pathogen invasion since external tissues are in contact with the external media and therefore with the potential pathogens. Mucous layer forms a viscoelastic gel that protects the epithelium against harmful components (Forstner *et al.*, 1995; Smirov *et al.*, 2005; Faure *et al.*, 2006) and it is considered that normal erosion of the mucus gel is likely to wash away adherent bacteria (Van der Marel *et al.*, 2008). Main structural components of mucus are mucins, but it also contains biological active components as lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and Immunoglobulin-M (Ig-M) (Alexander and Ingram, 1992; Rombout *et al.*, 1993; Nagashima *et al.*, 2001; Hellio *et al.*, 2002; Gómez and Balcázar, 2008) that help preventing the colonization of foreign agents.

1.2.1.2.1.1. Gut mucosal surface

The mucosal surface of the gastrointestinal tract is a complex system combining gastrointestinal epithelium, immune cells and resident microflora (McCracken and Lorenz, 2001)

and its optimal functioning relies on epithelial integrity, mucus production and the presence and equilibrium of commensal bacteria. All these compartments interact closely with each other and are essential for maintaining gut homeostasis and health, providing the front line of defence against pathogenic microorganisms (Faure *et al.*, 2006). Therefore, the final thickness, composition and protective effect of the mucous layer are determined by the dynamic balance between opposing anabolic (synthesis and secretion from goblet cells) and catabolic (physical and proteolytic degradation) processes (Faure *et al.*, 2006; Van der Marel *et al.*, 2008). Intestinal mucins, are the major structural components of the gut mucus layer. They are highly glycosylated macromolecules distinguished by the presence of dense O-glycosylation on the aminoacids serine and threonine, clustered in "mucin domains" (Van Klinken *et al.*, 1995; Magalhaes *et al.*, 2007). They are produced by goblet cells of the surface epithelium, where they help to contain commensal bacteria in the gut lumen by preventing their firm adhesion to epithelial cells (Wilson *et al.*, 1999; Schenk and Mueller, 2008).

Mucins are stored in apically located granules of goblet cells and are secreted at a slow baseline rate to maintain the mucus coat over the epithelium. In response to stimulation, intestinal goblet cells may accelerate the discharge of mucins by compound exocytosis or presumably by accelerating single granule exocytosis (Forstner and Forstner, 1994; Plaisancié *et al.*, 1998). Interactions between mucins and bacteria play a role in the integrity of the mucus barrier and thus may influence its protective properties (Corne *et al.*, 1974). In mammals, regulation of mucus secretion has been coupled to neural, hormonal and paracrine effects as well as to dietary and immunological factors. In fish very little is known about the stimulation of intestinal mucus secretion by dietary factors, although the interactions of dietary components with the secretory activity of goblet cells could represent new interesting possibilities for the manipulation of this important protective function (Barceló *et al.*, 2000; Claustre, *et al.*, 2002).

1.2.1.2.2. Cellular innate immune system

The cellular component of the innate immune system is formed by phagocytic cells (granulocytes, neutrophils, macrophages/monocytes) and the NCCs although epithelial cells and dendritic cells also participate in the innate defense in fish (Magnadóttir, 2006).

NCCs act on a wide variety of target cells as allogenic, xenogenic tumor cells, virus infected cells and protozoan parasites but also participate in antibacterial immunity by eliciting cytokine production and secretion (Jaso-Friedmann *et al.*, 2001; Gómez and Balcázar, 2008).

Phagocytic cells are essential in protection against unwelcomed entities in adverse conditions. Phagocytosis plays an important role in the destruction of microbes as well in the initiation and development of adaptive immune responses by antigen presentation. The initiation of phagocytosis is triggered by the interaction of a particle with a receptor on the phagocyte. Then the phagocyte normally forms pseudopods that engulf and ingest the particle. The ingested particle is contained in phagosomal vesicles that fuse with a lysosome forming a phagolysosome where the invader will be killed by the action of strong antimicrobial mechanisms (oxygen dependent and independent reactions). As a result of this intracellular killing, the residual body will be removed from inside the phagocyte by exocytosis. Recent studies in rainbow trout (*Oncorhynchus mykiss*) have demonstrated that trout B cells are able to perform phagocytosis and kill internalized bacteria (Li *et al.*, 2006), suggesting a close evolutionary step between B cells and macrophages.

Although antibodies (immunoglobulins) are an acquired immune parameter, natural antibodies (NAs) can also be classified as components of the innate system since can be secreted by B cells without prior antigen-specific activation or antigen-driven selection (Magnadóttir, 2006). NAs are polyreactive to phylogenetically conserved structures, such as nucleic acids, heat shock proteins, LPS, viral and parasitic products (Avrameas and Ternyck, 1995; Casali and Schettino, 1996; Bohn, 1999; Boes, 2000; Pashov *et al.*, 2002; Magnadóttir, 2006), and are important in maintaining apoptosis by clearing away apoptotic cells without an immune response (Meyorach *et al.*, 1998; Bohn, 1999; Chow *et al.*, 1999; Boes, 2000; Magnadóttir, 2006). In fish, although exists a high variability in NAs specificity between different species, they play an important role in innate/acquired defense (Vilain *et al.*, 1984; González *et al.*, 1988, 1989; Magnadóttir, 2006) by taking part in both viral and bacterial defense (González *et al.*, 1989; Sinyakov *et al.*, 2002) or by inhibiting specific antibody response when existing high non-specific antibody activity (Sinyakov *et al.*, 2002). It is suggested that the importance of NAs in fish may be greater than in higher vertebrates because fish have neither appreciable affinity maturation responses nor class switch capabilities (Magor and Magor, 2001).

1.2.1.2.3. Humoral innate immune system

Humoral components of fish innate immune system contain proteins and peptides that react against microorganisms and microbial products (Ellis, 1989, Magnadóttir, 2006; Gómez and Balcázar, 2008). Among them, transferrin is a molecule responsible for iron transport and

delivery to cells, acting as a pathogens´s growth inhibitor by chelating the essential available iron needed for their replication and, during acute phase response, removing iron from damaged tissue (Bayne and Gerwick, 2001) and activating fish macrophages (Sttaford and Belosevic, 2003; Magnadóttir, 2006).

Interferons (IFNs) are secreted by host cells in response to recognition of viral dsRNA intermediates (Haller *et al.*, 2006; Gómez and Balcázar, 2008). Two main families of IFNs are classified in function of the site of synthesis, IFN-α is synthesized by leucocytes and IFN-β by fribroblasts. INFs induce Mx expression and other antiviral proteins acting as growth inhibitor (Secombes *et al.*, 1996; Leong *et al.*, 1998; Magnadóttir, 2006). Fish serum and body fluids also have antiproteases, which main function is to inhibit the action of proteases that some microorganisms use to penetrate into the host, having also an important role in homeostasis (Ellis, 1987; Zuo and Woo, 1997; Armstrong and Quigley, 1999; Bayne and Gerwick, 2001; Gómez and Balcázar, 2008). Lytic enzymes are also important in defense against bacteria, even if they act individually or in cascade. They include hydrolases and lysozyme, chitinase, cathepsins, complement (lytic pathway) and other bacteriolytic enzymes (Alexander and Ingram, 1992). Lysozyme can act as a bactericidal hydrolysing β-[1,4] linked glycoside bonds of bacterial cell walls lysing bacteria, but also can act as an opsonin or activating the complement system and phagocytes (Jolles and Jolles, 1984; Grinde, 1989). It is present in mucus, lymphoid tissue, plasma and other body fluids of most fish species. Chitinase, although its role in fish immune defense is not clear, it seems that may be involved in the defense against bacterial and fungal pathogens (Lindsay and Gooday, 1985; Manson *et al.*, 1992).

Other natural lysins in fish serum, commonly detected by their spontaneous haemolytic effect on heterologous erythrocytes, are usually attributed to the activation of the alternative pathway of the complement system (Alexander and Ingram, 1992; Magnadóttir, 2006). The complement system consists in more than 35 plasma soluble proteins that are crucials to the innate and adaptive immunity (Gómez and Balcázar, 2008). Its activation via classical, lectin or alternative pathway, leads in a cascade of biochemical reactions accompanied by the generation of biologically active mediators that result in antigen elimination via cell membrane lysis and activation of nonspecific mediators of inflammation (Holland and Lambris, 2002; Gómez and Balcázar, 2008).

Mucosal or serum agglutinins and preciptins are lectins like C-type lectins and pentraxins. C-type lectins show binding specificity for different carbohydrates like mannose, N-acetyl glucosamine

or fucose in the presence of Ca^{+}, leading to opsonization, phagocytosis and activation of the complement system (Arason, 1996; Magnadóttir, 2006). Most widely studied is the mannose binding lectin (MBL), which shows specificity for mannose, N-acetyl glucosamine, fructose and glucose. This protein binds the capsule of bacteria and triggers the complement cascade. A lectin similar to ficolin has been isolated recently from cod (*Gadus morhua*) (Magnadóttir, 2006). Ficolin is believed to play a role in host defense through opsonization and complement activation in a similar manner to MBL (Matsushita and Teizo, 2001; Kakinuma *et al.*, 2003). Pentraxins (C-reactive protein (CRP) and Serum amyloid protein (SAP)) are commonly associated to acute phase response (Gómez and Balcázar, 2008). CRP main biological role is the ability to recognize pathogens and damaged cells of the host and to mediate their elimination by activating the complement system and phagocytic cells (Jiang *et al.*, 1991; De Haas *et al.*, 2000; Volanakis, 2001; Magnadóttir, 2006) and it is involved in the recognition and clearance of apoptotic cells (Nauta *et al.*, 2003).

1.2.2. Gut microbiota and fish health

Due to fish nature and its continuous contact with a wide variety of pathogenic and opportunistic bacteria that can colonize internal and external surfaces, fish health status depends or is conditioned to the immediate environment (Ellis, 2001; Gómez and Balcázar, 2008). Even though some authors have reported the presence of yeasts in fish microbiota (Gatesoupe, 2007), bacteria are the main constituents in fish microbiota (Spanggaard *et al.*, 2000; Pond *et al.*, 2006; Gómez and Balcázar, 2008). In marine fish the predominant endogenous microbiota are the Gram-negative facultative bacteria such as *Acinetobacter spp, Alteromonas spp, Aeromonas spp, Flavobacterium/Cytophaga spp, Micrococcus spp, Moraxella spp, Pseudomonas spp* and *Vibrio spp* (Cahill, 1990; Onarheim *et al.*, 1994; Blanch *et al.*, 1997; Gómez and Balcázar, 2008). This indigenous gut microbiota will inhibit non-indigenous microorganisms and certain microbial pathogens from proliferating in the mucosal habitats. In addition they will interact with the immunologic system throughout organism life since the “bacterokines” produced by bacterial members of the microbiota will communicate with the cytokine network resulting in an immunologic non-reactivity and tolerance to the microbiota (Savage, 2005). When this tolerance is broken, inflammation can result in mucosa damage. The establishment of protective microbiota is essential to maintain health by competitive exclusion and facilitates immune system development and maturation (Balcázar *et al.*, 2006, Gómez and Balcázar, 2008). For

example, in larvae fish gut microbial colonization takes part in a few hours post-hatching modulating the expression of some genes that will create a favourable habitat for themselves and will prevent invasion by other nondesirable bacteria (Rawls *et al.*, 2004; Balcázar *et al.*, 2006; Gómez and Balcázar, 2008).

It is important to take in mind that composition of endogenous microbiota can be conditioned by genetic, nutritional and environmental factors. To stablish a healthy microbiota plays an important role in the generation of immunophysiologic regulation in fish. Hence, the study of how nutritional factors can influence in the "adequate" microbiota establishment and health management is an actual interesting field of study in aquaculture.

1.2.3. Importance of diseases in the Mediterranean aquaculture: an overview

The fast development of aquaculture and its intensification has resulted inevitably in a progressive increase of the problems linked to the intensive animal production. Successful health maintenance and disease prevention and/or control do not depend on a single procedure but are the consequence of integrated concepts and a management based on the profound and inverse relationship between environmental quality and disease status of fish (Plumb, 1999a) (Fig. 1.3).

Under ideal environmental conditions, healthy looking fish without a clinical sign or lesion can carry pathogens that could occasionate a disease outbreak if stressful conditions, like overcrowding, nutritional deficiencies or inadequate handling, occurs (Fig. 1.4) (Plumb, 1999a). Therefore, avoid stress by maintaining good environmental quality through proper management is essential in order to maintain a healthy and disease free fish population.

Direct and indirect costs of preventing and controlling infectious diseases in aquaculture are estimated to exceed 10% of total production costs (Brown and Johnson, 2008). However, in some instances this estimation may be conservative because for example, some catfish (*Ictalarus punctatus*) operations report that nearly 75% of hatched swim-up fry die before reach marked size (Plumb, 1999b). Thus, infectious diseases cost to the aquaculture sector millions of dollars annually by reduced production, vaccines and prophylaxis.

Toranzo *et al.* (2005) revised the main bacterial pathologies affecting marine aquaculture systems, including Photobacteriosis, Furunculosis, Vibriosis, Marine flexibacteriosis, Pseudomniasis, Streptococcosis and Mycobacteriosis.

Photobacteriosis or pasteurellosis is caused by the halophilic bacterium *Photobacterium damselae subsp. piscicida (Pasteurella piscicida),* and has caused important economic losses in the Mediterranean marine aquaculture during the last two decades, especially in gilthead sea bream, European sea bass and Senegalese sole (*Solea senegalensis*) (Romalde *et al.*, 1999; Magariños *et al.*, 2001, 2003; Toranzo *et al.*, 2005). These pathogens are causing high mortalities together with acute septicemia especially in juveniles. When fish survives to this disease usually derives in "pseudotuberculosis" characterized by esplenomegaly and incidence of white granulomes inside spleen (Quaglio *et al.*, 1991).

Furunculosis is caused by *Aeromonas spp*. and includes acute or chronic septiceamia presenting frequently skin and muscle necrotic areas. It causes economically important loses in cultivated salmonids in fresh and marine waters and it can also affects a wide range of non-salmonid species (Toranzo *et al.*, 2005). Although *Aeromonas salmonicida subsp. salmonicida* is the main causative agent of this disease other subspecies as *masoucide*, *achromogenes* and *smithia* can cause ulcerative diseases in goldfish goldfish (*Carassius auratus*), carps (*Cyprinus spp*.), eel (*Anguilla sp.*), several marine flat fish and salmonids, mainly in Europe and Japan. This pathology caused by *Aeromonas salmonicida subsp. salmonicida* was first described in Spain (Canary Islands) by Real *et al.* (1994) affecting gilthead sea bream.

Vibriosis is caused by *Vibrionaceae* and there are several species considered as pathogens: *V. alginolyticus, V. parahaemolyticus, V. cholera* (non-O1), *V. vulnificus* (Biotype 2), *V. anguillarum, V. ordalii, V. damsela, V. carchariae,* V. *salmonicida* and *Photobacterium damselae subsp. damselae*. At high temperatures, vibriosis presents itself as a haemorrhagic septicaemia, but in colder waters the symptoms are reduced to inactivity, lethargy, melanosis and the presence of erythema in fins and abdomen. More detailed information about vibriosis is compiled in the next subsection.

Pseudomonisasis is mainly caused by *Pseudomonas angullis*eptica in cultured fish (Austin and Austin, 1993; Toranzo and Barja, 1993) and occurs at low temperatures during the winter months. The main fish species affected by this disease is eel, even though, this pathogen is also associated to winter syndrome in gilthead sea bream (Tort *et al.*, 1998), where temperature, stress associated to intensive production conditions, energetic disequilibrium and nutritional deficiencies derived from a long starving period play a crucial role (Zarza and Fonlut, 2002). Recently, *P. anguilliseptica* was also recovered as an emerging pathogen of turbot and black spot sea bream (*Pagellus bogaraveo*) cultured in Spain (López-Romalde *et al.*, 2003). Clinical signs

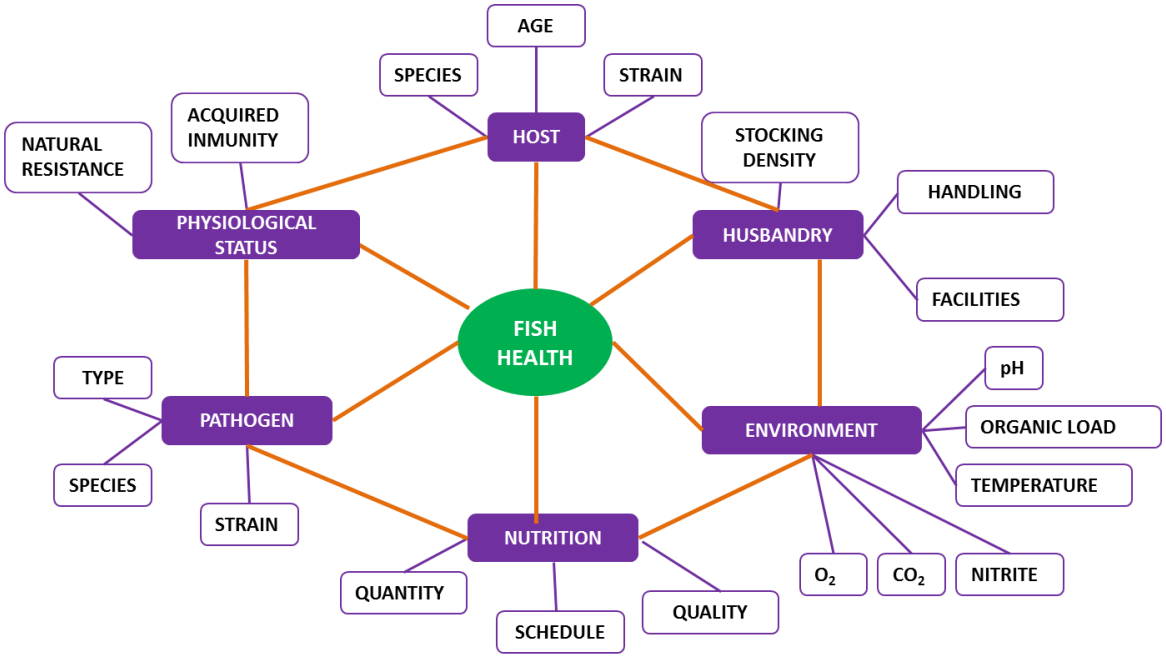


Figure 1.3. Relationship between environmental conditions, biological factors and management practices in aquaculture influencing health and infectious diseases outbreaks in fish. Adapted from Plumb (1999a).

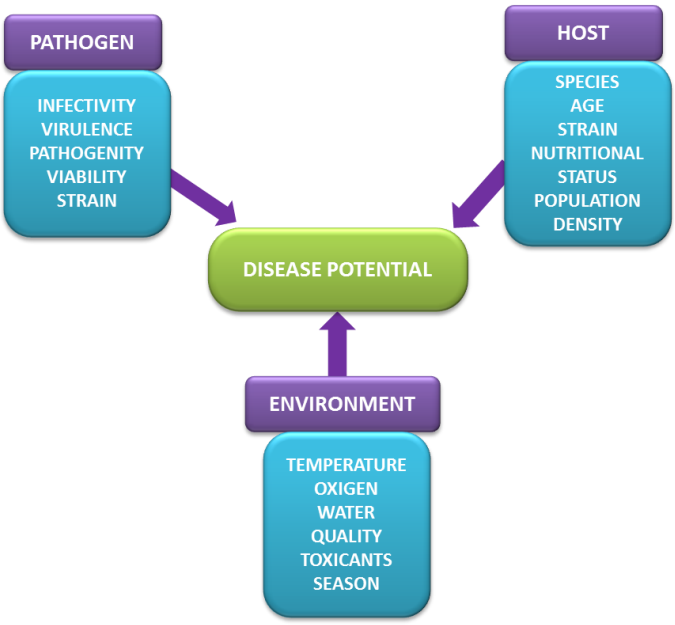


Figure 1.4. Some variables of the infectious agent, host and environment influencing the potential for diseases outbreaks (from Plumb, 1999a).

include haemorrahagic petechia in skin and internal organs as well as abdominal distension. Marine flexibacteriosis is mainly caused by *Tenacibaculum maritimum* (formerly, *Cytophaga marina*, *Flexibacter marinus* and *F. maritimus*) (Wakabayashi *et al.*, 1986; Bernardet and Grimont, 1989; Sukui *et al.*, 2001; Toranzo *et al.*, 2005). Among cultured fish, the disease has been reported in juveniles and adults of several species such as turbot, sole, gilthead sea bream, European sea bass, red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*), flounder and Salmonids (Toranzo *et al.*, 2005). Clinical symptomatology includes eroded and haemorrhagic mouth, ulcerative skin lesions, frayed fins and tail rot. This pathogen attacks gills producing necrotic focuses but also affects the skin, where derived lesions are frequently portals of entry for other bacterial or parasitic pathogens as *Monogea spp*.

Streptococcal infection of fish is considered a re-emerging disease affecting a wide variety of wild and cultured fish throughout the world (Kitao, 1993; Bercovier *et al.*, 1997; Romalde *et al*., 1999; Toranzo *et al.*, 2005). In marine aquaculture streptococcosis is normally caused by *Lactococcus garvieae*, *S. iniae*, *S. agalactiae* and *S. parauberis*. It is characterized by general septicaemia, suppurative, exophthalmia, meningo-encephalitis, and lesions in skin and fins.

Mycobacteriosis is commonly called fish tuberculosis and is considered a chronic wasting disease caused by *Mycobacterium* species, being *Mycobacterium marinum* its main causative agent. During the last decades it has caused important losses in European sea bass production in the Mediterranean area (Colorni, 1992; Colorni *et al.*, 1993, 1996; Toranzo *et al.*, 2005). This disease is considered a matter of concern in the turbot culture in Europe (dos Santos *et al.*, 2002). Internal signs vary according to fish species and mainly include granulomas in spleen, kidney and liver. External manifestations include scale loss accompanied by haemorrhagic lesions which could penetrate in the musculature in advanced cases (Toranzo *et al.*, 2005).

1.2.3.1. Vibriosis

Vibriosis is one of the most prevalent bacterial fish diseases caused by bacteria in aquaculture. Within the *Vibrionaceae* family, two main genera are found: *Vibrio spp* and *Photobacterium spp*. The *Vibrio* species are the most dominant heterotrophic bacteria in the marine environment and are widely distributed in coastal seawaters and/or brackish waters. They are also found on the surface and/or in the gastrointestinal tract of marine animals or other organisms (Jun and Woo, 2003) as well as on the mucosal surfaces and internal organs of

clinically healthy fish. Colwell and Grimes (1984) considered *V. alginolyticus, V. parahaemolyticus, V. cholera* (non-O1), *V. vulnificus* (Biotype 2), *V. anguillarum, V. ordalii, V. damsela, V. carchariae* and V. *salmonicida* as fish pathogens. Since then, more species have been considered to cause economically serious losses in marine culture including *V. harveyi, V. marinus, V. furnissii, V. rnimicus, V. Pelagius, V. splendidus* and *V. tapetis,* (Austin and Austin, 1993; Angulo *et al.,* 1994; Esteve *et al.,* 1995; Saeed, 1995; Álvarez *et al.,* 1998a; Benediktsdottir *et al.,*1998; Wu and Pan, 1997, 2000; Diggles *et al.,* 2000; Jensen *et al.,* 2003; Jun and Woo, 2003; Villamil *et al.,* 2003a). Detailed list of potential pathogenic *Vibrio* species and associated susceptible fish host are compiled and presented in Table 1.1 (Adapted from Jun and Woo, 2003).

Even though, all the *Vibrio* species detailed in Table 1.1 have been described to cause vibriosis in cultured fish, vibriosis is considered to be mainly caused by the pathogen *V. anguillarum* and has been particularly devastating in the marine culture of Salmonids and *Perciformes*. Outbreaks affecting more than 50 species of fresh- and salt-water species have been reported in several countries from the 70´s to nowadays, including Japanese and European eel (*Anguilla japonica* and *Anguilla anguilla*), ayu (*Plecoglossus altivelis*), European sea bass, gilthead sea bream, striped bass (*Morone saxatilis*), cod (*Gadus morhua*), turbot, Pacific salmon (*Oncorhynchus spp.*), Atlantic salmon and rainbow trout (Anderson and Conroy, 1970; Strout *et al.*, 1978; Tolmasky *et al.*, 1985; 1988; Toranzo and Barja,1990, 1993; Actis *et al.*, 1999, Toranzo *et al.*, 2005). Among *V. anguillarum* isolates, a total of 23 O serotypes (O1–O23, European serotype designation) are known to occur (Sørensen and Larsen, 1986; Pedersen *et al.*, 1999; Toranzo *et al.*, 2005) but only serotype O1 (antigenically homogeneous), O2 (antigenic heterogeneity O2a and O2b) and, to a lesser extent, serotype O3 (mainly eel and ayu; antigenic heterogeneity O3a and O3b) have been associated with mortalities in farmed and wild fish throughout the world (Tajima *et al.*, 1985; Toranzo and Barja, 1990, 1993; Larsen *et al.*, 1994; Santos *et al.*, 1995; Toranzo *et al.*, 1997; Toranzo *et al.*, 2005).

The remaining serotypes are considered to be environmental strains and only on rare occasions are isolated as responsible for vibriosis in fish. These serological and genetic studies have high epidemiological value in order to determine the possible origin of the *V. anguillarum* infections as well as to implement adequate vaccination programs in one particular country (Toranzo *et al.*, 2005). This is why some authors recommend identifying the serotype causing the disease outbreak when diagnosing a possible *V. anguillarum* case, in order to use it for future epidemiological purposes (Romalde *et al.,* 1995). Even more, the correlation between serotype

and pathogenity may reflect the ability of the bacterial surface antigens to interact with the host´s tissues, since some studies of surface components (outer-membrane proteins and lipopolysaccharides) demonstrated that these bacterial components are related to the serotypes of the pathogens (Aoki *et al.*, 1981; Nomura and Aoki, 1985; Actis *et al.*, 1999).

Another major fish pathogen within the genus *Vibrio* is *V. alginolyticus.* Some authors consider that the virulent properties of *V. alginolyticus* to fish cannot be firmly established because it varies between and within species and, normally, the onset of vibriosis caused by this pathogen is associated with non-optimal culture conditions or damaged fish (Jun and Woo, 2003). Nevertheless, *V. alginolyticus* has caused severe vibriosis with massive mortality in various fish species throughout the world (Colorni *et al.,* 1981; Austin *et al.,* 1993; Lee, 1995; Saeed, 1995; Woo, 1995; Álvarez *et al.,* 1998a; Balebona *et al.,* 1998; Zhu *et al.,* 2000). In the south of Europe *V. alginolyticus* uses to be isolated after a vibriosis disease outbreak (Balebona, 1994).

Table 1.1. Vibrio species and associated susceptible fish

Vibrio species	Susceptible fish	References
V. alginolyticus	Grouper, *Epinephelus alabaricuss*	Lee, 1995
	Gilthead sea bream, *Sparus aurata*	Balebona *et al.,* 1998
	Turbot juvenile, *Psetta maxima*	Austin *et al.,* 1993
	Silver sea bream, *Sparus sarba*	Li, 2002
V. alginolyticus *V. parahaernolyticus* *V. vulnificus*	Silver sea bream, *Sparus sarba*	Li *et al.,* 1999 Woo *et al.,* 1995
V. alginolyticus *V. parahaernolyticus*	Grouper, *Epinephelus salmoides*	Ong, 1988
V. alginolyticus *V. parahaemolyticus* *V. anguillarum*	Gilthead sea bream, *Sparus aurata*	Colorni *et al.,* 1981
V. anguillarum	Rainbow trout, *Salmo gairdneri*	
	Atlantic salmon, *Salmo salar*	Tiainen *et al.,* 1994
	Turbot, *Psetta maxima*	
	Coho salmon, *Oncorhynchus kisutch*	Toranzo *et al.,* 1987
	Rainbow trout, *Salmo gairdneri*	
	Sea bream, *Acanthopagrus cuvieri*	Rasheed, 1989
	Turbot, *Psetta maxima*	
	Coho salmon, *Oncorhynchus kisutch*	Santos *et al.,* 1991
	Rainbow trout, *Oncorhynchus mykiss*	
	Rainbow trout, *Salmo gairdneri*	Lamas *et al.,* 1994
	Atlantic salmon, *Salmo salar*	Svendsen and Bogwald, 1997
	Sea perch, *Lateolabrax japonicus*	Xiao *et al.,* 1999
V anguillarum *V. ordalii*	Pacific salmon, *Oncorhynchus tshawytscha*	Chart and Trust, 1984; Ransom *et al.,* 1984
V. carchariae	Summer flounder, *Paralichthys dentatus*	Soffientino *et al.,* 1999
	Brown shark, *Carcharhinus plumbeus*	Bertone *et al.,* 1996
	Grouper, *Epinephelus coioides*	Lee *et al.,* 2002

Table 1.1. Vibrio species and associated susceptible fish (Cont.)

Vibrio species	Susceptible fish	References
V. carchariae *V. damsela*	Brown shark, *Carcharhinus plumbeus* Spiny dogfish, *Squalus acanthias* Lemon sharks, *Negaprion brevirostris*	Grimes *et al.,* 1984, 1985
V. damsela	Turbot, *Psetta maxima* Damselfish, *Chromis punctipinnis* Gilthead sea bream, *Sparus aurata* Yellowtail, *Seriola quiniqueradiata* Sole, *Solea senegalensis*	Fouz *et al.,* 1992 Love *et al.,* 1981 Vera *et al.,* 1991 Sakata *et al.,* 1989 Zorrilla *et al.,* 2003
V. mimicus	Red sea bream, *Pagrus major*	Wu and Pan, 2000
V. parahaemolyticus	Iberian toothcarp, *Aphanius iberus*	Alcaide *et al.,* 1999
V. pelagius	Turbot, *Psetta maxima*	Villamil *et al.,* 2003 a, b
V. salmonicida	Atlantic salmon, *Salmo salar*	Totland and Nylund, 1988
V. furnissii	European eel, *Anguilla anguilla*	Esteve *et al.,* 1995
V. harveyi *V. marinus*	Silver mullet, *Mugil curema* Atlantic spadefish, *Chaetodipterus faber* Silver black porgy, *Acanthopagrus cuvieri* Brown spotted grouper, *Epinephelus tauvina* Rainbown trout, *Salmo gairdneri* Atlantic salmon, *Salmo salar* Yellowtail, *Seriola dumerili* Sole, *Solea senegalensis* Atlantic salmon, *Salmo salar*	Álvarez *et al.,* 1998a Saeed, 1995 Zhang and Austin, 2000 Wu and Pan, 1997 Zorrilla *et al.,* 2003 Benediktsdottir *et al.,* 1998
V. splendidus	Turbot, *Psetta maxima* Rainbow trout, *Oncorhynchus mykiss*	Angulo *et al.,* 1994
V. splendidus *V. campbellii-like*	Turbot, *Colistium nudipinnis* Brill, *Colistium guntheri*	Diggles *et al.,* 2000
V. splendidus *V. tapetis*	Corking-wrasse, *Symphodus melops*	Jensen *et al.,* 2003
V. viscosus	Atlantic salmon, *Salmo salar*	Salte *et al.,* 1994
V. vulnificus	Grouper, *Epinephelus sp.* Grouper, *Epinephelus awoara* Eel, *Anguilla anguilla*	Qin and Pan, 1996 Tison *et al.,* 1982 Dalsgaard *et al.,* 1999 Biosca *et al.,* 1991 Amaro *et al,* 1995 Collado *et al,* 2000 Fouz *et al.,* 2000

Both, *V. anguillarum* and *V .alginolyticus* can be diagnosed on standard biochemical tests. For *V. anguillarum*, specific selection media (VAM; Alsina *et al.*, 1994) and commercial diagnosis kits by Enzyme Linked Immune Sorbent Assay (ELISA) or agglutination are available. Besides, a PCR-based approach, having as target the gene rpoN, has been described by Osorio and Toranzo (2002) for the accurate detection of *V. anguillarum* in infected fish tissues.

Vibriosis presents itself as haemorrhagic septicaemia. Fish affected by this classical vibriosis "turns red" due to extensive skin haemorrhages. These are mainly located around the head, abdomen and the inflamed anus as well as the base of the fins. Bilateral exophthalmia, corneal opacity, reduced number of leucocytes (Ransom *et al.*, 1984) and distension of the abdomen are also common symptoms. Internal signs of typical septicaemia include visceral petechiation, splenomegaly and liquefactive renal necrosis (Noga, 2000). The intestine is distended full of transparent fluid (Cisar and Fryer, 1969), and the pathologic symptoms are more severe in the descending gastrointestinal tract and rectum than in the anterior region due to a pH gradient and the limited growth capacity of *V. anguillarum* in acidic medium (Ransom *et al.*, 1984). Frequently, fish affected by vibriosis are anorexic with excessive mucous secretions in gills, but usually not necrotic (Toranzo *et al.*, 2005). Young fry may not show any of the above clinical symptoms but widespread lethargy (large numbers of fry swimming sluggishly close to the surface and the cage nets possibly caused by a swim bladder distension derived from the infection) and darker colouration. In acute and severe epizootics the course of the infection is rapid and most of the infected fish die without showing any clinical signs (Actis *et al.*, 1999).

Lots of efforts have been done in order to minimize the effects on production caused by vibriosis. The uses of vaccination, other bacteria that inhibit the growth of *V. anguillarum* or antibiotics are the most common practices to control this disease. Vaccination by immersion and/or injection is usually successful to prevent the disease and is highly recommended (Qin and Pan, 1996; Woo *et al.,* 2001; Li, 2002). Since, *V. anguillarum* is the main *Vibrio* species causing loses in fish production, there are several commercial vaccines against this pathogen in the market (Newman, 1993; Toranzo *et al.*, 1997; Toranzo *et al.*, 2005). However, only one licensed bacterin (GAVA-3), developed at the University of Santiago (Spain), covers the three antigenic entities of *V. anguillarum* (O1,O2a and O2b) in marine aquaculture (Toranzo *et al.*,1997; Toranzo *et al.*, 2005). In the case of European sea bass two treatments at monthly intervals of aqueous *V. anguillarum* bacterins by bath exposure (1-2g fish) are also used (Toranzo *et al.*, 2005). From the producer's point of view, even vaccination is highly recommended to control this disease, it is necessary to select the appropriate vaccination protocol according to the requirements of each site, consider the extra costs, labour/time and stress derived situations, in particular regarding vaccine administration by intraperitoneal (ip) injection.

Once the pathology has appeared, antibiotics and other chemotherapeutic agents, as chloramphenicol, nitrofurazone, oxolinic acid, oxytetracycline and sulphamerazine are used in fish farms either as feed additives or as components in immersion baths to achieve either prophylaxis or therapy (Actis *et al.*, 1999; Jun and Woo, 2003). Daily administration of antibiotics, mixed in the feed, for 10 days is usually effective to eliminate mortalities (oxytetracycline at 100 $mg \cdot kg^{-1}$ biomass, flumequine at 80 $mg \cdot kg^{-1}$ biomass and oxolinic acid at 60 $mg \cdot kg^{-1}$ biomass, per day) and potentiated sulphonamides (trimethoprim+sulfadiazine) at 70 $mg \cdot kg^{-1}$ biomass per day. However, extensive use of antibiotics and other chemotherapeutic agents has resulted in an increase drug-resistant bacteria, especially *V. anguillarum* and *V. salmonicida* (Hjeltnes and Roberts, 1993) and nowadays some of them had lost effectiveness controlling vibriosis (Li, 2002; Jun and Woo, 2003).

Regarding European sea bass culture, the economic implications of vibriosis is considered severe and it occurs in fish farms once or twice yearly. Vibriosis outbreaks may occur at any time but it is more usual to appear after handling stress, adverse weather conditions or when seawater temperatures are unstable. There are some predisposing factors that should considered to fight against this disease like: overfeeding, overcrowding, fouling of cage nets, recent occurrence of the disease, daily water temperature instability and in particular, environmental stress of any kind. Therefore, reducing stress is imperative for long-term management. All age/size European sea bass classes are susceptible to vibriosis although young fish are more susceptible. The consequences of a vibriosis outbreak in terms of production costs are high, with fish mortalities ranging from 15% to 25% (35% in young fry). Effects on growth have also to be considered due to the prolonged loss of appetite. Extra costs comprise labour for the daily removal, transportation, and sanitary disposition of the dead fish. Time is also required to prepare the medicated feed on a daily basis. All these factors, together with the drug resistant arising bacteria, compromise the optimization of fish production and this is why there is an increasing economic and social concern to decrease the use of antibiotics and other therapeutic chemicals used in fish farming in favour of a more environmentally friendly approaches to control diseases (Hansen and Olafsen, 1999; Verschuere *et al.*, 2000; IUCN, 2007). For instance, there is a great interest in the use of different products and organisms to control potential pathogens by competitive exclusion (probiotics and prebiotics) that has been successful in preventing disease outbreaks in other areas of animal production, as could be mannan oligosaccharides (MOS).

1.2.4. Stress in fish: implications on immune system

In an intensive animal production system as aquaculture, stress is unavoidable due to the frequent exposure of fish to several stressors (transport, overcrowding, vaccination, etc.). Stress has been associated to decreased fish performance and increased susceptibility to infectious diseases whose directly have an economical effect on fish farming industry costs. Thus, lot of efforts have been done during the last decades to study the consequences of the stress derived situations (Molinero *et al.*, 1997; Rotllant *et al.*, 2000; Tort *et al.*, 2001; Acerete *et al.*, 2004; Barton *et al.*, 2005).

Stress can be defined as the condition in which the dynamic equilibrium of an organism is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimulus commonly named stressors (Chrousus and Gold, 1992). Under a stressful situation, the sensors of the immune system perceive the stressors being the primary response (alarm reaction) carried out at the hypothalamus where two major axes are stimulated: The Hypothalamus-Sympathetic nerves-Chromaffin axis (HSC) and the Hypothalamus-Pituitary-Interrenal cell axis (HPI) (Fig. 1.5). The first one is stimulated via the nervous fibers that innerve the chromaffin cells of the head kidney stimulating the release of cathecolamines (Adrenaline and Noradrenaline) via cholinergic receptors (Reid *et al.*, 1996). The second one is stimulated via an endocrine cascade where the corticotropin releasing hormone (CRH) is released by the hypothalamic neurons and stimulates the release from pituitary of adrenocorticotropic hormone (ACTH) which induces the secretion of cortisol by interrenal cells (Vijayan *et al.*, 2005; Alsop and Vijayan, 2008) and in a minor way triggering also the release of intermediate products of ACTH synthesis as α-melanocyte-stimulating hormone (g-MSH) and lipotropic hormone (b-LPH). Cortisol is considered the principal glucocorticoid secreted by the interrenal cells, which are embebbed in the head kidney of teleost fish (Patiño *et al.*, 1987; Balm *et al.*, 1989; Rotllant *et al.*, 2000). It has been shown to induce self-suppression by negative feed-back of its secretion directly at the interrenal cells, at the ventrodorsal hypothalamus suppressing the synthesis and/or release of CHR (Fryer and Peter, 1977), and at the pituitary inhibiting ACTH release (Fryer *et al.*, 1984).

The increase of plasma cortisol concentration in fish derives in a secondary response (stage of resistance) in which the organism tries to adjust its metabolism to cope with the disturbance by activating different metabolic pathways as could be the mobilization/reallocation of energy, osmotic disturbance, increasing cardiac input and oxygen uptake and transfer. This stage can lead in both an adaptive response (allostasis) and a poor-adaptative response. The first

one is a fundamental process through which organisms actively adjust to both predictable and unpredictable events (McEwen and Wingfield, 2003; Varsamos *et al.*, 2006). An allostatic state refers to altered and sustained activity levels of the primary mediators in response to a stressor. The cumulative result of an allostatic state is allostatic load, where, within limits an organism can cope with and adapt to or tolerate stressors to keep homeostasis (Varsamos *et al.*, 2006). If fish have a poor-adaptive response, problems will arise, since the system is not dealing correctly with the stressor and the increased allostatic load results in allostatic overload (McEven and Wingfield, 2003; Varsamos *et al.*, 2006) that could result in a severe stress situation, causing massive mortality or in a sub-lethal stress affecting physiological functions as osmoregulation, reproduction, growth and immune system (Ortuño *et al.*, 2002; Vijayan *et al.*, 2005; Aluru and Vijayan, 2009).

Effects of stress on fish immune system have been widely studied during the last years. The immunosuppressive effects are well known in mammals and in fish (Ellis, 1981) although the mechanisms implied are not completely clarified (Espelid *et al.*, 1996). In view of the fact that whether cortisol acts directly on the immune cells or indirectly through secondary agents is not yet clear. Some of the consequences of increased cortisol levels are direct cytolytic effect on the lymphocytes or as redistribution of immunological cells between lymphoid tissues (Pickering and Duston, 1983; Ellsaesser and Clem, 1986; Wiik *et al.*, 1989, Bly *et al.*, 1990; Varsamos *et al.*, 2006), suppression of mitogenic responses on peripheral blood leucocytes *in vitro* (Espelid *et al.*, 1996), cellular depletion in lymphoid organs, delayed first immune response, inhibition of intracellular phagocytes microbicidal activity and reduction of the humoral response (Pickering and Pottinger, 1989; Pulsford *et al.*, 1995; Narnaware and Baker, 1996; Harris and Bird, 2000). In addition, other physiological parameters like plasma glucose, lactate, ion concentrations and thyroid hormones have been associated to stress response in fish (Pickering *et al.*, 1982; Redding *et al.*, 1984; Laidley and Leatherland, 1988; Robertson *et al.*, 1988; Iwama *et al.*, 1989; Rotllant *et al.*, 2000).

All these suppressive effects on the immune system have been correlated to increased disease susceptibility (Ellis *et al*., 2007). Nevertheless, some authors argue that even if there is detrimental effect on immunological cells and mechanisms, fish seems to handle the endogenous cortisol produced post-stress with short-term suppression of some immune reactions which are not decisive for the total immunological status (Rotllant *et al.*, 2000).

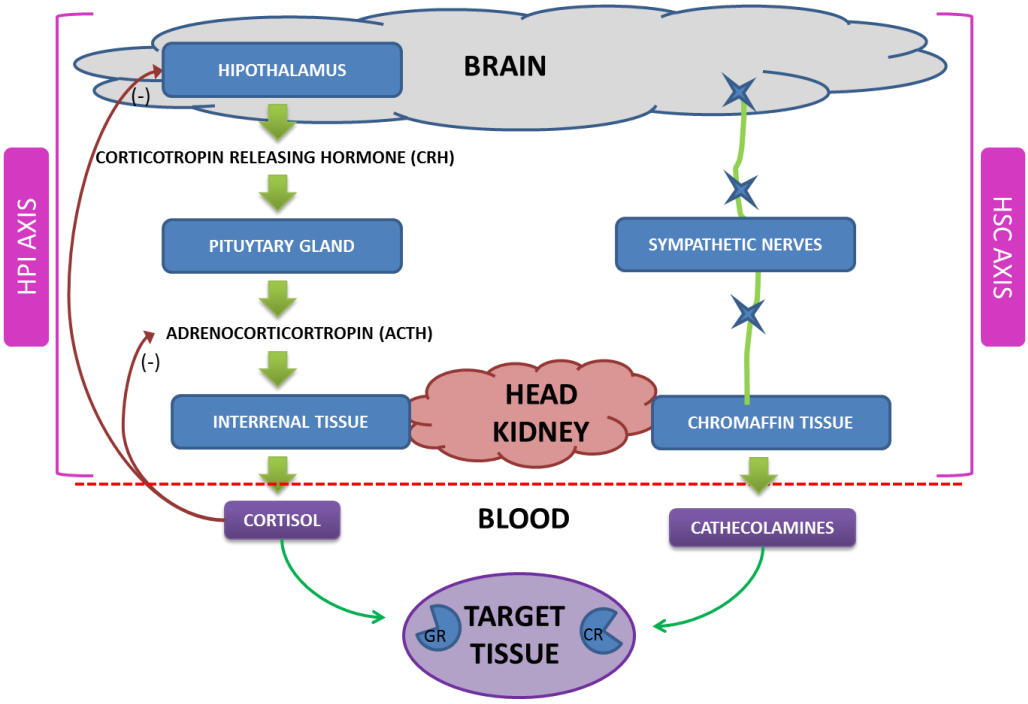


Figure 1.5. Simplified scheme of stress response in fish. Hypothalamus-Sympatehticnerves Chromaffin cell Axis (HSC Axis), Hypothalamus Pituitary-Interrenal Cell Axis (HPI Axis), Catecholamine receptor (CR), glucocorticoid receptor (GR). Adapted from Ganga (2010).

1.3. DIETARY NUTRIENTS UTILIZATION IN FISH

As mentioned previously, the reduction of production costs in terms of FCR is determinant to aquaculture sector. During the last years, fish nutritionists have invest an enormous research effort to understand how to optimize FCR, including studies of metabolic processes involved in dietary ingredients digestion/absorption as well as their hormonal/nervous regulation. This account will draw on various books (*Textbook of veterinary Physiology; Fish Nutrition; Fish Physiology)*, reviews and publications in lipids, protein and carbohydrate digestion, absorption and transport metabolism published on the last years (Buddington *et al.*, 1997; Izquierdo *et al.*, 2000; Bureau *et al.*, 2002; Wilson, 2002; Tocher, 2003; Krogdahl *et al*., 2005).

1.3.1 Nutrients in diets for nutritional requirements of carnivorous fish species

In marine fish nutrition dietary lipids are important to maintain cellular membranes integrity and function, as well as to provide energy. Marine fish lipids are rich in a great variety of saturated and monounsaturated fatty acids, which are "*de novo*" synthesized, whereas polyunsaturated fatty acids must be included in the diet. Three very long chain polyunsaturated fatty acids, namely arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and

docosahexaenoic acid (DHA, 22:6n-3) have a variety of important functions in fish species, as in most vertebrates, but in marine fish they must be provided in the diet since these species have very restricted Δ5 and Δ6 desaturase and elongase activities (Izquierdo, 2005). Inadequate contents of those dietary essential fatty acids (EFA) could produce several alterations such as poor feeding and swimming activities, poor growth and dropping mortality, fatty livers, abnormal pigmentation, disaggregation of gill epithelia, immune-deficiency and raise basal stress levels (Izquierdo, 1996, 2005).

As in other animals, the required dietary protein level for carnivorous fish is influenced by dietary protein digestibility and balance of essential and non-essential amino acids, dietary protein-to-energy ratio and the type and amount of non-protein ingredients included in the diet (Wilson, 2002). The first successful amino acid test diet for fish was developed for chinook salmon by Halver *et al.* (1957), indicating that essential aminoacids for this specie where: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. All other species that have been studied to date have been shown to require the same 10 amino acids (Wilson, 2002).

In carnivorous fish species the amount of carbohydrates present in natural diets is low and consequently, carbohydrate digestion efficiency is lesser compared to omnivorous and herbivorous fish (Cahu and Infante, 1995). Moreover, the metabolic capacity to utilize absorbed carbohydrates is very restricted in carnivorous fish. These facts together with the higher amount of proteins required by carnivorous species, markedly restricts the capacity to efficiently utilize the less costly high carbohydrate and low protein diets (Buddington *et al.*, 1997). Besides, the collateral presence of carbohydrates in dietary ingredients, such as the galactosidic oligosaccharides raffinose and stachyose in soybean meal, causes inflammatory responses denoting the activation of the enteric immune system.

Fish qualitative requirements for vitamins (Kaushik, 2002) and minerals (Gouillou-Coustans and Guillaume, 2001) are similar to those in other vertebrates but the specific quantitative requirements have not been completely determined.

1.3.2. Digestion, absorption and transport of main nutrients in fish gut

1.3.2.1. Digestion processes: general description

Digestion is a very complex process that combines physical, chemical and enzymatic actions specific for each kind of macronutrient, to reduce macromolecules into smaller and absorbable particles.

When food is ingested it may be lubricated with some mucus produced in mouth, pharynx and esophagus, but with a very weak and restricted enzymatic activity. Then, peristaltic movements controlled by and intrinsic nerve network contribute to bring ingested food through the esophagus to the stomach, where mucus is secreted and bolus is mixed with gastric juices before being transferred to the gut where pancreatic and enteric enzymes and juices will continue nutrients digestion.

In species with true stomach as European sea bass, endocrine and nervous system control the secretion of hydrochloric acid (HCl) and pepsinogen (activated to pepsin by HCl) by a single type of cells, the oxynticopeptic cells (Annex II). In addition, mucus is secreted in order to protect the stomach from being digested. As in mammals, NaCl reacts with H_2CO_3 to produce $NaHCO_3$ and HCl, to facilitate the acidic and enzymatic breakdown and nutrient release (Smith 1989; Chakrabarti *et al.* 1994; Rust, 2002). The pylorus controls the transport of food from the stomach to the gut and prevents retreat from the intestine. After digesta passes the stomach, and in order to, first, protect the enterocytes and second, to raise pH to an optimum value where intestinal digestive enzymes effectiveness is maximized, the bicarbonate produced in acinar cells of the exocrine pancreas is secreted by gallbladder bile release. In fish, as in other vertebrates, there is a pH range along the gut based on the enzymes limited ranges of pH to work properly. Usually, stomach pH ranges between 2 and 4, whereas immediately below the pylorus becomes alkaline (7-9), then, in the upper intestine decreases slightly to a maximum of 8.6 and finally became neutral in the posterior segment.

When digesta achieves the lumen, the digested nutrients are transferred from the lumen across the brush border into enterocytes, where absorption can occur by energy-dependent transport (active transport), simple or facilitated diffusion. Simple diffusion follows a non-asymptotic kinetics and it is not usual for hydrosoluble molecules but seems to be common for the resultant products of lipid degradation (Guillaume and Choubert, 2002). In the case of aminoacids and glucose they are absorbed by simple diffusion and active transport being dependant on the presence of protein molecules immersed on the lipidic bilayer. So,

hydrosoluble nutrients transport from the lumen to the blood stream requires both: the accumulation of aminoacids, glucose, nucleic acids, etc. in the enterocytes for the active transport (energy dependent) and the facilitated transport of ATP to the apical membrane (Casirola *et al.*, 1995) as well as the diffusion transporters localized at the basolateral membrane that will transport the nutrients by facilitated diffusion.

Enzymes and mucus secretions could be produced in both pancreatic tissue (released with alkaline solution by the bile duct and secreting the greater variety and quantities of enzymes in fish) or in the gut wall where secretory cells for mucus and enzymes developed in the depths of the folds, migrate to tops of the ridges (closest to the gut lumen), and discharge their products (Rust, 2002). So, in fish, digestion of nutrients could occur extracellulary by the enzymes released in the lumen, membrane linked by the brush border linked enzymes or intracellulary in the supranuclear vacuoles within the enterocytes. Enzymes storage is done in its inactive form (zymogens) and becomes active after they are secreted in presence of an acid or enzymatic catalyst (Annex I and II).

1.3.2.2. Protein digestion

Protein digestion in fish is carried out by proteases and peptidases. First, in the stomach proteins are denaturalized by HCl and partially hydrolyzed by pepsin. On gut, pancreatic trypsin and chymotrypsin and other pancreatic and enteric peptidases continue with the digestion (Linder, 1985). Trypsin and chemotrypsin are secreted as zymogens, then trypsinogen is activated by an enterokinase and the active trypsin it is involved in the activation of chymotrypsinogen to chemotrypsin but also take an important role in the activation of elastase and collagenase (Annex I and II). Both enzymes are endopeptidades and act over specific internal peptidic bonds, trypsin hydrolyzes bonds including lysine and arginine and chymotripsin those of aromatic amino acids (Hoar, 1983). The products of protein hydrolysis are short-chain peptides (di- and tripeptides) in the intestinal lumen (Verri *et al.*, 2009), which are either hydrolyzed to their constituent aminoacids by cytosolic hydrolases (di- and tripeptidasdes), mainly in the proximal segment of the intestine where gut pH is more appropriate for their activity (Buddington and Diamond, 1987a, 1987b; Bakke-McKellep *et al.*, 2000; Nordrum *et al.*, 2000) (Annex II).

The transport of these products across the epithelial barrier appears as a combination of transcellular and paracellular routes (Verri *et al.*, 2009). The transcellular transport is carried

out by a combination of a carrier-mediated route (PEPT1, Na^+ independent, H^+ dependent) and apparent diffusion (Verri *et al.*, 2009). Fish protein transporters distribution pattern seems to differ from mammals, having a lower number per area unit (Buddington *et al.*, 1987b; Storelli *et al.*, 1989) but being present along the whole digestive tube (Buddington *et al.*, 1997). The later route is probably accounting for the majority of total uptake at higher concentrations of peptides (Bakke-McKellep *et al.*, 2000; Nordrum *et al.*, 2000). So, absorbed peptides are either hydrolised to aminoacids within the cell before basolateral exit via amino acid transport systems or leave the cell intact via basolateral transporters (Verri *et al.*, 2009) (Annex II).

1.3.2.3. Lipids digestion

Lipolytic activity in fish is generally greatest in the proximal part of the intestine and the pyloric caeca if present, but can extend into the lower parts of the intestine with progressive activity decease (Tocher, 2003). However, some exceptions do occur as probably a consequence to an adaptation to a short gut as the case of turbot or plaice (*Pleuronectes platessa*) (Koven *et al.*, 1994; Olsen and Ringø, 1997). Low lipolytic activity has also been found in the stomach of several fish species, but usually does not contribute significantly to lipid digestion, so the physiological significance of gastric lipolytic activity in fish is unclear (Tocher, 2003) (Annex I vs II).

The pancreatic tissue is generally assumed to be the major source of digestive lipase enzymes in fish, but digestive lipases are also secreted by the intestinal mucosa although these lipase activities may actually be originally produced in pancreatic tissue, and later absorbed by pancreatic enzyme into the intestinal mucosa (Fänge and Grove, 1979; Smith, 1989; Tocher, 2003). It has been also reported that intestinal cells can secrete lipolytic enzymes (Koven *et al.*, 1994; Tocher, 2003) and also as occurs with carbohydrases, a bacterial origin cannot be excluded (Olsen and Ringo, 1997).

Several types of lipases have been recognized in fish and its pattern of activity over the various digestive tract segments seems to differ with species (Izquierdo and Henderson, 1998). Among them, data suggest that non-specific and bile salt dependant (BAL) is the main lipolytic enzyme in teleost fish, whereas the existence of a pancreatic lipase-colipase system is less certain (Tocher and Sargent, 1984; Lie *et al.*, 1987; Gjellesvik *et al.*, 1992; Izquierdo and Henderson, 1998) playing an important role in digestion of neutral lipids by catalyzing the hydrolysis of carboxyl esters bond of acyl-glycerols and minor dietary fats including cholesterol esters (CE) and vitamin esters (Wang and Hartsuck, 1993; Izquierdo *et al.*, 2000). Mammalian pancreatic lipase

(MPL) has also described in fish and acts over the triacylglicerides (TG) activated by colipase in presence of bile salts (Izquierdo *et al.*, 2000). Phospholipase A_2 (PLA_2) activity has been also widely reported in fish (Izquierdo and Henderson, 1998) and catalyzes the hydrolysis of the fatty acid ester bond at the sn-2 position of phosphoacylglycerides (PL) releasing fatty acids and lysophospholipids in both dietary and biliary PL. The zymogen is released in the gut and it is activated by trypsin only in presence of bile salts although its activity is stimulated in presence of sodium deoxycholate (Iijima *et al.*, 1997; Izquierdo *et al.*, 2000). It is important to mention that MPL does not seem to be the main responsible for the digestion of neutral lipid in marine fish since fats containing polyunsaturated fatty acids (PUFAs) are more resistant to its activity and are more susceptible to BAL (Izquierdo *et al.*, 2000). Then, the main products of lipid digestion in fish are free fatty acids produced by lipolytic action on all major lipid classes. In addition, there will be partial acyl glycerols, predominantly 2-monoacylglycerols, but perhaps also diacylglycerols and glycerol from the digestion of triacylglyerols, 1-acyl-lysoglycerophospholipids from the digestion of phosphoglycerides, and cholesterol and long chain alcohols from the hydrolysis of cholesteryl and wax esters, respectively (Tocher, 2003).

After lipid hydrolysis, dietary fat is uptake on the enterocytes by diffusion of a micellar form of monoglycerides and fatty acids (Izquierdo *et al.*, 2000). The reacilation process can occur by two pathways: the monoglyceride pathway in the smooth endoplasmic reticulum which synthesizes triglycerides and second the L-α-glycerophosphate pathway in both the rough and the smooth endoplasmic reticulum which synthesizes both TGs and PLs (Sire *et al.* 1981; Izquierdo *et al.*, 2000). These compounds are absorbed across the brush border primarily in the anterior and mid intestine and cecum (Sargent *et al.,* 1989; Higgs and Dong 2000) by mainly passive diffusion, and finally exported from the intestine into the submucosa as chylomicrons-like (from high dietary lipid and PUFA) and very-low-density lipoprotein (VDLD)-like particles from high dietary saturated fatty acids (Izquierdo *et al.*, 2000; Tocher, 2003). The lipid transport can occur by two ways: extracellulary and intracellulary. Extracellular transport carries lipoproteins to liver via the portal system (Tocher, 2003) or in a lower extent via lymphatic system (Sheridan *et al.*, 1985). Major plasma protein in fish is similar to mammalian serum albumin and presumably transports free fatty acids from adipose tissue to other tissues through the blood as in mammals (Tocher, 2003) (Annex I vs II). But fish plasma also contains chylomicrons, VLDL, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) formed by the actions of lipoprotein lipase (LPL) and lecithin:cholesterol acyl transferase (LCAT) on LDL (Babin and Vernier, 1989; Tocher,

2003). So, triacylglycerols in chylomicrons and VLDL are hydrolyzed by LPL and hepatic lipase at peripheral tissues with the hydrolysis products being absorbed (Tocher, 2003). Intracellular fatty acid transport in fish seems to work similar to mammals, so it would be facilitated by specific low-molecular-weight and highly conserved cytoplasmic proteins that bind long chain fatty acids and other hydrophobic ligands. Those fatty acid binding proteins (FABPs) in mammals have been characterized in liver, gut, adipose tissue, brain and heart (Tocher, 2003).

1.3.2.4. Carbohydrates digestion

Digestion and absorption of carbohydrates appear to take place along the same general routes in herbivorous, omnivorous and carnivorous fish species although with different efficiency. Polysaccharides are broken down by α- and β-endoglucosidases, whereas di- and tri-oligosaccharides are hydrolyzed by various brush border enzymes into their constituent monosaccharides associated to membrane-linked hydrolysis (Krogdahl *et al.*, 2005) in the anterior intestine (Kuz'mina, 1985; Olatunde *et al.,* 1991) and caeca (Glass *et al.*, 1987, 1989). α-amilase is the most important carbohydrase (Kaushik, 2002) and besides its origin is pancreatic it can be also produce by the digestive tract microflora (Sugita *et al.*, 1997; Krogdahl *et al.*, 2005). Its main function relies in its ability to catalyze the intrahydrolysis of α (1-4)-glucosidic linkages in starch and similar molecules to shorter oligosaccharides, including maltotriose and maltose (Cahu and Infante, 1995; Krogdahl *et al.*, 2005). The disaccharides or glucosides are then hydrolyzed by brush border disaccharidases and glucosidases delivering monosaccharides that can be transported across the villi (Annex II). Fish have other glucosidase activities, usually present only in the liver, where endogenous glycogen breakdown occurs (i.e. γ-amylase) (Mehrani and Storey, 1993; Krogdahl *et al.*, 2005) by a route that bypasses the better-known glycogen phosphorilase (Moon *et al.*, 1999).

Regarding chitin digestion, chitinase is usually associated to fish species consuming chitinous preys. Despite chitinase activity is not restricted to digestive tract (Lindsay *et al.*, 1984; Krogdahl *et al.*, 2005) its higher concentrations are found in stomach and pyloric tissue. Besides, it is possible that a teleostean lysozyme would contribute to digestive chitinase activity (Krogdahl *et al.*, 2005) as well as gut microflora. Cellulase activity has been observed in several fish species denoting the fish ability to digest cellulose and fibrous carbohydrates (Chakrabarti *et al.*, 1994), but it seems that microorganisms may contribute with an important fraction of this activity since this activity is reduced when fish diets are supplemented with antibiotics. Disaccharidases are

frequently localized in the brush border but can also be detected in the gut content and hydrolyze low molecular weight saccharides including the disaccharides maltose (α(1-4) glucopyranosyl glucose), sucrose (α (1-4)glucopyranosyl fructose), and trehalose (α1-α1glucopyranosyl glucose) (Buddington and Hilton, 1987; Harpaz and Uni, 1999; Krogdahl *et al.,* 1999, 2005). Their activity varies within species and exists evidence that their activity patterns do not respond to the amount of dietary carbohydrate (Buddington and Hilton, 1987; Shiau and Liang, 1995; Krogdahl *et al.,* 1999). Thus, the main products of carbohydrate hydrolysis are D-glucose, D-galactose and D-fructose arabinose. However, the way of transport of these products through the brush border in fish is still not completely well understood (Annex II).

In fish, in contrast with mammals, paracellular transport is almost negligible (Ferraris *et al.*, 1990) (Annex I vs II). Monosaccharides are transferred inside the cellular membrane by simple diffusion or by specific carriers located at the brush border and bilateral membrane (Krogdahl *et al.*, 2005). Inositol is transported by a specific carrier and may inhibit glucose transporter due to the existence of an allosteric inositol binding site (Vilella *et al.*, 1990; Krogdahl *et al.*, 2005). D-glucose can be transported by specific carriers or through the basolateral membrane, as in superior vertebrates, in both cases being the transport rate significantly lower than in mammals (Ferraris, 2001). Collie and Ferraris (1995) reported that D-glucose transporters (SGLT1; Sodium/glucose co-transporter 1) are dependent on Na^+ and that they are lower in number than in mammals, so lower glucose absorption occurs in fish intestine. As it occurs in mammals this glucose carrier also transports D-galactose and the competition for transport has been reported in some fish species (Sala-Rabanal, 2004), in fact D-galactose even has a higher affinity for this carrier than D-glucose (Sala-Rabanal, 2004). Molecular studies have evidenced the presence of both GLUT4 (Glucose transporter type 4) (Planas *et al.*, 2000) and GLUT2 (Glucose transporter type 2) (Krasnov *et al.*, 2001) in fish intestine. In carnivorous fish the main absorption of carbohydrates occurs in the anterior part of the intestine and decreases along the intestine. Hormonal control of carbohydrate digestion and absorption in fish also remains unclear. In mammals this system is under hormonal control by vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide (PACAP), cholecystokinin (CCK) and possibly other hormones activating pancreatic protein kinase A and protein kinase C pathways. However, in fish even though, for example, networks of VIP and PACAP-immnunoreactive cells have been reported in the gut (Olsson and Karila, 1995) as well as CCK activity (Rønnestad *et al.*, 2003), the relationship between them and carbohydrate metabolism has not been studied in detail. In the other hand, in

fish has been reported that intestinal glucose uptake can be triggered by glucagon, glucagon-like peptide (GLP) and testosterone (Krogdahl *et al.*, 2005) (Annex II).

Mineral absorption occurs at the gills, skin and gut through selective transport mechanisms. Since they compete for the same uptake site, the correct dose inclusion in the fish diets is essential in order to not saturate the site and reduce the uptake of other minerals.

1.3.3. Control and regulation of digestion

Control and regulation of digestion is a complex system where secretion of digestive juices, peristalsis, enzyme release and activation and hormonal and nervous control of appetite have to be correctly coordinated. Unfortunately, in fish this process is not as well understood as in mammals (Annex I vs II). Many regulators of metabolism are involved in control of appetite, transport and uptake of nutrients as well as in control of blood nutrient level and cell and tissue growth as can be insulin, insulin-like growth factor, glucagon, GLP, pancreastatin, somatostatin, growth hormone (GH), etc. (Rust, 2002). Besides there is a gastroenteropancreatic system (GEP) where neurotransmitters and hormones (Wendelaar-Bonga, 1993) and specific autonomic nerves (Smith, 1989) are involved on the regulation and control of the gastrointestinal processes. Nerves from the cranial, spinal and enteric sections of the autonomic system innervate various parts of the gastrointestinal tract, which in concert with GEP hormones influence in the detection of satiety, control of peristalsis, vasoconstriction/dilation, control of smooth muscle (for regurgitation or relaxation of the stomach to allow distension) and secretion of digestive fluids (Smith, 1989; Rust, 2002). Gut endocrine cells can produce the same major hormones (glucagon, GLP, somatostatin, pancreatic polypeptide, pancreastatin and in some species, insulin) as well as several others (gastrin, CCK, bombesin, enkephalin, VIP, tachykinins, neuropeptide Y-like peptides, neurotensin, secretin, gastric inhibitory peptide and serotonin) (Wendelaar-Bonga, 1993; Rust, 2002). For instance it is known that gastrin stimulates HCl and pepsinogen release in stomach, CCK stimulates gallbladder contraction and secretion of pancreatic digestive enzymes and hormones inhibiting gastric emptying. Bombesin and gastrin releasing peptide stimulates gastrin, CCK and GH secretion, inhibiting feeding behaviour. But compared to higher vertebrates little is known about how they work in fish (Rust, 2002) (Annex I vs II).

1.3.4. Glycogen metabolism in fish liver

Once the nutrients have been absorbed they are transported to the liver through the circulatory (hepatic portal vein) and lymphatic systems where they can be processed and sent to other body tissues, representing the central metabolic organ of the body. The most important role of liver is to regulate the circulating levels of amino acids, lipids and glucose, although it plays an important role in digestion, detoxification, waste removal and bile production. The principal end product of dietary carbohydrates is glucose, which arrives at liver and is metabolized via reversible Emdem-Meyerhoff pathway being phosphorylated to glucose-6-phosphate and stored as glycogen (Annex II). In fish, the reliance on tissue glycogen stores during starvation periods differs from mammals. In mammals, starvation quickly leads to glycogen´s liver breakdown, whereas in fish if hepatic lipids are present in significant amounts, they are the first reserves to be used during starvation (Black and Love, 1986; Lim and Ip, 1989; Blasco *et al.,* 1992; Böhm *et al.*, 1994; Dabrowski and Guderley, 2002), followed by muscle lipids and being muscle protein the last reserve to be mobilized in normal condition. Hepatic glycogen synthesis is based on both; the incorporation of blood glucose into glycogen via production of UDP-glucose (Uridin diphosphate glucose) from glucose-1-phosphate and UTP (Uridin triphosphate) and on gluconeogenesis from lactate and aminoacids which requires the reversal of many glycolytic reactions (Fig. 1.6). Once the glycogen is stored in the liver three main processes are involved in glycogen metabolism: glycolysis, pentose phosphate pathway and Krebs cycle and oxidative phosphorilation.

Glycolysis involves the gradual oxidation of glucose derived from glycogen in order to be exported to the blood, oxidative phosphorilation and other ATP (Adenosine-5´-triphosphate) yielding metabolic conversions (Dabrowski and Guderley, 2002). Pentose phosphate pathway is another process involved and it is related to tissue protection from free radical damage. It depends on the glucose-6-phosphate levels having as principal role the production of NADPH (Reduced Nicotamide-Adenine-Dinucleotide Phosphate) for biosynthetic purposes and to produce ribose sugar for nucleotide synthesis. On oxidative phosphorilation the pyruvate produced by glycolysis is fully oxidized to CO_2 and H_2O in the mitochondria through the combined action of Krebs cycle and the electron transport system (ETS) (Dabrowski and Guderley, 2002).

All these processes are influenced by hormones, being glucagon and GLPs, catecholamines and glucocorticoids the major hormones stimulating glucose liberation from glycogen, while Iinsulin and the insulin-like growth factors are the major hormones stimulating

storage (Fig. 1.7; Annex II). Although glucagon, GLP and cortisol could also stimulate hepatic gluconeogenesis from amino acids (Dabrowski and Guderley, 2002) (Fig. 1.7).

1.4. USE OF ALTERNATIVE IN FEED ANTIBIOTICS IN AQUACULTURE

Aquaculture represents the animal production sector with higher growth in the last decades. But in spite of this, as mentioned before, factors such infectious diseases continue to remain a limiting factor for the development, productivity and profitability of aquaculture production. Unfortunately the use of long term in-feed antibiotics, as treatment for disease outbreaks and growth promoters has been a common practice, leading in the appearance of resistant antibiotic bacteria, overgrowing of non-desirable bacteria or fungi and of course, causing a threat to the environment and human safety (Carrington and Secombes, 2006). Due to this concern, the use of antibiotics has been legislated around the world. In Europe the Council Regulation (EEC) 2377/90 of June 1990 revised by Commission Regulation (EC) 1353/2007 of 20 November 2007 forbids the use of chloramphenicol and nitrofurans as treatments in European aquaculture. Maximum Residue Limits (MRLs) for antibiotics allowed to use in the European Community for fish farming are indicated in Table 1.2.

Then, the increasing economic and social concern to reduce the use of antibiotics and other therapeutic chemicals used in fish farming has encouraged more environmentally friendly approaches to control disease (Hansen and Olafsen 1999; Verschuere *et al.*, 2000) by good management practices and sanitary prevention measures, such as the use of vaccines and the reinforcement of fish immune system (IUCN, 2007). Commonly, a substance that reinforces the immune system is called immunostimulant. During the last years, studies have been focused to get the optimal dose to achieve an immunomodulation stage, avoiding over stimulation of the immune system and the consequent immune suppression. An immunostimulant or immunemodulator can be defined as a chemical, drug, stressor or action that has a modulatory effect upon the innate or non-specific immune response by interacting directly with cells of the immune system. Practically, they are dietary supplements that potentially aid in disease control by the general improvement of innate immune response and even enhancing some parameters related with stress derived situations, originating a better fish welfare that usually results in a production optimization.

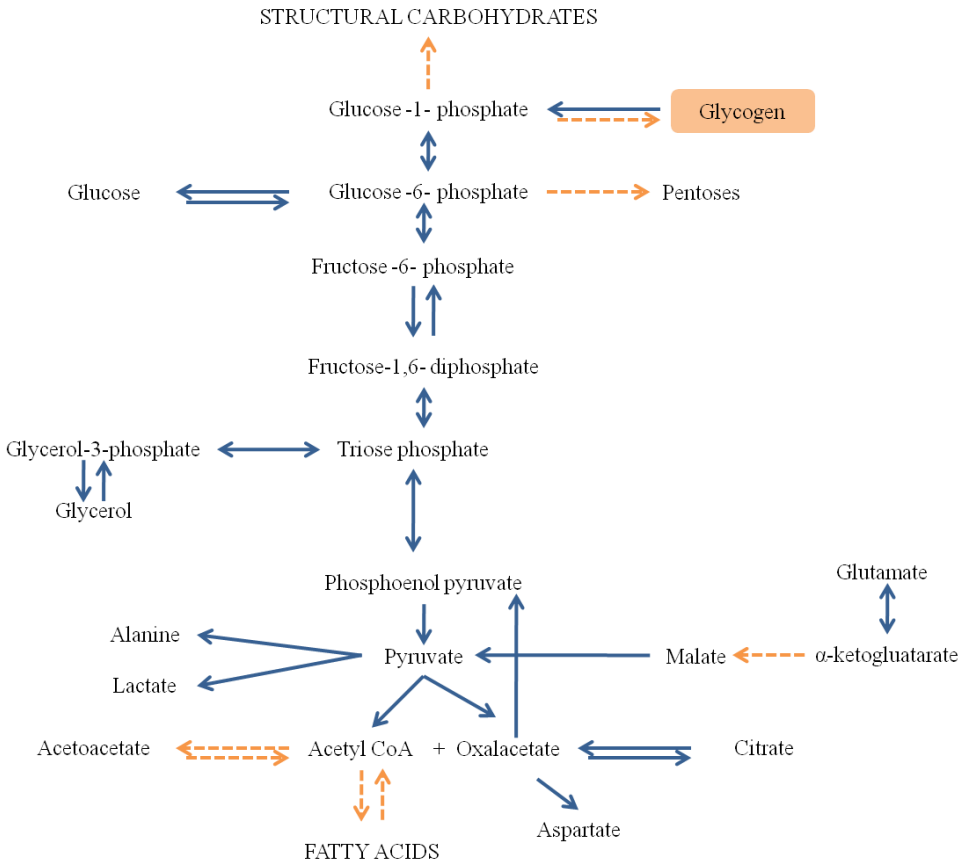


Figure 1.6. Glucose metabolism in liver. Reversible arrows show reaction step or steps catalyzed by same enzymes in both directions. Broken arrows show reactions over many intermediate steps. Paired solid arrows show different enzymes involved in the two directions of the reaction.

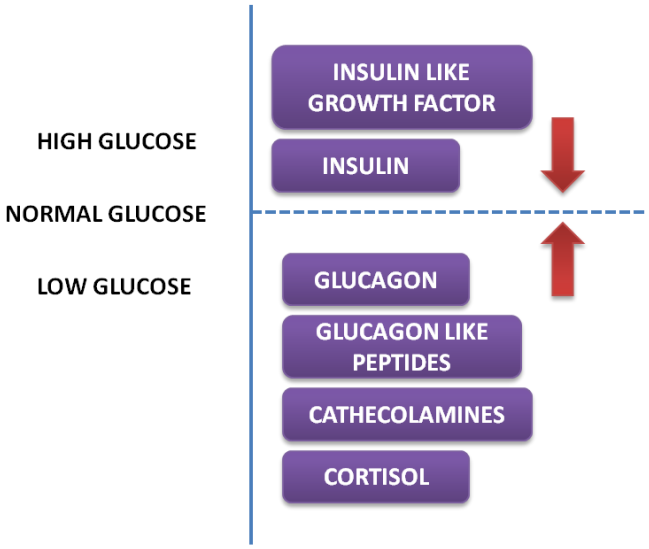


Figure 1.7. Main factors influencing glucose blood regulation levels

Table 1.2. Authorized antibiotics in European Aquaculture, indicating substance, Maximum Residue Limits (MRLs; μg/kg), fish species and Council Reglament that regulates it use. Adapted from the Council Regulation (EEC) 2377/90 of June 1990 revised by Commission Regulation (EC) 1353/2007 of 20 November 2007.

Antibiotic	MLRs	Specie	Council Regulation
Sulphonamide group	100	All food-producing species	
Trimethoprim	50	Fish	
Amoxicillin	50	All food-producing species	
Ampicillin	50	All food-producing species	
Benzilpenicillin	50	All food-producing species	508/1999/EC
Cloxacillin	300	All food-producing species	
Dicloxacillin	300	All food-producing species	
Oxacillin	300	All food-producing species	
Sarafloxacin	30	Salmonids	
Chlortetracycline	100	All food-producing species	
Oxytetracycline	100	All food-producing species	
Tetracycline	100	All food-producing species	
Flumenquine	600	Salmonids	2728/1999/EC
Oxolinic Acid	300	Fish	807/2001/EC
Florfenicol	1000	Fish	1322/2001/EC
Erythromycin	200	All food-producing species	1181/2002/EC

These compounds include a broad range of bioactive products, ranging from synthetic chemicals as levamisole to biological substances that include: (1) Prebiotics as bacterial derivatives or polysaccharides as chitin, chitosan, lentinan and oligosaccharides; (2) animal and plant extracts including for example extracts from abalone and tunicates; (3) nutritional factors as vitamins C and E, and finally (4) hormones, cytokines and others as can be lactoferrin, interferon, growth hormone or prolactin (for review see Sakai, 1999). Table 1.3 and 1.4 indicate a brief summary based on some of the immunostimulants studied in the last years and their effects on immune response and stress parameters respectively. Usually three ways of immunostimulant administration are used: intraperioneal injection (ip), immersion or dietary administration. The first one has the strongest response but entails a high manipulation stress and is not useful with small fish. Administration by immersion reduces manipulation stress but the effect is not as high as in the first case. Finally, the dietary administration is the most used route of supplementation since is the less stressful method and can be used with all fish sizes although higher doses are required.

Therefore, prevention of infectious diseases through effective alternative methods to in-feed antibiotics such as prebiotics, is receiving increased attention in fish production

Table 1.3. Some immunostimulants studied during the last years in fish culture and its effects on immune and biological parameters classified by species.

Species	*Immunostimulant*	*Dose*	*Immune response stimulated*	*Reference*
Gilthead sea bream (*Sparus aurata*)	Vit E	1200 mg/kg	Lysozyme	Cuesta *et al.*, 2002
	α-tocopherol	600-1800 mg/kg	Complement Phagocytes	Ortuño *et al.*, 2000
	Vit C	3000 mg/kg	Complement	Ortuño *et al.*, 1999
	Chitin	1 mg (ip)	Humoral + Cellular	Esteban *et al.*, 2000
	Yeast	1-10 g/kg	Cellular	Ortuño *et al*, 2002
Japanese flounder (*Paralichthys olivaceus*)	Astaxantin	100 mg/kg	Chemotaxis, Respiratory burst	Galindo-Villegas *et al.*, 2002
	Vit C	6100 mg/kg	Respiratory burst	Galindo-Villegas *et al.*, 2002
	VIt E	600 mg/kg	Lysozyme Phagocytosis	Galindo-Villegas *et al.*, 2002
	Arginin	150 mg/kg	Respiratory burst Lysozyme	Galindo-Villegas *et al.*, 2002
	β-Glucan	3000 mg/kg	Respiratory burst	Galindo-Villegas *et al*, 2002
	β-Glucan + Manose	1%	Respiratory burst Lysozyme	Honda *et al.*, 2004
Turbot (*Psetta maxima*)	Vit C	300-2000 mg/kg	Phagocytosis Lysozyme	Roberts *et al.*, 1995
	Vit E	500 mg/kg	Fagocitosis	Pulsford, 1995
Red sea bream (*Pagrus major*)	Vit C	10000 mg/kg	Phagocytosis	Yano *et al.*, 1990
European sea bass (*Dicentrarchus labrax)*	β-Glucan	2% body weight	Humoral	Bagni *et al.*, 2000
Common carp (*Cyprinus carpio*)	Chitin	1%	↓ Growth	Gopalakannan and Arul 2006
	Chitosan	1%	↑Growth Respiratory burst Lysozyme	Gopalakannan and Arul 2006
	Levamisole	250 mg/kg	↑Growth Respiratory burst Lysozyme	Gopalakannan and Arul 2006
Japanese amberjack (*Seriola quinqueradiata*)	α-tocopherol acetate	119-5950 mg/kg 122-6100 mg/kg	Phagocytosis Lysozyme	Hosokawa, 2000 Ito *et al.*, 2000
	β-Glucan	1 ml/ fish	Lysozyme Complement	Engstad *et al.*, 1992
	β-Glucan	0.2%	Cellular ↓ Mortality	Burrells *et al.*, 2001a
	β-Glucan +LPS	10 µg/ml	Respiratory burst	Paulsen *et al.*, 2001
	Levamisole	2,5 ml/l bath	Lysozyme Phagocytosis	Findlay and Munday 2000
	Nucleotides	0.03% 0.03%	↓ Mortality Antibody titers ↓ Mortality ↑Osmoregulation ↑Ht	Burrells *et al.*, 2001a Burrells *et al.*, 2001b

Table 1.3. Some immunostimulants studied during the last years in fish culture and its effects on immune and biological parameters classified by species (Cont.).

Species	*Immunostimulant*	*Dose*	*Immune response stimulated*	*Reference*
Hybrid striped bass (*Morone chrysops* x *Morone saxatilis*)	Levamisole	100-500 mg/kg	↑Growth ↑FCR Respiratory burst	Li *et al.*, 2006
	Yeast	1-4%	↑Growth ↑ Survival (*S.iniae)*	Li and Gatlin III, 2003
	Grobiotic	1-2%	↑ Survival (*S.iniae)* ↑Extracellular superoxide production	Li *et al.*, 2004
Philippine catfish (*Clarias batrachus*)	Bovine lactoferrin	100 mg/kg	Hemolytic activity Phagocytosis	Kumari and Sahoo, 2006
	β-Glucan	0.1%	Hemolytic activity Phagocytosis	Kumari and Sahoo, 2006
	Levamisole	50 mg/kg	Respiratory burst	Kumari and Sahoo, 2006

(Patterson and Burkholder, 2003; Genc *et al.*, 2007). Prebiotics are classified as non-digestible food ingredients that beneficially affect the host by stimulating growth or activity of certain intestinal bacteria, which include mannan oligosaccharides (MOS).

1.4.1. Prebiotics

Prebiotics are classified as non-digestible food ingredients that by stimulating growth or activity of certain intestinal bacteria such us *Lactobacillus spp.* and *Bifidobacterium spp.* beneficially affect health and growth of the host by decreasing the presence of intestinal pathogens and/or changing the production of health related bacterial metabolites. The non-digestible characteristic remains in the fact that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their glycosidic bonds non-digestible to the hydrolytic activity of the human/animal digestive enzymes (Roberfroid and Slavin, 2000; Ringø *et al.*, 2010).

The main described role of prebiotics regards in the potential change of the microbial community to one dominated by beneficial bacteria (Bieklecka *et al.,* 2002; Patterson and Burkholder, 2003; Manning and Gibson, 2004). By this change the fermentation products of the intestine can be altered by increasing the concentrations of volatile fatty acids (VFAs), as described for several terrestrial species (Tsukahara *et al.,* 2002; Smiricky-Tjardes *et al.*, 2003),

Table 1.4. Some immunostimulants studied during the last years in fish culture and its effects on stress parameters classified by species.

Species	*Inmunostimulant*	*Dose*	*Stress Challenge*	*Immune response*	*Reference*
Common carp (*Cyprinus carpio*)	Bovine Lactoferrin	20-200 mg/day	Crowding (30 kg/m^3)	↓ Cortisol	Kakuta, 1998
			Hypoxia	↓Cortisol, Adrenaline, Noradrenaline, Dopamine	Kakuta, 1998
European sea bass (*Dicentrarchus labrax*)	Probiotic (*L. delbrueckii delbrueckii*)	10 bact/ml: By rotifers (11-29 days) By artemia (30-70 days)	Culture conditions	↓ Cortisol	Carnevali *et al.*, 2006
	Ergosan (algal extract + alginic acid)	0.5%	Culture conditions	↑Complement (15 and 30 days) ↑Lysozyme ↑ HSP liver	Bagni *et al.*, 2005
	Macrogard (yeast extract B-glucans)	0.1%	Culture conditions	↑Lysozyme ↑ HSP liver ↑Complement (15 days)	Bagni *et al.*, 2005
White leg shrimp (*Litopenaeus vannamei*)	β-Glucan + Vit C	2g/kg + 0.2 g/kg	↓ Salinity (0‰)	↑Growth ↓ Cumulative stress index	López *et al.*, 2003.
Gilthead sea bream (*Sparus aurata*)	Vit E	0 g/kg	Physical stress (5 min.)	Faster rising of Cortisol ↑Mortality	Montero *et al.*, 2001
	Vit C Vit E Vit C+E	Vit E: 1.2 g/kg Vit C: 3g/kg	Water Stirring (15 min) Crowding (100kg/m^3) Air exposure (2 min)	↓ Glucose ↓ ACH50 ↓ Respiratory Burst	Ortuño *et al.*, 2003
	Nucleotides	Commercial diet	Severe confinement (2h)	↑Bacterilitic capacity, ↑ Lysozyme and stress reponse	Montero *et al.*, 2007
	Vit C and E	250 mg vit C/kg or 250 mg Vit E/kg	Crowding (40 kg/m^3)	↑ Lysozyme ↑ACH50 in Vit E supplemented diet	Montero *et al.*, 1999
Golden shiner (*Notemigonus crysoleucas*)	Vit C and E	Vit E: 0-38mg/kg Vit C: 23-222 mg/kg	Heat (36-37°C) 48 h	↑survival in diets with Vit C + E	Chen *et al.*, 2004
Rainbow trout (*Oncorhynchus mykiss*)	Vit C, E and n-3 HUFA	Vit E: 25.6 – 275.6 mg/kg Vit C: 0-1000 mg/kg n-3 HUFA: 12.5 – 30.5 g/kg	Crowding (100 kg/m^3)	↑Cortisol levels in diets: +Vit E+n-3HUFA -Vit C+Vit E+n-3HUFA	Trenzado *et al.*, 2007

then inhibiting the colonization of pathogens (Manning and Gibson, 2004; Vázquez *et al.,* 2005; Burr, 2008) which is of special interest in fish where gastrointestinal tract is a potential port of entry for some pathogenic bacteria. Even though, other effects have been attributed to the use of prebiotics such as, the enhancement of non-specific immune response and improved mineral

uptake or livestock performance. Prebiotics have been widely studied in terrestrial organisms (Patterson and Burkholder, 2003; Smiricky-Tjardes *et al.,* 2003; Konstantinov *et al.,* 2004).

However, research and application of orally administered prebiotics is in its infancy regarding fish and shellfish production compared to the progress that has been made in the development of prebiotics for terrestrial animals (Patterson and Burkholder, 2003; Ringø *et al.*, 2010). Until the date, the prebiotics studied in fish include inulin, fructo oligosaccharides (FOS), short-chain fructo oligosaccharides (scFOS), mannan oligosaccharides (MOS), galacto oligosaccharides (GOS), xylo oligosaccharides (XOS), arabinoxylo oligosaccharides (AXOS), isomalto oligosaccharides (IMO) and GroBiotic®-A.

1.4.1.1. Mannan oligosaccharides (MOS)

Oligosaccharides are simple carbohydrates containing a low number of monosaccharides, normally three to ten, upon hydrolysis. Bio-Mos® is a natural sugar derived from the other cell wall of a select strain of the yeast *Saccharomyces cerevisiae*, mainly composed by mannan oligosaccharides and produced by Alltech, Inc (Kentucky, USA). The way of action of Bio-Mos® is based in two main functions: blocking pathogen colonization and modulating immune system.

The use of MOS as blocking pathogen colonization evolves from the concept that some sugars as mannose could be used as inhibitors of pathogen adhesion to intestinal cells. Bacterial adhesion is a necessary step in microbial colonization and pathogenesis and it is mediated by interaction with carbohydrates present on cell surface (Bavington and Page, 2005) since bacteria will attach to particular receptor molecules on the cell surface mediated by specific carbohydrate groups. So, the idea of using MOS in animal feed in order to contain pathogenic bacteria attachment, is based in a product that survive the passage through the intestine and mimic these carbohydrates groups, then pathogens with specificity to bind mannose, will attach to the MOS product via MBLs instead to the intestinal villi, being flushed away from the intestine and reducing the incidence/severity of the potential disease.

In the other hand, the effect of MOS as immune modulator is not that clear, since it can be activated by different pathways. But the general hypothesized idea is based in the activation of the PRR and PRP triggering the innate immune response. This activation could be triggered by the MOS itself since for the fish is a non-self-substance, but also by stimulated immune system soluble components acting as PRR.

1.4.2.1. Effects of dietary MOS on fish culture

During the last decade MOS has been tested in a wide variety of farmed animals, ranging from mammals to fish, in order to test the possibility of optimizing production process by enhancing growth, feed conversion or reduce the risk of disease outbreaks by its dietary inclusion. Some of these studies are reported in Table 1.5, where a brief summary of MOS supplementation effects over biological and immune parameters of some terrestrial animal trials are indicated.

Table 1.5. Effects of dietary MOS supplementation on some terrestrial animals.

Species	Parameter	Reference
Poultry	↑FCR	Savage *et al.*, 1997 Sonmez and Eren, 1999 Spring, 1999 Spring *et al.*, 2000 Valancony *et al.*, 2001 Iji *et al.*, 2001 Fritts and Waldroup, 2003 Sims *et al.*, 2004
Poultry	↑ Growth	Savage *et al.*, 1997 Kumprecht *et al.*, 1997 Fairchild *et al.*, 2001 Spais *et al.*, 2003 Miguel *et al.*, 2004
Poultry	↓ Mortality	Stanley *et al.*, 2000 Fritts and Waldroup, 2003 Hooge *et al.*, 2003
Poultry	↑ Phagocytosis	Sisak, 1995
Poultry	↓ *Salmonella typhimurium* intestine colonization	Oyofo *et al.*, 1989a Spring *et al.*, 2000
Pigs	↑ Growth	Pettigrew, 2000 Miguel *et al.*, 2002
Calves	↑ Feed intake	Heinrichs *et al.*, 2001
Dogs	↑ Feed intake	Grieshop *et al.*, 2004

In the aquaculture sector, since it is a relatively new animal production compared with terrestrial animals, the number of studies regarding the effect of MOS is lower. In freshwater fish species, such as common carp, Jian carp (*Cyprinus carpio Var. Jian*.), European catfish (*Silurus glanis*) and hybrid red tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus*), growth and/or feed utilization has been significantly improved by feeding MOS supplemented diets (Zhou and Li, 2004; Hanley *et al.*, 2005; Staykov *et al.,* 2005; Bogut *et al.*, 2006; Culjak *et al.,* 2006). On the contrary, no effect of MOS supplementation was detected on growth of Gulf of Mexico sturgeon

(*Acipenser oxyrinchus desotoi*) or Nile Tilapia (*Oreochromis niloticus*) (Pryor *et al.*, 2003; Vendemiatti *et al.*, 2003). Feeding MOS has been found to modulate the immune response of fish species such as common carp and rainbow trout (Zhou and Li, 2004; Staykov *et al.*, 2005). In other species (White Sea bream larvae, *Diplodus sargus*) MOS supplementation to *Artemia* induced a higher resistance to stress and improved intestinal microvilli structure (Dimitroglou and Davies, 2004). However, few studies to date have demonstrated effects of feeding MOS on disease resistance in fish.

Therefore, most of the recent work on dietary alternative in feed antibiotics inclusion, and particularly MOS, has been focused on the reinforcement of fish defense mechanisms. In contrast, very little is known on the potential mechanisms implied on its improvement of growth or feed efficiency, even in terrestrial animals. The growth capacity of fish can be conditioned by several factors, including digestion and feed conversion efficiency or poor assimilation of nutrients.

Studies regarding the effect of dietary prebiotics and especially MOS, supplementation over the digestion/absorption of proteins in general are scarce, but especially in fish are largely unknown. A prebiotic-enzyme preparation added to a broiler diet containing poultry by-product meal up to 20% by weight was shown to increase protein efficiency and feed conversion (Kirkpinar *et al*., 2004). Iji *et al.* (2001) reported an increase of specific activities of leucine aminopeptidase and alkaline phosphatase, as well as higher L-tryptophan uptake on broiler chickens jejunum after MOS supplementation whereas no effect was found on ileum. Authors relate enhanced L-tryptophan uptake to a better functional integrity of membranes derived from the use of MOS.

Most effects of prebiotics on lipid metabolism are mainly related to changes on cholesterol absorption or reduced fat deposition but mechanisms implied are still poorly understood. A study on diabetic rats found that when dietary XOS was included in the diet, serum cholesterol and triglycerides were reduced in diabetic rats and liver triglycerides increased to a comparable level seen in healthy rats (Imaizumi *et al.*, 1991). Other studies have examined FOS, which were also found to reduce blood lipids (Bornet *et al.*, 2002; Roberfroid, 2002) probably through an inhibition of lipogenic enzymes in the liver, which may be a result of the action of propionate produced from the fermentation of prebiotics by gut bacteria (Wolever *et al.*, 1991). A reduction of fat deposition in liver and hypocholesterolemic effect in liver and eggs after fed laying hens 0.1% MOS (Young *et al.*, 2000) and abdominal fat pad percentage as

expressed as carcass weight in broilers (Kannan *et al.,* 2005) has been also reported. Other prebiotics have been suggested to decrease lipogenesis by a reduction of acetyl-CoA carboxylase activity in chickens (Santoso *et al.,* 1995; Kannan *et al.,* 2005). Nevertheless, reduction in fat deposition could be also related to an increase in lipolytic activity as described in rats (Gallaher *et al.,* 2000). Several studies have also shown chitosan as hypocholesterolemic in animal models (Sugano *et al.,* 1978, 1980; LeHoux and Grondin, 1993; Razdan and Pettersson, 1994; Gallaher *et al.*, 2000), but how prebiotics reduce cholesterol remains uncertain. Some authors argued that their action as weak anion exchanger could mediate in their hypocholesterolemic effect, whereas others related this property to a reduction of VLDL particles since hepatocytes isolated from rats fed FOS incorporate significantly less palmitate into triglycerides than control liver cells (Gibson and Roberfroid, 1995).

Besides, increased bile acid excretion caused by prebiotics could also reduce cholesterol absorption because plasmatic or liver cholesterol would be utilized to maintain bile acid pool (Jie and Shu-Sheng, 1997; Gallaher *et al.*, 1999, 2000). Nevertheless, bile acid binding within the small intestine could disrupt micelle formation leading to a reduced ability to solubilize cholesterol and, consequently reducing its absorption (Nauss *et al.*, 1983; Gallaher *et al.*, 2000). Han *et al.* (1999) reported an inhibition of mice pancreatic lipase *in vitro*, arguing that this inhibition could result in a higher accumulation of lipid emulsion then resulting into a greater fat excretion after treat rats with a chitin-chitosan mixture. Other authors have related changes in intestinal supernatants viscosity with decreased cholesterol absorption in rats after chitosan supplementation (Gallaher *et al.*, 1993a, b; Carr *et al.*, 1996). In this sense, Konjac mannan (Shimizu *et al.,* 1991) and glucomannan hypocholesterolemic effect in rats appears to be mediated through a viscosity-associated specific reduction in cholesterol absorption (Gallaher *et al.*, 2000).

The effects of prebiotics over the digestion/absorption systems of carbohydrates are also scarce, especially in fish. Iji *et al.* (2001) reported an increased specific activity of maltase in broiler chickens jejunum after MOS supplementation and in pigs, glucose uptake was significantly higher in the intestine of animals fed diets supplemented with prebiotics (Breves *et al.,* 2001; Gatlin *et al.*, 2006). An increase of the sodium D-Glucose co transporter-1 (SGLT-1) was related to the stimulation of the sodium dependent D-Glucose uptake into brush border membrane in humans after fed with prebiotics (Eberl, 2005). Many of the physiological effects of prebiotics are linked to the promotion of determinant bacterial species in gut, being this promotion linked to an

increased production of Short Chain fatty Acids (SCFAs). Tappenden and McBurney (1998) found that an increase in SCFA contributed to elevated serum GLUT2 and serum GLP-2 and proglucagon mRNA of rat intestine.

Then, based on these evidences, further studies are needed in order to elucidate the effects and possible mechanisms involved in the mode of action of MOS regarding the main produced marine fish species and especially those that seem to be more sensitive to culture conditions as could be European sea bass.

1.5. OBJECTIVES

The overall aim of this thesis was to investigate the effects of MOS dietary supplementation on European sea bass.

For that purpose several specific objectives were addressed:

1. To find out the effects of different levels of dietary MOS supplementation on European sea bass culture performance, determining the optimal dose of MOS supplementation for European sea bass juveniles during the most disease and stress sensitive production (30-120 g).
2. To investigate the effect of MOS dietary supplementation on European sea bass health in terms of immune system parameters and disease resistance, and the potential mechanisms implied such as those related to pathogen infection, gut morphology, mucus production, gut associated immune system (GALT) and gut prostaglandins production.
3. To study the effect of MOS dietary supplementation on European sea bass welfare, studding its response to stress challenges and their interactions with pathogen resistance.
4. To better understand the effects of MOS supplementation growth and fish metabolism, and the potential mechanisms involved, such as nutrients digestibility or liver functioning and metabolism.
5. To study the effects of MOS supplementation along the whole production cycle on the quality of the European sea bass fillet produced.

Objective 1 was addressed in Trials I (Chapter 3), II (Chapter 4), IV (Chapter 6) and V (Chapter 7). Objective 2 in Trials I, II, III, IV and V (Chapters 3-7). Objective 3 in Trial IV (Chapter 6), Objective 4 in Trials I and II (Chapters 3 and 4) and objective 5 in Trial II (Chapter 4).

Chapter 2

Materials and Methods

2.1. DIETS

In each experiment, different isonitrogenous and isolipidic diets based in a commercial formulation were designed, depending on the experiment, to contain 0 $g \cdot kg^{-1}$ (Control), 2 $g \cdot kg^{-1}$ (BM2), 4 $g \cdot kg^{-1}$ (BM4) and 6 $g \cdot kg^{-1}$ (BM6) of MOS (Bio-Mos©, Alltech Inc., USA) replacing standard carbohydrates. Diets covered the nutritional requirements for this species (Izquierdo, 2005). In Trials I, II and III (Chapters 3-5), diets were manufactured by a commercial feed producer (Graneros de Tenerife S.A., Tenerife, Spain), whereas in trial IV and V (Chapters 6 and 7) diets were produced at the GIA experimental feed production plant as described below. Table 2.1 reports formulation and proximate composition.

During the first trial, fish were fed 0 $g \cdot kg^{-1}$ (Control), 2 $g \cdot kg^{-1}$ (BM2) and 4 $g \cdot kg^{-1}$ (BM4) MOS in diets commercially manufactured. In the second experiment the same MOS levels were tested together with a higher inclusion level of 6 $g \cdot kg^{-1}$ (BM6). Results obtained in both studies determined the level of MOS inclusion in the last two trials, where 4 $g \cdot kg^{-1}$ (BM4) was choose as the optimal MOS dose to use in European sea bass juveniles ranging from 35 to 120 g (19-23.7°C).

2.1.1. Diet formulation

Prior to Trilas IV and V diets preparation, the ingredients, obtained from a commercial feed producer, were analysed for proximate composition in GIA laboratories, to prepare the diets based in a sea bass

commercial diet formulation (Table 2.1). Diets were tested for peroxide index before starting feeding.

2.1.2. Diet preparation

The first step in diet preparation was to mix the active product (MOS) with the wheat meal, and then all the vegetable meals were added, one by one, and mixed carefully. Afterwards, the vitamin premix was added to the mixture and homogenized, whereas, separately, fishmeal was mixed with the mineral premix. Then, both mixtures (vegetal + vitamins and fish meal + minerals) were mixed together until obtaining a homogeneous mixture. Next, the meal mixture was complemented with a mixture containing myoinositol, potassium phosphate, calcium carbonate and sodium chloride. Independently, vegetable oils, fish oils and antioxidants were mixed together and then added carefully to the meal mix, first manually and then using a horizontal mixer DANAMIX BM-330 (Danamac, S.A., Guipuzcoa, Spain). At this step choline chloride was dissolved in distilled water and mixed with the whole mixture using the same horizontal mixer. When the whole mixture was hydrated, the feed was pelletized using a MOBBA 2 HP (Mod. 8.3) pelletizer machine (Eriez Magnetics, UK) through a 3mm diameter mold. The pellets obtained were dried at 38°C during approximately 16h and stored at 4°C until feeding. Samples for biochemical composition were taken and stored at -80°C until analysis.

2.2. GENERAL DESCRIPTION: FISH, EXPERIMENTAL DESIGN AND SAMPLING PROTOCOLS

A general overview of the methodology and materials used in the five main feeding experiments are included in this section. These experiments represent a gradual and progressive research line on the effects of dietary MOS supplementation for European sea bass juvenile culture regarding growth performance, disease resistance and possible mechanisms involved.

All the European sea bass juveniles used along the study were obtained from a local farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain) between 2005-2009, and were transferred to the main facility of the Canarian Institute of Marine Sciences (Instituto Canario de Ciencias Marinas; ICCM, Las Palmas, Spain) where all the experiments were carried out. Along

the five trials performed, a general protocol of fish acclimation and distribution into the experimental tanks was followed. Fish were maintained in indoor stocking 1000 l fiberglass tanks until being well adapted to the environmental conditions (at least 3 weeks). Afterwards, fish were randomly distributed into the experimental tanks and fed the corresponding experimental diet depending on the trial.

In all the experiments, standardized culture conditions were followed. Tanks were supplied with filtered sea water (37 ppm salinity) in a water open system under natural photoperiod (aprox. 12L:12D). Temperature and water dissolved oxygen were daily measured using an Oxy Guard-Handy beta instrument (Zeigler Bros, Gardners, USA). Depending on the experiment, 500, 1000 l fiberglass or digestibility tanks (120 l) were used (Fig. 2.1a, 2.1b and 2.1c, respectively). A water micro-aeration system was used to reduce stress, following the recommendations of Carvalho *et al.* (2004) for European sea bass.

Detailed experimental designs and samplings followed protocols are described in the chapters corresponding to each experiment. Only a summarized schematic representation for each individual trial has been included in this Materials and Methods section (Fig. 2.2, 2.3, 2.4 and 2.5 corresponding to trials I, II, III, IV and V, respectively).

2.3. BIOLOGICAL PARAMETERS EVALUATION

2.3.1. Relative growth

In order to observe the potential differences in efficacy among the different levels of MOS inclusion tested, relative growth was evaluated. Relative growth was defined as the relation between the increases in biomass (g) compared to initial weight (g). This parameter could be expressed in absolute values as well in percentage and it is corrected in relation to the individual fish weight through the following equation:

Relative growth = [(Final weight – Initial weight) / Initial weight] x 100

2.3.2. Specific growth rate (SGR)

This parameter indicates the increase in weight gain in relation to the number of days of feeding period and it is expressed in percentage values.

SGR= [ln Final weight – ln Initial weight) / days] x 100

Figure 2.1. Tanks used along the study. (a) 500 l; (b) 1000 l; (c) Tanks provided with a faeces collection system described by Cho *et al.* (1985) and modified by Robaina *et al.* (1995).

2.3.3. Feed conversion ratio (FCR)

This parameter was evaluated in order to determine the efficiency of the diets to promote growth in terms of ingested food. It is defined as the relation between the ingested food (g) and the generated biomass (g).

FCR = Ingested food / Generated biomass

Table 2.1. Main ingredients and composition in the experimental diets

Ingredient	**Diet (g kg^{-1} dry weight)**										
	Trial I			**Trial II**				**Trial III**		**Trial IV and V**	
	C	**BM2**	**BM4**	**C**	**BM2**	**BM4**	**BM6**	**C**	**BM4**	**C**	**BM4**
Fish meal [1]	515.0	515.0	515.0	515.0	515.0	515.0	515.0	515.0	515.0	515.0	515.0
Soybean meal	97.8	97.8	97.8	97.8	97.8	97.8	97.8	97.8	97.8	100.0	100.0
Wheat	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	55.0	55.0
Wheat gluten	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	86.0	86.0
Corn meal	65.3	63.3	61.3	65.3	63.3	61.3	59.3	65.3	61.3	64.0	60.0
Fish oil [2]	126.9	126.9	126.9	126.9	126.9	126.9	126.9	126.9	126.9	135.0	135.0
Fats and oils	20.3	20.3	20.3	20.3	20.3	20.3	20.3	20.3	20.3	20.3	20.3
Mineral mix [3]	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3	14.3
Vitamin mix [4]	10.3	10.3	10.3	10.3	10.3	10.3	10.3	10.3	10.3	10.3	10.3
Antioxidant (BHT)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
MOS [5]	0	2	4	0	2	4	6	0	4	0	4
	Composition (% dry weight)										
Crude Lipids	28.62	27.38	28.89	24.07	24.82	24.04	23.43	24.07	24.04	20.37	20.32
Crude Protein	44.57	46.81	47.82	48.71	48.77	48.33	48.37	48.71	48.33	51.62	52.07
Ash	8.85	9.48	9.28	9.98	9.81	9.86	9.69	9.98	9.86	9.74	9.82

[1] Peruvian fish meal (65% protein). [2] Peruvian fish oil. [3] TROUW Seabream/Seabass (1g) Calcium carbonate (0.2g), Potassium monophosphate (0.19g) and NaCl 97% (0.04g).
[4] TROUW Seabream/Seabass (0.8g), Choline chloride (0.17g) and Inositol (0.06g). C= 0 g kg^{-1} MOS; BM2=2 g kg^{-1} MOS; BM4=4 g kg^{-1} MOS; BM6=6 g kg^{-1} MOS.

Figure 2.2. Schematic representation of experimental design and sampling followed along Trial I.

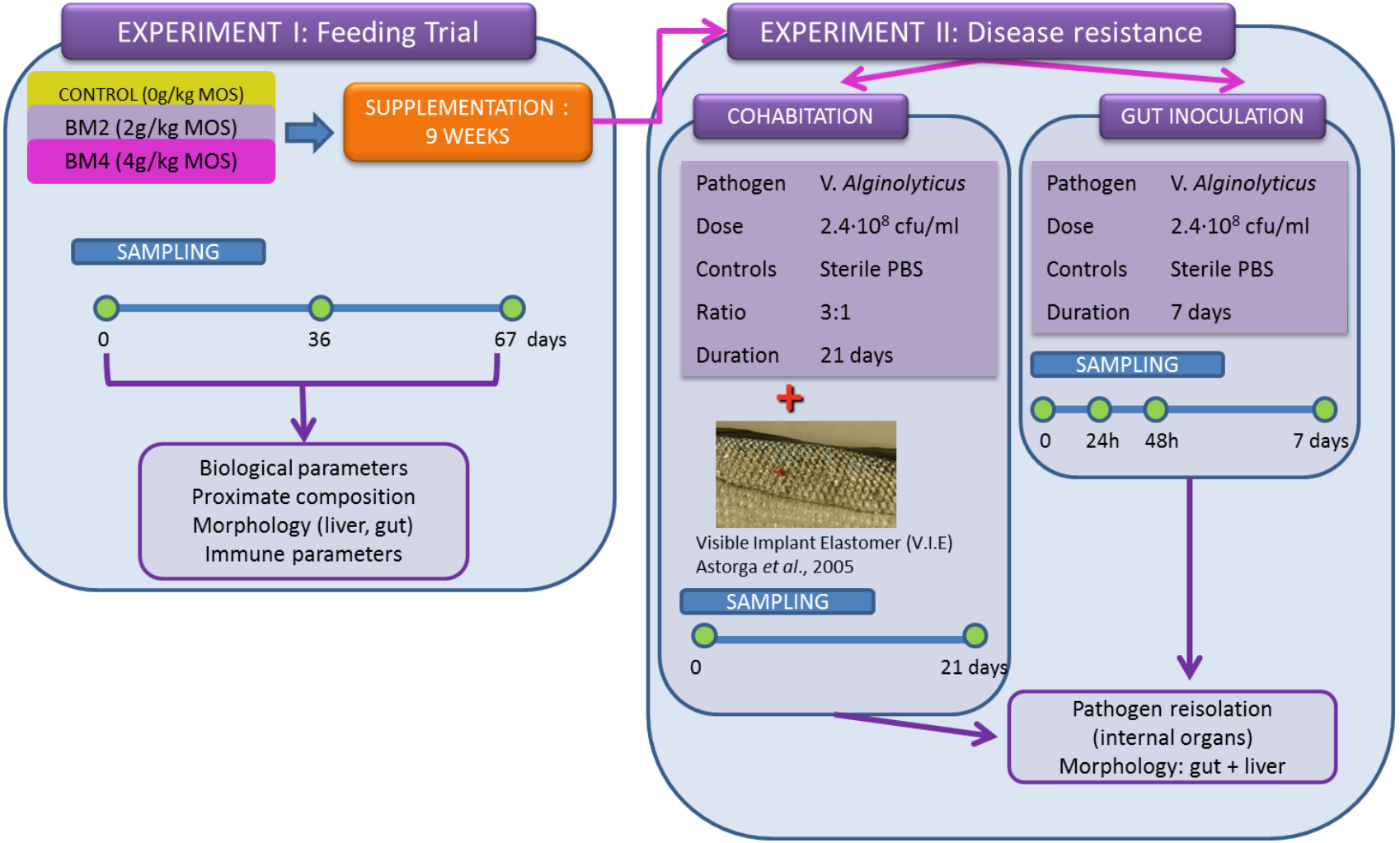


Figure 2.3. Schematic representation of experimental design and sampling followed along Trial II .

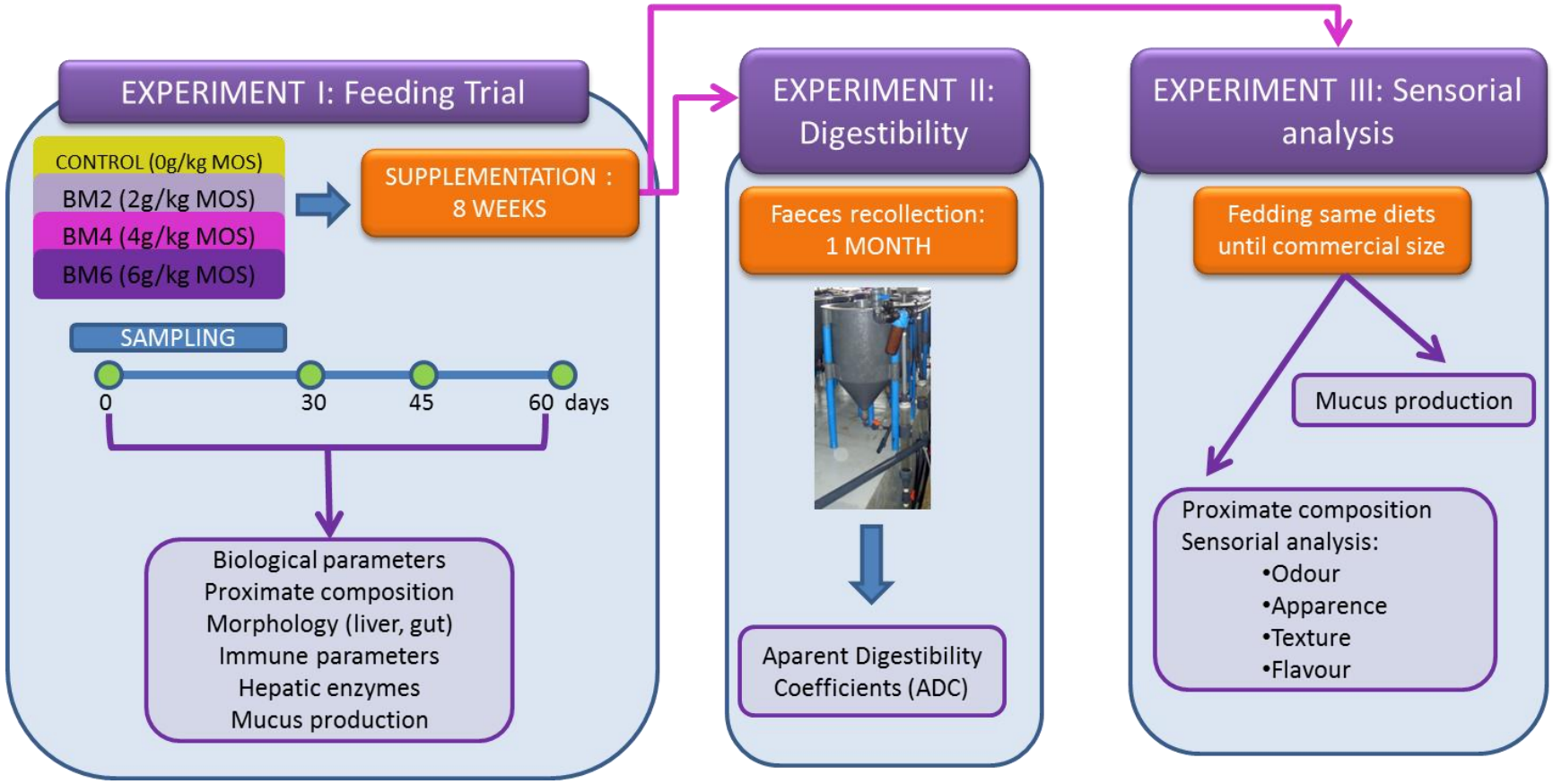


Figure 2.4. Schematic representation of experimental design and sampling followed along Trial III.

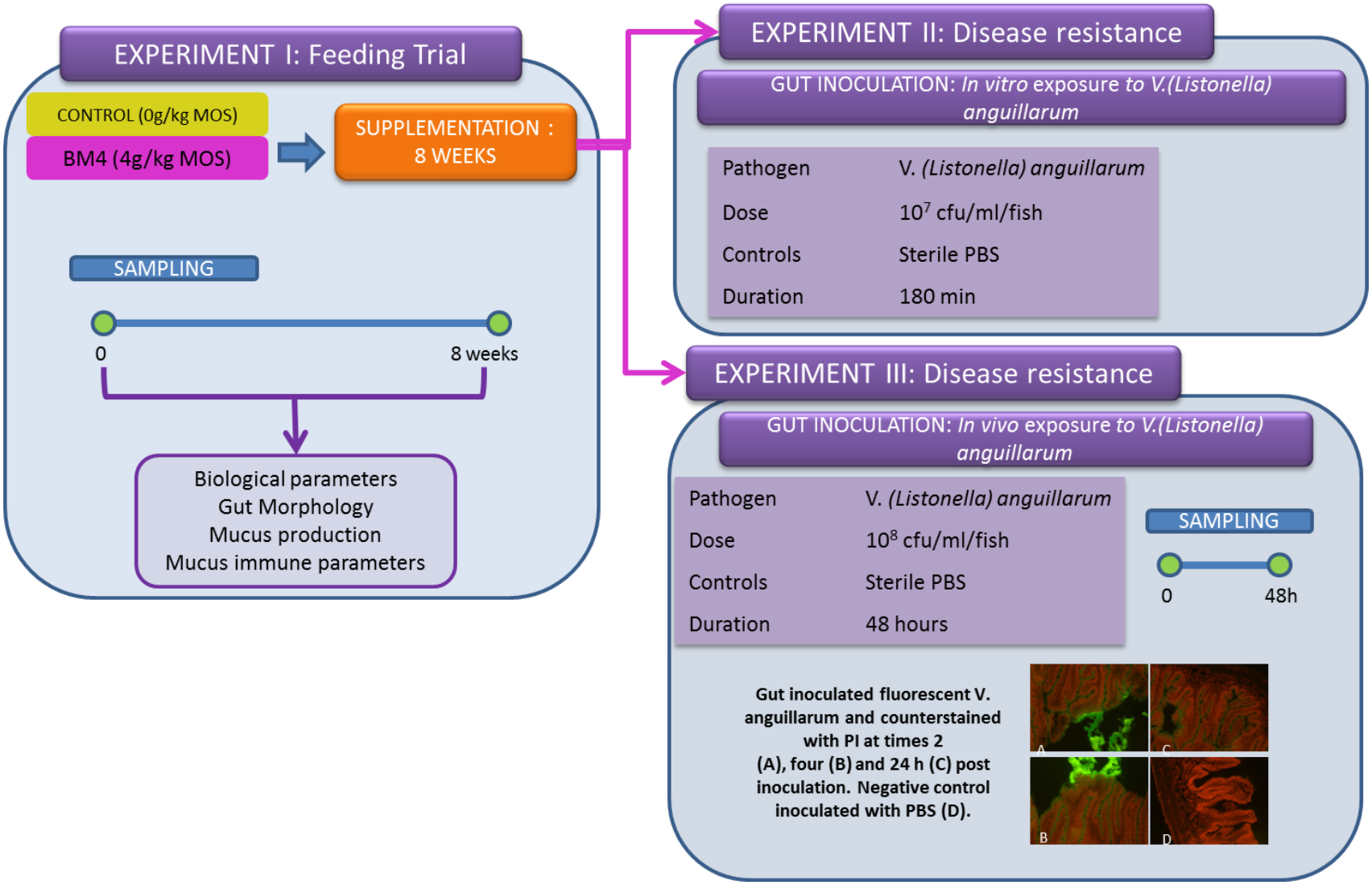
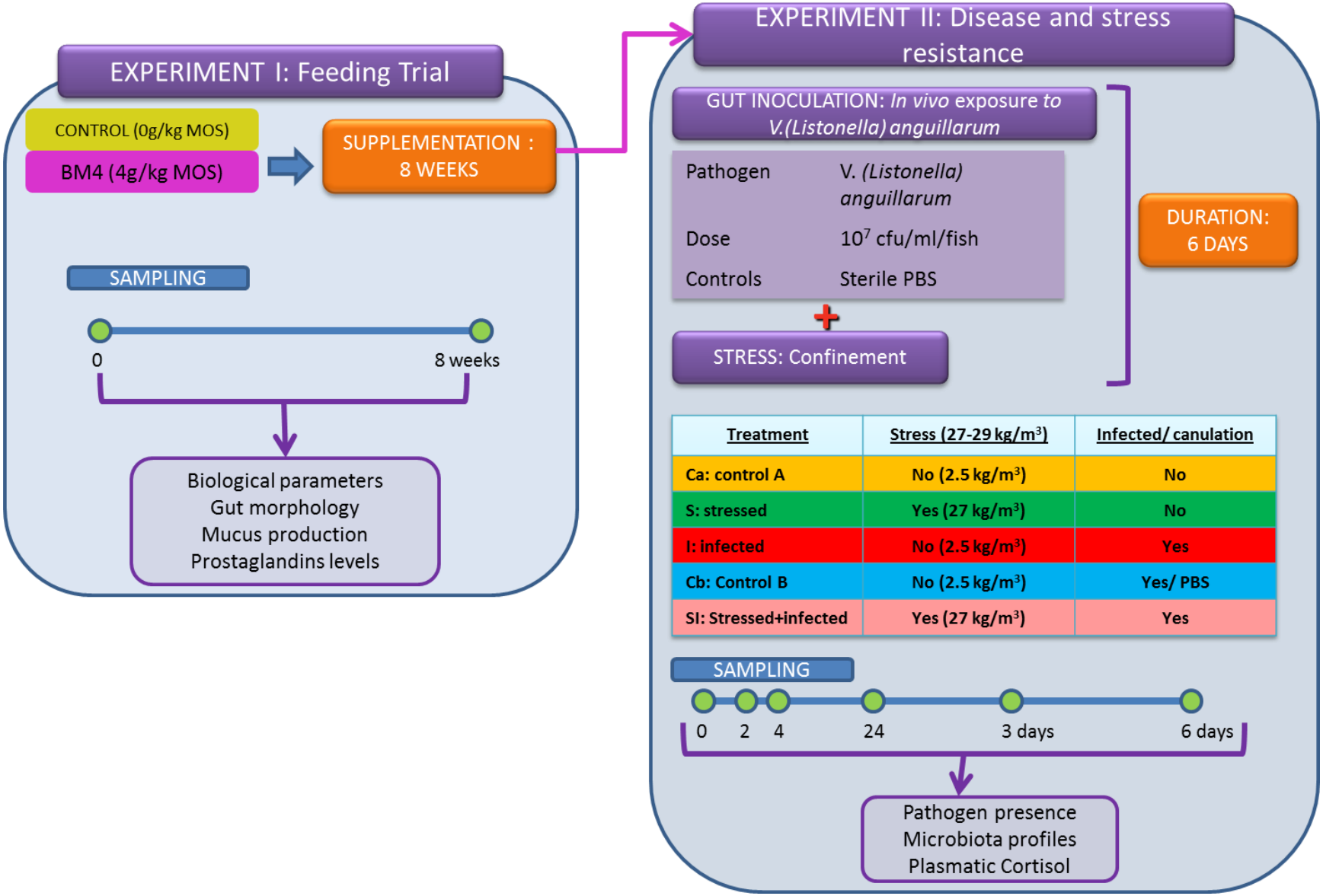


Figure 2.5. Schematic representation of experimental design and sampling followed along Trial IV and V.



2.4. BIOCHEMICAL ANALYSIS

Along the study, feed, faeces and fish biochemical composition was conducted following standard procedures (AOAC, 1995). Analyses were performed in triplicates.

2.4.1. Total crude lipid content

Crude lipids from weighted samples were extracted by a chloroform:methanol (2:1) mixture as described by Folch *et al*. (1957). Extracted lipids were diluted in chloroform and stored at -80°C under nitrogen atmosphere in order to avoid oxidation.

2.4.2. Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters were obtained by transmethylation with 1% sulphuric acid in methanol (Christie, 1989). The reaction was conducted in dark conditions under nitrogen atmosphere for 16h at 50°C. Afterwards, fatty acid methyl esters were extracted with hexane: diethyl ether (1:1 v/v) and purified by adsorption chromatography on NH2 Sep-Pack cartridges (Waters S.A., Massachussets, USA). Fatty acid methyl esters were separated by GLC (GC-14A, Shimadzu, Japan) in a Supercolovax-10-fused silica capillary column (Length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180°C for the first 10 min, increasing to 215°C at a rate of 2.5°C/min and then held at 215°C for 10 min, following the conditions described by Izquierdo *et al.* (1992). Fatty acid methyl esters were quantified by FID and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan).

2.4.3. Crude protein

Crude protein analyses were carried out according to the Kjeldahl Method (AOAC, 1995). Briefly, samples were digested with 37% sulphuric acid in presence of a cupper catalyst converting all the N_2 present in the sample to $(NH_4)_2SO_4$. After that, NH_3 was released from the digested sample by the addition of NaOH in excess. This ammoniac acid was distilled in boric acid and finally the ammoniac acid released was quantified by titration with chloridic acid 0.1 N. The total nitrogen in the sample is converted to total crude protein value by multiplying by the empirical factor 6.25.

2.4.4. Moisture content

Dry matter content was determined by thermal drying to constant weight in an oven at 110°C, with a first 24 h period followed by 1h periods until weight was not reduced any further. Sample weight was recorded before drying and after each drying period, previous to a cooling period in a desiccator until achieving ambient temperature. Moisture was expressed as a percentage of sample weight according to Official Methods of Analysis (AOAC, 1995).

2.4.5. Ash content

Ash content was determined by combustion in a muffle furnace at 600 °C for 12 hours (AOAC, 1995). Sample weight was recorded before combustion and after combustion period, previous to a cooling period in a desiccator until achieving ambient temperature. Ash content was expressed as a percentage of sample weight according to Official Methods of Analysis (AOAC, 1995).

2.5. HISTOLOGICAL STUDIES

These studies were mainly carried out in hepatic and enteric tissues. These tissues were selected based on the strong evidence of the protective function of MOS in gut morphology (Spring, 1999; Sonmez and Eren, 1999; Iji *et al.,* 2001; Valancony *et al.,* 2001; Dimitroglou and Davies, 2004) and since morphological changes on liver are a good indicator of the fish physiological status.

Liver samples were fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E) for optical examination (Martoja and Martoja-Pierson, 1970). Micrographs of liver were taken from the paraffin sections at a final magnification of 400X using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. Hepatocellular area as well as maximum and minimum longitude of cells taking hepatocyte nuclei as reference were measured with an analySIS® (Image Pro Plus®) software package using arbitrary units.

Six to eight transversal sections of each intestine segment were fixed in 10% neutral-buffered formalin and embedded in paraffin perpendicularly to the bottom of the mold. For each specimen ten serial sections (5 µm-thick) were stained with haematoxylin and eosin (H&E), Alcian Blue, Alcian Blue/PAS (pH=2.5) or May-Grünwald/Giemsa (MGG) (Martoja and Martoja-

Pierson, 1970) depending on the experiment. Micrographs were taken from the paraffin sections using a Nikon Microphot-FXA microscope and an Olympus DP50 camera.

For gut morphometric analysis, H&E/MGG stained sections were used. Mucosal folds height and width were analyzed using the measurement tool of an image analySIS® software package (Image Pro Plus®). In order to measure folds total surface area, MGG sections images (X50) were processed using Adobe Photoshop CS 8.0.1 (Adobe Systems, San Jose, CA). First, gut section was outlined, background removed and converted into a binary format where lumen area was removed. Then, images were converted to grey scale and muscular layer selected and removed. Finally, the remaining mucosal folds area (including lamina propria) was converted again to binary format and measured using an image analySIS® software package (Image Pro Plus®). The following commands were used for the conversion: GrayMode, Fill and Threshold. Binary black and white and grey scale images were compared with the original images to ensure an accurate conversion. Minor adjustments, if needed, were made with the following commands: Erode and Paintbrush.

The cellular infiltration level of a mixed leucocytes population (mainly eosinophilic granulocytes, ECGs) in the central lamina propria within the intestinal folds and consequent engrossment, was evaluated under a light microscope separately by two scientists unaware of the experimental treatments using MGG stained sections (X200, X400). A histological scoring system was established previously to the evaluation as follows: 0, not observed; 1 low; 2, moderate and 3, high. The number of goblet cells by unit of area was determined using an analySIS® (Image Pro Plus®) software package.

For determining the bacterial attachment to gut epithelium, fluorescence methods were followed. Briefly, fish were inoculated by anal canalization with 10^8 cfu·ml^{-1} fluorescent labelled *V. anguilllarum*. After 2, 4 and 24 h post inoculation fish were sacrificed with an anaesthetic overdose. The intestine, from the posterior pyloric caeca to the anus, was rapidly dissected out and separated into anterior and posterior regions, rinsed with PBS, embedded in *Tissue-Tek* (O.C.T, Sakura Finetek, Torrance, CA) and immediately frozen at -80°C. Cryosections (10µm) were stained with Propidium iodide (PI) (5 µg/ml), mounted with Fluoromount (Sigma, St.Louis, CA, USA) and inspected under fluorescence microscope (Olympus CX41, Olympus Optical, PA, USA).

2.6. ENZYMATIC ANALYSIS (G6PD and ME in liver)

Liver samples were homogenized in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4) and homogenates were centrifuged at 20000 g for 40 min at 4°C. Soluble protein content of liver homogenates was determined by the method of Bradford (1976) using Bovine Serum Albumin (BSA) as a standard. Enzyme activities of glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) were assayed using spectrophotometric procedures on the supernatant following the method described by Dias *et al.* (1998). Care was taken to ensure that initial rates were measured in all assays and that enzymes were stable in the buffer solution used during the time and temperature required to perform the assays (Álvarez *et al.*, 1998b). Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay temperature per minute, were expressed per mg of hepatic soluble protein (specific activity). All enzyme assays were at least assayed in duplicate.

2.7. IMMUNOLOGICAL ANALYSIS

2.7.1. Blood collection and sample preparation

Blood was obtained by caudal sinus puncture with a 1 ml plastic syringe. No anaesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize the stress effects. Blood was transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at –80°C until analysis.

2.7.2. Mucus collection and sample preparation

Mucus was isolated from skin and gut of healthy European sea bass. The skin mucus was collected from the body surface as described by Bordas *et al.* (1996) by scrapping with a plastic spatula and diluted in sterile seawater. Gastrointestinal mucus was prepared as described by Chambrillón *et al.* (2006) with small modifications. Fish were starved for 48 h and then intestines were removed and transferred to sterile Petri dishes where mucus was collected by scrapping the inner intestine surface with a sterile spatula. The mucus then was homogenized in phosphate buffered saline (PBS). All mucus preparations were centrifuged

twice at 20000 g for 30 min at 4°C to remove particulate and cellular material. Then they were sterilized under UV radiation and stored at -80°C until analysis.

2.7.3. Respiratory burst of circulating neutrophils (NBT)

Respiratory burst of blood circulating neutrophils was assayed as described by Siwicki *et al.* (1993). The objective of this methodology is to measure the production of superoxide anions generated by blood circulating neutrophils through the reduction of nitroblue tretrazolium (NBT) to formazan in presence of oxygen radicals.

2.7.4. Alternative complement pathway

Alternative complement pathway was performed as described by Sunyer and Tort (1995) for gilthead sea bream using rabbit red blood cells (RBC). The reciprocal of the serum dilution causing 50% lysis of RBC is designed as ACH50 and the results are presented as ACH50 units/ml.

2.7.5. Lysozyme

Lysozyme level in blood serum was determined by turbidimetric assay according to Anderson and Siwiki (1994) using hens egg white lysozyme in PBS as a standard. Results are presented as Lysozyme units/ml when analyzed in serum or as µg lysozyme/mg protein when determined in mucus.

2.7.6. Phagocytic activity of head kidney leucocytes

Head kidneys of fish were removed and leucocytes were isolated by density gradient centrifugation technique as follows. After collection, head kidneys were homogenized in supplemented Minimal Essential Medium (MEM) and filtered through a nylon membrane. Resulting cell solution was layered onto preformed gradient of 51%-34% Percoll in 10% Hank´s Balanced salt solution (HBSS) and centrifuged. The interface layer was harvested and diluted in 1 ml of supplemented MEM and centrifuged again in order to remove residual Percoll. The resulting pellet was diluted in 1 ml of supplemented MEM and viability and concentration of leucocytes suspension was determined. The solution was re-suspended in supplemented MEM adjusting to the desired final concentration. Leucocyte solution was incubated against *V. alginolyticus*, *V. anguilllarum* or against microparticles based on polystene depending on the

experiment as described by Esteban and Meseguer (1997) for *V. anguilllarum*. Ten series of one hundred leucocytes per fish were counted and the phagocytic index was determined as the percentage of leucocytes with phagocytic ability. Positive phagocytic activity was determined only for the leucocytes with presence of the microparticle of polyestyrene or vacuole/bacteria inside the cytoplasm.

2.7.7. Prostaglandins analysis

2.7.7.1. Tissue stimulation

Head kidneys and anterior and posterior gut segments were dissected out, weighed and digested in 4 ml of HBSS without Ca^{2+} plus 2% Collagenase during 30 minutes in continuous orbital agitation ($100U \cdot min^{-1}$). After digestion, samples were filtered through nylon gauze and washed with 4 ml of HBBS without Ca^{2+} and isolated cells recollected in a glass tube. Cells were concentrated in a pellet by centrifugation (5 min, 2500 g) and the resultant pellet re-suspended in 1 ml of HBBS without Ca^{2+} in an Eppendorf tube. Cells were stimulated during 30 min with 50 µM Ca^{+} PMA (Phorbol 12-myristate 13-acetate, Sigma Chemicals, CA, USA) and 10 µM A321 (Sigma Chemicals, CA, USA). Stimulation reaction was stopped by adding 50 µl of formic acid 2M. Samples were stored at -80°C until purification.

2.7.7.2. Purification of eicosanoids

The frozen stimulated fraction (pooled 4 fish tissues/tank) was centrifuged at 1000g for 5 min in order to precipitate any remaining debris. The supernatants were extracted using a octadecylsilyl (C18) “Sep-Pak” mini-columns (Millipore, Watford, UK) by the method of Powell (1982) and as described in detail by Bell *et al.* (1994). One milliliter of supernatants was applied to the column, which had been prewashed with 5 ml of methanol and 10 ml of MiliQ water. The column was successively washed with 10 ml of MiliQ water, 5 ml of 15% ethanol (v/v) and 5 ml of hexane/chloroform (65:35, v/v) before elution of prostanoids with 10 ml of ethyl acetate. The extracts were dried under nitrogen and re-suspended in 100 µl of methanol and stored at -80°C in small glass vials until analysis.

2.7.7.3. Prostaglandins immunoassay

For this analysis a Prostaglandin E2 EIA Kit- Monoclonal (Cayman Chemical Co., MI, USA) was used that it is based in the competition between PGE_2 and PGE_2-actylcholinesterase (AChE) conjugated PGE_2 (tracer) for a limited amount of PGE_2 monoclonal antibody.

2.8. STRESS PARAMETERS

2.8.1. Blood collection and sample preparation

Blood was obtained by caudal vein puncture with a 1 ml plastic syringe. No anesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize the stress effects. The sample was transferred to Eppendorf tubes coated with heparin as anticoagulant and plasma was separated immediately by centrifugation. Samples were stored at -80°C until analysis.

2.8.2. Cortisol measurements

Concentration of plasmatic cortisol was determined by radioimmunoassay (RIA) (Rotllant *et al.,* 2001). The antibody used in the assay was purchased from Biolink, S.L., (Costa Mesa, CA, USA) in a final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11.40% with 21-desoxycorticosterone, 8.90% with desoxycortisol and 1.60% with 17α-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels are expressed as ng cortisol /ml plasma.

2.9. FLESH QUALITY

Fish were starved for 24 h, slaughtered in a small tank with ice and seawater and gutted and kept at 4°C for 24 h until tests were carried out. Organoleptic tests were conducted on fish fillets cooked in aluminum boxes for 10 min in a steam oven (120°C). Immediately after cooking, fillets were offered to a panel of 8 selected and trained judges (ISO 1985, ISO 1993). Tests were conducted in isolated and air conditioned rooms with standardized light (ISO 1988). Judges were randomly offered closed food boxes labelled with codes containing the fillets (3x4 cm). Odour (marine, off-odour and oily), appearance (juicy, shininess and colour), texture (cohesiveness, hardness, and juiciness) and flavour (marine, off-flavour and oily) were tested for

samples of fish fed the experimental diets and classified by the judge in a continuous scale from 0 to 100 for each parameter (Ginés *et al*., 2004).

2.10. GUT i*n vivo* AND *ex vivo* EXPOSURE TO BACTERIAL PATHOGENS

2.10.1. Gut ex vivo exposure to V. anguillarum

After fish were sacrificed with an anaesthetic overdose and netted, the intestine, from the posterior pyloric caeca to the anus, was rapidly dissected out and separated into anterior and posterior regions, rinsed and placed in ice-cold Ringer solution (140 mM NaCl, 2.5 mM KCl, 15 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1mM KH_2PO_4, 0.8 mM $MgSO_4$, 10 mM glucose and 5 mM HEPES buffer; pH was set to 7.8). Fresh Ringer solution was prepared daily, continuously gassed with air and stored on ice. Then, the segments were reversed and closed at the distal and proximal ends using fine forceps and introduced in flasks filled with 10 ml Ringer solution continuously gassed with air for oxygenation and stirring. The intestinal sacs were kept in the flasks for a total of 120 min. After 60 min of stabilization, the Ringer solution was substituted with a Ringer solution containing 10^7 cfu/ml of fluorescent labelled *V. anguillarum*. Bacteria were labelled with fluorescein isothiocyanate (FITC; Sigma Chemicals, St.Louis, USA). Briefly, bacteria were incubated with 0.25 mg FITC/ml bacterial PBS suspension for 60 min at 20°C. Four steps of washing by centrifugation (3000 g, 5 min) and re-suspension with PBS ensured sufficient removal of dissolved FITC.

At termination of the exposure to fluorescently labelled *V. anguilllarum*, the outer side (mucosal side) of the intestines, in contact with the bacterial solution, was washed three times with Ringer solution in order to discharge the bacteria attached to the intestine and not translocated. Then, the inner side (serosal side) of the intestine sac was washed three consecutive times with Ringer´s solution to collect the translocated bacteria. Samples were immediately centrifuged at 6000 g for 10 min to concentrate particulate fluorescence, the supernatant discarded and the pellet was dissolved in Ringer´s solution. The fluorescence of the remaining solution was measured for 1 s at 480/535 nm on a microplate reader (Victor 1420 Multilabel Counter; Wallac). *V. anguilllarum* (strain VI- 88/09/03175, Universidad de Santiago de Compostela, Aquaculture Institute, Ictiopathology Department, Spain), used in the translocation studies was stored lyophilized. Previously, the bacteria was grown in a Tryptone Soybean Agar (TSA, Panreac Quimica SAU (Cultimed), Barcelona, Spain), and re-inoculated 24-

36 h prior analysis. Bacteria were dissolved in sterile PBS to an optical density of 1.0 at 600 nm, which equals 10^9 bacteria measured by plating and colony counting on BHI-agar (Brain heart infusion) plates.

2.10.2. Gut in vivo exposure to V. anguillarum/V.alginolyticus

Fish were subjected to a short-term *in vivo* exposure to *V. anguillarum* (10^7-10^8 cfu·ml^{-1} per fish) or *V.alginolyticus* ($2.4 \cdot 10^8$ cfu·ml^{-1} per fish) via anal cannulation using a catheter. After time exposure all fish were sacrificed and bacterial characterization of head kidney, spleen and liver was performed in order to determine the pathogen's capacity to pass through the intestinal epithelium by culturing samples on TSA, BHI or VAM (*V. anguilllarum* media; Alsina *et al.,* 1994) and incubated at 25°C for 24-48 h. The grade of pathogen infection was determined by the presence or absence of the pathogen in internal organs.

2.11. BACTERIAL ISOLATION AND BIOCHEMICAL CHARACTERIZATION

Samples of spleen, head kidney and liver from fish were cultured on TSA, BHI or VAM (*V. anguilllarum* media; Alsina *et al.,* 1994) and incubated at 25°C for 24-48 h. All strains isolated were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests using previously methods (Smibert and Krieg, 1981) in order to identify bacteria species present in the fish. Complementary API 20E (BioMérieux, Marcy l´Etoile, France) were also used.

2.12. GUT MICROBIOTA PROFILES

2.12.1. Bacterial DNA extraction and 16S rRNA amplification

The DNA was extracted from pooled 3 fish/tank posterior intestinal samples (3 tanks/treatment) with a lysozyme pretreatment (50 mg/ml of Tris-EDTA buffer (TE) for 30 min at 37°C) using Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA). DNA concentration checked by ordinary 1.5% electrophoresis gel and measured with Qubit™ Fluorometer (Invitrogen, CA, USA) using Quant-it ds DNA HS assay kit (Invitrogen, CA, USA). PCR amplification of the 16S rRNA genes was carried out using the reverse primer P1(5´-CCG TCA ATT C (AC) T TTG AGT TT-3´) and the forward primer P2 with GC clamp (5´-CGC CCG CCG CGC CCC GCG CCC CTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3´). Reagents in each

PCR tube (final volume 50µl) were: 2.0 µl BSA (10 µg/µl), 30µl of sterile distilled water, 5µl 10X PCR Buffer without $MgCl_2$ (Invitrogen, CA, USA), 1.75µl $MgCl_2$ (50mM, Invitrogen, CA, USA), 4µl dNTPs (2.5mM, Invitrogen, CA, USA), 0.25µl Taq DNA polymerase (5U/µl, Invitrogen, CA, USA), 4µl of primer P1 and P2 (10pmol/ µl; MWG-Biotech AG, Ebergberg, Germany). The touchdown thermal cycling was conducted as described by Casamayor *et al.* (2000) modified by Dhanasiri *et al.* (2011) using a personal Thermal Cycler MJ Mini (Bio-Rad, CA, USA) under the following conditions: 94°C for 5 min, 2 cycles starting at 94°C for 1 min, 66°C for 1 min, 72°C for 3 min, 2 cycles starting at 94°C for 1 min, 64°C for 1 min, 72°C for 3 min, 2 cycles starting at 94°C for 1 min, 62°C for 1 min, 72°C for 3 min, 2 cycles starting at 94°C for 1 min, 60°C for 1 min, 72°C for 3 min, 2 cycles starting at 94°C for 1 min, 58°C for 1 min, 72°C for 3 min, then 25 cycles starting at 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, final step 10 min at 72°C. PCR products were stored at -20°C until running denaturing gradient gel electrophoresis (DGGE).

2.12.2. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed using a DGGE-Bio-Rad system (Bio-Rad, CA, USA). Eight hundred ng of standardized PCR product per well were run on 6% acrylamide gels with a denaturing gradient of 40 to 60 % (where 100% denaturant is 7 M urea and 40% formamide). The gel was run at 65 V for 17 h at 60°C in 1×TAE buffer (50X Tris-Acetic acid-EDTA (TAE) Buffer, Merck, Darmstadt, Germany). Visualization of the DGGE bands was achieved by staining with a 1:10000 dilution of SYBR green (Invitrogen-Molecular Probes, Eugene, USA) for 45 min. The gel was scanned in a Bio-Rad universal hood II (Bio-Rad, CA, USA) and optimized for analyses by enhancing contrast and grayscale using Quantity One 4.6.6 software (Bio-Rad, CA, USA). Briefly, lanes were identified and their background intensities removed following program indications. Then, bands were detected and matched at 2% tolerance level and similarity was computed by comparing by Dice Coefficient between and within fish fed the different treatments.

2.13. STATISTICAL ANALYSIS

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Depending on the data one-way analysis of variance (ANOVA) or/and two-way ANOVA tests were performed. When F values showed significance,

individual means were compared using Tukey's or Scheffe test for multiple means comparison. Significant differences were considered for $P<0.05$-0.1 depending on the parameters studied. If the variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the data. Analyses were performed using Statgraphics software (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA).

2

Chapter 3

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Immune stimulation and improved infection resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides

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ABSTRACT

The objective of this study was to determine the effect of two levels of inclusion of mannan oligosaccharides derived from the outer cell wall of a select strain of *Saccharomyces cerevisiae* (Bio-Mos®, Alltech Inc, USA) on growth, feed utilization, immune status and disease resistance of European sea bass (*Dicentrarchus labrax*). Specimens of 35g at initial density of 3kg·m^{-3} were fed during 67 days at 0 g·kg^{-1}, 2 g·kg^{-1} and 4 g·kg^{-1} dietary MOS level of inclusion in a commercial sea bass diet. Food conversion rate, specific growth rate, whole body biochemical composition, phagocytic index of head kidney macrophages, NBT index, lysozyme and alternative complement pathway (ACP) activities as well as gut and liver histological structure were evaluated. Growth significantly increased at both MOS dietary inclusion levels. Histological features of the liver showed lower lipid vacuolization and regular-shaped morphology of hepatocytes around the sinusoidal spaces denoting a better utilization of dietary nutrients. No differences were found on gut histological evaluation. Statistical differences ($P<0.05$) on the phagocytic index were denoted with the inclusion of 4 g·kg^{-1} MOS group. A positive correlation

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was found between the levels of lysozyme and alternative complement pathway activities in blood and the level of inclusion of MOS in diets.

After the feeding trial, a cohabitation challenge test and direct gut inoculation were also performed with the pathogen *Vibrio alginolyticus* in a ratio 3:1. Twenty-one days post-challenge the number of cohabitant fish infected in the control group reached 33% comparing with none on the 4 $g \cdot kg^{-1}$ MOS group. Finally, new fish were infected with *Vibrio alginolyticus* by gut canalisation. After 24 hours post- infection no significant difference was denoted between groups and 48 hours post-infection total infected fish in the control group was twice that of the 2 $g \cdot kg^{-1}$ and 4 $g \cdot kg^{-}1$ MOS groups.

3.1. INTRODUCTION

Bacterial disease outbreaks impose a significant constraint in fish and shellfish production (Bachère *et al.*, 1995; Verschuere *et al.*, 2000). In commercial farms, unfavourable environmental conditions (availability of iron, osmotic strength, oxygen levels, pH, water quality, temperature) or poor management practices (inadequate nutrition, overcrowding, overfeeding) may stress the fish causing them a growth rate reduction and immune suppression, making them more susceptible to disease outbreaks (Hansen and Olafsen, 1999; Verschuere *et al.*, 2000; Winton, 2001). This is particularly significant for European sea bass (*Dicentrarchus labrax*), which despite its importance for Mediterranean aquaculture where it reaches up to 20% of total fish production, is a species very sensitive to stressors and infections may occasionally cause important losses (Izquierdo, 2005).

The increasing economic and social concern to decrease the use of antibiotics and other therapeutic chemicals used in fish farming has encouraged more environmentally friendly approaches to disease control (Hansen and Olafsen, 1999; Verschuere *et al.*, 2000). For instance, there is a great interest in the use of different products and organisms to control potential pathogens by competitive exclusion (probiotics and prebiotics), what has been successful in preventing disease outbreaks in other areas of animal production (Corrier *et al.*, 1995; Hansen and Olafsen, 1999; Nisbet *et al.*, 1994; Vanbelle *et al.*, 1990). Some of these products derive from *Saccharomyces cerevisiae*, like Bio-Mos® (Alltech Inc., Kentuky, USA) which is composed by a fraction of its cell wall rich in mannan oligosaccharides (MOS). The benefits of this product have been reported in a broad number of poultry species, where it improves feed efficiency (Sonmez

and Eren, 1999; Spring, 1999; Spring *et al.*, 2000; Valancony *et al.*, 2001; Iji *et al.*, 2001), promotes growth and seem to modulate immune response and preserve intestinal wall integrity. However, studies of MOS effect in fish are very scarce. Common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) growth and feed utilization significantly improved by feeding MOS supplemented diets (Staykov, 2004). Besides, MOS feeding markedly reduced mortality and improved antibodies levels, bactericidal and lysozyme activity and alternative complement pathway activity (ACP) (Staykov, 2004). MOS supplementation to *Artemia* improved intestinal microvilli structure in white sea bream larvae (*Diplodus sargus*) and induced a higher resistance to handling and stress (Dimitroglou and Davies, 2004).

In view of on these evidences for other fish species, inclusion of MOS on diets for European sea bass could improve stress and disease resistance together with growth and feed utilization. Hence, the objective of this study was to evaluate the effect of dietary inclusion of MOS on the rearing performance of on-growing European sea bass and its implications in certain immune parameters and resistance to infections.

3.2. MATERIALS AND METHODS

3.2.1. Diets

Three isonitrogenous and isoenergetic diets, based in a commercial formulation, were designed to contain 0 $g \cdot kg^{-1}$ (Control), 2 $g \cdot kg^{-1}$ (BM2) and 4 $g \cdot kg^{-1}$ (BM4) MOS (Bio-Mos®, Alltech Inc., USA) replacing standard carbohydrates. Diets covered nutritional requirements for this species (Izquierdo, 2005) and were manufactured by a commercial feed producer (Graneros de Tenerife, Tenerife, Spain) with the composition indicated in Table 3.1.

3.2.2. Experimental conditions

3.2.2.1. Experiment I

Eight hundred commercially reared European sea bass juveniles from a population which had not suffered apparent diseases originated from a local farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain), arrived at the main facility of the Canarian Institute of Marine Science (ICCM) on July 1st 2005. Fish were maintained in stocking tanks and fed a commercial extruded diet for 3 weeks (19-20.5°C) until being well adapted to the environmental conditions (3 $kg \cdot m^{-3}$ stoking density). Afterwards, 792 sea bass juveniles were randomly distributed in 9

indoor, cylindroconical 1000 l fibreglass tanks at an initial stocking density of 3 $kg \cdot m^{-3}$ (88 fish per tank). Fish average initial weight (g ± SD) and length (cm ± SD) were 33.75 ± 7.69 and 13.46 ± 0.97, respectively (n = 792). Tanks were supplied with filtered sea water (1.39 $l \cdot min^{-1}$), at a temperature of 20.5-23.4°C, and natural photoperiod (12L:12D). Water dissolved oxygen was kept at 8.0 ± 0.2 ppm. Fish were manually fed until apparent satiation with one of the three experimental diets for 9 weeks (3 times a day, 6 days a week). Each diet was assayed by triplicate.

Feed intake was calculated daily and growth parameters were determined at day 0, 36 and 67. Food conversion ration (FCR) defined as the amount of food ingested divided by the generated biomass, specific growth rate (SGR) defined as: [(ln final weight –ln initial weight) / number of days] x 100 and relative growth (%) defined as: [(final weight – initial weight)/ initial weight)] x 100. Nine fish at the beginning of the trial and 15 fish per tank at day 67 were sampled, after 24 h fasting, for body proximal composition and studies of hepatic and enteric tissue morphology. Blood samples were taken from 15 fish for plasma and serum collection at day 67 to determine NBT, lysozyme and ACP activities. In addition, the head kidney was removed from 15 fish per dietary treatment to determine phagocytic activity of macrophages.

Table 3.1. Main ingredients in the experimental diet

Ingredients	Diet (% dry weight)
Fish meal [1]	51.50
Soybean meal	9.78
Wheat	7.50
Wheat gluten	7.50
Corn meal	6.53
Fish oil [2]	12.69
Fats and oils	2.03
Mineral mix [3]	1.43
Vitamin mix [4]	1.03
Antioxidant (BHT)	0.01

[1]Peruvian fish meal (65% protein). [2]Peruvian fish oil. [3]TROUW Seabream/Seabass (0.8g), Choline chloride (0.17g) and Inositol (0.06g). [4]TROUW Seabream/Seabass (1g) Calcium carbonate (0.2g), Potassium monophosphate (0.19g) and NaCl 97% (0.04g).

3.2.2.2. Experiment II

After 9 weeks of feeding the experimental diets, 60 fish per diet from Experiment I were randomly selected and distributed among 12 indoor cylindrical 500 l fibreglass tanks in the pathogens incubation facility (3 kg of fish·m^{-3}, 20 fish per tank).Each group of fish were fed the same diet that they were fed in Experiment I. Initial fish average weight (g ± SD) and length (cm ± SD) were 99.37 ± 21.84 and 18.71 ± 6.30, respectively. A bacterial analysis was performed before the beginning of the challenge test to ensure that fish were not infected by bacteria. Two groups of 20 fish from each dietary treatment (Control, BM2, BM4) were exposed to a pathogen by cohabitation (3:1, healthy:infected) with fish intraperitoneally infected (ip) ($2.4 \cdot 10^8$ cfu/ml·fish) with *Vibrio alginolyticus*. Another two 20 fish groups per treatment were used as non-infected controls cohabitating (3:1) with fish intraperitoneally injected with sterile saline solution. Fish injected with *Vibrio alginolyticus* or sterile saline solution were marked by Visible Implant Elastomers (VIE, Northwest Marine Technologies, Shaw Island Washington, WA, USA) (Astorga *et al.*, 2005). Water temperature ranged from 22.0 to 23.0°C during the trial and artificial light photoperiod was adjusted to 12L:12D. Fish were manually fed their respective experimental diets until apparent satiation for 21 days (3 times a day, 6 days a week). At the end of this challenge test all fish were slaughtered and microbiological analysis of head kidney, spleen and liver were conducted in order to determine the infection spread grade.

3.2.2.3. Experiment III

In a third experiment, 80 fish per diet from Experiment I were randomly selected and distributed among 12 indoor cylindrical 500 l fibreglass tanks in the pathogens incubation facility (3.5 kg of fish·m^{-3}, 20 fish per tank). Each group of fish were fed the same diet that they were fed in Experiment I. Initial fish average weight (g ± SD) and length (cm ± SD) were 98.23 ± 5.63 and 18.51 ± 0.30, respectively. A bacterial analysis was performed before the beginning of the challenge test to ensure that fish were not infected by bacteria. Two groups of 20 fish from each dietary treatment (Control, BM2, BM4) were inoculated via anal canalisation with *Vibrio alginolyticus* ($2.4 \cdot 10^8$ cfu/ml·fish). Another two 20 fish groups per treatment were inoculated via anal canalisation with 1 ml of sterile saline solution. Water temperature ranged from 22.0 to 23.0°C during the trial and artificial light photoperiod was adjusted to 12L:12D. Fish were manually fed their respective experimental diets until apparent satiation for one week (3 times a day, 6 days a week). After 24 h and 48 h post inoculation, 33% of fish population was randomly

selected and bacterial characterization of head kidney, spleen and liver was performed in order to determine the pathogen's capacity to pass through the intestinal epithelium. Seven days after experimental infection, gut and liver samples were taken for histological studies.

3.2.3. Biochemical analysis

Feed and fish biochemical composition were conducted following standard procedures (AOAC, 1995), dry matter content was determined after drying the sample in an oven at 105°C to constant weight, ash by combustion in a muffle furnace at 600°C for 12 h, protein content (Nx6.25) was determined by Kjeldahl method and crude lipid was extracted following the method of Folch *et al.* (1957). Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo *et al.* (1992), being quantified by FID and identified by comparison to external standards. All analysis were conducted by triplicate.

3.2.4. Histological studies

On Experiment I, liver and gut samples were taken after 67 days of the feeding trial. Samples were fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E) for optical examination (Martoja and Martoja-Pierson, 1970). Micrographs of liver were taken from the paraffin sections at a final magnification of 400X using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. Hepatocellular area as well as maximum and minimum longitude of cells taking hepatocyte nuclei as reference were measured with an analySIS® (Image Pro Plus®) software package using arbitrary units. For each diet, twenty-seven micrographs originating from 9 fish (n = 27) were analyzed. On Experiment III, seven days after experimental infection, gut and liver samples were taken for histology analysis and routinely processed.

3.2.5. Blood collection and sample preparation

Blood was obtained by caudal sinus puncture with a 1 ml plastic syringe. No anaesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize the stress effects. The first aliquot of blood was

transferred to a coated Eppendorf with lithium heparin as anticoagulant and used for NBT (Nitro Blue Tetrazolium) index, measuring the activity of circulating neutrophils spectrophotometrically as described by Siwicki *et al.* (1993) by determining the reduction of NBT to formazan in presence of oxygen radicals. The second aliquot was divided in two portions, transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at –80°C for ACP and lysozyme activities determination.

3.2.6. Serum analysis

Alternative complement pathway was performed as described by Sunyer and Tort (1995) for gilthead sea bream (*Sparus aurata*) using rabbit red blood cells (RBC). The reciprocal of the serum dilution causing 50% lysis of RBC is designed as ACH50 and the results are presented as ACH50 units/ml.

Lysozyme level in blood serum was determined by turbidimetric assay according to the method described by Anderson and Siwiki (1994) using hens egg white lysozyme in PBS as a standard. Results were presented as Lysozyme units/ml.

3.2.7. Head kidney leucocytes phagocytic activity

Head kidneys of five fish per diet were removed and macrophages were isolated by density gradient centrifugation technique as follows. Following collection, head kidneys were homogenized in supplemented Minimal Essential Medium (MEM) (10µl·ml^{-1} heparin; 100µl·ml^{-1} penicillin/streptomycin; 2% fetal bovine serum) and filtered through a nylon membrane (100µm mesh syze, Sigma). Resulting cell solution was layered onto preformed gradient of 51%-34% Percoll in 10% Hank´s Balanced salt solution (HBSS) and centrifuged at 800 *g* for 30 min at 4°C. The interface layer was harvested with a Pasteur pipette, diluted in 1 ml of supplemented MEM and recentrifuged at 800 *g* for 5 min at 4°C to remove residual Percoll. The resulting pellet was diluted in 1 ml of supplemented MEM and viability and concentration of macrophage cell suspensions were determined in trypan blue. The final pellet with more than 93% viability was resuspended in supplemented MEM adjusting the final concentration to 10^6 macrophages·ml^{-1}. Macrophage solution was incubated with *Vibrio alginolyticus* as described by Esteban and Meseguer (1997) for *Vibrio anguillarum*. Phagocytic activity was measured as described by Blazer

(1991). One hundred macrophages per fish were counted and the phagocytic activity was determined as the percentage of macrophages with phagocytic ability.

3.2.8. Bacterial isolation and biochemical characterization

Samples of head kidney, liver and kidney from dead fish were cultured on TSA (Tryptone Soybean Agar, Cultimed) and incubated at 25°C for 24-48 hours. All strains isolated were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests using previously methods (Smibert and Krieg, 1981), in order to identify *Vibrio alginolyticus* present in the fish.

3.2.9. Statistical analysis

Means and Standard Deviations (S.D.) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of MOS inclusion. When F values showed significance, individual means were compared using Tukey's test for multiple means comparison. Significant differences were considered for $P<0.05$.

3.3. RESULTS

3.3.1. Experiment I

No mortalities were registered during the feeding experiment. Results for growth, biometry and feed utilization are shown in Table 3.2 and Figure 3.1. After 36 and 67 days of feeding, fish fed with diets BM2 and BM4 showed a significantly higher body weight and relative growth. Specific growth rate followed a similar trend (Fig. 3.1). Total length was also significantly higher in fish fed MOS containing diets after 36 days of feeding. Feed conversion ratio (FCR) was not significantly affected by MOS inclusion, but a positive correlation was found between the total amount of ingested food and the level of inclusion MOS inclusion ($y(t_{36}$ days) = 88.493x + 2366.6, R^2 = 0.9197; $y(t_{67}$ days) = 321.2x + 5255.2, R^2 = 0,869). No significant differences were found on the condition factor (K) (Table 3.2). MOS supplementation did not affected whole fish proximate composition (Table 3.3) or fatty acid composition (g fatty acids /100 g total fatty acids) (Table 3.4).

Morphological analyses for fish fed diets containing MOS resulted qualitatively in a regular-shaped morphology of the hepatocytes around sinusoidal spaces and a reduction on the lipid vacuolization of the cytoplasm that decreased the number of hepatocytes with the nuclei displaced to the cellular periphery. This effect was more pronounced in fish fed with the higher concentration of MOS (Fig. 3.2). According to these histological findings, quantitative morphometric analysis of the liver revealed significant differences ($P<0.05$) in the cells parameter studied. Thus, hepatocytes of fish fed BM4 presented significantly lower hepatocellular area as well as maximum and minimum cell length (Table 3.5). No differences were found on histological evaluation of guts of fish fed with the different diets.

Phagocytic activity of head kidney macrophages against *Vibrio alginolyticus* significantly increased ($P<0.05$) in fish fed the highest MOS inclusion level (4 $g \cdot kg^{-1}$) (Fig. 3.3). Resulting in a positive correlation between the phagocytic activity and the dietary MOS inclusion levels ($y = 4.33x + 19.043$, $R^2 = 0.9742$). No concluding results were obtained for neutrophils potential of reduction because significant differences were found between all treatments (Fig. 3.3). No significant differences ($P<0.05$) were found on lysozyme and ACH50 (Fig. 3.3), although a positive correlation was observed between the level of MOS inclusion and the level of these parameters in fish serum, ($y_{lysozyme} = 22.285x + 389.89$, $R^2 = 0.869$) ($y_{ACH50} = 7.925x + 89.903$, $R^2 = 0.9074$).

Table 3.2. Growth performance, nutrient utilization and somatic parameters of European sea bass fed commercial extruded diets with different levels of MOS inclusion

	Days of feed	CONTROL	BM2	BM4
Average Weight (g)	0	34.01±7.47	34.04±7.56	33.56±7.78
	36	59.17^{a}±2.80	63.30^{b}±2.56	62.17^{b}±3.95
	67	93.17^{a}±0.06	102.41^{b}±0.90	102.38^{b}±5.10
Long (cm)	0	13.48±1.03	13.55±0.92	13.41±0.95
	36	15.76^{a}±0.05	16.26^{b}±0.05	16.50^{b}±0.29
	67	18.16±0.13	18.73±0.15	18.63±0.25
Condition factor (k)	0	1.38±0.17	1.35±0.11	1.39±0.13
	36	1.51±0.01	1.47±0.02	1.48±0.02
	67	1.58±0.03	1.56±0.03	1.58±0.02
Relative growth (%)	36	73.18^{a}±0.90	84.62^{b}±3.38	83.81^{b}±8.71
	67	56.90^{a}±1.63	58.57^{b}±1.97	59.75^{b}±2.99

Different letters within a line denotes significant differences ($P<0.05$). Control= 0 $g \cdot kg^{-1}$ Bio-Mos; BM2=2 $g \cdot kg^{-1}$ Bio-Mos; BM4=4 $g \cdot kg^{-1}$ Bio-Mos. Values expressed in mean ± SD.

Table 3.3. Whole body final proximal composition *(g/100g dry weight)* of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 67 days)

	CONTROL	BM2	BM4
Crude protein	46.96±2.10	44.74±1.08	44.19±1.12
Crude lipids	44.81±3.54	43.56±2.5	42.99±3.35
Ash	3.60±0.65	3.06±0.27	2.48±0.14
Moisture	63.12±2.35	63.16±1.00	62.77±0.86

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} Bio-Mos; BM2=2 g·kg^{-1} Bio-Mos; BM4=4 g·kg^{-1} Bio-Mos. Values expressed in mean ± SD.

3.3.2. Experiment II

Despite all the intraperitoneally infected fish died in 72 h, there were no cohabitation mortalities by during the experiment. The grade of horizontal transmission was determined by the presence or absence of the pathogen in different internal organs, but the pathogen was only recovered on head kidney. After 21 days of challenge test the number of fish infected by *Vibrio alginolyticus* was significantly (P<0.05) reduced by the dietary MOS supplementation (Fig. 3.4). VIE mark allowed the correct identification of all injected fish.

Table 3.4. Composition of main fatty acids groups (*g fatty acids/100 g total fatty acids*) for whole body composition of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 67 days)

	CONTROL	BM2	BM4
Saturated	27.080±0.805	26.224±0.332	27.981±1.595
Monosaturated	31.059±0.154	29.640±1.076	31.217±1.828
Σn-3	24.585±0.114	23.582±1.360	24.586±0.878
Σn-6	8.496±0.586	8.486±0.370	7.820±0.613
Σn-9	19.886±0.033	18.823±0.573	19.884±1.266
Σn-3 HUFA	19.796±0.073	18.995±1.105	19.767±0.726

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} Bio-Mos; BM2=2 g·kg^{-1} Bio-Mos; BM4=4 g·kg^{-1} Bio-Mos. Values expressed in mean ± SD.

Table 3.5. Results of quantitative image analysis (µm) of hepatocytes from European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 67 days)

	DIETS		
	CONTROL	*BM2*	BM4
Maximum longitude	2.030^{ab}±0.451	2.165^{a}±0.498	1.610^{b}±0.311
Minimum longitude	1.416^{a}±0.240	1.352^{a}±0.261	1.087^{b}±0.256
Area	2.577^{a}±0.887	2.758^{a}±0.781	1.711^{b}±0.612

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} Bio-Mos; BM2=2 g·kg^{-1} Bio-Mos; BM4=4 g·kg^{-1} Bio-Mos. Values expressed in mean ± SD.

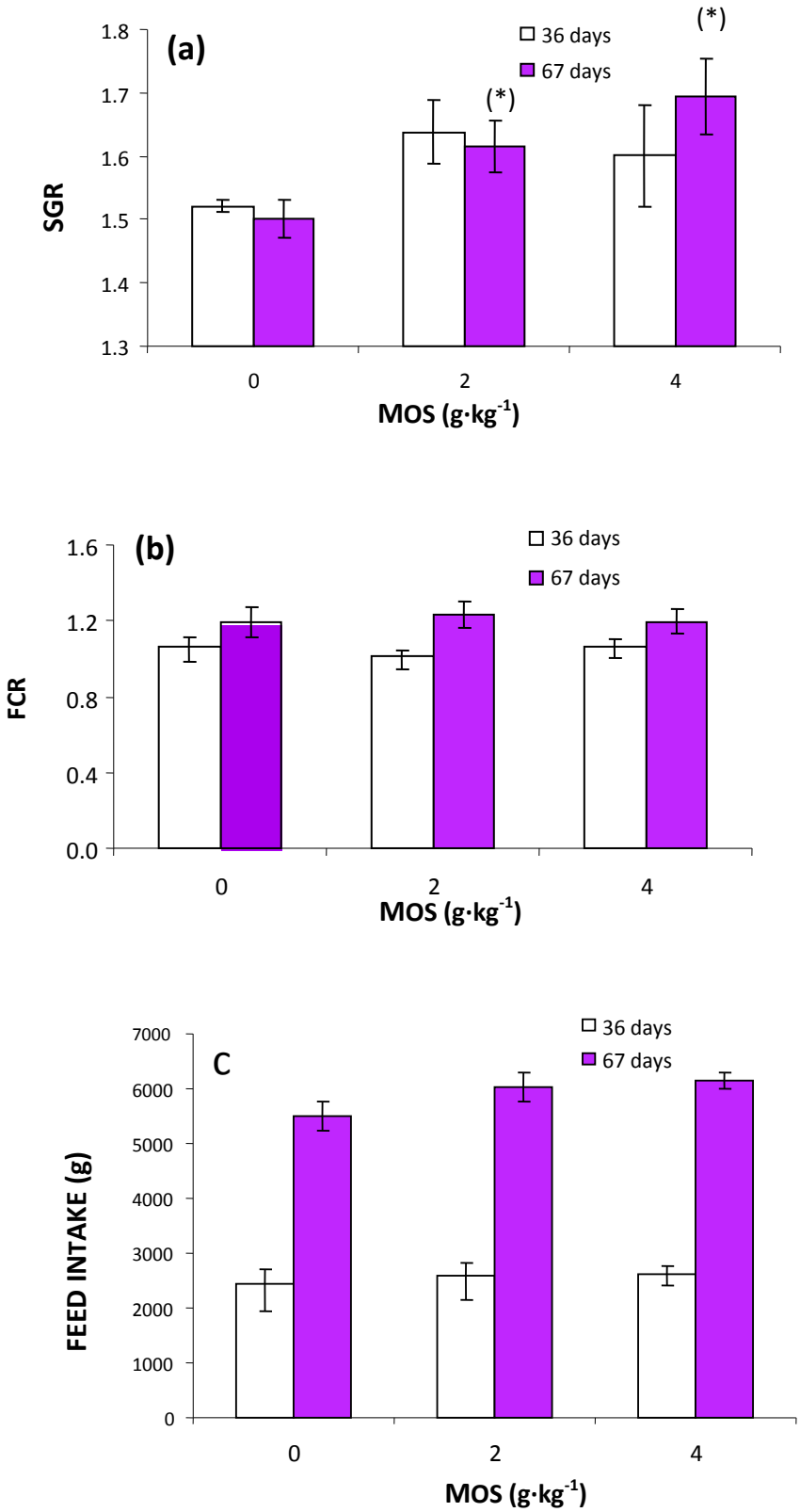


Figure 3.1. (a) Growth performance (SGR); (b) Feed efficiency (FCR); and (c) feed intake for European sea bass fed at different levels of MOS inclusion during Experiment I. Data represent the mean ±SD. (n36 days = 264; n67 days = 210). Significant differences ($P < 0.05$) among treatments for a given feeding period are indicated by (*).

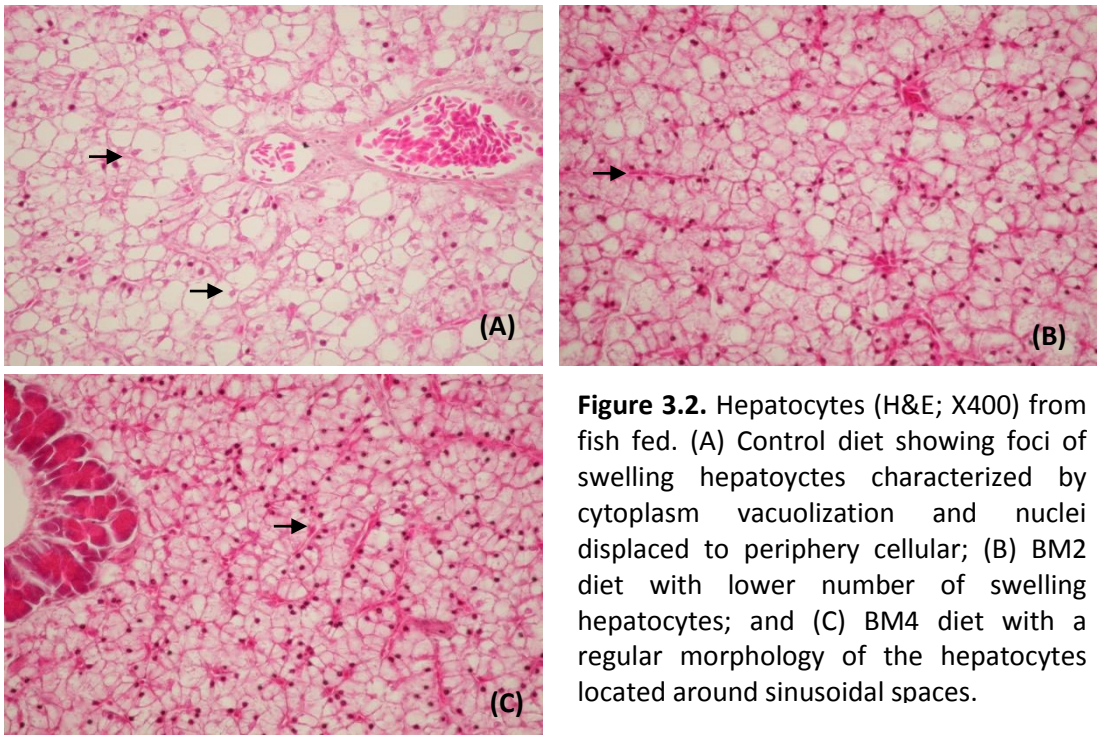


Figure 3.2. Hepatocytes (H&E; X400) from fish fed. (A) Control diet showing foci of swelling hepatoyctes characterized by cytoplasm vacuolization and nuclei displaced to periphery cellular; (B) BM2 diet with lower number of swelling hepatocytes; and (C) BM4 diet with a regular morphology of the hepatocytes located around sinusoidal spaces.

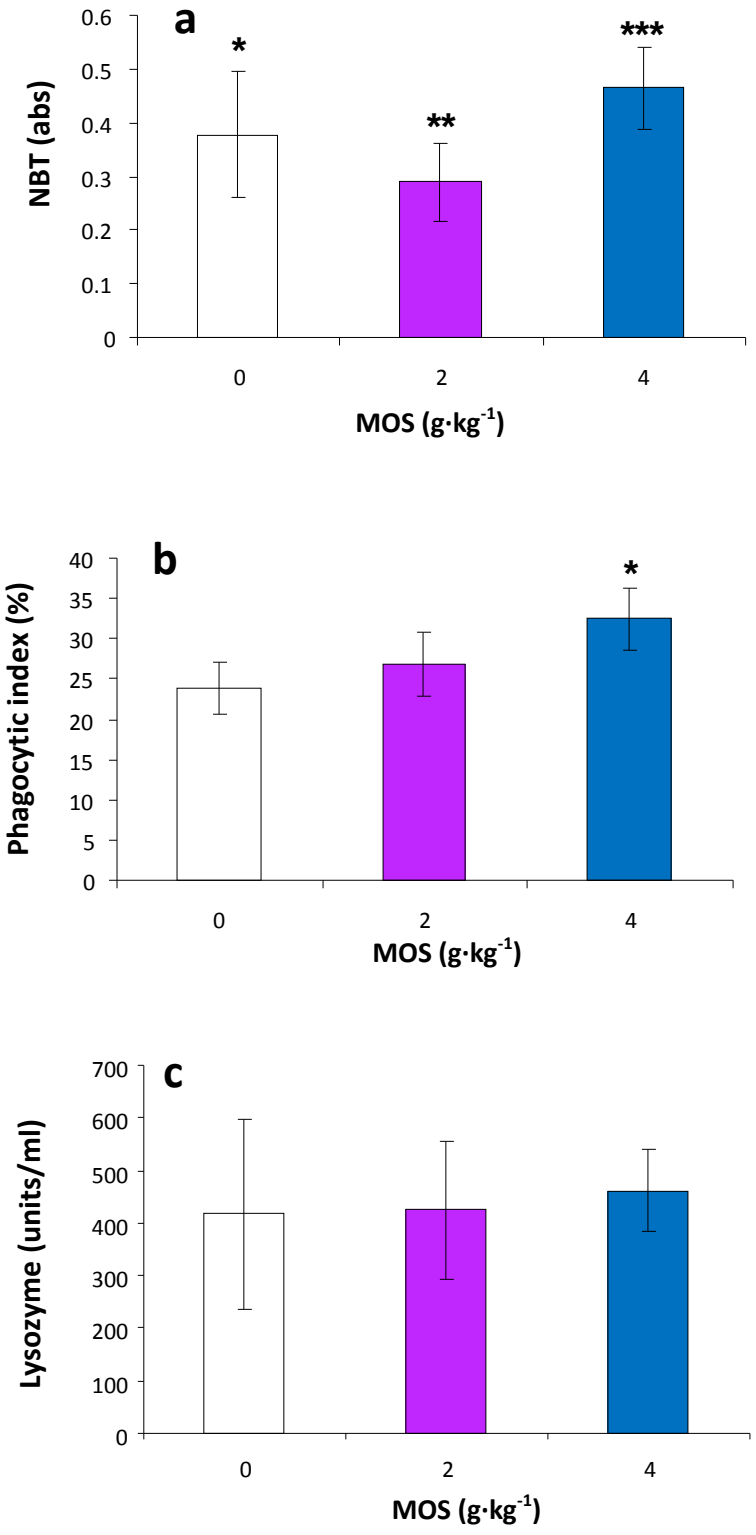
3.3.3. Experiment III

No mortalities were registered in this experiment. The grade of pathogen infection was determined by the presence or absence of the pathogen in internal organs and the pathogen was only recovered on head kidney. A 10% of infected fish were found in all pathogen induced fish at 24 h postinoculation regardless the dietary treatment. However, after 48 h the number of infected fish found in the control treatment was twice in the fish fed MOS (Fig. 3.5).

3.4. DISCUSSION

In the present study, despite inclusion of dietary MOS at 2 $g \cdot kg^{-1}$ and 4 $g \cdot kg^{-1}$ did not significantly affected diet proximate composition, fish fed MOS supplemented diets showed a significant growth improvement. Similar effects have been described for broiler chickens (Kumprecht *et al.*, 1997), pigs (Pettigrew, 2000, Miguel *et al.*, 2002), and common carp or rainbow trout (Staykov, 2004). Improved growth may be related with an enhanced aminoacid absorption as it has been shown in chicken (Iji *et al.*, 2001) and it is suggested by the improvement in the functional integrity of the enterocyte membrane in MOS fed animals (Spring *et al.*, 2000; Shane, 2001). In sea bass, dietary MOS did not affect fish gut at an optical microscopy level. However, some studies have reported a better microvilli alignment when MOS

was fed to fish (Dimitroglou and Davies, 2004) or broiler chickens (Iji *et al.*, 2001), where a significant increased jejunal villus height was described.



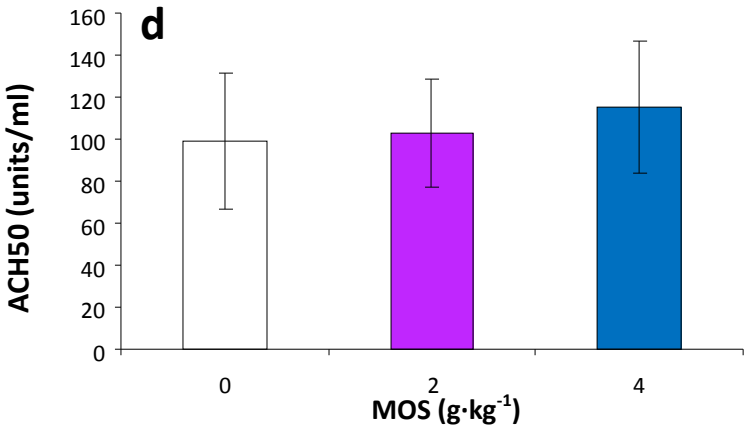


Figure 3.3. Influence of MOS on total (a) phagocytic activity of head kidney macrophages (n =24); (b) reduction potential of circulating neutrophils (n = 24); (c) lysozyme activity (n = 24); and (d) alternative complement pathway activity (n = 24) in European sea bass during Experiment I (mean ± SD; n = 24). Significant differences (P < 0.05) among treatments are indicated by (*).

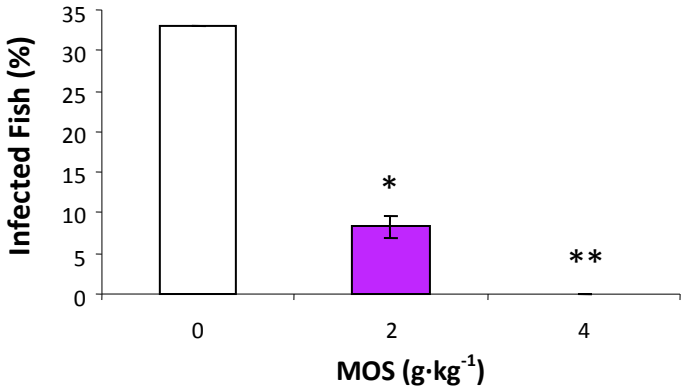


Figure 3.4. Presence of *Vibrio alginolyticus* presence on head kidney of European sea bass fed different levels of MOS inclusion and submitted to infection by cohabitation in Experiment II (mean ± SD; n = 60). Significant differences (P < 0.05) among treatments are indicated by (*).

A positive correlation was also observed for sea bass between the dietary MOS inclusion level and feed intake, in agreement with the results found in neonatal dairy calves (Heinrichs *et al.*, 2001) and senior dogs (Grieshop *et al.*, 2004). Besides increasing feed intake, MOS has been found also to reduce by 20% cholesterol absorption in rats (Gallaher *et al.*, 2000) and increase bile salt excretion (Jie and Shu-Sheng, 1997; Gallaher *et al.*, 1999). In turn, both reduced cholesterol absorption and increased bile salt excretion could reduce liver cholesterol in relation with the reduced liver fat deposition found in sea bass hepathocytes in the present experiment. This is in agreement with the results obtained by Young *et al.* (2000), who reported

a significant reduction of fat deposition in liver and a significant hypocholesterolemic effect in liver and eggs after fed laying hens 0.1% Bio-Mos.

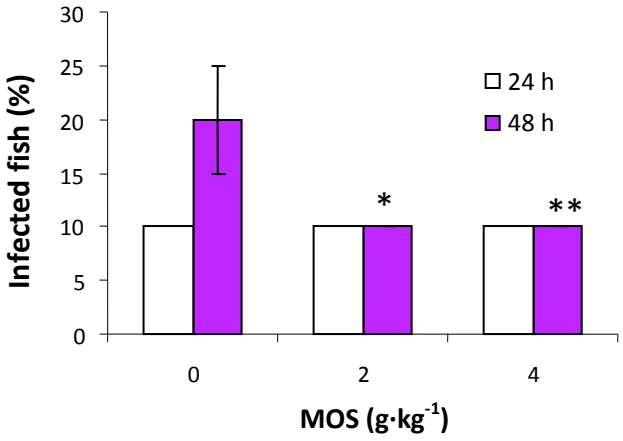


Figure 3.5. Presence of *Vibrio alginolyticus* presence on head kidney of European sea bass fed different levels of MOS inclusion and submitted to infection by anal canalisation in Experiment III (mean ± SD; n = 60). Significant differences (P < 0.05) among treatments are indicated by (*).

In the present study, dietary MOS incorporation at 4 g·kg^{-1} significantly improved head kidney macrophages phagocytic activity. Comparable results have been obtained for sea bass by Montero *et al.* (2005), who observed an improvement on immune status and stress resistance in fish fed with a mixture of MOS-β-glucans for 60 days. A dose-dependant enhancement of phagocytic activity by MOS has been also found in chicken macrophages (Sisak, 1995), what could be related to the presence of a mannose receptor involved in microbe recognition and phagocytosis in the absence of specific opsonization (Ofek *et al.,* 1995). The mannose receptor is the main molecule involved in antigen recognition and the binding process in antigen presenting cells (Engering *et al.,* 1997). Another possible action mechanism of MOS on the immune system could be the stimulation of mannose binding lectin (MBL) by liver secretion. This protein binds the capsule of bacteria and triggers the complement cascade (Janeway, 1993). In this respect, the results of the present study showed a trend to increase in the alternative complement pathway activity by elevation of MOS dietary levels. This is in agreement with the results obtained by Staykov (2004) who found higher levels of bactericidal activity, lysozyme, antibody levels and alternative complement pathway activity in rainbow trout and common carp fed MOS. Therefore, MOS could activate and facilitate antigen processing and serve to stimulate the initial stages of the immune response (Moran, 2004). Nevertheless, MOS effect on immune response

could be related with other factors. For instance, a selective absorption of tryptophan by gut brush-border membrane has been reported in chicks fed MOS (Iji *et al.*, 2001) and certain studies show that high doses of tryptophan cause abnormal white blood cell accumulation in tissues (Gross *et al.*, 1996, 1999), evidencing the active role that tryptophan or its metabolites plays is modulating immune system activity. Hence, further studies must be conducted to understand the role of MOS in fish immunomodulation.

Despite all the intraperitoneally infected fish rapidly died due to the endotoxic hiperagude shock produced by *Vibrio alginolyticus* extracelular products (ECPs) (Balebona *et al.*, 1998), no mortality was detected on cohabitants. In other species such as chinook salmon (*Oncorhynchus tshawytscha*) exposition to an *Aeromonas salmonicida* challenge showed a direct relationship between a rapid death of the experimentally infected fish and disease occurrence (Ogut and Reno, 2004). Hence, the lack of mortality in cohabitant fish seems to be related to a low density of bacteria released to the water from donors, being the receptors asymptomatic infected fish. However direct gut inoculation of *Vibrio alginolyticus* showed the reduction in the number of infected fish by feeding MOS. Among other carbohydrates, mannose constitutes an important surface component of cells. Mannose-specific lectins are utilized by many gastrointestinal pathogens as a means of attachment to the gut epithelium (Mirelman and Ofek, 1986). These molecules function as adhesins mediating the binding of some bacteria as *Campylobacter jejuni* (McSweegan and Walker, 1986) and *Aeromonas hydrophila* (Merino *et al.*, 1996) to epithelial cells, and the interaction of bacteria with phagocytic cells (Perry and Ofek, 1984; Wright *et al.*, 1989). Some studies reported that mannose inhibited the *in vitro* colonization of the chicken small intestine by *Salmonella typhimurium* (Oyofo *et al.*, 1989a) and reduced caecum colonization by *Salmonella typhimurium* following oral inoculation (Spring *et al.*, 2000).

In summary, these results showed that dietary incorporation of MOS at 4 $g \cdot kg^{-1}$ enhances sea bass growth, activates its immune system and increase its resistance to a bacterial infection directly inoculated in the gut, one of the main sites of infection in fish (Zapata and Cooper, 1990). Further experiments must be conducted to clarify the action mechanisms of MOS, as well as the optimum feeding period and dose.

Chapter 4

Aquaculture Nutrition

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Improved feed utilization, intestinal mucus production and immune parameters in sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS)

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ABSTRACT

In the present study, the effects of dose dietary administration of MOS (Bio-Mos®, Alltech Inc, USA) on growth, digestibility, liver morphology, lipid and carbohydrate metabolism enzymes, organoleptic properties, immune parameters and gastrointestinal tract mucus production were assessed. MOS dietary inclusion significantly reduced lipogenic enzymes activity in liver and resulted in a lower lipid vacuolization and more regular-shaped morphology of hepatocytes around the sinusoidal spaces, which could be associated to a better energy and feed utilization due to the improvement of feed utilization and the reduction of feed intake found in the current experiment. Moreover, MOS inclusion enhanced phagocytic activity of head kidney leukocytes in fish fed 4 and 6 $g \cdot kg^{-1}$ MOS diets at days 30, 45 and 60. Gut quantitative morphological analyses showed a marked dose-dependent enhancement in the number of cells secreting acid mucins by unit of area which could be related to the previous improvement in resistance to bacterial infection. No effects on sensorial parameters and biochemical composition of flesh were detected.

4.1. INTRODUCTION

Disease susceptibility in aquaculture is mainly linked to inadequate feeding and nutrition as well as detrimental environmental and welfare conditions. Even small and unavoidable deviations from optimum values in these

Keywords:
Flesh quality
Immune parameters
Lipogenic enzymes
Liver morphology
Mucus
Sea bass

factors, that are closely interlinked, can lead to reduced growth and increased fish vulnerability to disease (Verschuere *et al,.* 2000; Winton, 2001). The increasing economic and social concern to reduce the use of antibiotics and other therapeutic chemicals used in fish farming has encouraged more environmentally friendly approaches to control disease (Hansen and Olafsen, 1999; Verschuere *et al,.* 2000) by good management practices and sanitary prevention measures, such as the use of vaccines and the reinforcement of fish immune system (IUCN, 2007).

Therefore, prevention of infectious diseases through effective alternative methods to in-feed antibiotics, such as prebiotics, is receiving increased attention in fish production (Patterson and Burkholder, 2003; Genc *et al.,* 2007). Prebiotics are classified as non-digestible food ingredients that beneficially affect the host by stimulating growth or activity of certain intestinal bacteria, and include mannan oligosaccharides (MOS). Bio-Mos® (Alltech Inc., Kentaycky, USA) is a product composed by a fraction of *Saccharomyces cerevisiae* cell wall rich in these compounds, whose benefits have been reported in a broad number of poultry species, where it improves feed efficiency, promotes growth and preserves intestinal wall integrity (Hooge *et al,* 2003; Sims *et al.,* 2004; Waldroup *et al.,* 2003). In freshwater fish species, such as common carp (*Cyprinus carpio*) and Jian carp (*Cyprinus carpio Var. Jian*.), growth and feed utilization has been significantly improved by feeding MOS supplemented diets (Zhou and Li, 2004; Culjak *et al.,* 2006; Staykov *et al.,* 2007). As well, MOS feeding markedly reduced mortality and improved lysozyme, classical complement pathway (CCP) and alternative complement pathway (ACP) activities (Staykov *et al.,* 2007). However, the effects of MOS in marine fish have been scarcely studied. Torrecillas *et al.* (2007) reported significant improvement on growth and innate immune parameters for European sea bass (*Dicentrarchus labrax*) juveniles after fed fish with MOS. In the same study was reported an improvement in the disease resistance after inoculating bacteria directly in gastrointestinal tract, one of the main entry portals of bacteria in fish (Zapata and Cooper 1990). But studies about the mechanisms involved in this reduction of bacterial translocation after feeding MOS have not been studied. Many infectious diseases are initiated by bacterial colonization of mucosal surfaces, where in order to become pathogenic, bacteria must attach to host mucus, be able to use mucus for growth and multiply fast before being flushed out of the gut (Van der Marel *et al.* 2008), then, hijack the cellular molecules and signalling pathways of the host (Liévin-Le Moal and Servin, 2006). Indeed, it appears that an intact mucosal surface is capable of forming a protective barrier against some potentially pathogenic bacteria as *A.*

hydrophila (Behrendt, 2005). Therefore, despite it has not been yet determined, dietary MOS could improve the conditions of the protective barrier and reduce bacterial translocation.

In order to investigate this hypothesis and further investigate the dose effect of dietary inclusion of MOS on health and growth performance of juvenile European sea bass and the possible mechanisms implicated in those effects, growth response and its relation to diet digestibility, biochemical composition, activities of enzymes implicated in lipid and carbohydrate metabolism as well as liver morphology and morphometry was studied. Moreover, selected innate immune parameters and gastrointestinal tract mucus production were also studied. Finally, after achieving commercial size, organoleptic properties of fish fed MOS were evaluated in order to determine any potential effect of MOS supplementation in flesh quality.

4.2. MATERIALS AND METHODS

4.2.1. Diets

Four diets, based in a commercial formulation, were designed to contain 0 $g \cdot kg^{-1}$ (Control), 2 $g \cdot kg^{-1}$ (BM2), 4 $g \cdot kg^{-1}$ (BM4) and 6 $g \cdot kg^{-1}$ (BM6) (Bio-Mos®, Alltech Inc., USA) replacing standard carbohydrates. Diets covered nutritional requirements for this species (Izquierdo 2005) and were manufactured by a commercial feed producer (Graneros de Tenerife S.A. Tenerife, Spain) with the composition indicated in Table 4.1.

4.2.2. Experimental conditions

4.2.2.1. Experiment I: Feeding trial

Fifteen hundred commercially reared European sea bass originated from a local farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain), were transferred to the main facility of the Canarian Institute of Marine Science (ICCM) on October 1st, 2006. Fish were maintained in stocking tanks and fed a commercial extruded diet for 3 weeks until being well adapted to the environmental conditions. Since previous studies showing the positive effect of MOS on growth, immune parameters and disease resistance (Torrecillas *et al.* 2007) were conducted at experimental densities (3 $kg \cdot m^{-3}$), in order to test the MOS effect in more practical conditions, the present study was conducted at commercial densities (7-10 $kg \cdot m^{-3}$).

Then, 1392 sea bass juveniles were randomly distributed in 24 indoor, 500 l fiberglass tanks at an initial stocking density of 7 $kg \cdot m^{-3}$ (58 fish per tank). Fish average initial weight (g ± SD) and length

(cm ± SD) were 60.64 ± 0.85 and 16.57 ± 0.12, respectively. Tanks were supplied with filtered sea water, at a temperature of 20-23.7°C, and natural photoperiod (12L:12D). Water dissolved oxygen values ranged 6.8 ± 0.7 $mg \cdot l^{-1}$. Fish were manually fed until apparent satiation with one of the four experimental diets for 8 weeks (twice daily, 6 days a week). Six replicates of 58 fish each were used to assay each diet.

Table 4.1. Main ingredients and composition in the experimental diet

Ingredient	**Diet (g kg^{-1} dry weight)**			
	Control	**BM2**	**BM4**	**BM6**
Fish meal [1]	515.0	515.0	515.0	515.0
Soybean meal	97.8	97.8	97.8	97.8
Wheat	75.0	75.0	75.0	75.0
Wheat gluten	75.0	75.0	75.0	75.0
Corn meal	65.3	63.3	61.3	59.3
Fish oil [2]	126.9	126.9	126.9	126.9
Fats and oils	20.3	20.3	20.3	20.3
Mineral mix [3]	14.3	14.3	14.3	14.3
Vitamin mix [4]	10.3	10.3	10.3	10.3
Antioxidant (BHT)	0.1	0.1	0.1	0.1
MOS [5]	0	2	4	6
Composition (%, dry weight)				
Crude lipids	24.07	24.82	24.04	23.43
Crude protein	48.71	48.77	48.33	48.37
Ash	9.98	9.81	9.56	9.69

[1] Peruvian fish meal (65% protein). [2] Peruvian fish oil. [3] Mineral mix TROUW Seabream/Seabass (0.8g), Choline chloride (0.17g) and Inositol (0.06g) (Trouw Nutrition Spain, Madrid, Spain). [4] Vitamin mix TROUW Seabream/Seabass (1g) Calcium carbonate (0.2g), Potassium monophosphate (0.19g) and NaCl 97% (0.04g) (Trouw Nutrition Spain, Madrid, Spain). [5] Bio-Mos®, Alltech, Inc., USA

Feed intake was calculated daily and growth parameters were determined at day 0, 30 and 60. Nine fish at the beginning of the trial and 5 fish per tank at day 60 were sampled and pooled for body proximate composition, 15 fish per diet were sampled for liver enzyme activity and for studies of hepatic and enteric tissue morphology. Blood samples were taken from 15 fish per diet for serum collection at day 30 and 60 to determine lysozyme activity. In addition, the head kidney was removed from 15 fish per dietary treatment at days 30, 45 and 60 in order to determine phagocytic activity of head kidney leukocytes.

4.2.2.2. Experiment II: Digestibility study

At the end of the feeding trial, 15 fish per diet were randomly selected and distributed among 12 indoor 120 l fiberglass digestibility tanks. Each group of fish was fed the same diet as in the former trial. Water temperature ranged from 22.0 to 23.7°C during the trial with natural

photoperiod (12L: 12D). Fish were manually fed their respective diets until apparent satiation for one month (twice daily, 6 days a week). Faeces were collected following the faeces collection system described by Cho *et al.* (1985) modified by Robaina *et al.* (1995). Immediately after their daily collection, faecal matter was centrifuged, frozen and stored at -20°C. Pooled faeces from each group were freeze-dried prior to analysis. Apparent digestibility coefficients were calculated using the formula reported by Maynard and Loosly (1969). Each diet was assayed by triplicate.

Proximate composition of diets and faeces were used to calculate apparent digestibility coefficients (ADC) for protein and lipid. Acid insoluble ash, which served as a marker of feed digestibility, was determined using the method of Atkinson *et al.* (1984).

4.2.2.3. Experiment III: Sensorial analysis

At the end of the feeding trial, 25 fish per diet were randomly selected and distributed among 4 indoor 1000 l fiberglass tanks to continue the on-growing until commercial size. Each group of fish was fed the same diet as in the former trial. Fish were submitted to a water temperature of 19.3 to 24.2°C and a natural photoperiod of 12L:12D and fed manually their respective experimental diets until apparent satiation (twice daily, 6 days a week) until they achieved commercial size (14 months). Each diet was assayed in triplicate.

At the end of the feeding period fish were starved for 24 h and then slaughtered in a small tank with ice and seawater. A total of 12 fish from each treatment were randomly sampled, gutted and kept at 4°C for 24 h until tests were carried out. Organoleptic tests were conducted on fish fillets cooked in aluminum boxes for 10 min in a steam oven (120°C). Immediately after cooking, fillets were offered to a panel of 8 selected and trained judges (ISO 1985, ISO 1993). Tests were conducted in isolated and air conditioned rooms with standardized light (ISO 1988). Judges were randomly offered closed food boxes labelled with codes containing the fillets (3X4 cm). Odour (marine, off-odour and oily), appearance (juicy, shininess and colour), texture (adhesiveness, cohesiveness, hardness and juiciness) and flavour (marine, off-flavour and oily) were tested for samples of fish fed the experimental diets and classified by the judge in a continuous scale from 0 to 100 for each parameter. Samples of flesh were also analyzed for biochemical composition and fatty acid composition as described in the following paragraph.

4.2.3. Biochemical analysis

Feed, faeces and fish biochemical composition were conducted following standard procedures (AOAC 1995). Dry matter content was determined after drying the sample in an oven at 105 °C to constant weight, ash by combustion in a muffle furnace at 600 °C for 12 h, protein content (Nx6.25) was determined by Kjeldahl method and crude lipid was extracted following the method of Folch *et al.* (1957). Fatty acids from total lipids were prepared by transmethylation as described by Christie *et al.* (1989) and separated by gas chromatography under the conditions described by Izquierdo *et al.* (1992), being quantified by flame ionizator detector (FID) and identified by comparison to external standards. All analysis were conducted in triplicate.

4.2.4. Histological studies

On Experiment I, liver and gut samples were taken after 60 days of the feeding trial. On Experiment II, gut samples were taken after 14 months of MOS supplementation. Samples were fixed in 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E) for optical examination. For each specimen gut, five sets of five serial longitudinal sections (5 µm-thick) were stained with specific staining Alcian Blue or Alcian Blue/ PAS in order to determine cells secreting mucins (Martoja and Martoja-Pierson, 1970). Micrographs were taken from the paraffin sections at a final magnification of 400X and 200X using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. For liver, hepatocellular area as well as maximum and minimum longitude of cells taking hepatocyte nuclei as reference, and for gut the number of cells stained for specific mucin staining by unit of area, were measured with an analySIS® (Image Pro Plus®) software package using arbitrary units.

4.2.5. Blood collection and sample preparation

On Experiment I, blood was obtained by caudal vein puncture with a 1 ml plastic syringe. No anesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize the stress effects. The sample was transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at -80 °C for lysozyme activity determination.

4.2.6. Serum analysis

Lysozyme level in blood serum was determined by turbidimetric assay according to the method described by Anderson and Siwiki (1994) using hen egg white lysozyme in PBS as a standard. Results were presented as Lysozyme units·ml^{-1}.

4.2.7. Head kidney leucocytes phagocytic activity

Head kidneys of 15 fish per diet were removed and leucocytes were isolated by density gradient centrifugation technique as follows. After collection, head kidneys were homogenized in supplemented Minimal Essential Medium (MEM) (10µl·ml^{-1} heparin; 100µl·ml^{-1} penicillin/streptomycin; 2% fetal bovine serum and filtered through a nylon membrane (100 µm mesh size, Sigma). Resulting cell solution was layered onto preformed gradient of 51%- 34% Percoll in 10% Hank's balanced salt solution (HBSS) and centrifuged at 800 g for 30 min at 4°C. The interface layer was harvested with a Pasteur pipette, diluted in 1 ml of supplemented MEM and recentrifuged at 800 g for 5 min at 4°C to remove residual Percoll. The resulting pellet was diluted in 1 ml of supplemented MEM and viability and concentration of head kidney leucocytes cell suspensions were determined in trypan blue as described by Torrecillas *et al.* (2007). The final pellet with more than 92% viability was resuspended in supplemented MEM adjusting the final concentration to 10^6 leucocytes·ml^{-1}. Head kidney leucocytes solution was incubated in a ratio 1:1 with microparticles based on polystyrene (1µm, Fluka) as described by Esteban and Meseguer (1997) for *Vibrio anguillarum*. Phagocytic activity was measured as described by Blazer (1991). Ten series of one hundred leucocytes per fish were counted and the phagocytic index was determined as the percentage of leucocytes with phagocytic ability. Positive phagocytic activity was determined only for the leucocytes with presence of the microparticle of polyestyrene inside the cytoplasm.

4.2.8 Liver enzyme activities

Liver samples were homogenized in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4) and homegenates were centrifuged at 20000 g for 40 min at 4°C. Soluble protein content of liver homogenates was determined by the method of Bradford (1976). Enzyme activities of glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40) were assayed using spectrophotometric procedures on the supernatant following the method described by Dias *et al.* (1998). Care was taken to ensure that initial rates

were measured in all assays and that enzymes were stable in the buffer solution used during the time and temperature required to perform the assays (Álvarez *et al.* 1998b). Enzyme activity units (IU), defined as micromoles of substrate converted to product per minute at assay temperature per minute, were expressed per mg of hepatic soluble protein (specific activity). All enzyme assays were at least assayed in duplicate.

4.2.9. Statistical analysis

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of MOS inclusion. When F values showed significance, individual means were compared using Tukey's or Scheffe test for multiple means comparison. Significant differences were considered for $P<0.05$. If the variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the data. Analyses were performed using Statgraphics software (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA).

4.3. RESULTS

4.3.1. Growth and feed utilization

No mortalities appeared during any of the trials. After 30 and 60 days of feeding the experimental diets, mean body weight, relative growth, K and SGR were not significantly affected by MOS inclusion (Table 4.2). However, at the end of the trial control fish were slightly larger (1% higher) than BM2 fed fish. In addition, a reduction in feed intake was found with the inclusion of MOS, which was significantly lower in fish fed BM4 and BM6 diets in comparison with the control fish (Table 4.2). Accordingly, FCR were also significantly improved in fish fed the two highest MOS inclusion levels 4 $g \cdot kg^{-1}$ and 6 $g \cdot kg^{-1}$ (Table 4.2). Final eviscerated weight was higher and viscerasomatic index lower in fish fed BM4, although not significantly different from the other fish (Table 4.2).

Data on the activities of the two lipogenic enzymes assayed in fish liver showed a significant reduction of both activities with the inclusion of MOS compared to fish fed control diet ($P<0.05$, Table 4.3). MOS inclusion (Table 4.4) reduced whole body lipid content, and

significantly in fish fed BM6 diet in comparison to fish fed control diet. The fatty acid profile of fish was not affected by the diet (Table 4.5). Moisture of carcass was significantly reduced in fish fed BM4 and BM6 diets.

4.3.2. Histological studies

Liver morphological studies showed that inclusion of MOS produced a more regularly-shaped hepatocytes organized around sinusoidal spaces with a lower lipid vacuolization of the cytoplasm that decreased the number of hepatocytes with the nuclei displaced to the cellular periphery (Fig. 4.1). Accordingly, quantitative morphometric analysis of liver revealed significant ($P<0.05$) reductions in hepatocellular area and maximum and minimum cell lengths in hepatocytes of fish fed MOS (Table 4.6).

Table 4.2. Growth performance, nutrient utilization and somatic parameters of European sea bass fed commercial extruded diets with different levels of MOS inclusion on feeding trial (Experiment I)

	Dietary treatments			
	Control	***BM2***	***BM4***	***BM6***
Final weight (g)	92.35±3.30	88.96±2.53	92.91±2.16	91.18±4.27
Final length (cm)	19.17^{a}±0.18	18.89^{b}±0.16	18.93^{ab}±0.17	18.94^{ab}±0.19
Final relative growth (%)	50.85±5.39	47.35±6.49	53.63±4.09	50.88±6.76
Final eviscerated weight (g)	77.8±7.49	77.4±5.70	79.32±7.16	80.42±7.71
Final VSI (1) (%)	11.70±1.89	12.39±1.67	11.55±1.01	12.17±1.85
SGR (2) (t=30 days)	0.99±0.17	0.96±0.19	0.94±0.14	0.99±0.16
SGR (2) (t=60 days)	0.71±0.06	0.67±0.07	0.74±0.05	0.71±0.08
FCR (3) (t=30 days)	1.72±0.32	1.78±0.19	1.79±0.22	1.63±0.17
FCR (3) (t=60 days)	2.68^{c}±0.40	2.59^{bc}±0.76	1.74^{a}±0.25	2.19^{b}±0.34
FI (g)(4) (t=30 days)	1988.20±117.84	1948.78±237.97	1924.31±72.94	1844.70±187.16

Different letters within a line denotes significant differences ($P<0.05$). Control= 0 $g \cdot kg^{-1}$ MOS; BM2=2 $g \cdot kg^{-1}$ MOS; BM4= $g \cdot kg^{-1}$ MOS; BM6=6 $g \cdot kg^{-1}$ MOS. Values expressed in mean ± SD. (n=6 tanks per diet). [1]VSI= Visceralsomatic index. [2]SGR =((log average final weight - loge average initial weight)/no days) x 100. [3]FCR = feed consumption (g) / weight gain (g). [4] FI = Feed intake (g).

Morphological studies of the posterior gut showed a significant increase ($P < 0.05$, n=258) in the number of cells secreting acid mucins by unit of area after 8 weeks of supplementation with MOS (Fig. 4.2a). Thus, a positive correlation was observed between the level of MOS inclusion and the amount of cells secreting mucus in posterior gut ($y = 132.32x + 794.17$; $R^2 = 0.9628$). After 14 months of supplementation at the end of the Experiment III there was also an increase ($P < 0.05$, n=230) in the number of cells secreting acid mucins by 10^6 units of area for fish fed 2 and 4 $g \cdot kg^{-1}$ MOS diet (Fig. 4.2b) and a positive correlation between the level of MOS inclusion and the

amount of cells secreting mucus (y = 79.311 x + 855.42, R^2 = 0.9069). The same tendency was observed in the anterior gut (y = 40.698 x + 606.48, R^2 = 0.801) (Fig. 4.2b).

Table 4.3. Results for lipogenic enzymatic activities in liver of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 60 days)

	Dietary treatments			
	Control	***BM2***	***BM4***	***BM6***
G6PD				
IU/mg protein	$0.606^{a} \pm 0.108$	$0.373^{b} \pm 0.007$	$0.401^{b} \pm 0.042$	$0.443^{b} \pm 0.037$
ME				
mIU/mg protein	$11.60^{a} \pm 1.40$	$8.20^{b} \pm 4.17$	$7.93^{b} \pm 1.32$	$9.40^{ab} \pm 0.31$

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} MOS; BM2=2 g·kg^{-1} MOS; BM4=4 g·kg^{-1} MOS; BM6=6 g·kg^{-1} MOS. Values expressed in mean ± SD (n=15).

Table 4.4. Whole body final proximate composition *(g/100g dry weight)* of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 60 days).

	Dietary treatments			
	Control	***BM2***	***BM4***	***BM6***
Crude protein	18.99 ± 0.30	18.49 ± 0,82	18.39 ± 0.39	18.21 ± 0.29
Crude Lipids	$20.74^{a} \pm 0.89$	$19.00^{ab} \pm 1.05$	$19.37^{ab} \pm 0.66$	$17.13^{b} \pm 1.17$
Ash	9.50± 0.76	9.45± 0.67	8.99 ± 0.81	9.19 ± 0.94
Moisture	$15.82^{a} \pm 0.45$	$15.73^{a} \pm 0.17$	$10.05^{b} \pm 0.01$	$10.60^{b} \pm 0.63$

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} MOS; BM2=2 g·kg^{-1} MOS; BM4= g·kg^{-1} MOS; BM6=6 g·kg^{-1} MOS. Values expressed in mean ± SD. (n=6)

Table 4.5. Composition of main fatty acids groups (g fatty acids/100 g total fatty acids) for whole body composition of European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 60 days)

	Dietary treatments			
	Control	***BM2***	***BM4***	***BM6***
Saturated	33.12 ± 0.97	34.98 ± 2.41	34.64 ± 0.79	34.22 ± 1.06
Monosaturated	31.75 ± 0.61	30,60 ± 0.89	31.32 ± 1.36	31.32 ± 0.68
∑n-3	21.28 ± 0.42	21.38 ± 1.26	20.41 ± 0.78	21.05 ± 1.09
∑n-6	10.73 ± 0.39	10.01 ± 0.16	10.68 ± 0.18	10.35 ± 0.27
∑n-9	28.02 ± 0.60	26.91 ± 0.83	27.53 ± 1.28	27.5 ± 0.67
∑n-3 HUFA	17.56 ± 0.31	17.68 ± 1.09	16.77 ± 0.71	17.40 ± 1.00

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} MOS; BM2=2 g·kg^{-1} MOS; BM4=4 g·kg^{-1} MOS; BM6=6 g·kg^{-1} MOS. Values expressed in mean ± SD.

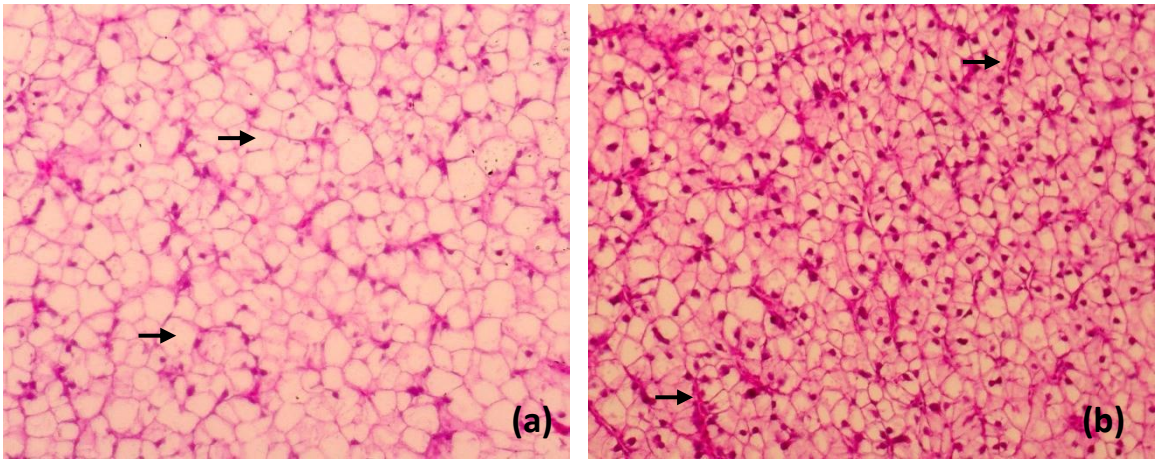

Figure 4.1. Hepatocytes (H&E, X400) from fish fed. (a) Control diet showing foci of swelling hepatoyctes characterized by cytoplasm vacuolization and nuclei displaced to periphery cellular, (b) mannan oligosaccharides diets with a regular morphology of the hepatocytes located around sinusoidal spaces at the end of Experiment I.

Table 4.6. Results of quantitative image analysis (µm) of hepatocytes from European sea bass fed commercial extruded diets with different levels of MOS inclusion at the end of Experiment I (t = 60 days)

	Dietary treatments			
	Control	***BM2***	***BM4***	***BM6***
Maximum longitude	$0.37^{a} \pm 0.02$	$0.26^{b} \pm 0.01$	$0.25^{b} \pm 0.03$	$0.26^{b} \pm 0.05$
Minimum longitude	$0.25^{a} \pm 0.02$	$0.19^{b} \pm 0.01$	$0.17^{b} \pm 0.03$	$0.18^{b} \pm 0.01$
Area	$1.87^{a} \pm 0.31$	$1.01^{b} \pm 0.05$	$0.70^{b} \pm 0.08$	$0.81^{b} \pm 0.08$

Different letters within a line denotes significant differences (P<0.05). Control=0 g·kg^{-1} MOS; BM2=2 g·kg^{-1} MOS; BM4=4 g·kg^{-1} MOS; BM6=6 g·kg^{-1} MOS. Values expressed in mean ± SD (n=75 x 5 x 6; hepatocytes x fish x tank).

4.3.3. Immune parameters

Phagocytic activity of head kidney leucocytes increased significantly in fish fed BM4 and BM6 diets after 30, 45 and 60 days of MOS supplementation ($P < 0.05$, Fig. 4.4), resulting in a positive correlation between the phagocytic activity and the dietary MOS inclusion levels after 60 days ($y=2.632x + 18.56$, $R^2 = 0.9474$). No differences were found on lysozyme activity (Fig. 4.5), although a positive correlation was observed between the level of MOS inclusion and the level of this parameter in fish serum at day 60 ($y = 18.103 x + 403.61$, $R^2 = 0.9044$).

4.3.4. Digestibility study

Visual inspection of faeces collected every day confirmed that there were no signs of contamination from uneated food. ADC of lipids (%) and protein (%) ranged from 81.96 to 82.84 and from 73.31 to 75.5, respectively. No significant differences were found in protein and lipid digestibility coefficients among the experimental diets.

4.3.5. Sensorial analysis

When fish reached commercial size, there were no significant differences in proximate composition and fatty acid profile of fish fillets (Table 4.7). Further, MOS inclusion did not affect any of the sensory attributes studied in European sea bass fillet (Fig. 4.6).

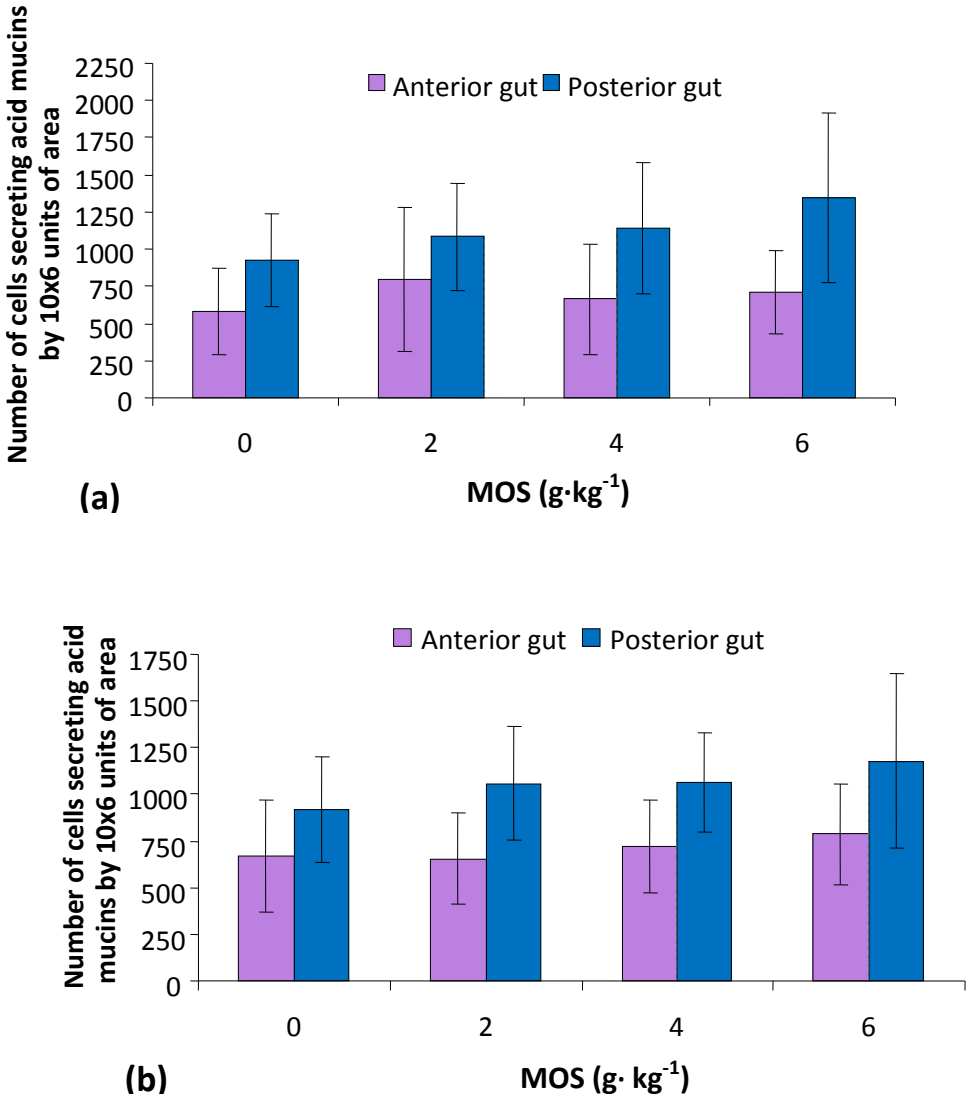

Figure 4.2. (a) Number of cells secreting acid mucins by 10^6 units of area for European sea bass anterior gut and posterior gut fed at different levels of mannan oligosaccharides (MOS) inclusion at the end of Experiment I (t = 60 days). (b) Number of cells secreting acid mucins by 10^6 units of area for European sea bass anterior gut and posterior gut fed different levels of MOS inclusion at the end of Experiment III (14 months of supplementation). Data represent the mean ± SD. Different letters denote significant differences ($P < 0.05$) among treatments.

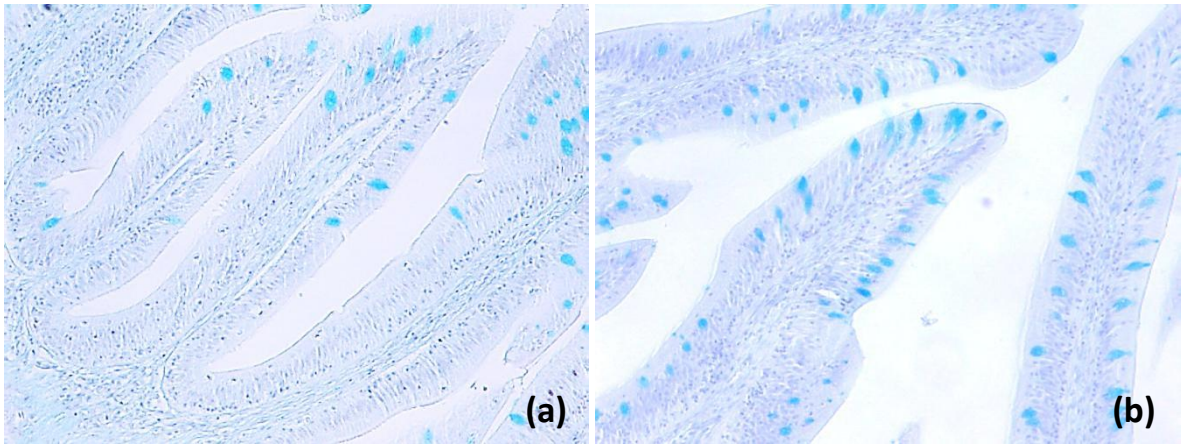

Figure 4.3. Posterior Gut (Alcian Blue x200) from fish fed, (a) Control diet, (b) 6 g·kg^{-1} MOS diets showing a significant improvement in the number of cells secreting acid mucins during Experiment I.

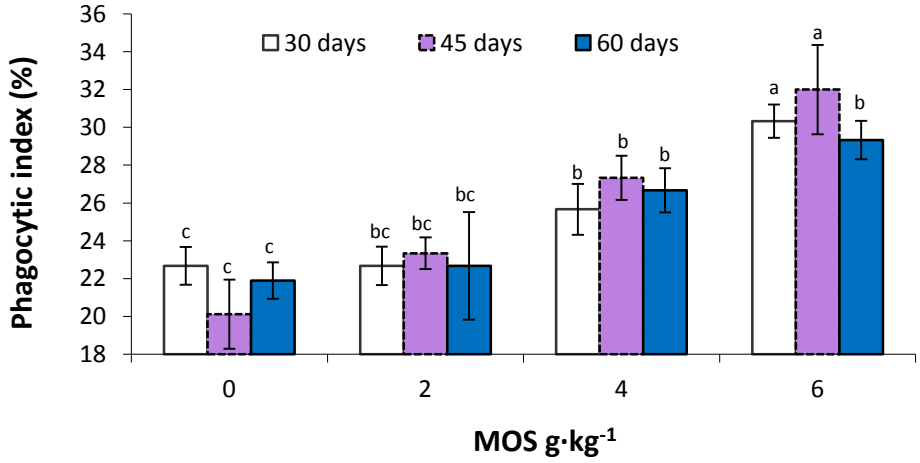

Figure 4.4. Influence of MOS on phagocytic index (%) of head kidney leukocytes in European sea bass in Experiment I at 30, 45 and 60 days of supplementation (mean ± SD; n=15). Different letters denotes significant differences (P<0.05).

Table 4.7. Fillet proximal composition *(g/100g dry weight)* of European sea bass fed commercial extruded diets with different levels of MOS inclusion (commercial size).

	Dietary treatments			
	Control	***BM2***	***BM4***	***BM6***
Crude protein	22.129 ± 0.692	22.511± 0.832	22.544 ± 0.405	23.127 ± 0.897
Crude Lipids	20.97 ± 0.117	20.813 ± 0.031	20.863± 0.025	20.891 ± 0.191
Ash	9.50± 0.76	9.45± 0.67	8.99 ± 0.81	9.19 ± 0.94
Moisture	10.57^{a} ± 0.03	10.41^{b} ± 0.04	10.51^{ab} ± 0.10	10.47^{ab} ± 0.01

Different letters within a line denotes significant differences (P<0.05). Control= 0 g·kg^{-1} MOS; BM2=2 g·kg^{-1} MOS; BM4=4 g·kg^{-1} MOS; BM6=6 g·kg^{-1} MOS. Values expressed in mean ± SD (n=9).

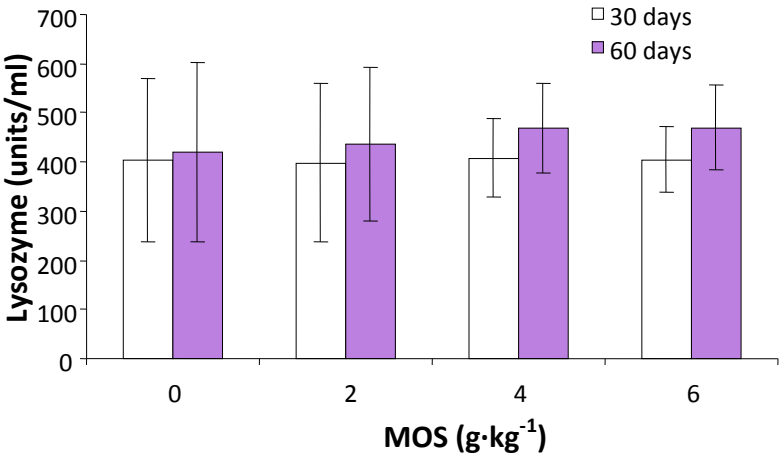

Figure 4.5. Influence of MOS on total lysozyme activity (n =15) in European sea bass during Experiment I (mean ± SD; n=15). Different letters denotes significant differences (P<0.05).

4.4. DISCUSION

Dietary inclusion of MOS markedly improved feed utilization in juvenile European sea bass together with a reduction in feed intake in agreement with the studies in poultry (Waldroup *et al.,* 2003), broiler chickens (Hooge *et al.,* 2003), turkeys (Fritts and Waldroup, 2003; Sims *et al.,* 2004), common carp (Zhou and Li, 2004; Culjak *et al.,* 2006) and rainbow trout (Staykov *et al.*, 2007). Feed intake and satiation in fish, as in other vertebrates, appears to be regulated via complex mechanisms involving elaborate interactions between the brain and peripheral signals (De Pedro and Bjornsson, 2001; Terova *et al.,* 2008). However, these mechanisms have not been clearly defined yet (Stanley *et al.*, 2005; Volkoff *et al.,* 2005). The effect of MOS on feed intake could be related either to the peripheral satiation system (satiation and appetite signals) or to the long-term system (body energy stores) (Jensen, 2001; Terova *et al.*, 2008) that provide information for the hypothalamic central feeding system. Further experiments are being conducted to elucidate this effect of MOS on feed intake in sea bass.

Liver histology revealed a lower lipid vacuolization and subsequently a reduction in hepatocytes size in European sea bass fed MOS, which is in agreement with the significant reduction in the lipogenic enzymes activities found in this tissue and the tendency to reduced viscerasomatic index and increased eviscerated weight of these fish. MOS has been also reported to reduce fat deposition in liver of laying hens (Young *et al.,* 2000) and abdominal fat pad percentage as expressed as carcass weight in broilers (Kannan *et al.,* 2005). Other probiotics have been also suggested to decrease lipogenesis by a reduction of acetyl-CoA carboxylase activity in

chickens (Santoso *et al.,* 1995; Kannan *et al.,* 2005). Nevertheless, reduction in fat deposition could be also related to an increase lipolytic activity as described in rats (Gallaher *et al.,* 2000). In fish, the hepatic tissue has been recognized as a main site for the novo fatty acid synthesis (Dias *et al.,* 1998), G6PD and ME being key regulatory enzymes in the lipogenic and glucolytic pathways. In agreement with these results, Laíz-Carrión *et al.* (2005) also reported a reduction in G6PD together with an increase on the hepatic glucogenolysis and gluconeogenesis after feeding

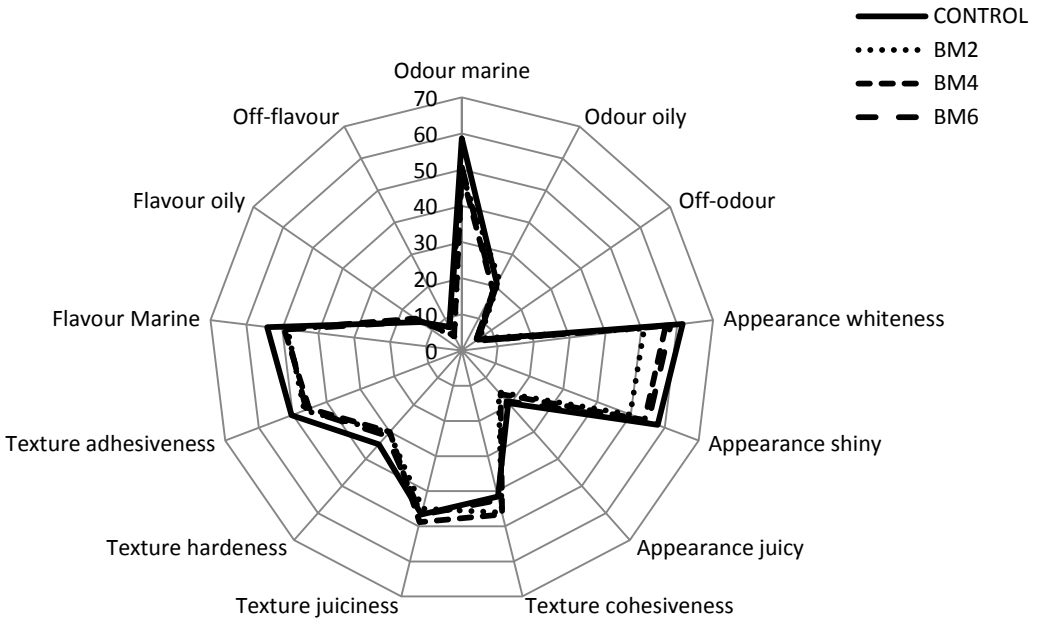

Figure 4.6. Scores of principal organoleptic parameters evaluated of sea bass fillets fed experimental diets during 14 months. Odour (marine, off-odour and oily), appearance (juicy, shininess and colour), texture (adhesiveness, cohesiveness, hardness, and juiciness) and flavour (marine, off-flavour and oily). Values are presented as percent of variation from fish fed dose dietary MOS. Different letters denotes significant differences (P<0.05, n=12).

immunestimulants to gilthead sea bream (*Sparus aurata*), indicating a higher production of internal glucose. Hence, feeding these immunestimulants seems to increase hepatic glucose production, providing an internal energy fuel for body tissues which allows better dietary energy utilization, promoting growth (Torrecillas *et al.,* 2007) and feed conversion ratios as shown in the present experiment. Thus, the present study has shown that the lower lipid vacuolization and

regular-shaped morphology of hepatocytes found in sea bass fed MOS is mediated by a reduction in hepatic lipogenic activity, which could be related to a better energy and feed utilization.

Increase in dietary inclusion of MOS from 4 $g \cdot kg^{-1}$ and above significantly improved head kidney leucocytes phagocytic activity, in agreement with our previous studies where a dose-dependent enhancement of this parameter was described in sea bass reared at low fish densities (Torrecillas *et al.,* 2007) and those found in chicken macrophages (Sisak, 1995). Enhanced phagocytic activity in sea bass could be related to the presence of a mannose-receptor in sea bass head kidney leucocytes involved in microbe recognition and phagocytosis. A mannose-receptor present at the phagocyte surface has been described for gilthead sea bream head kidney leucocytes (Rodríguez *et al.,* 2003), similar to the one present on mammalian macrophages which constitutes the main molecule involved in antigen recognition and binding process in antigen presenting cells (Engering *et al.,* 1997). As well, MOS could enhance sea bass innate immune system by stimulation of mannose binding lectin (MBL) by liver secretion (Torrecillas *et al.,* 2007). MBL is a collectin, that shows specificity for mannose, N-acetyl-D-glucosamine, fucose and glucose, and plays a crucial role in the complement system (Turner, 1996) activating the lectin pathway of complement through the MBL-associated serine proteases (MASPs) (Nikolakopoulou and Zarkadis, 2006) and representing an ancient mechanism of host defense. Indeed, Staykov *et al.* (2007) reported an improvement in classical complement pathway as well as alternative complement pathway in rainbow trout (*Oncorhynchus mykiss*) after feeding MOS for 90 days suggesting a possible involvement of MOS in the activation of these mechanisms of defense. In sea bass, alternative complement pathway activity was also positively correlated to the increase in dietary MOS (Torrecillas *et al.,* 2007). In addition, in the present study, a positive correlation between lysozyme activity and MOS level of inclusion was detected, although no significant differences were found. These results are in agreement with those found in common carp and rainbow trout (Staykov, 2004; Staykov *et al.,* 2007), suggesting that MOS activates and facilitates antigen processing and stimulates the initial stages of the immune response (Moran, 2004).

The results showed an enhancement in the number of cells secreting acid mucins on posterior gut of sea bass fed MOS in the present study, which could explain the lower number of infected sea bass by gut canalization observed in the previous study (Torrecillas *et al.,* 2007). Bacterial adhesion is a necessary step in microbial colonization and pathogenesis, being mediated by interaction with carbohydrates present on cell surfaces (Bavington and Page, 2005). Epithelial

mucosal surfaces have a number of defense mechanisms to prevent bacterial adhesion, which include mucus secretion and sloughing (Bavington *et al.,* 2004), mucociliary action and antiadhesive action of mucins (Carlstedt and Davies, 1997). Mucins are the major antiadhesive components of mucus providing the primary antiadhesive defense against colonizing organisms targeting carbohydrates, as well as providing bulk physical properties used in clearing bacteria (Sanberg *et al.,* 2000). Mucins demonstrate a high level of structural diversity and determine the physical properties of mucus (Pérez-Vilar and Hill, 1999), which may reflect their function as antiadhesives and may contribute to susceptibility to infection (Bavington and Page, 2005). Hence, the improvement in mucus secretion in fish fed MOS in the present study could be directly related with the lower number of infected sea bass after direct bacterial inoculation in the gut (Torrecillas *et al.,* 2007) via antiadhesive properties of the mucus. The improvement in mucus secretion could be also directly related with the enhanced disease resistance found in other vertebrates. For instance, mannose has been found to inhibit the *in vitro* colonization of the chicken small intestine by *Salmonella typhimurium* (Oyofo *et al.,* 1989b) and reduced caecum colonization by *Salmonella typhimurium* following oral inoculation (Spring *et al.,* 2000).

In summary, the present study has shown that dietary incorporation of MOS at 4 $g \cdot kg^{-1}$ and 6 $g \cdot kg^{-1}$ enhances sea bass FCR, activates its immune system and increases mucus secretion in gut, one of the main sites of infection in fish (Zapata and Cooper, 1990), with no effect on sensorial parameters and biochemical composition of flesh.

Chapter 5

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Reduced gut bacterial translocation in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS)

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ABSTRACT

The objective of this study was to determine the effect of mannan oligosaccharides derived from the outer cell wall of a select strain of *Saccharomyces cerevisiae* (Bio-Mos®, Alltech Inc., USA) on mucus production, selected mucus immune parameters activity, gut morphology and *in vivo* and *ex vivo* gut bacterial translocation for European sea bass (*Dicentrarchus labrax*). Specimens were fed 4g ·kg^{-1} dietary MOS level of inclusion in a commercial sea bass diet for eight weeks. At the end of this period, anterior gut mucosal folds height, width and folds surface area were increased by MOS supplementation ($P<0.05$, n=240). Posterior gut presented shorter folds ($P<0.05$, n=240) but wider that those fed control diet ($P<0.05$, n=240) resulting in increased total surface area ($P<0.05$, n=240). For rectum, feeding MOS reduced fold length ($P<0.05$, n=240). Gut morphological analyses showed an enhancement in the number of cells secreting acid mucins by area unit, higher density of eosinophilic granulocytes (ECGs) in the mucosa for fish fed MOS together with an improvement in gut mucus lysozyme activity which could be related to the reduced *in vivo* and *ex vivo* gut bacterial translocation found. No differences were found for the skin mucus immune parameters evaluated.

Keywords:
Sea bass
Bacterial translocation
Mucus
Gut Morphology

5.1. INTRODUCTION

The epithelial surfaces of fish, such as those of skin, gills or gastrointestinal tract are the first contact areas for potential pathogens (Ijima *et al.,* 2003; Narvaez *et al.,* 2010). In particular, many infectious diseases are initiated by gut bacterial colonization of mucosal surfaces (Abraham *et al.,* 1999; Ringø *et al.,* 2007; Schroers *et al.,* 2009). The mucosal surface of the gastrointestinal tract constitutes a complex ecosystem composed by gastrointestinal epithelium, immune cells and resident microflora (McCracken and Lorenz, 2001). Gut mucosal immune system, despite being present in fish, differs from that of mammals (Swan *et al.,* 2008), lacking, for instance, Peyer's patches and antigen-transporting M cells (Buddington *et al.,* 1997). The existence of a Gut-Associated Lymphoid Tissue (GALT), made up of granulocytes, lymphocytes, macrophages and plasma cells, spread through the digestive system has been reported in many fish species including rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*) or Atlantic cod (*Gadus morhua*) among others (Joosten *et al.,* 1996; Bakke-McKellep *et al.,* 2007; Inami *et al.,* 2009). Although these leucocytes are present in and under the epithelium at all levels of the fish digestive tract, various studies have indicated that regional differences occur in organization of GALT at different levels of the digestive tract possibly reflecting specialized immunological functions (Abelli *et al.,* 1997). In particular for sea bass, and based on the existence of an apparent gradient to concentrate the GALT toward the anus (Abelli *et al.,* 1997), the most important immunological function has been attributed to hindgut, where antigens appear to be taken up by enterocytes and transported to intraepithelial macrophages for processing and antigen presentation (Joosten *et al.,* 1996; Petrie and Ellis, 2006; Swan *et al.,* 2008).

Intestinal barrier efficiency depends on mucus production, epithelial integrity and the presence and balance among commensal bacteria. These three features are closely related with each other being essential to maintain gut homeostasis and health and providing the front line of defence against pathogenic microorganisms (Faure *et al.,* 2006). Regarding the first factor, the mucous layer forms a viscoelastic gel that protects the gastrointestinal epithelium against harmful intraluminal components (Ellis, 2001; Olafsen, 2001; Smirnov *et al.,* 2005; Faure *et al.,* 2006; Van der Marel *et al.,* 2008). Regular erosion of the mucus gel allows routine washing away of adherent bacteria (Van der Marel *et al.,* 2008). Thus, the dynamic balance between catabolic (both physical and proteolytic degradation) and anabolic (expression, synthesis and secretion from goblet cells) processes would determine the final thickness, composition and protective effect of the mucous

layer as it is well described in other vertebrates (Faure *et al.,* 2006; Van der Marel *et al.,* 2008). Major structural components of the gut mucus layer are intestinal mucins produced by goblet cells that help to hold commensal bacteria in the gut lumen preventing their firm adhesion to epithelial cells (Ellis, 2001; Olafsen, 2001; Schenk and Mueller, 2008). Mucins are stored in apically located granules of goblet cells and are secreted at a slow baseline rate in order to maintain the mucus coat over the epithelium (Ellis, 2001; Olafsen, 2001) and in response to stimulation their production may be increased or their discharge accelerated (Forstner and Forstner, 1994; Plaisancié *et al.,* 1998; Torrecillas *et al.,* 2007; 2011a; Schroers *et al.,* 2009). This stimulation has been coupled to neural, hormonal and paracrine changes (McCool *et al.,* 1990; Ogata and Podolsky, 1997; Plaisancié *et al.,* 1998; Buyse *et al.,* 2001; 2002; Ducroc *et al.,* 2005; Hokari *et al.,* 2005; Plaisancié *et al.,* 2006) or even to dietary factors that affect goblet cells numbers, mucin heterogeneity and modulate the secretory activity of goblet cells (Laburthe *et al.,* 1989; McCool *et al.,* 1990; Satchithanandam *et al.,* 1990; Sharma *et al.,* 1995; McCullogh *et al.,* 1998; Deplancke *et al.,* 2001; Smirnova *et al.,* 2003; Schroers *et al.,* 2009, Torrecillas *et al.,* 2011a). Interactions between mucus secretion and microorganisms also affect the mucus barrier integrity influencing its protective properties (Corne at al., 1974; Ottesen *et al.,* 1997; Schroers *et al.,* 2009). In poultry, dietary probiotic supplementation changes intestinal bacterial populations altering mucin biosynthesis and/or degradation (Smirnov *et al.,* 2005) and yeast extracted MOS increases mucin-producing goblet cells and lamina propria thickness (Solis de los Santos *et al.,* 2007). In fish, peroral application of a specific strain of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*) alters gut mucus secretion and produces changes mucin glycosylation patterns (Schroers *et al.,* 2009) and dietary MOS supplementation affected mucus production by increasing gut goblet cells number for European sea bass (Torrecillas *et al.,* 2011a), but no apparent changes on mucin composition patterns were detected when included in gilthead sea bream diets (Dimitroglou *et al.,* 2010). Besides, MOS supplementation to on-growing diets enhances innate cellular and humoral immune parameters (Torrecillas *et al.,* 2007, 2011a; Staykov *et al.,* 2007) and reduces infection percentages in gut inoculated fish (Torrecillas *et al.,* 2007). However, despite that the increased infection resistance in fish fed MOS could be related to enhanced mucus production and immune parameters, a potential reduction in gut bacterial translocation could be an important factor that has not been studied yet. Therefore, the purpose of this research was to determine in European sea bass juveniles the effects of dietary MOS supplementation on infection resistance and the potential mechanisms implied, including *in vivo*

and *ex vivo* intestinal translocation of bacteria, as well as gut morphology, mucus production and selected immune parameters activity in mucus.

5.2. MATERIALS AND METHODS

5.2.1 Diets

Two diets, based on a commercial formulation, were designed to contain 0 g·kg^{-1} (Control), and 4 g·kg^{-1} MOS (Bio-Mos®, Alltech Inc., USA), replacing standard carbohydrates (corn meal). Diets covered nutritional requirements for this species (Izquierdo, 2005) and were manufactured by a commercial feed producer (Graneros de Tenerife, Tenerife, Spain) with the composition showed in Table 5.1.

Table 5.1. Main ingredients and composition in the experimental diet

Ingredient	**Diet (g kg^{-1} dry weight)**			
	Control	**BM2**	**BM4**	**BM6**
Fish meal [1]	515.0	515.0	515.0	515.0
Soybean meal	97.8	97.8	97.8	97.8
Wheat	75.0	75.0	75.0	75.0
Wheat gluten	75.0	75.0	75.0	75.0
Corn meal	65.3	63.3	61.3	59.3
Fish oil [2]	126.9	126.9	126.9	126.9
Fats and oils	20.3	20.3	20.3	20.3
Mineral mix [3]	14.3	14.3	14.3	14.3
Vitamin mix [4]	10.3	10.3	10.3	10.3
Antioxidant (BHT)	0.1	0.1	0.1	0.1
MOS [5]	0	2	4	6
Composition (%,dry weight)				
Crude lipids	24.07	24.82	24.04	23.43
Crude protein	48.71	48.77	48.33	48.37
Ash	9.98	9.81	9.56	9.69

[1] Peruvian fish meal (65% protein). [2] Peruvian fish oil. [3] Mineral mix TROUW Seabream/Seabass (0.8g), Choline chloride (0.17g) and Inositol (0.06g) (Trouw Nutrition Spain, Madrid, Spain). [4] Vitamin mix TROUW Seabream/Seabass (1g) Calcium carbonate (0.2g), Potassium monophosphate (0.19g) and NaCl 97% (0.04g) (Trouw Nutrition Spain, Madrid, Spain). [5] Bio-Mos®, Alltech, Inc., USA

5.2.2 Fish and biological parameters

Juvenile European sea bass were transferred from a local farm (ADSA, San Bartolomé de Tirajana, Canary Islands, Spain), to the main facility of the Canarian Institute of Marine Science (ICCM) where they were acclimatized during 8 weeks in indoor 1000 l fibreglass tanks until achieving the initial experimental size (116 g). Afterwards, fish were pooled and divided in two

groups, Control and MOS, and reared at a commercial density (10 $kg \cdot m^{-3}$). Tanks were supplied with filtered sea water, at a temperature of 22.3-23°C, and natural photoperiod (12L:12D). Fish were fed until apparent satiation with one of the two experimental diets for 8 weeks (2 times a day, 6 days a week). After 8 weeks of supplementation, fish were sampled for body weight and length, histological and mucus studies and subjected to a short-term in vivo exposure to *V. anguillarum* (10^8 $cfu \cdot ml^{-1} \cdot fish$) via anal canalisation as previously described by Torrecillas *et al.* (2007).

5.2.3. Histological studies

Fish intestines were dissected out and divided into anterior (from the end of the pyloric caeca to the first diffused sphincter), posterior (from the first diffused sphincter to rectum sphincter) and rectum sections. Six to eight transversal sections of each intestine segment were fixed in 10% neutral-buffered formalin and embedded in paraffin perpendicularly to the bottom of the mold. For each specimen ten serial sections (5 µm-thick) were stained with haematoxylin and eosin (H&E), Alcian Blue, Alcian Blue/PAS (pH=2.5) or May-Grünwald/Giemsa (MGG) (Martoja and Martoja-Pierson, 1970). Micrographs were taken from stained sections using an Olympus CX41 microscope (Olympus Optical, PA, USA).

For gut morphometric analysis, H&E/MGG stained sections were used. Mucosal folds height and width were analyzed using the measurement tool of an image analySIS® software package (Image Pro Plus®). In order to measure folds total surface area, MGG sections images (X50) were processed using Adobe Photoshop CS 8.0.1 (Adobe Systems, San Jose, CA). First, gut section was outlined, background removed and converted into a binary format where lumen area was removed. Then, images were converted to grey scale and muscular layer selected and removed. Finally, the remaining mucosal folds area (including lamina propria) was converted again to binary format and measured using an image analySIS® software package (Image Pro Plus®). The following commands were used for the conversion: GrayMode, Fill and Threshold. Binary black and white and grey scale images were compared with the original images to ensure an accurate conversion. Minor adjustments, if needed, were made with the following commands: Erode and Paintbrush. Forty replicates measurements of each variable were measured for each fish.

The cellular infiltration level of a mixed leucocytes population (mainly eosinophilic granulocytes, ECGs) in the central lamina propria within the intestinal folds and consequent engrossment, was evaluated under a light microscope separately by two scientists unaware of the experimental treatments using MGG stained sections (X200,X400). A histological scoring system was established previously to the evaluation as follows: 0, not observed; 1 low; 2, moderate and 3, high. The number of goblet cells by unit of area was determined using an analySIS® (Image Pro Plus®) software package (Torrecillas *et al.,* 2007).

5.2.4. Mucus immune parameters

Mucus was isolated from skin and gut of healthy sea bass. The skin mucus was collected from the body surface according to the method described by Bordas *et al.* (1996), by scrapping with a plastic spatula and dilution in sterile seawater. Gastrointestinal mucus was prepared by a modification of the method described by Chambrillón *et al.* (2006). Fish were starved for 48 h and then intestines were removed and transferred to sterile Petri dishes where mucus was collected by scrapping the inner intestine surface with a sterile plastic spatula. The mucus then was homogenised in phosphate buffered saline (PBS). All mucus preparations were centrifuged twice at 20000 g for 30 min at 4°C to remove particulate and cellular material, sterilized under UV radiation and stored at -80°C until analysis. The protein content was determined by the method described by Bradford (1976) using BSA as a standard.

Lysozyme activity in mucus was determined by turbidimetric assay as previously described by Anderson and Siwiki (1994) using hen egg white lysozyme (Sigma Chemicals, St.Louis, USA) as a standard. The bactericidal activity was determined in flat bottom 96 well microtiter plates. OD of a fresh *Vibrio anguillarum* culture in PBS was adjusted to 10^8 cfu·ml^{-1}. The assay was carried out by adding an equal volume (100 µL) of mucus and bacterial suspension to each well and mixing with a micropipette. Control wells contained PBS instead of mucus. During incubation for 1 h at 25°C, the bactericidal activity was measured by spectrometry at 540 nm. The bactericidal activity was determined as the decrease in absorbance at t=10, 20, 30, 40, 50 and 60 minutes of each sample vs. absorbance of sample control at t = 0.

5.2.5. Vibrio anguillarum ex vivo exposure

After 8 weeks of MOS supplementation, six fish per diet were randomly selected, netted and sacrificed with an anaesthetic overdose. The intestine, from the posterior pyloric caeca to

the anus, was rapidly dissected out and separated into anterior and posterior regions, rinsed and placed in ice-cold Ringer solution (140 mM NaCl, 2.5 mM KCl, 15 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1mM KH_2PO_4, 0.8 mM $MgSO_4$, 10 mM glucose and 5 mM HEPES buffer; pH was set to 7.8). Fresh Ringer was made daily, continuously gassed with air and stored on ice. The segments were then reversed and closed at the distal and proximal ends using fine forceps and introduced in flasks filled with 10 ml Ringer solution continuously gassed with air for oxygenation and stirring. The intestinal sacs were kept in the flasks for a total of 120 min. After 60 min of stabilization, the Ringer solution was substituted with a Ringer solution containing 10^7 cfu·ml^{-1} of labelled fluoresced labelled *V. anguillarum*. Bacteria were labelled with fluorescein isothiocyanate (FITC; Sigma Chemicals, St.Louis, USA). Briefly, bacteria were incubated with 0.25 mg FITC/ml bacterial PBS suspension for 60 min at 20°C. Four steps of washing by centrifugation (3000 g, 5 min) and re-suspension with PBS ensured sufficient removal of dissolved FITC. At termination of the exposure to fluorescently labelled *V. anguillarum*, the outer side (mucosal side) in contact with the bacterial solution of the intestines was washed three times with Ringer solution in order to discharge the bacteria attached to the intestine and not translocated. Then, the inner side (serosal side) of the intestine sac was washed three consecutive times with Ringer solution to collect the translocated bacteria. Samples were immediately centrifuged at 6000 g for 10 min to concentrate particulate fluorescence and the supernatant, containing dissolved fluorescence, was discarded. The fluorescence of the remaining solution was measured for 1 s at 480/535 nm on a microplate reader (Victor 1420 Multilabel Counter; Wallac). *V. anguillarum* (strain VI-88/09/03175, Universidad de Santiago de Compostela, Aquaculture Institute, Ictiopathology Department, Spain), used in the translocation studies was stored lyophilized. Previously, the bacteria was grown in a Tryptone Soybean Agar (TSA, Panreac Quimica SAU (Cultimed), Barcelona, Spain), and re-inoculated 24-36 h prior analysis. Bacteria was dissolved in phosphate-buffered saline (PBS) sterile to an optical density of 1.0 at 600 nm, which equals 10^9 bacteria measured by plating and colony counting on BHI-agar plates.

5.2.6. Vibrio anguillarum in vivo exposure

After 8 weeks of MOS supplementation, fish were subjected to a short-term *in vivo* exposure to a sub-lethal dose of *V.anguillarum* (10^8 cfu·ml^{-1} per fish) via anal canalisation as previously described by Torrecillas *et al.* (2007). Fourty disease-free fish per diet were randomly selected and transferred to the pathogens incubation facility of GIA and randomly distributed

among 8 indoor cylindrical 350 l fibreglass tanks, at an initial stocking density of 4.2 kg·m^{-3}. Initial fish average weight (g ± SD) and length (cm ± SD) were respectively 155.19 ± 16.33 and 23.21 ± 0.87 for control diet and 156.89± 15.30 and 23.75 ± 0.98 for fish fed MOS supplemented diet. Water temperature ranged from 21.5 to 22.3°C during the trial and artificial light photoperiod was adjusted to 12L:12D. Fish were manually fed their respective experimental diets until apparent satiation (2 times a day). After 48 h post inoculation, all fish were sacrificed and bacterial characterization of head kidney, spleen and liver was performed in order to determine the pathogen's capacity to pass through the intestinal epithelium by culturing samples on TSA (Panreac Quimica SAU (Cultimed), Barcelona, Spain) and incubating at 25°C for 24-48 h. All strains isolated were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests (Smibert and Krieg, 1981) in order to identify *V. anguillarum* presence in fish tissues.The grade of pathogen infection was determined by the presence or absence of the pathogen in internal organs.

At the same time, fish fed control and MOS diets were inoculated by anal canalization with fluorescent-labelled *V. anguillarum* in order to confirm the attachment to gut epithelium. Briefly, fish were inoculated via anal canalization with 10^7 cfu·ml^{-1} fluorescent labelled *V. anguillarum*. Fluorescence labelling of bacteria was conducted as described before. After 2, 4 and 24 h post inoculation fish were sacrificed with an anaesthetic overdose. The intestine, from the posterior pyloric caeca to the anus, was rapidly dissected out and separated into anterior and posterior regions, rinsed with PBS, embedded in *Tissue-Tek* (O.C.T, Sakura Finetek, Torrance, CA) and immediately frozen at -80°C. Cryosections (10µm) were stained with propidium iodide (PI) (5 µg·ml^{-1}), washed two times in PBS for five minutes, air dried in dark, mounted with Fluoromount (Sigma, St.Louis, CA, USA) and inspected under fluorescence microscope (Olympus CX41, Olympus Optical, PA, USA) (Fig. 1), showing presence of labelled pathogen attached to eithelium during the sampling times.

5.2.7. Statistical analysis

All data were tested for normality and homogeneity of variance. Means and Standard Deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods described by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of MOS inclusion. When F values showed significance, individual means were compared using Tukey's or Scheffe test for

multiple means comparison. Significant differences were considered for $P < 0.05$ and $P < 0.1$ depending on the parameters studied.

5.3. RESULTS

5.3.1. Biological parameters

No mortalities were recorded during feeding or *V. anguillarum in vivo* exposure periods in either Control or MOS groups. There was no difference on growth parameters between the two groups. Both increased their body weight by a 35% from the beginning until the end of the experiment.

5.3.2. Histological studies

Microscopic evaluation of H&E stained sections of fish anterior and posterior intestine revealed an intact epithelial barrier, organized microvilli, lack of cell debris in the lumen and no signs of oedema or vasodilatation for both dietary treatments. However, gastrointestinal morphology differed between treatments. Regarding the anterior gut, after 8 weeks of MOS supplementation all the enteric morphometric characteristics evaluated including mucosal folds height, width and folds area were increased ($P<0.05$, $n=240$) (Table 5.2). The posterior gut presented shorter folds ($P<0.05$, $n=240$) but wider that those fed control diet ($P<0.05$, $n=240$), significantly increasing the total surface area (Table 5.2). In rectum, in fish fed MOS diet the fold length was decreased ($P<0.05$, $n=240$) compared to fish fed control diet (Table 5.2).

Quantitative morphological studies of posterior and anterior gut showed an increase ($P < 0.05$, $n=120$) in the number of cells secreting acid mucins by unit of area after 8 weeks of supplementation with MOS (Table 5.2; Fig. 5.2 A, B). MGG staining revealed the presence of ECGs both in epithelium and lamina propria. The intestinal lamina propria of fish fed MOS was markedly expanded by infiltrated ECGs compared to fish fed control diet, particularly in the posterior gut (Table 5.2, Fig. 5.2 C, D).

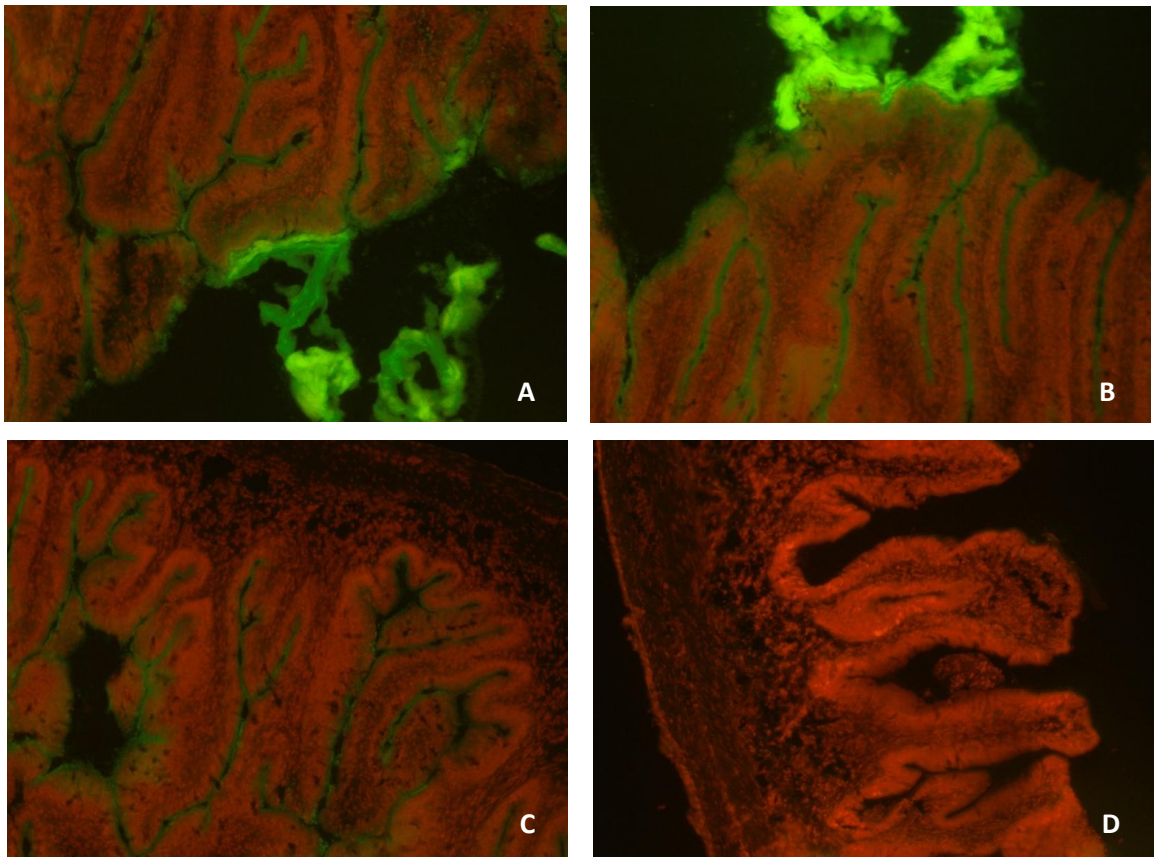

Figure 5.1. Gut inoculated fluorescent *V. anguillarum* and counterstained with PI at times 2 (A), four (B) and 24 hours (C) post inoculation. Negative control inoculated with PBS (D).

5.3.3. Selected mucus immune parameters: lysozyme and bactericidal activity

In terms of gut mucus quality, lysozyme activity was improved ($P<0.05$) after fed fish with MOS diet (Table 5.3) but no effect on bactericidal activity was detected (Fig. 5.3). Regarding skin mucus no effect of MOS supplementation was found on lysozyme and bactericidal activity (Table 5.3).

5.3.4. Vibrio anguillarum ex vivo exposure

Regarding *ex vivo* bacterial translocation of FITC-labelled *V.anguillarum*, a significant reduction ($P<0.1$) was found in the number of bacteria capable of crossing through the gut in fish fed MOS, both in the anterior and posterior intestine (Fig. 5.4).

Table 5.2. Folding intestine patterns, quantitative analysis of cells secreting acid mucins by unit of area and evaluation of the ECGs infiltration level in the central lamina propria within the intestinal folds for sea bass intestine after 8 weeks of MOS supplementation

Variable	Control diet	MOS diet
Folds height (µm)		
Anterior gut	1026.91 [a] ± 315.97	1209.01 [b] ± 344.07
Posterior gut	512.05 [a] ± 100.94	476.83 [b] ± 92.36
Rectum	705.22 [a] ± 189.40	679.28 [b] ± 103.97
Folds width (µm)		
Anterior gut	173.87 [a] ± 46.21	191.03 [b] ± 59.55
Posterior gut	119.77 [a] ± 34.19	142.08 [b] ± 46.85
Rectum	109.93 ± 53.04	100.69 ± 28.39
Folds surface area (μm^2)		
Anterior gut	576028.60 [a] ± 284280.38	747476.80 [b] ± 396013.06
Posterior gut	195342.47 [a] ± 75401.42	218406.35 [b] ± 101235.76
Rectum	252137.00 [a] ± 172243.1	214944.13 [b] ± 225388.47
Number cells secreting acid mucins / 10^6 area units		
Anterior gut	406.09 [a] ± 125.36	480.84 [b] ± 167.55
Posterior gut	697.46 [a] ± 355.50	869.29 [b] ± 321.76
Lamina propria engrossment		
Anterior gut	1	2
Posterior gut	1-2	2-3

Different letters within a line denotes significant differences ($P<0.05$). Control= 0 $g \cdot kg^{-1}$ MOS; MOS=4 $g \cdot kg^{-1}$ MOS. Values expressed in mean ± SD. Lamina propria engrossment: 0= not observed, 1= low, 2= moderate, 3= high.

5.3.4. Vibrio anguillarum in vivo exposure

In fish fed control diet, gut inoculated *V. anguillarum* was recovered from head kidney and liver in a 25% and 16% respectively, while in fish fed MOS diet no presence of the inoculated pathogen was detected on internal organs (Table 5.4). Associated to the reisolation of *V. anguillarum* on infected groups, other genera of bacteria were found in internal organs in both dietary treatments: *Aeromonas spp.*, *Pseudomonas spp*. and other *Vibrio spp*. (Table 5.4).

There were no evident differences in the bacterial presence percentages between fish fed different diets, except for *Pseudomonas spp*., which was only found in fish fed control diet. No bacteria were found in tissues of fish inoculated only with PBS (negative control), regardless the diet fed (data not shown).

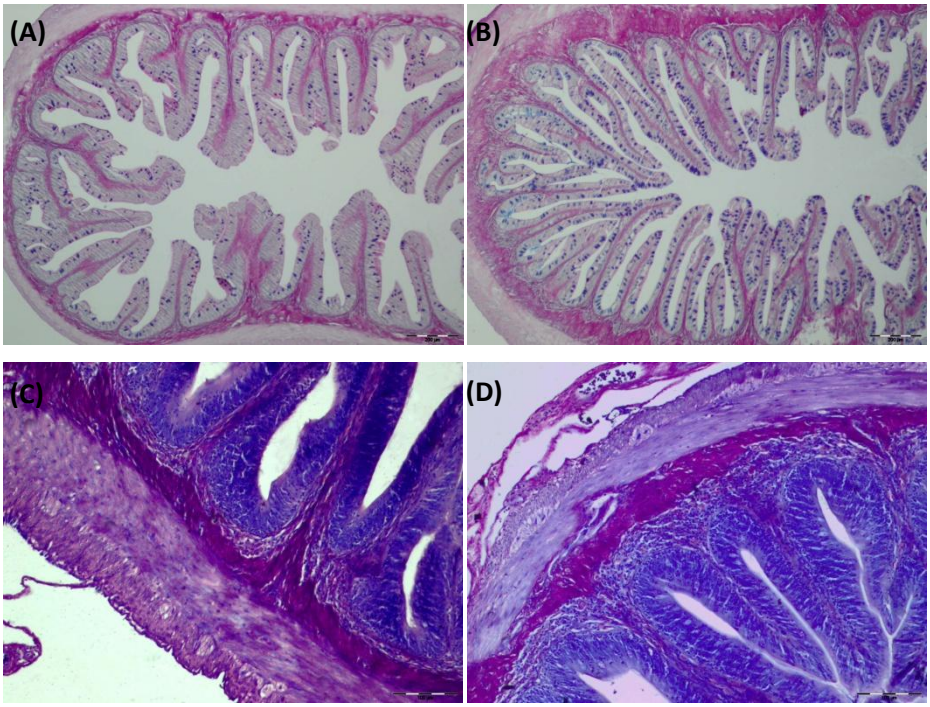

Figure 5.2. Posterior Gut (Alcian Blue-PAS) from fish fed, (A) Control diet, (B) 4g kg^{-1} MOS diets. Note the higher presence of cells secreting mucins by unit of area for fish fed MOS. Posterior Gut (May-Grünwald Giemsa) from fish fed (C) Control diet and (D) 4g kg^{-1} MOS diets. Note the higher cellular infiltration of a mixed leucocytes population, mainly ECGs, in the lamina propria within the intestinal folds for fish fed MOS.

Table 5.3. Lysozyme activities in intestinal and skin mucus of sea bass after 8 weeks of MOS supplementation

	Control Diet	**MOS Diet**
Lysozyme activity (U/mg protein) in skin mucus	195.82 ± 137.49	374.07 ± 355.98
Lysozyme activity (U/mg protein) in gut mucus	129.42 ^{a} ± 58.05	256.42 ^{b} ± 109.32

Different letters within a line denotes significant differences (P<0.05). Control= 0 $g \cdot kg^{-1}$ MOS; MOS=4 $g \cdot kg^{-1}$ MOS. Values expressed in mean ± SD.

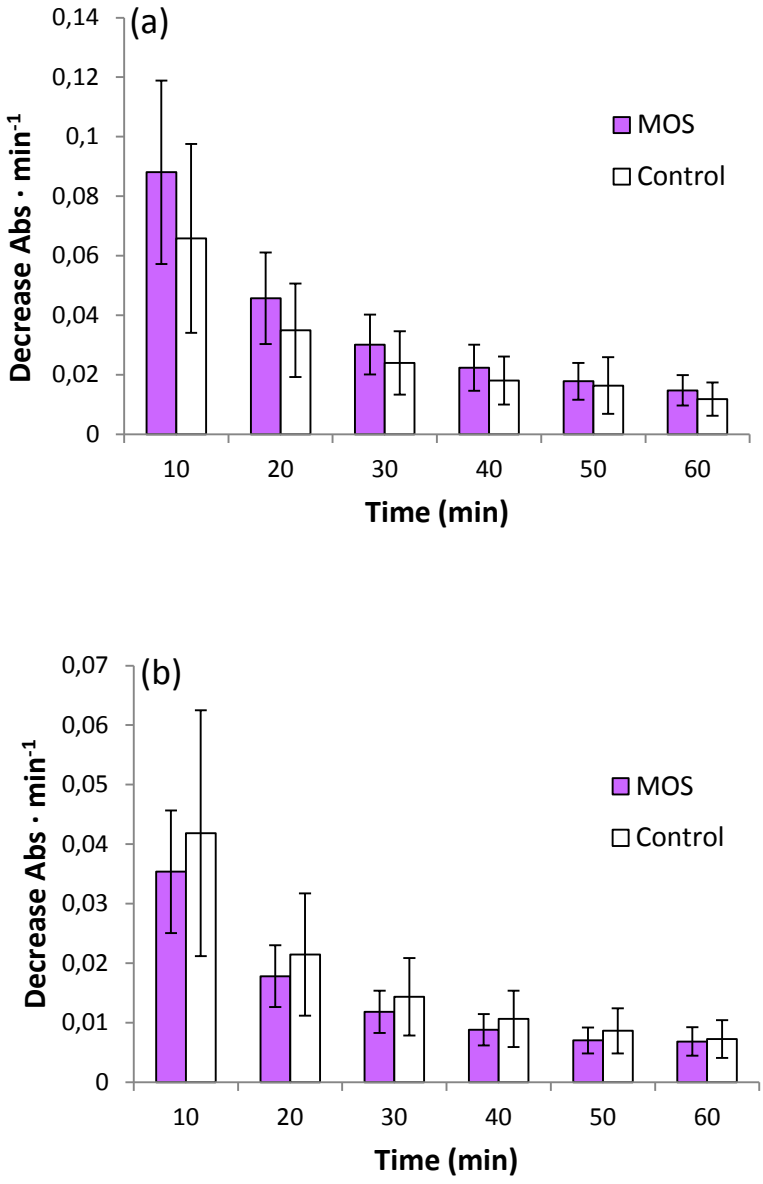

Figure 5.3. Bactericidal activity of (A) gut isolated mucus and (B) skin isolated mucus of European sea bass fed mannan oligosaccharides. Data represent the mean ± SD. Different letters denotes significant differences (P<0.05) among treatments.

5.4. DISCUSSION

Dietary supplementation with MOS for 8 weeks increased European sea bass resistance to *V. anguillarum* infection by *in vivo* gut inoculation, in agreement with our previous studies (Torrecillas *et al.,* 2007). Dietary yeast-based prebiotics enhance resistance to mycobacteria and vibrios challenges in species such as hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (Li and Gatlin III, 2005), whereas does not affect resistance in others. For instance,

catfish (*Ictalurus punctatus*) survival was not affected by yeast-based prebiotics after an immersion-challenge with *Edwardsiella ictaluri* (Welker *et al.,* 2007), what can be related to the complex host-pathogen-environment relations which affect the infection process as well as factors related to the prebiotic selected.

Table 5.4. Percentage presence of inoculated pathogen and associated bacteria on internal organs after *in vivo V. anguillarum* direct gut inoculation.

		Bacterial reisolation (%)		
		Liver	Spleen	Head kidney
CONTROL	***V. anguillarum***	**16.6**	**X**	**25**
	Aeromonas sp.	16.6	8.3	8.3
	Pseudomonas spp.	8.3	X	X
	Vibrio sp.	8.3	X	X
MOS	***V. anguillarum***	**X**	**X**	**X**
	Aeromonas sp.	16.6	X	16.6
	Pseudomonas spp.	X	X	X
	Vibrio sp.	8.3	X	X

Control= 0 g·kg^{-1} MOS; MOS=4 g·kg^{-1} MOS. Data expressed in percentage (%). X=not detectable

In the present study, the reduction in the number of bacteria capable of crossing the intestinal epithelium *ex vivo* by feeding MOS, may partly explain the lower re-isolation rate found on internal organs after the *in vivo* pathogen challenge in the present and previous studies (Torrecillas *et al.,* 2007). Despite the lack of bacterial translocation studies in fish fed MOS, in poultry, mannose reduces *in vitro Salmonella typhimurium* adherence to epithelial cells (Oyofo *et al.,* 1989a) and *in vivo* intestinal colonization (Oyofo *et al.,* 1989b) and mannan oligosaccharides also reduced caecum colonization by *S. typhimurium* following oral inoculation (Spring *et al.,* 2000). Among other carbohydrates, mannose constitutes an important surface component of cells. Mannose-specific lectins are utilized by many gastrointestinal pathogens as a mean of attachment to the gut epithelium (Mirelman and Ofek, 1986), acting as adhesins by mediating the binding of bacteria to epithelial cells. Therefore, the lower bacterial translocation ability in fish fed MOS in the present study could be related to a reduced adhesive rate to the fish intestinal mucosal surface by bacteria being specifically attracted to attach dietary mannan oligosaccharides. Nevertheless, this lower bacterial translocation could be also associated to a reduced bacterial ability to physically cross the intestinal barrier or to survive this translocation.

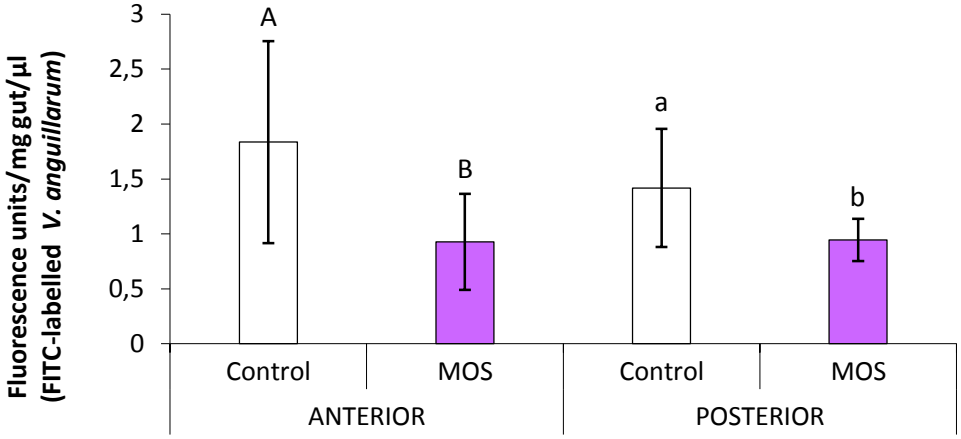

Figure 5.4. *In vitro* bacterial translocation of FITC labeled *Vibrio anguillarum* for anterior and posterior intestine of sea bass fed MOS and control diet expressed in fluorescence units·mg gut^{-}1 per µl. Data represent the mean ± SD. Different letters denotes significant differences ($P < 0.1$; n=6) among treatments.

Intestinal barrier efficiency markedly depends on mucus production. In the present study, MOS supplementation increased goblet cells density and, thus, mucus production in agreement with previous studies (Torrecillas *et al.,* 2011a) and further possibly reducing bacterial adhesion to the enterocytes, being bacterial adhesion a necessary step in microbial colonization and pathogenesis. Epithelial mucosal surfaces have a number of defence mechanisms to prevent bacterial adhesion, which include mucus secretion (Ellis, 2001; Olafsen, 2001) and antiadhesive action of mucins (Bavington *et al.,* 2004). In turn, increased mucus production could be responsible for improved gut integrity. In fact, MOS supplementation has been found to improve microscopic integrity of the gut in Senegal sole (*Solea senegalensis*) (Sweetman and Davies, 2005), rainbow trout and larval white sea bream (*Diplodus sargus*) (Yilmaz *et al.,* 2007) and cobia (*Rachycentron canadum*) (Salze *et al.,* 2008). Moreover, in the present study, MOS supplementation significantly enlarged intestine folds height and width, further increasing the intestinal barrier. In agreement with these findings recent studies in red drum (*Sciaenops ocellatus*) gut morphology revealed increased dimensions for intestinal fold height when supplemented with MOS (Zhou *et al.,* 2010). This quantified enlargement would be related to the lamina propria engrossment observed, as a result of the higher infiltrated ECGs seen on posterior gut lamina propia of fish fed MOS diet. Thus, increased gut ECGs, suggests the influence of MOS supplementation on GALT regulation in sea bass juveniles. In agreement, in other vertebrates, MOS supplementation stimulates gut immune system, promotes gut maturation and engrosses

lamina propia thickness (Solis de los Santos *et al.,* 2007), providing a more efficient anatomical barrier against bacterial translocation (Berg, 1992; Ringø *et al.,* 2007).

Besides mucous layer thickness, its composition and protective effect (i.e. lysozyme and antibacterial activity) are also essential components against pathogen infection that would contribute to reduce bacterial translocation (Berg, 1992). Indeed, a higher lysozyme activity was found in gut mucus of European sea bass fed MOS, further enhancing the front line of defense against pathogenic microorganisms in relation to the reduction bacterial translocation found in this group of fish.

In summary, the present study has shown the positive effect of two months dietary MOS supplementation for European sea bass on several mechanisms related to the gut mucosal barrier protective efficiency. Results revealed reduced gut bacterial translocation and higher intraepithelial ECGs presence, mucus production and lysozyme activity, suggesting the reinforcement of the mucosal surface of the gastrointestinal tract as the front line of defence against pathogenic microorganisms. Nevertheless, other mechanisms may be also implicated on the protection effect of MOS against pathogen infections in fish and deserve further studies. For instance, in poultry, probiotic and antibiotic dietary supplementation affects the intestinal bacterial populations (Smirnov *et al.,* 2005). In other vertebrates, MOS and other related complex carbohydrates inhibit viral replication, stimulate bone marrow activity, promote wound healing, inhibited tumor growth and cholesterol absorption and induced macrophage activation and interleukin-1 release (Tizard *et al.,* 1989; Nollet *et al.,* 2007).

Chapter 6

Stress response and disease resistance in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS)

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ABSTRACT

The objective of this study was to determine the effect of dietary mannan oligosaccharides (MOS), derived from the outer cell wall of a select strain of *Saccharomyces cerevisiae* (Bio-Mos®, Alltech Inc., USA), in diets for European sea bass (*Dicentrarchus labrax*) juveniles in relation to disease and stress resistance, combining intestinal infection with *Vibrio anguillarum* and stress challenge by confinement. Studies of potential changes in microbiota profiles and plasma cortisol levels were also included. Specimens were fed 4g·kg^{-1} dietary MOS level of inclusion in a commercial sea bass diet for eight weeks. At day 60, fish fed MOS presented a 10% higher weight gain ($P<0.05$) than fish fed control diet as well as increased ($P<0.05$) total final length. Specific and relative growth rates followed a similar trend, increasing by about 18% ($P<0.05$) in fish fed dietary MOS compared to non-supplemented fish.

After the feeding trial, fish were exposed to a pathogen challenge test against *V. anguillarum* combined with a confinement stressor panel. Cumulative mortality of fish fed MOS caused by anally inoculated *V. anguillarum* decreased from 66% to 12.5% and from 54.1% to 25% in infected and infected + stressed fish, respectively, compared to fish fed control diet. Results for European sea bass revealed a positive effect of MOS dietary inclusion (at 4g·kg^{-1}) on disease resistance against inoculated *V. anguillarum* as well as reduced effects of stress on

Keywords:
Bacterial translocation
Disease resistance
Mannan oligosaccharides
Sea bass
Stress response

microbiota diversity.Both of these findings, together with the higher gut mucus production and density of eosinophil granulocytes in gut mucosa obtained in previous studies, suggest that reinforcement of the intestinal barrier efficiency is the front line of defense against pathogenic microorganisms of European sea bass fed MOS.

6.1. INTRODUCTION

In intensive aquaculture, unfavorable environmental conditions or poor management practices can significantly affect homeostasis and lead to physiological stress which may unbalance the delicate equilibrium between host-environment-pathogen and compromise farmed fish welfare (Ellis *et al*., 2002; Conte, 2004; Turnbull *et al*., 2005; Huntingford *et al*., 2006; North *et al*., 2006; Di Marco *et al*., 2008). Among other welfare parameters, cortisol is commonly accepted as an indicator of fish stress (Barton and Iwama, 1991; Wendelaar Bonga, 1997). An increase of plasma cortisol concentration in fish results in a secondary response (stage of resistance) in which the organism tries to adjust its metabolism to cope with the disturbance. A mal-adaptive fish response results in allostatic overload (McEven and Wingfield, 2003; Varsamos *et al*., 2006), which negatively affects several physiological functions such as osmoregulation, reproduction, growth or immune defense (Ortuño *et al*., 2002; Vijayan *et al*., 2005) and even causes cellular alterations in different tissues such as those of the gastrointestinal tract (Peters, 1982; Olsen *et al*., 2003, 2005; Ringø *et al*., 2007). This situation may facilitate the induction of infectious diseases by enhanced uptake of potentially noxious materials, e.g. increased bacterial translocation (Tamayo *et al*., 1996; Olsen *et al*., 2005).

Vibriosis is one of the most prevalent bacterial fish diseases. In particular, certain pathogens such as *Vibrio anguillarum*, frequently isolated from farmed populations, cause important disease outbreaks (Zorrilla *et al*., 2003, Afonso *et al*., 2005; Chambrillón *et al*., 2006; Ringø *et al*., 2010). This is especially significant for European sea bass (*Dicentrarchus labrax*), a species very sensitive to stressors and pathogens, where infections may occasionally cause important financial losses (Izquierdo, 2005). Conventional approaches to prevent and control bacterial diseases in aquaculture, such as the use of long term in-feed antibiotics, have led to the appearance of detrimental side effects such as resistant antibiotic bacteria and proliferation of undesirable bacteria or fungi (Teuber, 2001; Carrington and Secombes, 2006). Therefore, besides vaccine development, prevention of infectious diseases through effective dietary supplements,

including probiotics and prebiotics, is receiving increased attention in fish production (Genc *et al*., 2007). Prebiotics, such as mannan oligosaccharides (MOS), are classified as non-digestible food ingredients that stimulate the growth of beneficial bacteria that contribute to the overall health of the host. Prebiotics are mainly oligosaccharides which have shown positive effects on the beneficial microbiota of terrestrial and aquatic organisms (Gibson and Roberfroid, 1995; Gibson *et al*., 2003; Sang and Fotedar, 2010; Dimitriglou *et al*., 2011).

Inclusion of MOS in fish diets has been found to improve growth performance (Zhou and Li, 2004; Staykov *et al*., 2005; Bogut *et al*., 2006; Culjak *et al*., 2006; Daniels *et al*., 2006; Genc *et al*., 2007; Staykov *et al*., 2007; Torrecillas *et al*., 2007; Sang and Fotedar, 2009), gut function and health by enhancing the ultrastructure of the intestinal mucosa (Dimitriglou *et al*., 2009, 2010; Salze *et al*., 2008; Zhou *et al*., 2010), promoting general non-specific immune response (Zhou and Li, 2004; Staykov *et al*., 2005, 2007; Daniels *et al*., 2006, 2007; Torrecillas *et al*., 2007, 2011a, 2011b; Terova *et al*., 2009; Sang and Fotedar, 2010) or mucus production (Torrecillas *et al*., 2011a, 2011b). Reduced *in vivo* and *ex vivo* gut bacterial translocation has been found in fish fed MOS, as a result of an enhancement in gut mucus quantity and mucus lysozyme activity (Torrecillas *et al*., 2011). However, other factors such as possible changes in microbiota richness or gut paracellular permeability could also be involved. Moreover, the effects of MOS supplementation in relation to stress response and disease resistance have not been widely studied (Dimitroglou and Davies, 2004; Welker *et al*., 2007; Andrews *et al*., 2009; Buentello *et al*., 2010). Therefore the purpose of this research was to investigate the effects of MOS supplementation in diets for European sea bass juveniles in relation to disease and stress resistance, combining *in vivo* intestinal infection with *V. anguillarum* and stress challenge by confinement. Effects of this treatment on microbiota profiles and plasma cortisol levels were also examined.

6.2. MATERIALS AND METHODS

6.2.1. Diets

Two experimental dry pelleted diets, based on a commercial formulation, were prepared in order to contain 0 (Control) and 4 $g \cdot kg^{-1}$ MOS (Bio-Mos® Aquagrade, Alltech, Inc., USA) replacing standard carbohydrates. Diets were pelleted in an industrial mixer, crumbled to

the desired size, and air-dried prior to storage at 4°C until feeding. Diet ingredients and biochemical composition are shown in Table 6.1.

Table 6.1. Main ingredients and composition in the experimental diets

Ingredient	Diet (g kg^{-1} dry weight)	
	Control	**BM4**
Fish meal [1]	515.0	515.0
Soybean meal	97.8	97.8
Wheat	75.0	75.0
Wheat gluten	75.0	75.0
Corn meal	65.3	61.3
Fish oil [2]	126.9	126.9
Fats and oils	20.3	20.3
Mineral mix [3]	14.3	14.3
Vitamin mix [4]	10.3	10.3
Antioxidant (BHT)	0.1	0.1
MOS (Bio-Mos Aquagrade)[5]	0	4
Composition (%, dry weight)		
Crude lipids	20.37	20.32
Crude protein	51.62	52.07
Moisture	7.84	7.88
Ash	9.74	9.82

[1]Peruvian fish meal (65% protein). [2]Peruvian fish oil. [3] Mineral mix TROUW Seabream/Seabass (0.8g), Choline chloride (0.17g) and Inositol (0.06g) (Trouw Nutrition Spain, Madrid, Spain). [4] Vitamin mix TROUW Seabream/Seabass (1g) Calcium carbonate (0.2g), Potassium monophosphate (0.19g) and NaCl 97% (0.04g) (Trouw Nutrition Spain, Madrid, Spain). [5] Bio-Mos® Aquagrade, Alltech, Inc., USA

6.2.2. Experimental conditions

6.2.2.1. Experiment I: Feeding trial

Five hundred and fifty commercially reared European sea bass juveniles were maintained in stocking tanks and fed a commercial extruded diet for 3 weeks until being fully adapted to the environmental conditions (4 $kg \cdot m^{-3}$ stocking density). Afterwards, fish were randomly distributed in 6 indoor 1000 l fiberglass tanks at an initial stocking density of 4 $kg \cdot m^{-3}$ (90 fish per tank). Fish average initial weight and length were 45.95 ± 0.60 g and 15.05 ± 0.05 cm, respectively (mean ± SD). Tanks were supplied with filtered sea water, at a temperature of 22.8-23.3°C and natural photoperiod (12L:12D). Water dissolved oxygen ranged between 6.5-7.2 ppm. Fish were manually fed until apparent satiation with one of the two experimental diets for 8 weeks (3 times a day, 6 days a week). Each diet was assayed in triplicate and growth parameters were determined at day 60.

6.2.2.2. Experiment II: pathogen and stress challenges

After the feeding trial, the fish were exposed to a pathogen challenge test plus a confinement stressor panel. Bacterial analysis of fish prior the pathogen challenge test ensured that fish were free of *V. anguillarum*. Two hundred and fifty two fish from each dietary treatment were randomly distributed among 18 indoor cylindrical 500 l fiberglass tanks (2.5 kg of fish·m^{-3}, 14 fish per tank) in a pathogen incubation facility. Five subgroups for fish fed each experimental diet were submitted to the following treatments: CA (Control A: no stress + not infected), CB (Control B: not stressed + inoculated with sterile PBS), S (stressed + not infected), I (not stressed + infected with *V. anguillarum*), and SI (stressed + infected with *V. anguillarum*) (Table 6.2). Infected fish were anally inoculated as described by Torrecillas *et al*. (2007) with *V. anguillarum* (10^7 colony forming units (CFU)·ml^{-1} per fish; strain VI- 88/09/03175). Stressed fish were subjected to a confinement stressor by increasing the stocking density x10 times (25-27 kg·m^{-3}) in relation to CA treatment (2.5 kg·m^{-3}). After 2h, 4h, 24h and 6 days of confinement, nine fish per treatment were sampled for bacterial characterization of head kidney and liver. Plasma was collected for cortisol determination after 2 and 4 h of confinement (acute response) and after one week (adaptive response). Posterior gut tissue samples were rinsed with PBS in order to eliminate fecal matter, frozen in liquid N_2 and stored at -80°C in order to evaluate gut microbiota profiles by denaturing gel electrophoresis (DGGE) at times 0h, 2h, 4h, 24h, 72h and 6 days for CA, CB and S groups. For the duration of the confinement study fish were fed the same diet that they were fed in Experiment I (3 times a day, 6 days a week).

6.2.3 Bacterial isolation and biochemical characterization

Samples of fish head kidney and liver were cultured on TSA (Tryptone Soybean Agar, Cultimed) and *V. anguillarum* media (VAM) (Alsina *et al*., 1994) and incubated at 25°C for 24-48 h. All strains isolated were subjected to taxonomic analysis by standard morphological, physiological and biochemical plate and tube tests (Smibert and Krieg, 1981). If necessary, these diagnostic tests were complemented with API 20E systems.

6.2.4. Supplementation effectiveness measurements against V. anguillarum intestinal infection

Relative Percent Survival (RPS) was calculated as described by Ellis (1988) for determining the efficacy of vaccines, following the equation: RPS= [1-(Mortality in fish fed MOS diet (%) / Mortality in fish fed Control diet (%)]*100

6.2.5. Blood collection and sample preparation

Blood was obtained by caudal vein puncture with a 1 ml plastic syringe. No anesthetic was used in order to avoid any possible effect in blood parameters and handling time was less than 1 min in order to minimize the stress effects. Blood samples were transferred to Eppendorf tubes coated with heparin as anticoagulant and plasma was separated immediately by centrifugation. Samples were stored at -80°C until analysis.

Table 6.2. Treatments combining stress confinement and experimental inoculation via anal cannulation with *V. anguillarum* evaluated during Experiment II.

Treatment	Stress	Inoculation
CONTROL A (CA)	NO (2.5 kg·m^{-3})	NO
CONTROL B (CB)	NO (2.5 kg·m^{-3})	YES (Phosphate buffered saline; PBS)
STRESSED (S)	YES (25 kg·m^{-3})	NO
INFECTED (I)	NO (2.5 kg/m^{3})	YES (10^{7} cfu *V. anguillarum*·ml^{-1})
INFECTED+STRESSED (SI)	YES (25 kg·m^{-3})	YES (10^{7} cfu *V. anguillarum*·ml^{-1})

6.2.6. Cortisol measurements

Plasma cortisol content was determined by RIA (Rotllant *et al*., 2001). The antibody used in the assay was purchased from Biolink, S.L. (Costa Mesa, CA, USA) in a final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11.4% with 21-desoxycorticosterone, 8.9% with desoxycortisol and 1.6% with 17α-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels were expressed as ng cortisol /ml plasma.

6.2.7. Bacterial DNA Extraction and 16S rRNA Amplification

DNA was extracted from pooled posterior intestinal samples from 3 fish per tank (3 tanks per treatment (CA and S) at times 2h, 24h, 3days and 6 days for both diets) with a lysozyme pretreatment (50 mg/mL of TE buffer for 30 min at 37°C) using Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA). DNA concentration was checked by standard 1.5% electrophoresis gel and measured with a Qubit™ Fluorometer (Invitrogen, CA, USA) using Quant-it ds DNA HS assay kit (Invitrogen, CA, USA). PCR amplification of the 16S rRNA genes was carried out using the reverse primer P1(5´-CCG TCA ATT C (AC) T TTG AGT TT-3´) and the forward primer P2 with GC clamp (5´-CGC CCG CCG CGC CCC GCG CCC CTC CCG CCG CCC CCG CCC GCC TAC GGG

AGG CAG CAG-3´). Reagents in each PCR tube (final volume 50µL) were: 2.0 µl BSA (10 µg/µl), 30 µl of sterile distilled water, 5µl 10X PCR Buffer without $MgCl_2$ (Invitrogen, CA, USA), 1.75µl $MgCl_2$ (50mM, Invitrogen, CA, USA), 4µl dNTPs (2.5mM, Invitrogen, CA, USA), 0.25µl Taq DNA polymerase (5U/µl, Invitrogen, CA, USA), 2.5 µl of primer P1 and P2 (10pmol/ µl; MWG-Biotech AG, Ebergberg, Germany). The touchdown thermal cycling was conducted as described by Casamayor *et al*. (2000) modified by Dhanasiri *et al*. (2011) using a personal Thermal Cycler MJ Mini (Bio-Rad, CA, USA). PCR products were stored at -20°C until running DGGE.

6.2.8. Denaturing Gradient Gel Electrophoresis

The denaturing gradient gel electrophoresis (DGGE) was performed using a DGGE-Bio-Rad system (Bio-Rad, CA, USA). Seven hundred ng of PCR product per well were run on 6% acrylamide gels with a denaturing gradient of 40 to 60 % (where 100% denaturant is 7 *M* urea and 40% formamide). The gel was run at 65 V for 17 h at 60°C in 1×TAE buffer (50X TAE Buffer, Merck, Darmstadt, Germany). Visualization of the DGGE bands was achieved by staining with a 1:10000 dilution of SYBR green (Invitrogen-Molecular Probes, Eugene, USA) for 45 min. The gel was scanned in a Bio-Rad DCode™ system (Bio-Rad, Hercules, USA) and optimized for analyses by enhancing contrast and grayscale using Quantity One 4.6.6 software (Bio-Rad, CA, USA). Briefly, lanes were identified and their background intensities removed following program indications. Then, bands were detected and matched at 2% tolerance level and similarity was computed by comparing by Dice Coefficient between and within fish fed control and MOS diets for CA and S treatments along the stressor panel.

6.2.9. Statistical analysis

All data were tested for normality and homogeneity of variance. Statistical analyses followed methods described by Sokal and Rolf (1995). Biological parameters, percentage relative survival and bacterial presence data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of MOS inclusion. The data obtained in terms of mortality, relative percentage survival, plasmatic cortisol and bacterial presence on internal organs were compared through two-way ANOVA in order to examine the combined effect of the fixed factors “diet” and “treatment”, using Statgraphics software (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA). When F values showed significance, individual means were compared using Tukey/Duncan tests for multiple means comparison. Significant differences

were considered for P <0.05 and P< 0.07 depending on the parameters studied. Means and standard deviations (SD) were calculated for each parameter measured.

6.3. RESULTS

6.3.1. Experiment I: Feeding trial

No mortalities were registered during the feeding experiment. After 60 days of feeding, fish fed with MOS diet showed higher (P<0.05) body weight, total length and condition factor (Table 6.3). Specific and relative growth rates followed a similar trend, by increasing by about 18% (P<0.05) in fish fed dietary MOS compared to non-supplemented fish (Table 6.3).

Table 6.3. Growth performance and somatic parameters of European sea bass fed experimental diets for 60 days (Experiment I).

	Dietary treatments	
	Control	**MOS**
Final weight (g)	81.46^{a} ±2.40	90.76^{b} ±1.16
Final length (cm)	18.08^{a} ±0.19	18.59^{b} ±0.06
Final condition factor	1.38^{a}±0.01	1.42^{b}±0.02
Final relative growth	77.44^{a}±2.38	97.49^{b}±1.44
SGR	1.02^{a}±0.02	1.21^{b}±0.01

Different letters within a line denote significant differences (P<0.05). Control= 0 $g \cdot kg^{-1}$ MOS; MOS= 4 $g \cdot kg^{-1}$ MOS. Values expressed in mean ± SD. (n=3 tanks/diet)

6.3.2. Experiment II: Pathogen and stress challenges

6.3.2.1 Mortality, relative percent survival and pathogen presence

No mortality occurred in the CA, CB and S treatments. On the contrary, an increasing mortality rate up to day 3 was registered in fish of treatments I and SI (Figure 6.1). Mortality in these fish occured from 24h after inoculation regardless of the diet received. However, the majority of mortality for fish fed control diet appeared within 24 h of the pathogen inoculation, whereas for the MOS supplemented fish mortality slowed after 72h (Fig. 6.1). The total cumulative mortality during the challenge test in fish fed MOS diet for SI and I treatments (25.0% and 12.5%) was reduced (P<0.02) compared to fish fed the control diet (66.6% and 54.1%) independently from the treatment (P≥0.29). Bacterial cultures obtained from head kidney and liver confirmed *V. anguillarum* as the causative agent for all the mortalities recorded. The RPS values ranged between 94.4-81.9% and 83.3-53.3% on I and SI treatments, respectively (Table

6.4). A reduction of the RPS values (P<0.08) after 3 days and up to the end of the challenge was detected for the SI treatment compared to I treatment.

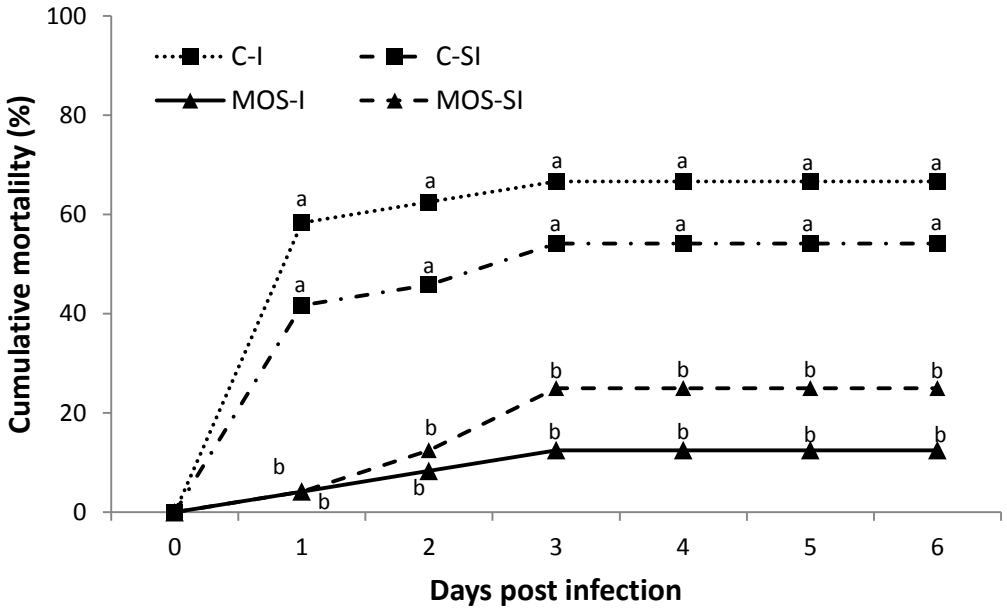

Figure 6.1. Cumulative percentages of mortality of the different treatments whose presented mortality (I (not stressed + infected) and SI (stressed + infected)) after direct gut inoculation (10^7 cfu·ml^{-1}) with the pathogen *V. anguillarum* for European sea bass (*Dicentrarchus labrax*) fed with Control and MOS diets. Different letters indicate significant differences among diets (P<0.05). No mortality was recorded in the CA, CB and S treatments.

MOS supplementation reduced (P<0.05) total bacterial presence in survivor fish after 4h of inoculation for CB and I treatments in liver (Table 6.5) and for I treatment in head kidney compared to fish fed control diet. Within the same diets after 2 hours of inoculation, livers of fish fed the control diet for I and SI treatments presented higher percentages of bacterial presence (P<0.05) than CA, CB and S treatments. Livers of fish fed MOS followed the same pattern of infection but after 24h. At time 2h post inoculation livers of fish fed MOS presented with a higher bacterial presence (P<0.05) in the SI treatment compared to the CA, CB and S treatments but no differences were found among I and SI treatments. After 24h of inoculation, head kidneys of fish fed MOS presented higher bacterial presence (P<0.05) on I treatment compared to CA and CB treatments. No effect was detected among treatments on bacterial presence for head kidney of fish fed control di*et al*ong the experiment. After 2 h of inoculation the bacterial presence in internal organs was affected by treatments but not by diet supplementation (Table 6.5). Instead,

diet supplementation affected total bacterial content on internal organs for 4h, 24h and 6 days post-inoculation (P<0.05) (Table 6.5). *V. anguillarum* was only recovered from internal organs on I and SI treatments for both diets (Table 6.5; Fig. 6.2). *V. anguillarum* presence on liver was affected by the treatment applied (P<0.05) along the trial, regardless of the diet. The same pattern was followed for head kidney, although MOS reduced *V. anguillarum* presence (P<0.05) on SI treatment after 4 h of inoculation (Table 6.5; Fig. 6.2) compared to fish fed control diet. Presence of other *Vibrio sp*. in liver was reduced (P<0.05) for fish fed MOS after 6d of inoculation. Instead, the presence of these species in head kidney was affected by treatment regardless of the diet, presenting after 24 h a higher abundance (P<0.05) in the S treatment compared to CA and CB treatments (Table 6.5). Abundance of *Pseudomonas spp*. in liver after 4h was increased in S treatments (P<0.05) independently of the diet fed compared to CA, I and SI treatments. Instead, after 6 days livers of fish fed MOS presented lower re-isolation percentages (P<0.05) of *Pseudomonas spp*. compared to livers of fish fed control diet, being present in all treatments except for CA.

6.3.2.2. Cortisol levels

Increased stocking density, experimental bacterial infection and the combination of both of these stressors elicited a stress acute response that was evident as elevated plasma cortisol levels (P<0.05) in both dietary treatments. However, the response pattern differed between diets (Figures 6.3A, 6.3B, 6.3C). At 2 h post-stress no differences in mean plasma cortisol levels were found among diets for the different stressors analyzed. Instead, similar patterns of response were observed for both diets, with plasma cortisol levels being greater (P<0.05) in the I and SI compared to the CA, CB and S treatments (Figure 6.3A). At 4 h post-stress/post-inoculation (Figure 6.3B), effects of diet were evident with mean plasma cortisol levels in fish fed the MOS diet being significantly lower that plasma cortisol levels in fish fed the control diet in the infected (I) and combined infection and confinement stressor (SI) treatment groups presented (P<0.05). In contrast, stressed non-inoculated fish (S) fed the MOS diet exhibited a higher cortisol response (P<0.05) to crowding stress at 4h compared to fish receiving the control diet (Figure 6.3B). Within the two diet groups, for fish receiving the control diet, the I and SI groups showed higher (P<0.05) plasma cortisol levels than fish in the CA and CB treatment groups at 4h, although no differences were observed between the CB and S treatments. At 4h, fish fed the MOS diet showed higher (P<0.05) levels of cortisol in the I, SI and S groups compared to the untreated CA group but for

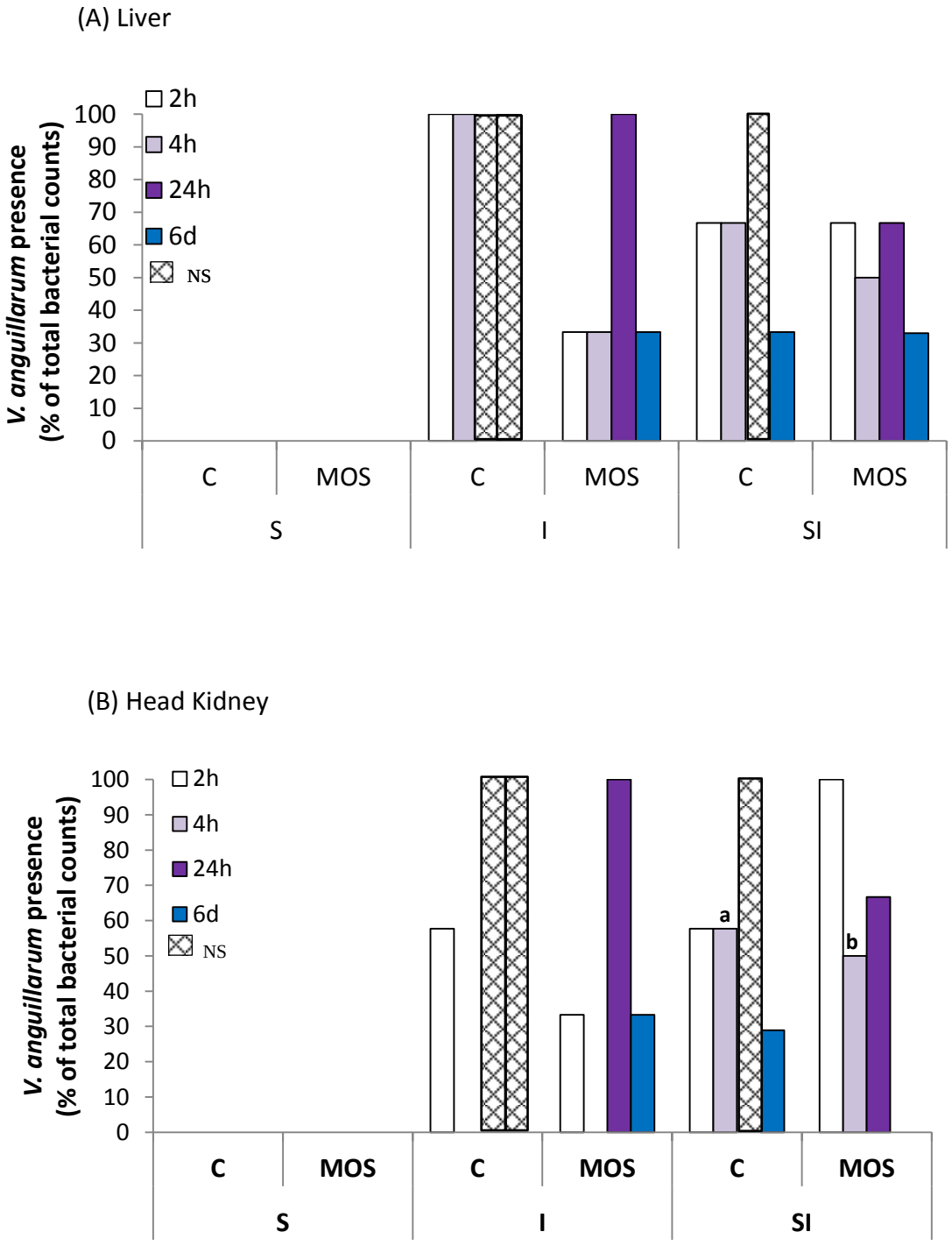

Figure 6.2. European sea bass (*Dicentrarchus labrax*) *V. anguillarum* abundance patterns (% of infected survivor population) on (A) liver and (B) head kidney along the pathogen-stressor panel. *V. anguillarum* was only recovered from internal organs on the I and SI treatments for both diets. Different letters indicate significant differences ($P<0.05$) among fish fed the different diets for a given time and treatment. Control= 0 g·kg^{-1} MOS; MOS= 4 g·kg^{-1} MOS; S: stressed + not infected; I: not stressed+ infected; SI: stressed + infected. (n=3 tanks per treatment).

Table 6.4. MOS dietary effectiveness against *V. anguillarum* intestinal infection measured as Relative percent survival (RPS).

	Days post infection					
Treatment	**1**	**2**	**3**	**4**	**5**	**6**
I	94.4 ± 9.6	88.6 ± 10.3	81.9[a] ± 20.3	81.9[a] ± 20.3	81.9[a] ± 20.3	81.9[a] ± 20.3
SI	83.3 ± 28.9	60.0 ± 52.9	53.3[b] ± 5.8	53.3[b] ± 5.8	53.3[b] ± 5.8	53.3[b] ± 5.8

Different letters within a line denotes significant differences (P≤0.07). I= Infected; SI= Infected + Stressed. Values expressed in mean ± SD

the MOS-fed fish the CB and I treatment groups were not significantly different (Fig. 6.3B). At the end of the experiment, after 6 days, cortisol levels had decreased in all the treatment groups and were indistinguishable from untreated controls (Fig. 6.3C).

6.3.2.3. Microbial community analysis

Posterior gut of non-stressed fish fed control diet (Fig. 6.4I) displayed distinctly different microbial profiles than stressed fish fed control diet (Fig. 6.4II) and stressed and non-stressed fish fed MOS (Fig. 6.4III) and subsequently microbial profiles were clustered into three distinct groups. Posterior gut of fish fed MOS showed similar microbial profiles for stressed and non-stressed fish (Fig. 6.4III) whereas microbial profiles of stressed fish fed control diet differed from those belonging to non-stressed treatments (Fig. 6. 4II and 6.4III).

6.4. DISCUSSION

In the present study, dietary MOS supplementation reduced infection incidence in agreement with previous studies in the same species (Torrecillas *et al*., 2007, 2011b) and increased effectiveness in terms of relative percent survival against *V. anguillarum* gut bacterial translocation for European sea bass juveniles. Dietary yeast-based prebiotics enhance resistance to mycobacteria and *Vibrio spp* challenges in species such as hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (Li and Gatlin III, 2004), reduce parasitic infection against *Amyloodinium ocellatum* in juvenile red drum (*Sciaenops ocellatus*) (Buentello *et al*., 2010) and increase survival of Indian major carp (*Labeo rohita*) challenged against *Aeromonas hydrophila* (Andrews *et al*., 2009), rainbow trout (*Oncorhynchus mykiss*) challenged against *V. anguillarum* (Rodrigues-Estrada *et al*., 2008) and Nile tilapia (*Oreochromis niloticus*) challenged against *Streptococcus agalactiae* (Sado *et al*., 2008). However no effect was detected in channel catfish (*Ictalarus punctatus*) when challenged against *Edwardsiella ictaluri* (Welker *et al*., 2007). A better integrity of the enterocytes

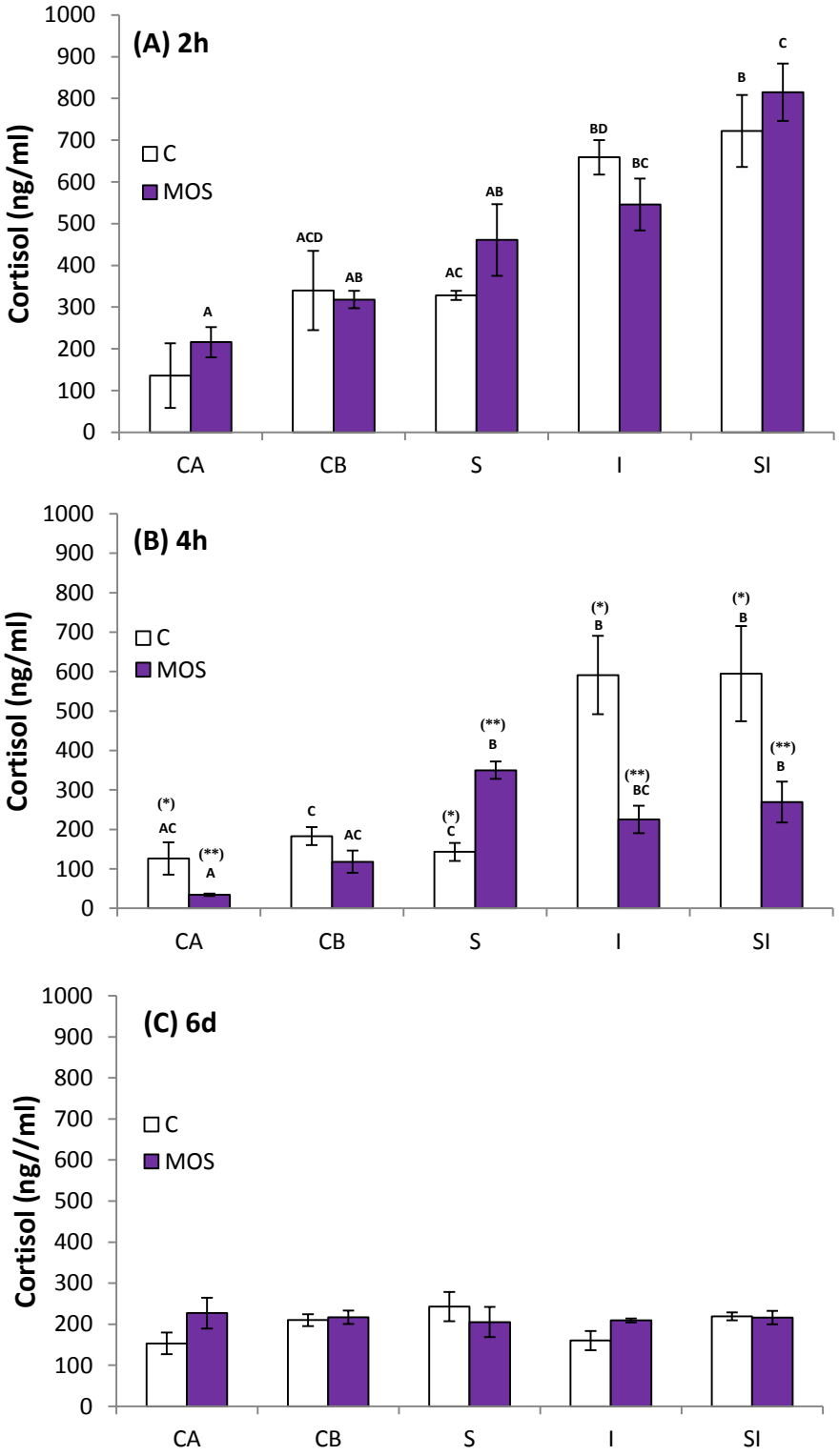

Figure 6.3. Plasma cortisol evolution along the stressor-infection panel at (A) 2 h and (B) 4 h (acute stress) and (C) 6 days (adaptive response) for European sea bass fed Control (C) and MOS diets and submitted to the different treatments. Control= 0 g·kg^{-1} MOS; MOS= 4 g·kg^{-1} MOS; CA: not stressed (2.5 kg·m^{-3}) + not infected; CB: not stressed+ inoculated with PBS; S: stressed (25 kg·m^{-3}) + not infected; I: not stressed+ infected (10^7 cfu *V.anguillarum*·ml^{-1}); SI: stressed (25 kg·m^{-3}) + infected (10^7 cfu *V.anguillarum*·ml^{-1}). Comparisons for a given time between fish fed control and MOS diet (symbols) and within same diet among different treatments (capital letter). Different letters/symbols indicate significant differences among groups (P<0.05).

membrane may contribute partially to explain the lower disease incidence found in the present study since paracellular bacterial translocation increases when loss of enterocytes or loosening of their cell junctions occurs (Ringø *et al*., 2007). In fact, MOS supplementation has been found to increase fish microvilli density (Dimitroglou *et al*., 2009, 2010) and length (Salze *et al*., 2008; Yilmaz *et al*., 2007; Dimitriglou *et al*., 2009) and increase intestinal fold length (Zhou *et al*., 2010; Torrecillas *et al*., 2011b) evidencing its role on the functional integrity of the intestine. This better gut health status could be related the enhanced gut mucus production found in fish after MOS supplementation (Torrecillas *et al*., 2011a, 2011b) which could be protecting enterocytes from damaging, then increasing regularity, height and integrity of gut villi, which consequent better utilization and absorption of dietary nutrients. In parallel, an increased mucus secretion is considered to be an effective defense mechanism of the host, since invading bacteria are flushed out of the gut lumen together with the mucus (Ellis, 2001; Smirnova *et al*., 2003, Van der Marel *et al*., 2008) therefore preventing their firm adhesion. It is known that pathogenic bacteria produce a wide array of virulent factors (including haemolysins, cytotoxins, enterotoxins, endotoxins and adhesins) that could produce exotoxin-induced disruption of the tight junctions (Jutfelt *et al*., 2008), which can affect intestinal barrier function and facilitate translocation (Chopra *et al*., 2000; Ringø *et al*., 2010) and could be counterbalanced by mucus secretion (Jutfelt *et al*., 2008).

Thus, the reduced cumulative mortality found in I and SI treatments of fish fed MOS could be also partially related to the increased gut mucus production of European sea bass fed MOS (Sweetman *et al*., 2010; Torrecillas *et al*., 2011a, 2011b) by reducing the adhesion rate of the inoculated pathogen to the gut epithelium and preventing their translocation. Indeed, MOS supplementation was found to increase gut mucus lysozyme and antibacterial activities (Torrecillas *et al*., 2011b), which are also essential components in the defense against pathogen infection that would contribute to reduce the bacterial translocation rates (Berg, 1992). In addition, other factors such as enhanced cellular and humoral fish innate immune parameters after MOS supplementation (Zhou and Li, 2004; Genc *et al*., 2007; Montero *et al*., 2007; Staykov *et al*., 2005, 2007; Terova *et al*., 2009; Torrecillas *et al*., 2007, 2011a, 2011b) could also contribute to the positive effects of the MOS diet, by reducing both, gut bacterial translocation or the capacity of translocated bacteria to survive this translocation (Sweetman *et al*., 2010;

Table 6.5. Bacterial species isolated from internal organs (liver and head kidney) of European sea bass fed Control and MOS diets for the different treatments.

		LIVER											HEAD KIDNEY										
		CA		CB		S		I		SI		P≤0.05	CA		CB		S		I		SI		P≤0.05
Bacterial abundance (%)		C	MOS	C	MOS	C	MOS	C	MOS	C	MOS		C	MOS	C	MOS	C	MOS	C	MOS	C	MOS	
2h	**Total**	**(--)^{A}**	**(--)^{A}**	**(--)^{A}**	**(--)^{A}**	**(--)^{A}**	**11^{A}**	**44.4^{B}**	**22^{AB}**	**22.2^{B}**	**55^{B}**	**T**	**(--)**	**(--)**	**(--)**	**(--)**	**(--)**	**(--)**	**33.3**	**22**	**19.2**	**33**	**T**
	V. anguillarum	(--)	(--)	(--)	(--)	(--)	(--)	100	33.3	66.7	66.7	T	(--)	(--)	(--)	(--)	(--)	(--)	57.7	33.3	57.7	100	T
	Vibrio spp.	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	16.7	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)
	Aeromonas spp.	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	16.7	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)
	Pseudomonas spp	(--)	(--)	(--)	(--)	(--)	33.3	(--)	16.7	(--)	16.7	(--)	(--)	(--)	(--)	(--)	(--)	(--)	57.7	16.7	(--)	(--)	(--)
4h	**Total**	**11.1**	**(--)**	**44.4^{a}**	**(--)^{b}**	**33.3**	**33**	**77.8^{a}**	**11.0^{b}**	**44.3**	**33**	**D**	**(--)**	**(--)**	**19.1**	**(--)**	**57.7**	**11**	**33^{a}**	**(--)^{b}**	**33**	**33**	**D**
	V. anguillarum	(--)	(--)	(--)	(--)	(--)	(--)	100	33.3	66.7	50	T	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	57.7	50	D/T
	Vibrio spp.	33.3	(--)	66.7	(--)	33.3	16.7	22	(--)	(--)	16.7	(--)	(--)	(--)	57.7	(--)	7.5	33.3	57.7	(--)	(--)	16.7	(--)
	Aeromonas spp.	(--)	(--)	16.7	(--)	(--)	(--)	(--)	(--)	(--)	16.7	(--)	(--)	(--)	28.8	(--)	(--)	(--)	(--)	(--)	(--)	16.7	(--)
	Pseudomonas spp	(--)	(--)	27.9	(--)	22.2	50	(--)	(--)	(--)	(--)	T	(--)	(--)	57.7	(--)	19.1	33.3	(--)	(--)	(--)		(--)
24h	**Total**	**(--)**	**11^{A}**	**11.1**	**11.0^{A}**	**22.2**	**22^{A}**	**NS**	**66.0^{B}**	**NS**	**66.0^{B}**	**(--)**	**(--)**	**(--)^{A}**	**(--)**	**(--)^{A}**	**19.2**	**22^{AB}**	**NS**	**66^{B}**	**NS**	**44^{AB}**	**D**
	V. anguillarum	(--)	(--)	(--)	(--)	(--)	(--)	NS	100	NS	66.7	(--)	(--)	(--)	(--)	(--)	(--)	(--)	NS	100	NS	66.7	(--)
	Vibrio spp.	(--)	33.3	(--)	33.3	33.3	66.7	NS	16. 7	NS	33.3	(--)	(--)	(--)	(--)	(--)	57.7	66.6	NS	33.3	NS	(--)	T
	Aeromonas spp.	(--)	(--)	33.3	(--)	33.3	(--)	NS	16. 7	NS	(--)	(--)	(--)	(--)	(--)	(--)	(--)	(--)	NS	(--)	NS	(--)	(--)
	Pseudomonas spp	(--)	(--)	(--)	(--)	33.3	(--)	NS	(--)	NS	(--)	(--)	(--)	(--)	(--)	(--)	57.7	(--)	NS	(--)	NS	(--)	(--)
6d	**Total**	**33.3**	**(--)**	**66.7**	**16.7**	**66.7**	**16.7**	**NS**	**50**	**66.7**	**16.7**	**D**	**(--)**	**(--)**	**50**	**16.7**	**(--)**	**16.6**	**NS**	**16.7**	**50.9**	**(--)**	**(--)**
	V. anguillarum	(--)	(--)	(--)	(--)	(--)	(--)	NS	33.3	33.3	33.3	(--)	(--)	(--)	(--)	(--)	(--)	(--)	NS	33.3	28.9	(--)	(--)
	Vibrio spp.	66.7	(--)	100	33.3	83.3	16.7	NS	(--)	100	(--)	D	(--)	(--)	57.7	33.3	(--)	(--)	NS	(--)	57.7	(--)	T
	Aeromonas spp.	66.7	(--)	(--)	33.3	16.7	33.3	NS	(--)	33.3	(--)	(--)	(--)	(--)	(--)	33.3	(--)	33.3	NS	(--)	50	(--)	(--)
	Pseudomonas spp	(--)	(--)	100	(--)	100	16.7	NS	100	33.3	(--)	D/T	(--)	(--)	57.7	(--)	(--)	(--)	NS	16.7	(--)	(--)	(--)

Control= 0 g·kg^{-1} MOS; MOS= 4 g·kg^{-1} MOS; CA: not stressed+ not infected; CB: not stressed+ PBS; S: stressed + not infected; I: not stressed+ infected; SI: stressed + infected. "T" and "D" indicates significant difference according to Treatment and Diet respectively (P<0.05) for two way ANOVA. Lower case indicates significant differences among groups (P<0.05) for the same organ between fish fed control and MOS diet and capital letters indicate significant differences among groups (P<0.05) within same diet among different treatments for a given time (one way ANOVA). NS= no sample availability due to mortality; (--) = not detected.

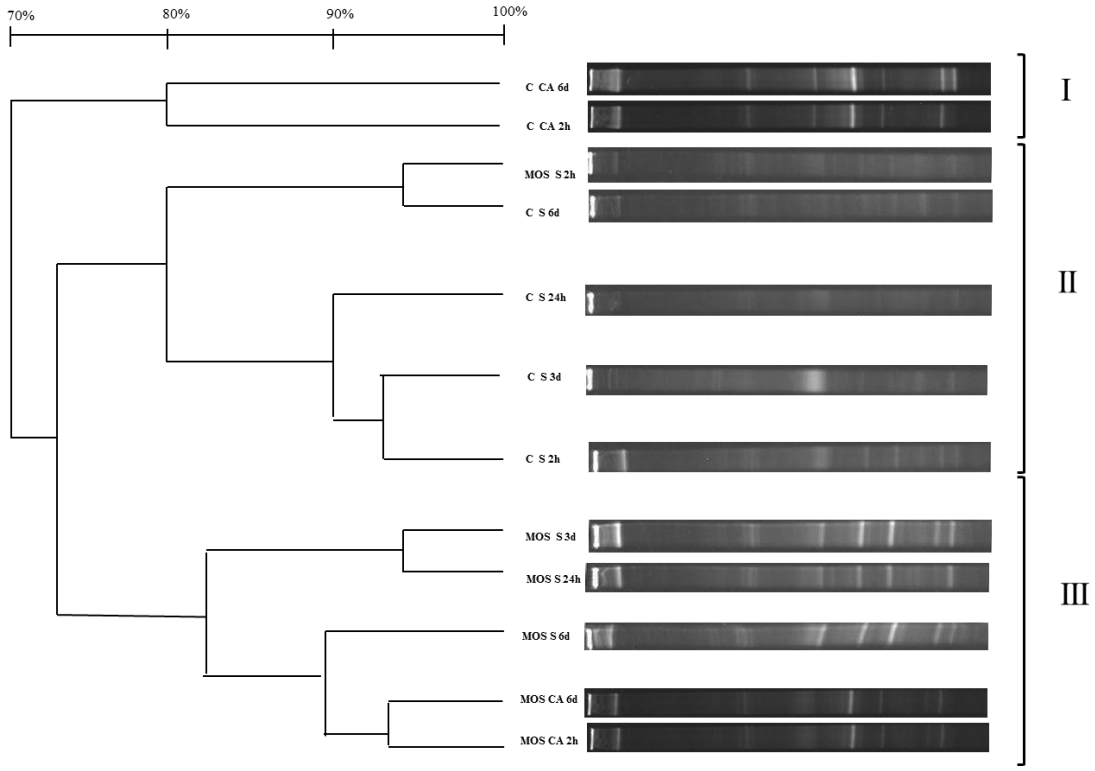

Figure 6.4. WPGAMA dendogram developed using the Dice Coefficient of gel electrophoresis (DGGE) bands for European sea bass posterior gut microbial populations fed with the different diets and subjected to a stressor panel.

Torrecillas *et al*., 2007, 2011a, 2011b). Since, translocated bacteria are usually neutralized by the local immune system in the lamina propria of mammals (Sansonetti 2002) and probably also in fish (Jutfelt *et al*., 2008).

Infection levels markedly affected plasma cortisol levels. Thus, at 4h post stress/infection in fish fed the control diet, the higher disease incidence observed in infected (I), and stressed and infected (SI) fish was associated with plasma cortisol levels higher than those seen in the MOS-treated fish at the same time. While it is likely that higher infection levels contributed to this difference in cortisol levels between the dietary groups it is also the case that fish with higher levels of cortisol have an increased susceptibility to disease (Pickering and Pottinger, 1985; Harris *et al*., 2000; Kenneth *et al*., 2003; Wang *et al*., 2005; Ellis *et al*., 2007). In our study these higher levels of cortisol found in infected fish may also have further facilitated bacterial infection by affecting the permeability of the gastrointestinal tract or by more general immunosuppressive actions. In fact, it seems that in the SI treatment groups the potentially

complex interaction of the effects of the stressor on plasma cortisol, which will lead to immunosuppressive effects and will enhance the bacterial infection, will in turn result in further cortisol secretion combined with the impact of the bacterial challenge itself. Acute stress has been associated with the loss of intestinal goblet cells and detachment of enterocytes in carp (Szakolczai, 1997) and to damage of the intercellular junctional complexes in Atlantic salmon (*Salmo salar*) (Olsen *et al*., 2003) and rainbow trout (Olsen *et al*., 2005) 4h after stress. Interestingly in the present study, after 2h hours of inoculation, *V. anguillarum* started to appear on internal organs regardless of the diet fed, but it is after 4 hours post-stress when the higher percentages of bacterial translocation appear in fish fed control diet, then denoting a possible effect. Therefore, this higher bacterial presence in internal organs of fish fed control diet might provide evidence of the role of cortisol on fish gut paracellular permeability as it occurs in fish and higher vertebrates (Kiliaan *et al*., 1998; Söderholm and Perdue, 2001; Olsen *et al*., 2003, 2005).

In this sense, not only alterations on paracellular activity should be considered, but also the decrease in disease resistance attributable to the immnunosuppresive effects of elevated levels of cortisol (Pickering and Pottinger, 1985; Barton and Iwama, 1991; Vazzana *et al*., 2002; Ellis *et al*., 2007). Particularly in sea bass, confinement stress for 3h reduced peritoneal leukocytes innate cytotoxic activity (Vazzanna *et al*., 2002), which could be related to the marked increase in cortisol levels and disease incidence levels 4h post stressor/infection exposure in fish fed control the diet for the infected (I) and stressed and infected (SI) treatments compared to fish fed MOS diet. Mannose receptors (MRs) are localized in macrophages and endothelial cell subsets whose natural ligands include self-glycoproteins and microbial glycans (Ringø *et al*., 2010), even they are also expressed by immature cultured dendritic cells (DC), where it mediates high efficiency uptake of glycosylated antigens (Linehan *et al*., 2000; Ringø *et al*., 2010). Receptors similar to mammalian MRs (Rodríguez *et al*., 2003) and a C-type lectin possessing MR features (Zhao *et al*., 2009) have been characterized in fish and shellfish (Ringø *et al*., 2010). Besides, mannose-containing ligands may also bind to other receptors such as DC-SIGN and dectin-2 resulting in leucocyte activation (Ringø *et al*., 2010) and induce intracellular signaling that may increase production of mucus (Rogers, 2001) or/and proinflammatory cytokines (Nimmerjahn and Ravetch, 2006; Baccan *et al*., 2010). The cytokine microenvironment at an inflammatory site determines a leucocyte´s functional capacity to respond to a treatment with glucocorticoids (Heasman *et al*., 2004). In mouse, corticosterone inhibits phagocytosis in resting macrophages for

Fcγ, mannose and β-glucan receptors, whereas is triggered by catecholamines in activated macrophages for the 3 types of receptors (Baccan *et al*., 2010). Thus, since MOS supplementation has been shown to influence positively phagocytic cells activity (Torrecillas *et al*., 2007, 2011a) possibly mediating its activation through the presence of MRs (Torrecillas *et al*., 2011a), a possible change in the phagocytes response to glucocorticoids and its contribution to the lower disease incidence found in the present study for infected groups fed MOS should not be discarded. All these factors together with a better intestinal integrity, enhanced mucus production and stimulated gut associated lymphoid tissue response (Torrecillas *et al*., 2011a, 2011b) derived from MOS supplementation, had a protective effect against intestinal infection that not only delayed the incidence of infection to 24h post stress but also reduced mortality following the challenge.

The isolation of other genera of bacteria on internal organs for the CB, S, I and SI treatments for both dietary formulations from 4h post stress, could be the consequences of exposure to the stressor, since other bacteria from common gut microbiota could have also translocated as a consequence of an immune-compromised host. Even taking this into account, MOS supplementation reduced total bacterial species isolated from internal organs from 4h post stress/infection to the end of the challenge when particularly in liver, *Vibrio spp* and *Pseudomononas spp* counts were reduced after MOS supplementation. These reduced percentages could be due to either enhanced gut first barrier defense, or to a possible reduction of total gut *Vibrio spp* or *Pseudomonas spp* relative and/or absolute abundance after dietary MOS inclusion, leading to lower translocation percentages. Other prebiotics in fish diets have been demonstrated to have effects on microflora species relative/absolute abundances but results are dependable mainly on the prebiotic tested (Ringø *et al*., 2006; Bakke-McKellep *et al*., 2007; Hui-Yan *et al*., 2007; Zhou *et al*., 2009). Short chain fructoligosaccharides (scFOS) increased *V. parahemolyticus, Aeromonas hydrophila, Lactobacillus spp.* and *Streptococcus faecalis* (Hui-Yan *et al*., 2007; Zhou *et al*., 2009) in red drum whereas MOS reduced viable intestinal bacterial population, especially *Aeromonas/Vibrio spp*. but increased enterococci counts, after culture-based evaluation of rainbow trout intestinal microbiota (Dimitroglou *et al*., 2009).

WPGAMA analysis showed a shift of stressed fish intestinal microbial profiles compared to non-stressed fish fed control diet with each treatment clustered into distinct groups. Whereas intestinal microbial profiles of fish fed MOS diet remained in the same cluster, regardless of the treatment. In fact, results obtained suggest that stress exerted a clear greater

effect on gut microbiota for fish fed control diet than for fish fed MOS diet. Alterations on microbial profiles induced by stress in fish fed MOS diet appeared to be reduced or eliminated, although these profiles are more similar to those belonging to stressed fish fed control diet. The reason for this is not clear, however, appears that dietary MOS inclusion minimized stress-derived-effects regarding this parameter and further studies should be conducted in order to clarify the mechanisms involving these changes.

In summary, the present study has confirmed, in European sea bass, the positive effect of two months dietary MOS supplementation (at $4g \cdot kg^{-1}$) on infection and disease resistance against *V. anguillarum* after gut inoculation. Results revealed a reduced gut bacterial translocation *in vivo* in fish fed MOS as well as reduced effects of stress on microbiota diversity. Both of these findings, together with the higher gut mucus production and density of eosinophil granulocytes in gut mucosa obtained in previous studies (Torrecillas *et al*., 2011a, 2011b) suggest that reinforcement of the intestinal barrier efficiency is the front line of defense against pathogenic microorganisms of European sea bass fed MOS.

Chapter 7

Regulation of intestinal mucus production in European sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS)

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ABSTRACT

The objective of the present study was to better understand the regulation of intestinal mucus production in European sea bass (*Dicentrarchus labrax*) by dietary mannan oligosaccharides (MOS) and if this process is mediated by the potential production of prostaglandins (PGs), since these eicosanoids are important modulators of mucus synthesis and secretion in other vertebrates. For this purpose, specimens were fed 4g ·kg^{-1} MOS included in a commercial diet for eight weeks. After that period fish fed MOS presented significantly higher weight gain and total length ($P<0.05$) than fish fed the non-supplemented control diet. Specific and relative growth rates were also higher ($P<0.05$) in fish fed dietary MOS. Besides the number of goblet cells secreting acid mucins per area unit in both anterior and posterior gut was increased by dietary MOS supplementation. Finally, stimulated posterior gut of fish fed MOS showed higher ($P<0.05$) PGs production than fish fed control diet, whereas no differences were found in anterior gut or head kidney. The study showed that MOS supplementation enhanced the potential production of PGs in posterior intestine of European sea bass in relation to a higher presence of goblet cells and leucocytes in this tissue.

Keywords:
Eicosanoids levels
Goblet cells
Mannan oligosaccharides
Mucus production
Sea bass

7.1. INTRODUCTION

The epithelial surfaces of fish, such as skin, gills or gastrointestinal tract are the first contact areas for potential pathogens (Ijima *et al*., 2003; Narvaez *et al*., 2010). In fact, many infectious diseases are initiated by gut bacterial mucosal colonization (Abraham *et al.*, 1999). Mucus gel layer is an integral structural component of the intestine, acting as a medium for protection, lubrication and transport between the luminal contents and the epithelial lining (Deplancke and Gaskins, 2001; Smirnov *et al*., 2005). The viscoelastic polymer-like properties of mucus are derived from the major gel-forming glycoprotein components called mucins. Mucins are involved in a variety of cytoprotective functions against a number of hazards including mechanical insults, colonization of potential pathogenic bacteria and their toxins or luminal proteases arising from bacterial and mucosal cells (Barceló *et al*., 2000), playing a key role in the protection of the underlying epithelium (Ellis, 2001). Normally, mucins are secreted by goblet cells at a slow baseline rate in order to maintain the mucus coat over the intestinal epithelium but, in response to stimulation, these cells may accelerate their discharge (Plaisancié *et al*., 1998).

Mucus secretion regulation in fish has been scarcely studied. In other vertebrates these variations have been coupled to neural, hormonal and paracrine changes or dietary factors that affect goblet cells number and mucin heterogeneity (McCraken *et al*., 1995; Sharma *et al*., 1995; Ganessunker *et al*., 1999; Deplancke and Gaskins, 2001) and modulate the secretory activity of goblet cells (Satchithanandam *et al*., 1990; McCullogh *et al*., 1998; Barceló *et al*., 2000; Deplancke and Gaskins, 2001). Short chain fatty acids (SCFA) (Willemsen *et al*., 2003), carbachol (Laburthe *et al*., 1989; McCool *et al*., 1990), probiotic supplementation (Smirnov *et al*., 2005), vasoactive intestinal peptide (VIP) (McCool *et al*., 1990; Ogata and Podolsky, 1997; Plaisancié *et al*., 1998; Hokari *et al*., 2005), peptide YY (PYY) (Plaisancié *et al*., 1997, 1998), serotonin (Menguy, 1967; Moore *et al*., 1996, Plaisancié *et al*., 1998), interleukin (IL)-1 (Jarry *et al*., 1996; Plaisancié *et al*., 1998; Enss *et al*., 2000), leptin (Buyse *et al*., 2001, 2002; Guilmeau *et al*., 2003; Ducroc *et al*., 2005; Plaisancié *et al*., 2006) and prostaglandin E_2 (PGE_2) (McQueen *et al*., 1983; Phillips *et al*., 1993, Tani *et al*., 1997; Belley and Chadee, 1999; Akiba *et al*., 2000; Krause and DuBois, 2000; Mohajer and Ma, 2000; Riehl *et al*., 2000; Ohnishi *et al*., 2001; Cho *et al*., 2005; Hisatsune *et al*., 2007; Shao *et al*., 2007; Tetaert *et al*., 2007) among other substances,, have been shown to influence mucus production.

PGE_2 is considered to play an important role in the gastrointestinal tract of higher vertebrates, by its beneficial physiological functions on epithelial cytoprotection. Among other processes, enhancement of gut motility and bicarbonate secretion, reduced acid secretion (Dey *et al*., 2009) and restore of epithelial barrier function through closure of tight junctions (Belley and Chadee, 1999) are influenced by PGE_2. The actions of PGE_2 are mediated by four G-protein-coupled (EP) receptors that are encoded by different genes and referred to as EP_1, EP_2, EP_3 and EP_4 receptors (Breyer *et al.*, 2001). On human and rat colonic epithelial cells, EP_4 couples to PGE_2 evoking mucin exocytosis (Belley and Chadee, 1999), an important constituent for epithelial barrier function. Dey *et al*. (2009) reported that PGE_2 couples through high affinity prostanoid receptors of the EP_4 subtype expressed by human colonic epithelial cells to stimulate the output of IL-8 by a cAMP-dependent mechanism, evidencing the theoretical role of PGE_2 as major pro-inflammatory mediator in the gut.

Considering the regulatory role of eicosanoids on mucus production in higher vertebrates and since the reduction in the disease incidence after experimental bacterial challenge on European sea bass (*Dicentrarchus labrax*) caused by dietary MOS supplementation has been related to the reinforcement of the gastrointestinal tract mucosal surface through enhanced mucus production and gut associated lymphoid tissue stimulation (Torrecillas *et al*., 2011a, 2011b), the objective of this study was to determine if dietary supplementation effect on mucus production is related to the potential PGs production by the intestinal tract.

7.2. MATERIALS AND METHODS

7.2.1. Diets

Two experimental pelleted diets, based on a commercial formulation, were prepared in order to contain 0 (Control) and 4 $g \cdot kg^{-1}$ MOS (MOS; Bio-Mos® Aquagrade, Alltech, Inc., USA) replacing standard carbohydrates. Diets were pelleted in an industrial mixer, crumbled to the desired size, and air-dried prior to storage at 4ºC until feeding. Diet ingredients and biochemical composition are shown in Table 7.1.

7.2.2. Experimental conditions

Five hundred and fifty commercially reared European sea bass juveniles were maintained in stocking tanks and fed a commercial extruded diet for 3 weeks until being fully

adapted to the environmental conditions (4 kg·m^{-3} stocking density). Afterwards, fish were randomly distributed in 6 indoor cylindroconical 1000 l fiberglass tanks at an initial stocking density of 4 kg·m^{-3} (90 fish per tank). Fish average initial weight and length were 45.95 ± 0.60 g and 15.05 ± 0.05 cm, respectively (mean ± SD). Tanks were supplied with filtered sea water, at a temperature of 22.8-23.3°C and natural photoperiod (12L:12D). Water dissolved oxygen ranged between 6.5-7.2 ppm. Fish were manually fed until apparent satiation with one of the two experimental diets for 8 weeks (3 times a day, 6 days a week). Each diet was assayed in triplicate and growth parameters, histological studies and PGs analysis were performed at day 60.

Table 7.1. Main ingredients and composition in the experimental diets

Ingredient	Diet (g kg^{-1} dry weight)	
	Control	**BM4**
Fish meal [1]	515.0	515.0
Soybean meal	97.8	97.8
Wheat	75.0	75.0
Wheat gluten	75.0	75.0
Corn meal	65.3	61.3
Fish oil [2]	126.9	126.9
Fats and oils	20.3	20.3
Mineral mix [3]	14.3	14.3
Vitamin mix [4]	10.3	10.3
Antioxidant (BHT)	0.1	0.1
MOS (Bio-Mos Aquagrade)[5]	0	4
Composition (%, dry weight)		
Crude lipids	20.37	20.32
Crude protein	51.62	52.07
Moisture	7.84	7.88
Ash	9.74	9.82

[1] Peruvian fish meal (65% protein). [2] Peruvian fish oil. [3] Mineral mix TROUW Seabream/Seabass (0.8g), Choline chloride (0.17g) and Inositol (0.06g) (Trouw Nutrition Spain, Madrid, Spain). [4]Vitamin mix TROUW Seabream/Seabass (1g) Calcium carbonate (0.2g), Potassium monophosphate (0.19g) and NaCl 97% (0.04g) (Trouw Nutrition Spain, Madrid, Spain). [5] Bio-Mos, Alltech, Inc., USA

7.2.3. Histological studies

Fish intestines were dissected out and divided into anterior (from the end of the pyloric caeca to the first diffused sphincter) and posterior (from the first diffused sphincter to rectum sphincter) sections. Six to eight transversal sections of each intestine segment were fixed in 10% neutral-buffered formalin and embedded in paraffin perpendicularly to the bottom. For each specimen five serial sections (5 µm-thick) were stained with specific staining Alcian Blue/PAS in order to determine cells secreting mucins (Martoja and Martoja-Pierson, 1970).

Micrographs were taken from stained sections using an Olympus CX41 microscope (Olympus Optical, PA, USA). The number of cells stained for specific mucin staining by unit of area, was determined with an analySIS® (Image Pro Plus®) software package (Torrecillas *et al*., 2011a, 2011b).

7.2.4. Lipid analysis

Crude lipid was extracted following the method of Folch *et al*. (1957). Fatty acids were prepared by transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo *et al*. (1992), being quantified by FID and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan).

7.2.5. Prostaglandin analysis

7.2.5.1. Tissue stimulation

Head kidneys and anterior and posterior gut segments were dissected out, weighted and digested in 4 ml of Hank´s Balanced Salt Solution (HBSBS) without Ca^{2+} plus 2% collagenase during 30 minutes under continuous orbital agitation ($100U \cdot min^{-1}$). After digestion, samples were filtered through nylon gauze and washed with 4 ml of HBBS without Ca^{2+} and isolated cells recollected in a glass tube. Cells were concentrated in a pellet by centrifugation (5 min, 2500 g) and the resultant pellet re-suspended in 1 ml of HBBS without Ca^{2+} in an Eppendorf tube. Cells were stimulated during 30 min with 50 µM Ca^{2+} PMA (Phorbol 12-myristate 13-acetate, Sigma Chemicals, CA, USA) and 10 µM A321 (Sigma Chemicals, CA, USA). Stimulation reaction was stopped by adding 50 µl of formic acid 2M. Samples were stored at -80°C until purification.

7.2.5.2. Purification of eicosanoids

The frozen stimulated fraction (pooled 4 fish tissues/tank) was centrifuged at 1000g for 5 min in order to precipitate any remaining debris. The supernatants were extracted using a octadecylsilyl (C18) “Sep-Pak” mini-columns (Millipore, Watford, UK) by the method of Powell (1982) and as described in detail by Bell *et al*.(1994). One milliliter of supernatants was applied to the column, which had been prewashed with 5 ml of methanol and 10 ml of MiliQ water. The column was successively washed successively with 10 ml of MiliQ water, 5 ml of 15% ethanol (v/v) and 5 ml of hexane/chloroform (65:35, v/v) before elution of prostanoids with 10 ml of

ethyl acetate. The extracts were dried under nitrogen and re-suspended in 100 µl of methanol and stored at -80ºC in small glass vials until analysis.

7.2.5.3. Prostaglandin E_2 immunoassay

Measurement of prostaglandins was performed using an enzyme immunoassay (EIA) kit for PGE_2 according to manufacturer's protocol (Cayman Chemical Co., MI, USA), that is based in the competition between PGE_2 and PGE_2-actylcholinesterase (AChE) conjugated PGE_2 (tracer) for a limited amount of PGE_2 monoclonal antibody.

7.2.6. Statistical analysis

All data were tested for normality and homogeneity of variance. Means and standard deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods described by Sokal and Rolf (1995). Biological parameters, number of mucus producing cells, individual fatty acids percentages and PGs levels data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of MOS inclusion. Aditionally, a two-way ANOVA analysis was applied to fatty acids pecentages in order to examine the combined effect of the fixed factors "diet" and "tissue", using Statgraphics software (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA). When F values showed significance, individual means were compared using Tukey/Duncan tests for multiple means comparison. Significant differences were considered for $P < 0.05$.

7.3. RESULTS

7.3.1. Growth parameters and biometry

No mortalities were registered during the feeding experiment. After 60 days of feeding, fish fed with MOS diet showed higher ($P<0.05$) body weight, total length and condition factor (Table 2). Specific and relative growth rates followed a similar trend, increasing about 18% ($P<0.05$) in fish fed dietary MOS compared to non-supplemented fish (Table 7.2).

7.3.2. Histological studies

The number of cells secreting acid mucins·$10^6 \mu m^{-2}$ in anterior gut was 1735.56 ± 211.23 for fish fed control diet and 2027.12 ± 197.65 for MOS supplemented diet. For posterior

gut values obtained were 2821.58 ± 283.94 and 3230.54 ± 538.87 for fish fed control and MOS diets respectively.

Table 7.2. Growth performance and somatic parameters of European sea bass fed experimental diets for 60 days (Experiment I).

	Dietary treatments	
	Control	**MOS**
Final weight (g)	81.46^{a} ±2.40	90.76^{b} ±1.16
Final length (cm)	18.08^{a} ±0.19	18.59^{b} ±0.06
Final condition factor	1.38^{a}±0.01	1.42^{b}±0.02
Final relative growth	77.44^{a}±2.38	97.49^{b}±1.44
SGR	1.02^{a}±0.02	1.21^{b}±0.01

Different letters within a line denote significant differences (P<0.05). Control= 0 g·kg^{-1} MOS; MOS=4 g·kg^{-1} MOS. Values expressed in mean ± SD (n=3 tanks/diet).

7.3.3. Gut Fatty Acids profile

MOS supplementation in diets for European sea bass reduced (P<0.05) total saturated fatty acids, 18:0 and 22:5n-3 contents in the intestinal tract, regardless the gut region studied, as well as DHA/EPA and ARA/EPA ratios. In the anterior gut, dietary MOS inclusion decreased (P<0.05) ARA/EPA ratio from 0.449 in fish fed control diet to 0.330 in fish MOS diet (Table 7.3). Fish fed MOS diet also showed lower (P<0.05) contents of 22:5n-3 in anterior gut with no changes on the total n-3 fatty acids levels. In relation to the anterior gut, the posterior region had higher 22:5n-3 and total n-6 percentages as well as reduced n-3/n-6 ratio, regardless the diet supplemented (Table 7.3). In the posterior gut, dietary MOS inclusion reduced (P<0.05) total saturated fatty acids as well as DHA/EPA ratio in comparison to fish fed control diet (Table 7.3).

7.3.4. Prostaglandins levels in gut and head kidney

Stimulated posterior gut of fish fed MOS showed higher (P<0.05) levels of PGs compared to fish fed control diet. No differences were found for stimulated head kidney and anterior gut after 8 weeks of MOS supplementation (Figure 7.1).

Table 7.3. Anterior and posterior gut fatty acids profile *(% of total area)* of European sea bass fed 0 and 4 g·kg^{-1} MOS during 8 weeks.

	Anterior gut		Posterior gut		P<0.0
	Control	MOS	Control	MOS	
14:0	1.562±0.527	1.900±0.440	2.286±1.469	2.618±0.935	
14:1n-7	0.043±0.010	0.117±0.121	0.244±0.366	0.200±0.235	
14:1n-5	0.043±0.012	0.131±0.120	0.250±0.366	0.192±0.221	
15:0	0.301±0.096	0.322±0.153	0.561±0.454	0.514±0.216	
15:1n-5	0.016±0.015	0.114±0.168	0.121±0.210	0.225±0.315	
16:0ISO	0.453±0.103	0.370±0.183	0.174±0.248	0.600±0.496	
16:0	16.271±0.826	15.804±0.301	16.002±1.118	14.976±0.243	
16:1n-7	2.449±0.716	2.743±0.614	2.549±0.748	3.310±0.638	
16:1n-5	0.147±0.009	0.170±0.045	0.192±0.101	0.143±0.105	
16:2n-6	0.166±0.006	0.241±0.078	0.269±0.172	0.201±0.163	
16:2n-4	0.362±0.059	0.411±0.059	0.466±0.203	0.577±0.021	
17:0	0.264±0.058	0.321±0101	0.183±0.155	0.399±0.071	
16:3n-4	0.000±0.000	0.000±0.000	0.007±0.012	0.012±0.021	
16:3n-3	0.234±0.093	0.222±0.067	0.140±0.105	0.356±0.156	
16:3n-1	0.358±0.210	0.419±0.029	0.440±0.102	0.766±0.446	
16:4n-3	0.400±0.107	0.291±0.110	0.581±0.452	0.467±0.228	
16:4n-1	0.029±0.049	0.000±0.000	0.250±0.349	0.150±0.088	
18:0	11.241±1.398	9.018±1.190	11.391±0.827	9.089±2.373	D
18:1n-9	12.626±2.038	14.912±2.459	12.796±2.203	16.883±2.174	D
18:1n-7	2.497±0.652	1.978±0.114	2.652±0.23	2.795±0.259	
18:1n-5	0.168±0.015	0.168±0.036	0.166±0.011	0.182±0.035	
18:2n-9	0.122±0.018	0.129±0.063	0.159±0.068	0.208±0.013	
18:2n-6	14.306±2.329	16.362±1.383	13.781±2.050	16.999±2.134	D
18:2n-4	0.129±0.058	0.175±0.034	0.158±0.038	0.168±0.022	
18:3n-6	0.149±0.025	0.190±0.028	0.120±0.040	0.185±0.011	D
18:3n-4	0.055±0.044	0.078±0.011	0.074±0.070	0.047±0.041	
18:3n-3	1.228±0.369	1.467±0.272	1.186±0.313	1.696±0.332	
18:4n-3	0.289±0.099	0.358±0.139	0.295±0.103	0.473±0.119	
18:4n-1	0.053±0.014	0.070±0.024	0.049±0.009	0.076±0.029	
20:0	0.192±0.054	0.214±0.021	0.205±0.032	0.221±0.056	
20:1n-9+n-7	1.413±0.230	1.560±0.161	1.493±0.278	1.812±0.092	
20:1n-5	0.171±0.011	0.170±0.012	0.207±0.066	0.252±0.055	
20:2n-9	0.014±0.004	0.018±0.008	0.014±0.012	0.029±0.004	
20:2n-6	0.961±0.059	0.851±0.062	1.046±0.111	0.972±0.242	
20:3n-6	0.190±0.065	0.190±0.024	0.206±0.065	0.180±0.039	
20:4n-6	2.794±0.658	2.307±0.403	2.765±0.673	1.912±0.701	
20:3n-3	0.071±0.025	0.072±0.012	0.043±0.039	0.079±0.013	
20:4n-3	0.265±0.044	0.304±0.056	0.258±0.027	0.333±0.072	
20:5n-3	6.191±1.132	6.995±0.120	6.103±1.253	5.671±0.335	
22:1n-11	0.666±0.207	0.859±0.165	0.632±0.131	0.795±0.163	
22:1n-9	0.118±0.076	0.064±0.029	0.142±0.047	0.096±0.064	
22:4n-6	0.105±0.036	0.123±0.052	0.110±0.038	0.166±0.042	
22:5n-6	0.265±0.018	0.221±0.025	0.292±0.011	0.246±0.068	
22:5n-3	**1.516 ^{a} ±0.062**	**1.362 ^{b} ±0.067**	1.576±0.043	1.537±0.066	D/T
22:6n-3	19.108±3.175	16.210±2.947	17.366±5.446	11.195±2.386	
Saturated	29.831±1.797	27.578±1.053	**30.628^{a}±0.208**	**27.817^{b}±1.367**	D/T
Monoenes	20.189±3.505	22.819±2.954	21.237±4.291	26.632±3.371	
Σn-3	29.303±3.996	27.280±2.669	27.548±60.09	21.805±2.610	
Σn-6	18.936±1.598	20.484±1.048	32.077±3.412	37.614±3.496	T
Σn-9	14.292±2.271	16.683±2.661	14.603±2.525	19.027±2.113	D
Σn-3 HUFA	27.152±4.236	24.943±2.964	25.346±6.575	18.814±2.689	
DHA/EPA	3.095±0.139	2.316±0.405	**2.819 ^{a} ±0.435**	**1.963 ^{b} ±0.308**	D
ARA/EPA	**0.449 ^{a} ±0.036**	**0.330 ^{b} ±0.057**	0.451±0.023	0.333±0.105	D
ARA/DHA	0.145±0.010	0.143±0.006	0.162±0.019	0.167±0.034	
n3/n6	1.566±0.346	1.338±0.191	0.876±0.283	0.588±0.128	T

Control= 0 g·kg^{-1} MOS; MOS=4 g·kg^{-1} MOS; "T" and "D" indicates significant difference according to Tissue and Diet respectively (P<0.05) for two way ANOVA. Lower case indicates significant differences among fish fed the different diets (P<0.05) for the same intestine section (one way ANOVA).

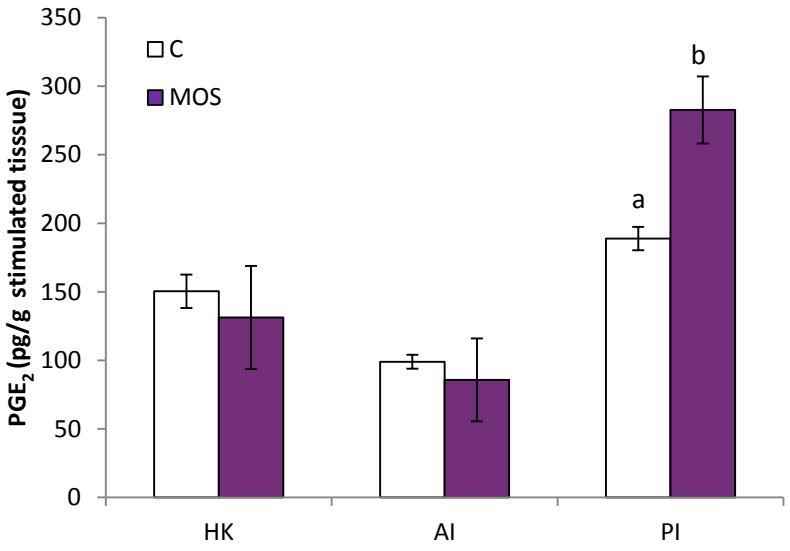


Figure 7.1. PGs levels of stimulated head kidney (HK), anterior intestine (AI) and posterior intestine (PI) of sea bass fed experimental diets for 8 weeks. Different letters indicate significant differences among dietary treatments for a given organ ($P<0.05$). C= 0 g·kg^{-1} MOS; MOS=4 g·kg^{-1} MOS. Values expressed in mean ± SD (n=3 x 4). The specificity of the antibody was 100% for PGE_2.

7.4. DISCUSION

In the present study, despite inclusion of dietary MOS did not affected diet proximate composition, fish fed MOS supplemented diet showed a significant growth improvement according to previous results in the same species (Torrecillas *et al*., 2007) and other fish species (Zhou and Li, 2004; Hanley *et al*., 2005; Staykov *et al*., 2005, 2007; Bogut *et al*., 2006; Culjak *et al*., 2006). The positive effect of MOS supplementation over the functional integrity of the intestine has been demonstrated in terms of increased microvilli density (Dimitroglou *et al*., 2009, 2010) and length (Yilmaz *et al*., 2007; Salze *et al*., 2008; Dimitriglou *et al*., 2009) as well as increased intestinal fold length (Zhou *et al*., 2010; Torrecillas *et al*., 2011b). Improved gut integrity could be directly related to an enhanced gut mucus production as reported in previous studies for European sea bass fed MOS (Torrecillas *et al*., 2011a, 2011b) and in agreement with the 15% increased number of cells secreting mucins detected in the present study. Then, an enhanced mucus production could be the protecting the enterocytes from damaging, either increasing regularity, height or integrity of gut villi, what would lead to a better utilization and absorption of dietary nutrients, but also to a reduced infection rate after direct gut bacterial inoculation via reinforcement of the gut mucosal surface as seen in previous studies (Torrecillas *et al*. 2007, 2011b).

Mucins are the major antiadhesive components of mucus providing the primary antiadhesive defence against colonizing organisms targeting carbohydrates (Sanberg *et al*., 2000), their protective properties relying on the abundance and composition of high molecular weight glycoproteins (HMG) (Enss *et al*., 1995). The viscosity and resistance to bacterial degradation of HMG are related to their contents to sialic acids and sulphate esters, terminally linked to the carbohydrate chains (Corfield *et al*., 1992; Enss *et al*., 1995). Generally, mucins are secreted by goblet cells at a slow baseline rate in order to maintain the mucus coat over the intestinal epithelium but, in response to stimulation, these cells may accelerate their discharge (Plaisancié *et al*., 1998). Unfortunately, mucus secretion regulation in fish has been scarcely studied. In other vertebrates literature, not only some substances as carbachol (Laburthe at al., 1989; McCool *et al*., 1990) or probiotic supplementation (Smirnov *et al*., 2005) have been demonstrated to influence goblet cells activity, but also both: hormones (McCool *et al*., 1990; Ogata and Podolsky, 1997; Plaisancié *et al*., 1997, 1998, 2006; Buyse *et al*., 2001, 2002; Guilmeau *et al*., 2003; Ducroc *et al*., 2005; Hokari *et al*., 2005) and some inflammation regulators (Plaisancié *et al*., 1998; Belley and Chadee, 1999; Akiba *et al*., 2000; Enss *et al*., 2000; Riehl *et al*., 2000; Ohnishi *et al*., 2001; Cho *et al*., 2005; Hisatsune *et al*., 2007; Shao *et al*., 2007; Tetaert *et al*., 2007) have been established as factors influencing this protective barrier. In particular, PGs exert beneficial physiological effects on epithelial cytoprotection including enhancement of gut motility and mucosal barrier functions that comprise mucus secretion and bicarbonate release as well as reduced gastric acid secretion (Dey *et al*., 2009). PGE_2 stimulates HMG release in gastric, duodenal and colonic cells of higher vertebrates (Tani *et al*., 1997; Belley and Chadee, 1999; Akiba *et al*., 2000; Riehl *et al*., 2000; Ohnishi *et al*., 2001; Cho *et al*., 2005; Hisatsune *et al*., 2007; Shao *et al*., 2007; Tetaert *et al*., 2007) via EP_4 receptor (Belley and Chadee, 1999). PGs have been demonstrated not only to increase mucus secretion but also to alter the pattern of terminal mucosal monosaccharides by decreasing oligosaccharide chains with incomplete glycosilation (terminal GlcNAc and GalNAc) and increasing chains with terminal s-linked fucose and sialic acids groups and then directly influencing their protective properties (Enss *et al*., 1995) by increasing viscoelasticity (Tsukise *et al*., 2000; Schroers *et al*., 2009) and inhibiting or retarding the HMG bacterial degradation (Hoskins *et al*., 1985; Hoskins, 1991; Enss *et al*., 1995). These alterations on HMG patters could be directly linked to the lower *in vitro* and *in vivo* gut bacterial translocation found in previous studies (Torrecillas *et al*., 2007, 2011b). Further studies are being conducted in this sense in order

to determine the effects of MOS supplementation on HMG glycosylation patterns and its relation to enhanced disease resistance.

The importance of PGs in fish physiology has been demonstrated with roles in ion transport (Van Praag *et al*., 1987; Beckman and Mustafa, 1992; Oxley *et al*., 2010), vasoactivity (Mustafa and Agnisola, 1994; Stenslokken *et al*., 2002; Oxley *et al*., 2010) and intestinal muscular tone (Shahbazi *et al*., 2001, 2002; Oxley *et al*., 2010). The gastro-protective properties of exogenous PGs were demonstrated in eel (*Anguilla anguilla*), where indomethacin/aspirin-induced mucosal erosion was prevented by stimulation of serosal to mucosal HCO_3^- secretion (Faggio *et al*., 2000; Oxley *et al*., 2010). In the present study, posterior gut isolated cells of fish fed MOS presented a higher capacity of PGs production when stimulated compared to fish fed control diet but no effect was found in anterior gut and head kidney isolated cells. From mammalian literature, EPs have been found along the gastrointestinal tract (Ding *et al*., 1997; Cho *et al*., 2004) and are highly expressed on the mucus secreting cells of the gut (Northey *et al*., 2000). In particular EP_4, which has been related with mucin exocytosis via coupling PGE_2, is highly expressed in the ileum (Bastien *et al*., 1994) equivalent to posterior gut in fish (Guillaume *et al*., 2001). In fact, Holland *et al*. (1999) correlated the occurrence of increased numbers of goblet cells with the eicosanoid generating capacity in rainbow trout (*Oncorhynchus mykiss*) gills. Hence, the higher presence of mucus secreting cells on distal intestine compared to proximal region of fish fed MOS could be one factor influencing the PGs production potential of this intestinal region.

However, other factors, such as the inflammatory status of the epithelium, could be affecting PGs release levels. For example, different basal and ARA-induced eicosanoid release patterns depending on the inflammatory status of the epithelium have been found in human bronchial specimens, where after stimulation, specimens with the inflamed tissue released more TXB_2 and PGE_2 than normal tissue (Yaqoob and Calder, 2007). Thus, the abundance of gut leucocytes infiltrated in the tissue at the time of stimulation could be also influencing released PGs levels, since, MOS supplementation has been found increase the presence of infiltrated eosinophils in the posterior gut (Torrecillas *et al*., 2011b) and the majority of intestinal PGs are produced by lamina propria and submucosa leucocytes, together with the lesser PGs production potential of the enterocytes (Mohajer and Ma, 2000; Oxley *et al*., 2010).

Nevertheless, besides the increased mucus production found in MOS fed fish may be related to a higher PGs production potential by the increased leucocyte and goblet cells present

in the intestinal tract of these fish, MOS could also be inducing the activation of PLA_2 and COX, the primary enzymes initiating PGs production. In one hand, the expression level of the mRNA novel phospholipase detected in red sea bream (*Pagrus major*) goblet cells and responsible for the triggering of PGE_2 production in these cells, the IN PLA_2 is elevated by intravenous injection of lipopolysaccharides (LPS), suggesting that IN PLA_2 is secreted in response to bacterial infection, contributing to antimicrobial defence (Fujikawa *et al*., 2009). If the continuous passing through of the indigestible MOS along the fish gut emulates a bacterial infection and promotes IN PLA_2 synthesis has not been yet determined. On the other hand, COX activity depends on the availability of polyunsaturated fatty acids (PUFA) in the tissue released by PLA_2, the observed differences on the COX product analysed, the PGEs, could reflect different levels of available substrate in the membranes of the posterior gut cells. Although analysis of the total lipid content did not reveal any significant differences in the levels of substrate PUFA, we consider that this observation does not rule out the possibility of substrate availability differences as individual phospholipids were not separated prior to GC analysis. In fact, the lower levels of n-3/n-6 ratio on intestine of fish fed MOS before stimulation, could suggest a higher production of eicosanoids synthesized from C20 PUFA in cellular membranes (Schmitz and Ecker, 2008; Oxley *et al*., 2010) and could be correlated with the higher amount of gut infiltrated leucocytes found in previous studies (Torrecillas *et al*., 2011b). Further studies must to be conducted in order to clarify if MOS supplementation affects the fatty acid composition of individual phospholipids, such as phosphatidylinositol, the main PUFA substrate for PLA_2, favoring the ARA cascade in the posterior intestine of European sea bass.

Besides, based in the dependence of intracellular Ca^{2+} as mediator for PGs induced intestinal secretion (Beubler *et al*., 1986; Bukahve and Rask-Madsen, 2004), the influence of secondary mediators after PGs release on secretion of fluid, Cl^-, Na^+ and $HCO3^-$ and subsequent inhibition of sodium and Cl^- absorption, could be also implied in this higher activity. Indeed, prebiotics supplementation has been related to an enhanced $Ca2^+$ uptake (Cashman, 2003), but the implication of PGs has not been yet considered and this effect is attributed to a reduced intestinal pH caused by increased concentration of SCFA (Cashman, 2006).

Even if the influence in modulation of gut mucus secretion vía PGs release by MOS supplementation could be influenced by the number goblet cells, EP receptors and leucocytes presence in the tissue or intracellular Ca^{2+} availability, other mechanisms could also be implicated in the triggering of mucus production by MOS supplementation, as variations in SCFA

composition caused by microflora profiles changes (Barceló *et al*., 2000), enhanced VIP secretion (Ogata and Podolsky, 1997; Hokari *et al*., 2005) or gut leptin implication through the activation of protein kinase C (PKC) and phosphatydilinositol 3 kinase (PI3K) dependent pathways (Plaisancié *et al*., 2006) and further studies have to be performed in order to clarify the mechanism or mechanisms implicated in the enhanced potential of fish fed MOS in disease resistance via gut inoculation through better status of the gut mucosal surface.

Chapter 8

General discussion

See Annex III for graphical representation of this section.

The use of prebiotics in terrestrial farmed animals has been proved to have benefits on health and performance, in terms of production optimization, stimulation of immune system and changes in microbiota profiles, among others, whereas the use of prebiotics in the fish, and specifically in marine fish, has been less investigated. Among the different commercial prebiotics tested in fish until now, MOS is one of the most studied.

Is MOS efficient as preventive for disease occurrence in fish?

MOS dietary supplementation reduced *in vivo* gut bacterial translocation against *V. alginolyticus* (Torrecillas *et al*., 2007) and *V. anguillarum* both *ex vivo* (Torrecillas *et al*., 2011a) and *in vivo* (Torrecillas *et al*., 2011b), as well as under combined both *in vivo* intestinal infection with *V. anguillarum* and continued stress challenge (Chapter 6) for European sea bass juveniles. Protection against disease occurrence has been also potentiated by the use of MOS (Chapter 6). Similar results have been found for other species supplemented with dietary yeast-based prebiotics and challenged against potential pathogens (Li *et al*., 2004; Rodrígues-Estrada *et al*., 2008; Sado *et al*., 2008; Andrews *et al*., 2009; Buentello *et al*., 2010). As discussed in detail on Chapters 4 and 6, a better status of the enterocytes membrane of fish fed MOS (Dimitroglou *et al*., 2007, 2009, 2010; Yilmaz *et al*., 2007; Salze *et al*., 2008; Zhou *et al*., 2010; Torrecillas *et al*., 2011b) may partially explain this lower disease incidence, since bacterial translocation increases when loss of enterocytes or loosening of their cell junctions occurs (Ringø *et al*., 2007). As debated along the manuscript, a reduction on the adhesive rate of potential pathogens to gut epithelium and their related exotoxines may be also

contributing to the enhanced gut integrity of fish fed MOS. Mannose constitutes an important surface component of cells and MBLs are utilized by many gastrointestinal pathogens as a mean of attachment to the gut epithelium (Mirelman and Ofek, 1986), acting as adhesins by mediating the binding of bacteria to epithelial cells. Therefore, mannose protects fish intestinal mucosal surface of potential pathogens, which will be specifically attracted to attach dietary MOS and flushed out of the lumen with feces. Moreover, other factors, as the enhanced intestinal mucus production of fish fed MOS (Torrecillas *et al*., 2011a, 2011b; Chapter 7), could partially contribute to a better gut integrity of fish fed MOS. Increased mucus production protects enterocytes from damaging, promoting regularity, height and integrity of gut villi, which in turn would improve utilization of dietary nutrients, enhancing growth (Torrecillas *et al*., 2007; Chapter 6; Chapter 7) and optimizing feed conversion ratio (Torrecillas *et al*., 2011a).

Is MOS acting only as a barrier or it also enhances the immune potential of fish?

An increased mucus secretion is an effective defense mechanism of the host, flushing invading bacteria out of the gut lumen (Ellis, 2001; Smirnova *et al*., 2003, Van der Marel *et al*., 2008), therefore reducing the side-effects of bacterial exotoxin-induced disruption of the tight junctions (Jutfelt *et al*., 2008) and reducing translocation rates (Ringø *et al*., 2010) as discussed in detail in Chapters 5 and 6. Indeed, as reported in Chapter 5, MOS dietary inclusion in fish feed was found to increase gut mucus lysozyme and antibacterial activities (Torrecillas *et al*., 2011b). Both parameters, which are essential components in the defense against pathogen infection, could partially avoid the establishment and proliferation of the adhered bacteria, then promoting gut integrity as discussed in Chapter 5. In addition, enhanced cellular and humoral fish innate immune parameters found in fish fed MOS (Torrecillas *et al*., 2007, 2011a, 2011b) may be also contributing to the positive effects of MOS over gut integrity and disease incidence, by reducing both, bacterial translocation and the capacity of translocated bacteria to survive this translocation. An enhancement of these immune parameters may be related to MBL stimulation by liver secretion which activates the lectin pathway of complement through the MBL-associated serine proteases (MASPs) (Nikolakopoulou and Zarkadis, 2006) as discussed in Chapters 3 and 4.

As discussed in detail in Chapter 6, translocated bacteria are usually neutralized by the local immune system in the lamina propria of mammals and, probably, also in fish (Jutfelt *et al*., 2008). European sea bass fed MOS presented higher number of infiltrated ECGs in posterior

gut mucosa, resulting in a lamina propria engrossment (Torrecillas *et al*., 2011b). In this sense, as discussed in detail along Chapters 3 and 6, mannose receptors (MRs) are localized in macrophages and endothelial cell subsets whose natural ligands include self-glycoproteins and microbial glycans (Ringø *et al*., 2010). Receptors similar to mammalian MRs (Rodríguez *et al*., 2003) and a C-type lectin possessing MR features (Zhao *et al*., 2009) have been characterized in fish and shellfish (Ringø *et al*., 2010). In fact, mannose containing ligands may also bind to other receptors such as DC-SIGN and dectin-2 resulting in leucocyte activation (Ringø *et al*., 2010) and inducing intracellular signaling that may increase production of proinflammatory cytokines and mucus production (Rogers, 2001).

In addition, other aspects such as tissue prostaglandins (PGs) production, could be involved on the positive role of MOS over gut mucus production and disease resistance after intestinal infection. Mucins provide the primary anti-adhesive defense against colonizing organisms targeting carbohydrates (Sanberg *et al*., 2000) and their protective properties rely on the amount and composition of high molecular weight glycoproteins (HMG) (Enss *et al*., 1995). The viscosity and resistance to bacterial degradation of HMG are related to their contents in sialic acids and sulphate esters terminally linked to the carbohydrate chains (Corfield *et al*., 1992; Enss *et al*., 1995) contributing to infection susceptibility (Bavington and Page, 2005). In fact, in other vertebrates PGs not only stimulate HMG release, but also alter the pattern of terminal mucosal monosaccharides by increasing viscosity and viscoelasticity of mucus (Tsukise *et al*., 2000; Schroers *et al*., 2009), thus inhibiting or retarding the HMG bacterial degradation. Indeed, MOS supplementation increased posterior gut capacity of producing challenged PGs (Chapter 7). As discussed in detail in Chapter 7, higher levels of PGs could be the result of a higher presence of infiltrated immune cells in gut lamina propria and submucosa after dietary MOS supplementation. Further, a higher presence of mucus producing cells after dietary MOS supplementation could be influencing PGs levels, in terms of number of EPs or regarding the activity of intestinal PLA_2 or COX as discussed in Chapter 7. But also other mechanisms could be implicated in the triggering of mucus production by MOS, as it could be the variations in SCFA composition caused by changes on the microflora profiles (Barceló *et al*., 2000), enhanced VIP secretion (Ogata and Podolsky, 1997; Hokari *et al*., 2005) or gut leptin implication (Plaisancié *et al*., 2006).

Is MOS-induced disease resistance only due to a direct effect on the immune system or to its influence on other mechanisms?

As discussed in Chapter 6, there is a greater effect of stress on gut microbiota in fish fed control diet than in fish fed MOS diet. Although the mechanisms implied are not clear yet, this fact, together with the lesser disease incidence and the lower levels of plasma cortisol (Chapter 6) on infected and stressed plus infected fish, suggests that dietary MOS inclusion minimized gut stress-derived effects in fish fed MOS. Nevertheless, further studies focused on microbial communities' diversity and characterization of SCFA profiles for European sea bass fed MOS must be conducted in order to clarify other possible mechanisms involving enhanced mucus production. Moreover, it would be interesting to study if dietary MOS is increasing nutrient absorption by promoting the growth of certain bacteria capable to affect intestinal pH.

Has the use of MOS a positive effect in fish production?

The capacity of fish for growth can be conditioned by several factors, including assimilation of nutrients rate or digestion and feed conversion efficiency. In fact, prebiotics are dietary supplements that have shown to increase nutrient digestibility and growth in some terrestrial organisms (Burr *et al*., 2008). MOS supplemented in diets for fish held at low stocking densities, improved growth of European sea bass (Torrecillas *et al*., 2007, Chapters 6 and 7) and other fish species (Zhou and Li, 2004; Hanley *et al*., 2005; Staykov *et al*., 2005, 2007; Bogut *et al*., 2006; Culjak *et al*., 2006; Daniels *et al*., 2006, 2007; Genc *et al*., 2007), even though dietary inclusion of MOS did not significantly affected diet proximate composition (Torrecillas *et al*., 2007). Improved growth performance may be associated to the improvement in the functional integrity of the enterocyte membrane in MOS fed animals, as discussed before. Besides, growth improvement could be also related to other factors as could be the enhanced specific activities of alkaline phosphatase and leucine aminopeptidase as it occurs in broiler chickens (Iji *et al*., 2001), in turn promoting protein digestion or absorption and transport of lipids and carbohydrates. Burr *et al.* (2008) reported an increased apparent digestibility coefficient (ADC) for protein, energy and organic matter and reduced lipid ADC after feeding red drum (*Sciaenops ocellatus*) with MOS and galacto-oligosaccharides (GOS). A possible interference of prebiotics with the uptake of dietary lipids by down-regulating other enzymes involved in lipid digestion/absorption was argued by those authors (Burr *et al.*, 2008). Further studies are required to determine if in fish, as in terrestrial organisms, bile acid excretion, digesta

supernatants viscosity or the possible changes in microflora are affecting the intestinal uptake of lipids.

As reported and discussed in Chapter 3, a positive correlation between dietary MOS inclusion level and feed intake was observed after two months of MOS supplementation, in agreement with the results found other fish species (Zhou and Li, 2004; Staykov *et al*., 2005, 2007; Bogut *et al*., 2006; Culjack *et al*., 2006). Nevertheless, when fish were given dietary MOS and stocked at higher densities the opposite effect was observed with no effect on growth performance, resulting in a better feed efficiency (Chapter 4). As discussed in Chapter 4, the effect of MOS on feed intake could be related either with the peripheral satiation system (satiation and appetite signals) or the long-term system (body energy stores) (Jensen, 2001; Terova *et al.*, 2008) that provide information for the hypothalamic central feeding system that controls feed intake. In this sense, an effect of MOS over CCK levels through reduced intestinal fat content could be stimulating gall bladder motility, delaying gastric emptying or even affecting NYY levels, which in turn reduces feed intake (Chapter 4). In fact, other mannans have been found to reduce cholesterol absorption or bile salt excretion. XOS supplementation to diabetic rats reduced serum cholesterol and triglycerides to a comparable level seen in healthy rats (Imaizumi *et al*., 1991) and FOS supplementation reduced blood lipids in healthy rats (Bornet *et al*., 2002; Roberfroid, 2002). Even more, several studies have shown chitosan and MOS to be hypocholesterolemic in animal models (Sugano *et al*., 1978, 1980; LeHoux and Grondin, 1993; Razdan and Pettersson, 1994; Gallaher *et al*., 2000; Young *et al*., 2000), but how this reduction takes place remains unclear, although a higher viscosity of digesta supernatants or disruption of micelle formation by a higher bile acid binding within the small intestine have been the highlighted argues. In fact, secretin stimulates the production of the enzymatic component of bilis, but also CCK or gastrin levels.

But would also MOS influence leptins levels and the insulin-glucagon balance? The secreted level of leptin is proportional to body fat level, and, though its action on hypothalamic centers, leptin suppresses food intake and increases energy expenditure (Frederich *et al*., 1995; Ganga *et al*., 2005). Glucagon and glucagon-like peptides, catecholamines and glucocorticoids favor the glucose liberation from glycogen, while insulin and the insulin-like growth factor stimulate storage (Dabrowski and Guderley, 2002). In this sense, prebiotic supplementation in humans increased GLP-1 and NYY levels (Cani *et al*., 2009). In fact, an inhibition/reduction of lipogenic enzymes in the liver, which could be, among others, a result of the action of propionate

produced from the fermentation of prebiotics by gut bacteria (Wolever *et al*., 1991), may also affect fat deposition in tissues. As shown in Chapters 3 and 4, liver histology revealed a lower lipid vacuolization and, subsequently, a reduction in hepatocytes size in European sea bass fed MOS, which is in agreement with the significant reduction in the G6PD and ME enzymes activity and the tendency to reduced viscerosomatic index and increased eviscerated weight of fish fed MOS (Chapter 4). These findings denote an increase in hepatic glucose production, and a better dietary energy utilization related to the improved growth and feed conversion ratios (Chapters, 3, 4 and 7) as suggested also in gilthead sea bream fed different dietary immunestimulants (Laíz-Carrión *et al*., 2005).

In summary, MOS supplementation (at $4g \cdot kg^{-1}$) effectively prevent disease occurrence in European sea bass, acting as a barrier and reducing gut bacterial translocation either *in vivo*, *ex vivo* or under *in vivo* plus continued stress conditions. Besides, MOS also markedly enhances innate immune parameters, particularly gut mucus production and density of eosinophil granulocytes in gut mucosa. In addition, MOS is modulating other mechanisms that interact with immune system, such as reducing gut stress-derived effects. Finally, dietary MOS has also a positive effect on fish production by promoting growth and feed efficiency. All together suggest that reinforcement of the intestinal barrier efficiency is the front line of defense against pathogenic microorganisms of European sea bass fed MOS.

Chapter 9

Conclusions

1. Dietary MOS supplementation up to 4 g·kg^{-1} to European sea bass juveniles enhanced sea bass growth (Chapter 3, 6 and 7) when reared at low experimental densities (3-4 kg·m^{-3}), in relation to higher feed intake. However, at higher experimental densities (7-10 kg·m^{-3}), MOS supplementation at 4 and 6 g kg^{-1}, did not affected feed intake and resulted in a better feed efficiency (Chapter 4).

2. MOS inclusion in European sea bass diets resulted in lower lipid vacuolization and a reduction in hepatocytes size, which is in agreement with the significant reduction in the G6PD and ME enzymes activities and the tendency to reduced viscerosomatic index and increased eviscerated weight (Chapter 4).

3. Sensorial parameters and biochemical composition of flesh were not affected by dietary MOS when supplemented along the whole production cycle, regardless the level of inclusion.

4. Dietary incorporation of MOS at 4 and 6 g kg^{-1} activates non-self cellular and humoral immune parameters in terms of phagocytic activity of head kidney leucocytes (Chapter 3 and 4) and enhanced the potential of posterior gut to produce challenged PGs. MOS dietary supplementation produced a positive dose dependent effect over European sea bass serum lysozyme and alternative complement pathway activities when supplemented at 4 g kg^{-1} (Chapter 3).

5. Dietary incorporation of MOS up to 4 g·kg^{-1} reduced gut bacterial translocation against *V. alginolyticus* (Chapter 3) and *V. anguillarum* both *ex vivo* (Chapter 5) and *in vivo* (Chapter 5) as well as under combined both *in vivo* intestinal infection with *V. anguillarum* and continued stress challenge (Chapter 6).

6. Two months of dietary MOS supplementation at 4 g kg^{-1} enhanced several mechanisms involved on gut mucosal barrier protection in terms of increased intraepithelial gut ECGs presence, mucus production and lysozyme activity and reduced the effects of stress by confinement on gut microbial communities profiles, suggesting the reinforcement of the mucosal surface of the gastrointestinal tract as the front line of defense against pathogenic microorganisms (Chapter 5) of fish fed MOS.

Chapter 10

Resumen

10.1. INTRODUCCIÓN

10.1.1. PRODUCCIÓN ACUÍCOLA DE LUBINA (*Dicentrarchus labrax*)

Actualmente la producción acuícola mundial suministra el 50% del consumo humano de pescado, lo que revela no solo el desarrollo económico global del sector sino también los continuos avances realizados en el marketing y el procesado del producto final (APROMAR, 2010). En 2008, la producción total acuícola para consumo humano alcanzó las 68,4 MTm frente a las 67 MTm provenientes de la pesca extractiva, y se espera que alcance las 100 MTm en 2030. Es por esto que la acuicultura, como sector productivo y a diferencia de la pesca de captura, posee un ritmo de crecimiento anual muy elevado. A modo de ejemplo, cabe destacar que la producción del sector aumentó de 0,6 MTm en los años 50 a 68,4 MTm en 2008, alcanzando un valor de mercado de 847791 millones de euros. Este aumento de producción a su vez se ha visto reflejado en un aumento del consumo humano de pescado, desde 11kg/persona que se consumían en 1970 hasta un consumo actual superior a los 20kg/persona. En 2008, la producción total acuícola europea alcanzó las 626650 Tm, lo que representa aproximadamente un valor de mercado de 2562 millones de euros, del que la producción acuícola española representó el 10,2%, alcanzando un valor comercial de 279 millones euros.

En relación a la producción de especies marinas en las regiones del sur de Europa y Mediterránea, la dorada (*Sparus aurata*), la lubina (*Dicentrarchus labrax*) y el rodaballo (*Psetta maxima*) son las especies más cultivadas. En concreto, la producción mundial de lubina alcanzó las 66738 Tm en 2009 representando un crecimiento del 6,9%

respecto al ejercicio anterior, y las previsiones apuntan a un mayor crecimiento en los próximos años (APROMAR, 2010).

Los principales países productores de lubina se encuentran situados en la región mediterránea e incluyen Grecia, Turquía, España e Italia. En España, la producción de lubina durante el 2009 alcanzó las 13840 toneladas, suponiendo un aumento del 40,7% respecto al 2008 y representando el 96% de total de lubina destinada a consumo humano. En este sentido el archipiélago canario, aprovechando las privilegiadas condiciones físico-químicas de las aguas que lo rodean y basándose en la búsqueda de soluciones y en la puesta a punto de la tecnología adecuada, es el mayor productor de lubina a nivel estatal, alcanzando el 32% de la producción total nacional. El resto de la producción se encuentra repartida entre Murcia, Andalucía, València y Catalunya (APROMAR, 2010) (Figura 10.1).

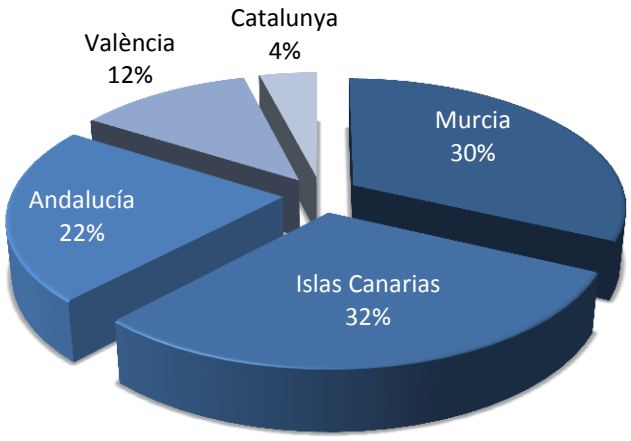


Figura 10.1. Producción porcentual de lubina en España divida por regiones (APROMAR, 2010)

Sorprendentemente, y a pesar de la importancia del cultivo de lubina en la acuicultura mediterránea, la lubina es una especie particularmente susceptible a problemas derivados de las propias condiciones de cultivo y a la incidencia de patologías. Esta sensibilidad da lugar a la aparición de dos principales limitaciones en su cultivo:

1. **Sensibilidad al manejo y a la aparición de brotes patológicos.** Partiendo que las principales pérdidas económicas en el sector acuícola son ocasionadas por la aparición de brotes patológicos, mantener la salud de los peces es determinante para conseguir un óptimo éxito productivo. La patología de origen bacteriano constituye una limitación en cultivo de la lubina ya que es una especie extremadamente sensible a situaciones de estrés, y en condiciones de cultivo intensivo el equilibrio existente entre huésped-patógeno-medio puede verse alterado fácilmente, favoreciendo la aparición de patologías que causan mortalidades masivas y reducen las tasas de crecimiento. En este sentido, la potenciación de sistema inmune innato en esta especie a través del uso de ingredientes alternativos al uso de antibióticos puede prevenir en gran medida la aparición de brotes patológicos y con ello a su vez minimizar el uso de sustancias terapéuticas.

2. **Limitación en la eficiencia de utilización de nutrientes**. La optimización en el engorde juega un papel fundamental en la producción acuícola y se están realizando innumerables esfuerzos por lograr un índice de conversión lo más reducido posible, y de esta manera abaratar los costes de producción que actualmente suponen de un 40-60% de los costes totales. En el caso de la lubina, como en la mayoría de casos, no existe una dieta especial y únicamente formulada para esta especie, compartiendo la formulación con la de la dorada, pese a que son dos especies con hábitos alimenticios diferentes y posiblemente con también diferentes requerimientos nutricionales (Izquierdo, 2005). Este hecho junto con la extrema sensibilidad de esta especie a los factores externos estresantes, conlleva la obtención de tasas de conversión alimenticias no tan óptimas como las obtenidas para la dorada. Está ampliamente demostrado que las situaciones de estrés alteran el metabolismo de los peces, ya que la instauración de una situación estresante se traduce en una demanda transitoria de energía con movilización de ciertas rutas metabólicas como la gluconeogenesis. Así, especies tan susceptibles al manejo como la lubina dedican, particularmente, parte de la energía suministrada a través del pienso para luchar contra esta situación reduciendo su crecimiento e incrementando los costes de producción.

10.1.2. IMPORTANCIA DE LAS ENFERMEDADES DE ORIGEN BACTERIANO EN EL MEDITERRÁNEO

El rápido desarrollo de la acuicultura como sector y su intensificación, al igual que la mayoría de explotaciones intensivas, ha traído como consecuencia un aumento progresivo de los problemas ligados a la producción controlada. Mantener la salud de los peces de forma satisfactoria y prevenir y/o controlar la aparición de brotes patológicos, no depende de un solo proceso, sino que es el resultado de una serie de conceptos integrados y de una gestión basada en mantener el profundo e inverso equilibrio existente entre la calidad del entorno y el estado sanitario del pescado (Plumb, 1999a). Las altas densidades de producción junto con las deficiencias y/o desequilibrios en la nutrición y el manejo inadecuado, conllevan situaciones de estrés físico y químico, que suelen traducirse en una mayor sensibilidad de las especies cultivadas frente a los agentes patógenos presentes en el medio. Evitar estas situaciones estresantes mediante una gestión adecuada es esencial para mantener una población de peces saludable y libre de enfermedades.

Se estima que los costes directos e indirectos derivados de la prevención y control de las enfermedades infecciosas en acuicultura supera el 10% de los costes totales de producción (Brown y Johnson, 2008). En algunos cultivos se ha informado de casos de mortalidades asociadas, por ejemplo, al cultivo de pez gato (*Ictalarus punctatus*) de un 75% de mortalidad en alevines previo a su comercialización (Plumb, 1999b). Por tanto, los brotes patológicos suponen al sector acuícola perdidas del orden de millones de euros en términos de mortalidad, reducción de la producción, vacunas y profilaxis.

Toranzo *et al.* (2005) revisó las principales patologías bacterianas que afectan a la maricultura, entre las que se encuentran: Pasteurelosis, Furunculosis, Vibriosis, Flexibacteriosis marina, Pseudomniasis, Estreptococcosis y Micobacteriosis.

La Pasteurelosis está causada por la bacteria *Photobacterium damselae subsp. piscicida* (anteriormente *Pasteurella piscicida*) y ha causado pérdidas económicas en el sector de la maricultura mediterránea en las últimas décadas, especialmente en dorada, lubina y lenguado senegalés (*Solea senegalensis*) (Romalde *et al.*, 1999; Magariños *et al.*, 2001, 2003; Toranzo *et al.*, 2005). Esta enfermedad causa altas mortalidades en juveniles con cuadros septicémicos agudos, que en el caso de sobrevivir, pueden transformarse en una lesión típica de

esplenomegalia con granulomas blanquecinos en su interior, también denominada "pseudotuberculosis" (Quaglio *et al.*, 1991).

La Furunculosis está causada por *Aeromonas spp*., conlleva el desarrollo de procesos septicémicos agudos y/o crónicos y se caracteriza por presentar, en algunos casos, lesiones necróticas en piel y musculatura. Aunque las infecciones causadas por estas bacterias producen pérdidas muy importantes en el cultivo de salmónidos, puede aparecer en condiciones de salinidades y/o temperaturas elevadas en las granjas marinas. Todo y que, *Aeromonas salmonicida subsp. Salmonicida* es el principal agente causante de esta enfermedad, otras subespecies como *A. masoucide*, *A. achromogenes* y *A. smithia* pueden causar cuadros ulcerativos en la carpa dorada (*Carassius auratus*), otras carpas (*Cyprinus spp*.), anguila (*Anguilla sp.*), salmónidos y varias especies de peces planos marinos, principalmente en Europa y Japón. Fue en las Islas Canarias dónde Real *et al.* (1994), describieron la primera infección por *Aeromonas salmonicida subsp. salmonicida* en dorada en España.

La Vibriosis está causada por la bacteria del género *Vibrionaceae* y hay varias especies que se consideran patógenas tales como: *V. alginolyticus, V. parahaemolyticus, V. cholera* (no-O1), *V. vulnificus* (Biotipo 2), *V. anguillarum, V. ordalii, V. damsela, V. carchariae* y *V. salmonicida*. La clínica y lesiones causadas por este tipo de bacterias origina un cuadro de septicemia hemorrágica ulcerativa a temperaturas altas, mientras que cuando las temperaturas son más frías la sintomatología se reduce a inactividad, letargia, melanosis y presencia de eritemas en vientre y aletas. En la siguiente sección se amplía la información respecto a esta patología de origen bacteriano dado que ha sido la patología a estudio en esta tesis.

La Pseudomniasis en peces cultivados es causada principalmente por *Pseudomonas angulliseptica* (Austin y Austin, 1993; Toranzo y Barja, 1993) en los meses de invierno cuando las temperaturas del agua son más bajas. La principal especie afectada por esta patología es la anguila, aunque también se ha asociado al síndrome de invierno de la dorada (Tort *et al.*, 1998), dónde la temperatura, el estrés asociado al cultivo intensivo, la inmunosupresión, el desequilibrio energético y las deficiencias nutricionales causadas por el ayuno prolongado juegan un rol decisivo (Padrós *et al.*, 1996; Zarza y Fonlut, 2002). En España, *P. Anguilliseptica* se considera como un patógeno emergente en el cultivo de rodaballo y besugo (*Pagellus bogaraveo*) (López-Romalde *et al.*, 2003). La sintomatología causada por esta bacteria se

caracteriza por la aparición de petequias hemorrágicas en piel y órganos internos así como distensión abdominal.

La Flexibacteriosis marina es causada principalmente por *Tenacibaculum maritimum* (anteriormente, *Cytophaga marina*, *Flexibacter marinus* y *F. maritimus*) (Wakabayashi *et al.*, 1986; Bernardet y Grimont, 1989; Sukui *et al.*, 2001; Toranzo *et al.*, 2005) y afecta a un amplio rango de especies cultivadas, tales como, rodaballo, lenguado, dorada, lubina, dorada japonesa (*Pagrus major*), chopa (*Acanthopagrus schlegeli*), platija y salmónidos (Toranzo *et al.*, 2005). El cuadro clínico se presenta con hemorragias en la boca, úlceras en la piel y aletas y cola putrefactas. A su vez, este patógeno ataca las branquias y la piel produciendo focos necróticos en estos tejidos que facilitan la entrada de patógenos secundarios oportunistas.

La estreptococcosis se considera una patología reemergente que afecta a un gran número de especies cultivadas (Kitao, 1993; Bercovier *et al.*, 1997; Romalde y Toranzo, 1999; Toranzo *et al.*, 2005). En la maricultura, es generalmente producida por *Lactococcus garvieae*, *Streptococcus iniae*, *S. agalactiae y S. parauberis* y se caracteriza por la aparición de septicemias generales, exoftalmia y aparición de lesiones en la piel y en las aletas.

La Micobacteriosis es causada por el género *Mycobacterium* spp., siendo *Mycobacterium marinum* el principal agente causante. En las últimas décadas ha causado pérdidas importantes en cultivo de la lubina en la cuenca mediterránea (Colorni, 1992; Colorni *et al.*, 1993, 1996; Toranzo *et al.*, 2005) y se considera un problema serio en el cultivo de rodaballo (dos Santos *et al.*, 2002). La sintomatología interna depende de la especie pero generalmente se caracteriza por presencia de granulomas en bazo, riñón e hígado. Externamente se puede apreciar descamación y lesiones hemorrágicas que pueden llegar a la musculatura en casos muy graves (Toranzo *et al.*, 2005).

10.1.2.1. LA VIBRIOSIS

La vibriosis es una de las patologías de origen bacteriano con mayor prevalencia en el sector acuícola marino. Las especies de *Vibrio* son las bacterias heterotróficas predominantes en el medio marino y están ampliamente distribuidas a las aguas marinas y/o salobres. También las

podemos encontrar en la superficie o tracto intestinal de los animales marinos y otros organismos (Jun y Woo, 2003) así como en las superficies mucosas y órganos internos de peces en perfecto estado sanitario. Colwell y Grimes (1984) consideraron *V. alginolyticus, V. parahaemolyticus, V. cholera* (no-O1), *V. vulnificus* (Biotipo 2), *V. anguillarum, V. ordalii, V. damsela, V. carchariae* y *V*. *salmonicida* como patógenos de peces. Desde entonces, otras especies se han considerado como los agentes causantes de importantes pérdidas en la maricultura como pueden ser: *V*. *harveyi, V. marinus, V. furnissii, V. rnimicus, V. pelagius, V. splendidus* y *V. tapetis,* (Austin y Austin, 1993; Angulo *et al.,* 1994; Esteve *et al.,* 1995; Saeed, 1995; Wu y Pan, 1997, 2000; Álvarez *et al.,* 1998a; Benediktsdottir *et al.,* 1998; Diggles *et al,* 2000; Jensen *et al.,* 2003; Jun y Woo, 2003; Villamil *et al.,* 2003a). En la Tabla 10.1 se presenta un listado detallado de las especies causantes de esta patología y las especies de peces susceptibles a ellas (adapatado de Jun y Woo, 2003).

Aunque todas las especies del género *Vibrio* descritas en la Tabla 10.1 han sido descritas como causantes de brotes de vibriosis en especies cultivadas, se considera a *V. anguillarum* como el principal agente causante de brotes patológicos de vibriosis y ha sido especialmente devastador en la maricultura de salmónidos y Perciformes. Desde los años 70, estos brotes han afectado a más de 50 especies de agua dulce y marina, incluyendo anguila europea y japonesa (*Anguilla anguilla* y *A. japonica*), ayu (*P. altivelis*), lubina, dorada, lubina estriada (*Morone saxatilis*), bacalao (*Gadus morhua*), fletán (*Hippoglossus hippoglossus*), salmón plateado (*Oncorhynchus spp.*), salmón atlántico (*Salmo salar*) y trucha arcoíris (*Oncorhynchus mykiss*) (Anderson y Conroy, 1970; Strout *et al.*, 1978; Tolmasky *et al.*, 1985; 1988; Toranzo *y* Barja, 1990, 1993; Actis *et al.*, 1999; Toranzo *et al.*, 2005).

Entre las cepas aisladas de *V. anguillarum* se han encontrado un total de 23 O serotipos (O1–O23) (Sørensen y Larsen, 1986; Pedersen *et al.*, 1999; Toranzo *et al.*, 2005) pero únicamente el serotipo O1 (homogénea antigénica), O2 (heterogenia antigénica O2a y O2b) y, en menor medida, el serotipo O3 (principalmente en anguila y ayu; heterogénea antigénica O3a y O3b) han sido asociados a mortalidades de peces cultivados a nivel mundial (Tajima *et al.*, 1985; Toranzo y Barja, 1990, 1993; Larsen *et al.*, 1994; Santos *et al.*, 1995; Toranzo *et al.*, 1997; 2005). Los serotipos restantes están considerados como cepas existentes en el medio y raramente son aislados en casos clínicos de vibriosis en peces. Los estudios serológicos y genéticos respecto al posible origen de las infecciones causadas por *V. anguillarum* tienen un alto valor

epidemiológico, además de ayudar en la determinación del correcto protocolo de vacunación en una zona geográfica en particular (Toranzo *et al.*, 2005). Es por ello que algunos autores recomiendan la identificación del serotipo cuando ocurre un brote de *V. anguillarum* para en un futuro poder utilizar estos datos con fines epidemiológicos (Romalde *et al.,* 1995). Es más, la correlación entre serotipo y patogenicidad puede reflejar la capacidad de interacción entre los antígenos de la superficie bacteriana y los tejidos del huésped, ya que estudios sobre los componentes de la pared celular bacteriana (proteínas de la membrana celular y lipopolisacáridos) han demostrado su relación con los serotipos de los patógenos (Aoki *et al.*, 1981; Nomura y Aoki, 1985; Actis *et al.*, 1999).

Tabla 10.1. Vibrios y especies de peces susceptibles a ellos.

Vibrio species	Susceptible fish	References
V. alginolyticus	Mero malabárico, *Epinephelus malabaricus* Dorada, *Sparus aurata* Rodaballo (juvenil), *Psetta maxima* Sargo dorado, *Sparus sarba*	Lee, 1995 Balebona *et al.,* 1998 Austin *et al.,* 1993 Li, 2002
V. alginolyticus *V. parahaernolyticus* *V. vulnificus*	Sargo dorado, *Sparus sarba*	Li *et al.,* 1999 Woo *et al.,* 1995
V. alginolyticus *V. parahaernolyticus*	Mero lutria, *Epinephelus salmoides*	Ong, 1988
V. alginolyticus *V. parahaemolyticus* *V. anguillarum*	Dorada, *Sparus aurata*	Colorni *et al.,* 1981
V. anguillarum	Trucha arcoiris, *Oncorhynchus mykiss* Salmón atlantico, *Salmo salar* Rodaballo, *Psetta maxima* Salmón plateado, *Oncorhynchus kisutch* Trucha, *Salmo gairdneri* Sargo sobaito, *Acanthopagrus cuvieri* Rodaballo, *Psetta maxima* Salmón plateado, *Oncorhynchus kisutch* Trucha arcoiris, *Oncorhynchus mykiss* Trucha, *Salmo gairdneri* Samón atlántico, *Salmo salar* Perca gigante, *Lateolabrax japonicus*	Tiainen *et al.,* 1994 Toranzo *et al.,* 1987 Rasheed, 1989 Santos *et al.,* 1991 Lamas *et al.,* 1994 Svendsen *y* Bogwald, 1997 Xiao *et al.,* 1999
V. carchariae	Falso halibut de Canadá, *Paralichthys dentatus* Tiburón trozo, *Carcharhinus plumbeus* Mero de pintas naranjas, *Epinephelus coioides*	Soffientino *et al.,* 1999 Bertone *et al.,* 1996 Lee *et al.,* 2002

Tabla 10.1. Vibrios y especies de peces susceptibles a ellos (Cont.).

Vibrio species	Susceptible fish	References
V.. carchariae *V. damsela*	Tiburón trozo, *Carcharhinus plumbeus* Galludo, *Squalus acanthias* Tiburón galano, *Negaprion brevirostris*	Grimes *et al.,* 1984, 1985
V. damsela	Rodaballo, *Psetta maxima* Castañeta herrera, *Chromis punctipinnis* Dorada, *Sparus aurata* Medregal del Japón, *Seriola quinqueradiata* Lenguado senegalés, *Solea senegalensis*	Fouz *et al.,* 1992 Love *et al.,* 1981 Vera *et al.,* 1991 Sakata *et al.,* 1989 Zorrilla *et al.,* 2003
V. furnissii	Anguila , *Anguilla anguilla*	Esteve *et al.,* 1995
V. harveyi	Lisa, *Mugil curema* Paguara, *Chaetodipterus faber* Sargo sobaito, *Acanthopagrus cuvieri* Mero lutria, *Epinephelus tauvina* Trucha, *Salmo gairdneri* Salmón atlántico, *Salmo salar*	Alvarez *et al.,* 1998 Saeed, 1995 Zhang *y* Austin, 2000 Wu *y* Pan, 1997
V. marinus	Seriola, *Seriola dumerili* Lenguadosengalés, *Solea senegalensis* Salmón atlántico, *Salmo salar*	Zorrilla *et al.,* 2003 Benediktsdottir *et al.,* 1998
V. mimicus	Pargo japonés, *Pagrus major*	Wu *y* Pan, 2000
V. parahaemolyticus	Fartet, *Aphanius iberus*	Alcaide *et al.,* 1999
V. pelagius	Rodaballo, *Psetta maxima*	Villamil *et al.,* 2003a, b
V. salmonicida	Salmón atlántico, *Salmo salar*	Totl*y y* Nylund, 1988
V. splendidus	Rodaballo, *Psetta maxima* Trucha arcoiris, *Oncorhynchus mykiss*	Angulo *et al.,* 1994
V. splendidus *V. campbellii-like*	Rodaballo, *Colistium nudipinnis* Brill, *Colistium guntheri*	Diggles *et al.,* 2000
V. splendidus *V. tapetis*	Porredana, *Symphodus melops*	Jensen *et al.,* 2003
V. viscosus	Salmón Atlántico, *Salmo salar*	Salte *et al.,* 1994
V. anguillarum *V. ordalii*	Salmón real, *Oncorhynchus tshawytscha*	Chart *y* Trust, 1984; Ransom *et al.,* 1984
V. vulnificus	Mero, *Epinephelus sp.* Mero amarillo, *Epinephelus awoara* Anguilla, *Anguilla anguilla*	Qin y Pan, 1996 Tison *et al.,* 1982 Dalsgaard *et al.,* 1999 Biosca *et al.,* 1991 Amaro *et al.,* 1995 Collado *et al.,* 2000 Fouz *et al.,* 2000

V. alginolyticus es otro de los patógenos mayoritarios relacionados con el cultivo de peces dentro del género *Vibrio*. Algunos autores consideran que las propiedades virulentas de este patógenos no pueden establecerse claramente ya que *V. alginolyticus* está asociado a condiciones de cultivo sub-óptimas o peces dañados (Jun y Woo, 2003). En cualquier caso, *V. alginolyticus* ha causado casos de vibriosis con mortalidades severas asociadas a distintas especies alrededor del mundo (Colorni *et al.,* 1981; Austin *et al.,* 1993; Lee, 1995; Saeed, 1995; Woo, 1995; Álvarez *et al.,* 1998a; Balebona *et al.,* 1998; Zhu *et al.,* 2000) siendo aislado en el sur de Europa, asociado a brotes de vibriosis (Balebona, 1994).

Ambos patógenos, *V. anguillarum* y *V. alginolyticus* pueden ser diagnosticados mediante métodos bioquímicos estandarizados. En el caso de *V. anguillarum*, existe un medio especializado de aislamiento (VAM; Alsina *et al.*, 1994) además de kits comerciales basados en análisis ELISA o de aglutinación. Además Osorio y Toranzo (2002) describieron, basándose en una técnica PCR y teniendo como diana el gen rpoN, el protocolo de detección precisa de esta bacteria en órganos internos de peces infectados.

Los síntomas clínicos de la vibriosis son los típicos de una septicemia hemorrágica. Los peces infectados por esta bacteria suelen presentar hemorragias alrededor de la cabeza, abdomen y cloaca, así como en la base de las aletas. Asociados a este cuadro de septicemia hemorrágica suelen aparecer exoftalmia bilateral, opacidad de la córnea, reducción en el número de leucocitos (Ransom *et al.*, 1984) y distensión abdominal. La sintomatología interna incluye petequias en las vísceras, esplenomegalia y necrosis licuefactiva renal (Noga, 2000). Además se suele observar distensión intestinal con el intestino lleno de líquido transparente (Cisar *y* Fryer, 1969), acentuándose estos síntomas en el intestino posterior y recto debido al gradiente de pH existente que limita el crecimiento de *V. anguillarum* en medio ácido (Ramson *et al.*, 1984). Con frecuencia los peces afectados por vibriosis presentan anorexia y anemia, además de secreciones excesivas de mucus en branquias aunque con ausencia de necrosis (Toranzo *et al.*, 2005). Los alevines infectados por vibriosis pueden no presentar los síntomas clínicos descritos anteriormente, pero se puede observar claramente un estado de letargia generalizado (se observan gran número de alevines nadando lentamente cerca de la superficie posiblemente como consecuencia de una distensión de la vejiga natatoria derivada de la infección) además de coloración oscura. En casos agudos, el curso de la infección es rápido, y la mayoría de los peces infectados mueren sin presentar síntomas clínicos (Actis *et al.*, 1999).

El sector acuícola ha dedicado mucho tiempo y esfuerzo para minimizar los efectos de la vibriosis sobre la producción. El uso de vacunas, otras bacterias que inhiban el crecimiento de *V. anguillarum* o antibióticos están altamente recomendados y han sido las prácticas más comunes para evitar los brotes patológicos causados por este agente. De entre ellos, a nivel de prevención se recomienda la vacunación por inmersión y/o inyección para controlar los brotes de vibriosis (Qin y Pan, 1996; Woo *et al.,* 2001; Li, 2002) y existiendo diferentes tipos de vacunas comerciales actualmente en el mercado (Newman, 1993; Toranzo *et al.*, 1997; 2005). Todo y que, únicamente una de ellas, GAVA-3, desarrollada en la Universidad de Santiago (España) cubre las 3 entidades antigénicas en maricultura para *V. anguillarum* (O1, O2a y O2b) (Toranzo *et al.*, 1997; 2005). En el caso concreto de la lubina, además se suelen aplicar dos tratamientos por baño de inmersión en intervalos mensuales de bacterinas de *V. anguillarum* para peces de 1-2 g (Toranzo *et al.*, 2005). Desde el punto de vista del productor, ya que la vacunación está altamente recomendada, es muy importante seleccionar el protocolo correcto de vacunación en función de los requerimientos del emplazamiento de la instalación además de tener en cuenta los costes adicionales en términos de mano de obra (coste y tiempo) y situaciones de estrés derivadas del proceso de manipulación. En particular los derivados de la vacunación por inyección intraperitoneal.

Una vez que la patología aparece, la profilaxis más utilizada en las granjas de cultivo suele ser el uso de antibióticos y otros agentes quimioterapéuticos como pueden ser cloranfenicol, nitrofurazonas, ácido oxolínico, oxitetraciclinas y sulfamerazinas, bien impregnados en el pienso o administrados mediante baños de inmersión (Actis *et al.*, 1999; Jun y Woo, 2003). La suplementación con antibióticos impregnados en el pienso durante 10 días normalmente es suficiente para eliminar mortalidades (oxitetraciclina a una concentración de 100 $mg \cdot kg^{-1}$ biomasa por día, flumenquina a una concentración de 80 $mg \cdot kg^{-1}$ biomasa o ácido oxolinico a 60 $mg \cdot kg^{-1}$ biomasa) además de las sulfamidas potenciadas a 70 $mg \cdot kg^{-1}$ biomasa por día. No obstante, el uso excesivo de antibióticos y otros agentes quimioterapéuticos ha derivado en la aparición de cepas bacterianas resistentes, especialmente de *V. anguillarum* y *V. salmonicida* (Hjeltnes y Roberts, 1993) y hoy en día algunos de estos han perdido su efectividad frente al control de la vibriosis (Li, 2002; Jun *y* Woo, 2003).

En el cultivo de la lubina en concreto, las implicaciones económicas derivadas de la vibriosis están consideradas como severas y suele aparecer una o dos veces al año en las granjas

de cultivo. Estos brotes pueden aparecer en cualquier época del año, pero son más frecuentes después de episodios de manejo, condiciones ambientales adversas o temperaturas del agua inestables. Hay algunos factores de predisposición que hay que tener en cuenta para luchar contra esta patología, tales como: sobrealimentación, altas densidades de cultivo, estado de limpieza de las redes de las jaulas, la aparición reciente de la patología, la inestabilidad de la temperatura del agua y en particular cualquier tipo de estrés medioambiental. La reducción de cualquier tipo de estrés es, por lo tanto, imperativa para una gestión adecuada a largo plazo. En general, todas las tallas cultivadas de lubina son susceptibles a la aparición de esta patología, aunque los alevines y juveniles suelen tener mayor susceptibilidad. Las consecuencias de un brote de vibriosis a nivel de pérdidas de producción son elevadas considerando que las mortalidades suelen oscilar entre el 15-25% (35% en alevines) además de los efectos negativos sobre el crecimiento que conlleva debido a la falta de apetito asociada a la patología. Los costes extra asociados al tiempo dedicado a la retirada de los peces muertos, transporte, la correcta disposición sanitaria de las bajas y el tiempo dedicado a la preparación de la medicación tienen que ser también considerados en términos de gastos económicos. Todos estos factores junto con la aparición de cepas bacterianas resistentes a determinados antibióticos, comprometen la optimización de la producción de pescado y es por ello que actualmente existe una tendencia económica y social enfocada a la minimización del uso de antibióticos y otros quimioterapéuticos usados en acuicultura y potenciando la utilización de medidas más respetuosas con el medioambiente (Hansen y Olafsen, 1999; Verschuere *et al.*, 2000; IUCN, 2007). Por ejemplo, existente un gran interés en el uso de diferentes productos y organismos basados en el control de patógenos potenciales mediante exclusión competitiva (probióticos y prebióticos). Estos productos han sido probados en otras áreas de producción animal intensiva con resultados prometedores e incluyen los mananoligosacáridos (MOS) que es la sustancia sometida a estudio en esta tesis.

10.1.3. USO DE PRODUCTOS ALTERNATIVOS A LOS ANTIBIÓTICOS EN ACUICULTURA

Todo y que la acuicultura representa el sector de producción animal con mayor crecimiento en las últimas décadas, como ocurre en otros sectores, se encuentra con factores

que continúan limtando su desarrollo, productividad y rentabilidad, y entre ellos se encuentra la aparición de patologías. Desafortunadamente el uso prolongado de antibióticos como tratamiento frente a los brotes patológicos así como promotores del crecimiento ha desencadenado la aparición de cepas bacterianas resistentes a estos, crecimiento de bacterias y hongos no deseados, y por supuesto, sigue constituyendo una amenaza para el medioambiente y la seguridad del consumidor (Carrington y Secombes, 2006). La suma de todos estos factores ha derivado en la legislación del uso de antibióticos en todo el mundo. En Europa concretamente se encuentra regulado por Reglamento del Consejo (EC) 1353/2007 del 20 de noviembre de 2007 en el cual se establece la prohibición del uso de cloranfenicol y nitrofuranos como medidas de tratamiento en la acuicultura europea. Los límites máximos de residuos (LMRs) para los antibióticos permitidos en la Unión Europea en acuicultura se indican en la Tabla 10.2.

La creciente preocupación económica y social sobre el uso de antibióticos y otros agentes terapéuticos en acuicultura ha potenciado la puesta en práctica de métodos más respetuosos con el medioambiente para controlar los brotes patológicos (Hansen y Olafsen 1999; Verschuere *et al.*, 2000) mediante prácticas de manejo y medidas sanitarias de prevención adecuadas como son el uso de vacunas y la potenciación del sistema inmune (IUCN, 2007). Comúnmente, una sustancia que potencia el sistema inmune recibe el nombre de immunoestimulante. En los últimos años se han hecho muchos esfuerzos para determinar la dosis óptima de suplementación de estos compuestos y llegar a un estado de inmunomodulación, en el cual se evite la sobre estimulación por lo tanto la subsecuente inmunosupresión.

Un inmunoestimulante/inmunomodulador puede ser definido como una sustancia química, droga, estresante o acción que tiene un efecto modulador sobre la respuesta inmune innata mediante la interacción directa con las células del sistema inmune. En la práctica, son suplementos dietéticos que potencialmente ayudan a controlar los brotes patológicos mediante la estimulación general del sistema inmune innato, e incluso mejoran los parámetros relacionados con el estrés. Esta modulación positiva suele derivar en un mejor estado de bienestar animal que en general se traduce en una optimización de la producción.

Tabla 10.2. Antibióticos autorizados en la Acuicultura Europea. Se indica la sustancia, el límite máximo de residuo (LMRs; µg/kg), la especie frente a la que está legislado y el Reglamento del Consejo que lo regula. Adaptado de la Regulación del Consejo (EEC) 2377/90 de Junio de 1990 revisado por la Comisión Reguladora 1353/2007 el 20 de Noviembre de 2007.

Antibiótico	LMRs	Especie cultivada	Reglamento
Sulfamidas	100	Todas las especies para consumo humano	508/1999/EC
Trimetoprima	50	Peces	
Amoxicilina	50	Todas las especies para consumo humano	
Ampicilina	50	Todas las especies para consumo humano	
Benzilpenicilina	50	Todas las especies para consumo humano	
Cloxacilina	300	Todas las especies para consumo humano	
Dicloxacilina	300	Todas las especies para consumo humano	
Oxacilina	300	Todas las especies para consumo humano	
Sarafloxacina	30	Salmónidos	
Clorotetraciclina	100	Todas las especies para consumo humano	
Oxitetraciclina	100	Todas las especies para consumo humano	
Tetraciclina	100	Todas las especies para consumo humano	
Flumenquina	600	Salmónidos	2728/1999/EC
Ácido oxolínico	300	Peces	807/2001/EC
Florfenicol	1000	Peces	1322/2001/EC
Eritromicina	200	Todas las especies para consumo humano	1181/2002/EC

Los inmunoestimulantes/inmunomoduladores envuelven un amplio rango de productos bioactivos, que incluyen desde químicos sintéticos como levamisole a sustancias biológicas tales como: (1) Prebióticos como derivados de bacterias o polisacáridos como quitina, chitosan, lentinan y oligosacáridos; (2) extractos animales y vegetales como por ejemplo extractos de Haliotis o tunicados; (3) factores nutricionales como la vitamina C y la vitamina E y por último (4) hormonas, citoquinas y otros compuestos como la lactoferrina, interferón, hormona del crecimiento o prolactina (para revisión ver Sakai, 1999). En las tablas 10.3 y 10.4 se presenta un pequeño resumen sobre el uso y los efectos de estas sustancias en la respuesta inmune y sobre los parámetros de estrés realizados en los últimos años, respectivamente. Normalmente los inmunoestimulantes se administran por tres vías principalmente: mediante inyección intraperitoneal (ip), baño de inmersión o mediante su inclusión en la dieta. La inyección intraperitoneal presenta el mayor patrón de respuesta pero conlleva un alto estrés derivado de la manipulación y no es muy práctico en peces de pequeño tamaño. El baño de inmersión reduce el efecto causado por la manipulación pero su respuesta no es tan alta como

en el primer caso. La administración mediante la dieta, que requiere dosis más altas, es la ruta más usada ya que minimiza la manipulación y puede ser usado en todas las tallas de peces.

Por tanto, la prevención de la aparición de brotes patológicos mediante métodos alternativos a los antibióticos, como el uso de prebióticos, está recibiendo una especial atención en los últimos años en el sector acuícola (Patterson y Burkholder, 2003; Genc *et al.*, 2007). Los prebióticos se definen como compuestos dietéticos no digeribles que afectan positivamente al huésped mediante la estimulación del crecimiento y/o actividad de determinados tipos de bacterias e incluyen los manano oligosacáridos (MOS).

10.1.3.1 PREBIÓTICOS

Los prebióticos se definen como ingredientes dietéticos no digeribles que a través de la potenciación del crecimiento y/o la actividad de determinadas bacterias intestinales tales como las pertenecientes al género *Lactobacillus* y *Bifidobacterium*, benefician la salud y el crecimiento del huésped mediante la reducción del número de patógenos intestinales presentes en éste y/o mediante la producción de metabolitos bacterianos por parte de estas bacterias. Su incapacidad a ser digeridos viene dada por la configuración del átomo de carbón anómero (CI o C2) de las unidades de monosacáridos de algunos oligosacáridos que hace que sus enlaces glucosídicos no sean digeridos por la actividad hidrolítica de las enzimas digestivas humanas/animales (Roberfroid y Slavin, 2000; Ringø *et al.*, 2010).

La principal función de los prebióticos reside en el cambio potencial de la comunidad bacteriana intestinal a una dominada por bacterias beneficiosas (Bieklecka *et al.,* 2002; Patterson *y* Burkholder, 2003; Manning y Gibson, 2004). Mediante este cambio los productos resultantes de la fermentación intestinal pueden ser alterados a través del aumento de la concentración de ácidos grasos volátiles, como se ha descrito para especies terrestres (Tsukahara *et al,.* 2002; Smiricky-Tjardes *et al.*, 2003), y que favorece la inhibición de la colonización intestinal por organismos patógenos (Manning y Gibson, 2004; Vázquez *et al.* 2005; Burr, 2007), hecho que es de especial interés en peces ya que el intestino se considera uno de los principales portales de entrada de bacterias patógenas. Asociados a la mejora de la microbiota intestinal, se han descrito otros efectos derivados de la suplementación prebiótica, tales como la potenciación de

la respuesta inmune innata y la mejora de la asimilación de minerales o del rendimiento de la producción (Patterson y Burkholder, 2003; Smiricky-Tjardes *et al.,* 2003; Konstantinov *et al.,* 2004). Respecto al uso de estos productos en especies acuáticas, podemos considerar que la investigación y aplicación está en su infancia si la comparamos con el estado de la investigación en otras especies terrestres (Patterson y Burkholder, 2003; Ringø *et al.*, 2010). Hasta la fecha, los prebióticos estudiados en peces han sido: inulina, fructo oligosacáridos (FOS), fructoo ligosacáridos de cadena corta, mannano oligosacáridos (MOS), galacto oligosacáridos (GOS), xylo oligosacáridos (XOS), arabinoxylo oligosacáridos (AXOS), isomalto oligosacáridos (IMO) y GroBiotic®-A.

10.1.3.1.1 MANANOLIGOSACARIDOS (MOS)

Los oligosacáridos son carbohidratos simples que contienen un bajo número de monosacáridos, normalmente de tres a diez. Bio-Mos® es un azúcar natural derivado de la pared celular de una cepa seleccionada de la levadura (*Saccharomyces cerevisiae*), y está principalmente compuesto por MOS y es producido por Alltech, Inc (Kentucky, USA). El modo de acción de Bio-Mos® se basa en dos principales funciones: el bloqueo de la colonización intestinal por parte de patógenos potenciales y la modulación del sistema inmune.

El uso de MOS como agente bloqueante frente a la colonización de bacterias patógenas evoluciona del concepto de que algunos azúcares como la manosa pueden ser utilizados como inhibidores de la adhesión de patógenos a las células intestinales. La adhesión bacteriana es un paso obligado en la colonización y patogénesis y esta mediada por la interacción entre los carbohidratos presentes en la superficie celular (Bavington y Page, 2005) dado que las bacterias se unen a receptores moleculares específicos de la superficie celular a través de grupos de carbohidratos específicos. Por lo tanto, la idea del uso de MOS en alimentación animal con objeto de contener la unión de las bacterias patógenas a las superficies celulares se basa en un producto que sobrevive al paso por el tracto intestinal y mimetiza determinados grupos de carbohidratos de superficie celular a los que se suelen adherir los patógenos bacterianos, por lo que los patógenos con especificidad por la manosa, se adherirán al MOS en vez de a las microvellosidades intestinales, siendo evacuados del intestino y reduciendo la incidencia o severidad de la patología potencial.

Tabla 10.3. Algunos immunoestimulantes estudiados en los últimos años y sus efectos sobre los parámetros inmunes y biológicos de algunas de las especies cultivadas.

Especie	Inmunoestimulante	Dosis	Respuesta immune estimulada	Referencia
Dorada (*Sparus aurata*)	Vit E	1200 mg/kg	Lisozima	Cuesta *et al.*, 2002
	α-tocoferol	600-1800 mg/kg	Complemento Fagocitosis	Ortuño *et al.*, 2000
	Vit C	3000 mg/kg	Complemento	Ortuño *et al.*, 1999
	Quitina	1 mg (ip)	Humoral + Celular	Esteban *et al.*, 2000
	Levadura	1-10 g/kg	Celular	Ortuño *et al*, 2002
Fletán (*Paralichthys olivaceus*)	Astaxantin	100 mg/kg	Quimiootaxis, Brote respiratorio	Galindo-Villegas *et al.*, 2002
	Vit C	6100 mg/kg	Brote respiratorio	Galindo-Villegas *et al.*, 2002
	VIt E	600 mg/kg	Lisozima Fagocitosis	Galindo-Villegas *et al.*, 2002
	Arginin	150 mg/kg	Brote respiratorio Lisozima	Galindo-Villegas *et al.*, 2002
	β-Glucanos	3000 mg/kg	Brote respiratorio	Galindo-Villegas *et al.*, 2002
	β-Glucanos + Manosa	1%	Brote respiratorio Lisozima	Honda *et al.*, 2004
Rodaballo (*Psetta maxima*)	Vit C	300-2000 mg/kg	Fagocitosis Lisozima	Roberts *et al.*, 1995
	Vit E	500 mg/kg	Fagocitosis	Pulsford, 1995
Medregal del Japón (*Seriola quinqueradiata*)	α-tocoferol acetato	119-5950 mg/kg 122-6100 mg/kg	Fagocitosis Lisozima	Hosokawa, 2000 Ito *et al.*, 2000
	β-Glucanos	1 ml/ pez	Lisozima Complemento	Engstad *et al.*, 1992
	β-Glucanos	0,2%	Celular ↓ Mortalidad	Burrells *et al.*, 2001a
	β-Glucanos +LPS	10 µg/ml	Brote respiratorio	Paulsen *et al.*, 2001
	Levamisole	2,5 ml/l baño	Fagocitosis Lisozima	Findlay *y* Munday, 2000
	Nucleótidos	0,03%	↓ Mortalidad	Burrells *et al.*, 2001a
		0,03%	Anticuerpos ↓ Mortalidad ↑Osmoregulación ↑Ht	Burrells *et al.*, 2001b
Carpa común (*Cyprinus carpio*)	Quitina	1%	↓ Crecimiento	Gopalakannan *y* Arul, 2006
	Chitosan	1%	↑Crecimiento Brote respiratorio Lisozima	Gopalakannan *y* Arul, 2006
	Levamisole	250 mg/kg	↑Crecimiento Brote respiratorio Lisozima	Gopalakannan *y* Arul, 2006

Tabla 10.3. Algunos immunoestimulantes estudiados en los últimos años y sus efectos sobre los parámetros inmunes y biológicos de algunas de las especies cultivadas (Cont.)

Especie	Inmunoestimulante	Dosis	Respuesta immune estimulada	Referencia
Pargo japonés (*Pagrus major*)	Vit C	10000 mg/kg	Fagocitosis	Yano *et al.*, 1990
Hibrido de lubina americana (*Morone chrysops* x *Morone saxatilis*)	Levamisole	100-500 mg/kg	↑Crecimiento ↑FCR Brote respiratorio	Li *et al.*, 2006
	Levadura	1-4%	↑Crecimiento ↑ Supervivencia (*S.iniae)*	Li *y* Gatlin III, 2003
	Grobiotic	1-2%	↑ Supervivencia (*S.iniae)* ↑Producción superoxido extracelular	Li *et al.*, 2004
Pez gato (*Clarias batrachus*)	Lactoferrina bovina	100 mg/kg	Actividad hemolitica Fagocitosis	Kumari *y* Sahoo, 2006
	β-Glucanos	0.1%	Actividad hemolitica Fagocitosis	Kumari *y* Sahoo, 2006
	Levamisole	50 mg/kg	Brote respiratorio	Kumari *y* Sahoo, 2006
Lubina (*Dicentrarchus labrax)*	β-Glucanos	2% peso corporal	Humoral	Bagni *et al.*, 2000

Por otro lado, el efecto de MOS como modulador del sistema inmune no está tan definido, ya que este sistema puede ser activado por diferentes rutas. Pero la idea generalizada se basa en la activación de los receptores patrón de reconocimiento de las líneas germinales codificadas o las proteínas patrón de reconocimiento que disparan la respuesta inmune innata. Esta activación puede ser iniciada por el MOS en sí, ya que para los peces es una sustancia no propia, o bien por los componentes solubles resultantes de la respuesta inmune estimulada que actúen como las proteínas patrón de reconocimiento.

Tabla 10.4. Algunos immunoestimulantes estudiados en los últimos años y sus efectos sobre los parámetros relacionados con el estrés de algunas de las especies cultivadas.

Especie	Inmunostimulante	Dosis	Estrés	Respuesta inmune	Referencia
Carpa común (*Cyprinus carpio*)	Lactoferrina bovina	20-200 mg/día	Confinamiento (30 kg/m^3)	↓ Cortisol	Kakuta, 1998
			Hipoxia	↓ Cortisol, Adrenalina, Noradrenalina, Dopamina	Kakuta, 1998
Lubina (*Dicentrarchus labrax*)	Probiótico (*L. delbrueckii delbrueckii*)	10 bact/ml: Vía rotiferos (11-29 days) Vía artemia (30-70 days)	Condiciones de cultivo	↓ Cortisol	Carnevali *et al.*, 2006
	Ergosan (extracto de algas + ác. alginico)	0,5%	Condiciones de cultivo	↑Complemento(15 y 30 días) ↑Lisozima ↑ HSP hígado	Bagni *et al.*, 2005
	Macrogard (extracto levadura B-glucanos)	0,1%	Condiciones de cultivo	↑Lisozima ↑ HSP hígado ↑Complemento (15 días)	Bagni *et al.*, 2005
Camarón patiblanco (*Litopenaeus vannamei*)	β-Glucanos + Vit C	2g/kg + 0,2 g/kg	↓ Salinidad (0‰)	↑Crecimiento ↓ Índice cumulative estrés	López *et al.*, 2003
Trucha arcoiris (*Oncorhynchus mykiss*)	Vit C, E y n-3 HUFA	Vit E: 25,6 – 275.6 mg/kg Vit C: 0-1000 mg/kg n-3 HUFA: 12,5 – 30,5 g/kg	Confinamiento (100 kg/m^3)	↑Niveles de cortisol en dietas: +Vit E+n-3HUFA -Vit C+Vit E+n-3HUFA	Trenzado *et al.*, 2007
Dorada (*Sparus aurata*)	Vit E	150 mg/kg	Estrés físico (5 min.)	Dimisminución del incremento del cortisol ↓Mortalidad	Montero *et al.*, 2000
	Vit C Vit E Vit C+E	Vit E: 1,2 g/kg Vit C: 3g/kg	Agitación del agua (15 min) Confinamiento (100kg/m^{-3}) Exposición al aire (2 min)	↓ Glucosa ↓ ACH50 ↓ Brote respiratorio	Ortuño *et al.*, 2003
	Nucleotidos	Dieta comercial	Confinamiento severo (2h)	↑ Capacidad bacteriolítica, ↑ Lisozima y respuesta al estrés	Montero *et al.*, 2007
	Vit C y E	250 mg vit C/kg or 250 mg Vit E/kg	Confinamiento (40 kg/m^3)	↑ Lisozima ↑ ACH50 en dieta suplementada con Vit E	Montero *et al.*, 2000

10.1.3.1.2. MOS EN ACUICULTURA

A lo largo de la última década la inclusión en dietas de MOS ha sido ensayada en varias especies de animales de producción intensiva, con objeto de optimizar la el proceso de producción mediante la mejora del crecimiento, tasas de conversión alimenticias o mediante la reducción del riesgo de aparición de patologías. Algunos de estos estudios se encuentran detallados en la Tabla 10.5, donde se incluye un breve resumen de los efectos derivados de la suplementación con MOS sobre los parámetros biológicos e inmunes de algunas especies de organismos terrestres.

En el sector acuícola, basándonos en el hecho de que es un sector relativamente nuevo en comparación con la producción de organismos terrestres, el número de estudios sobre el efecto de la suplementación con MOS es significativamente menor. En especies de agua dulce, tales como carpas (*Cyprinus carpio* y *C. carpio Var. Jian*.), siluro (*Silurus glanis*) o hibrido de tilapia de Mozambique x tilapia del Nilo (*Oreochromis mossambicus* x *O. niloticus*), el crecimiento y/o la utilización del alimento se han visto mejorados significativamente mediante la suplementación dietética de MOS (Hanley *et al.*, 1995; Zhou y Li, 2004; Staykov *et al.,* 2005; Bogut *et al.*, 2006; Culjak *et al.* 2006). Por otro lado, en otras especies como el esturión del Golfo de Méjico (*Acipenser oxyrinchus desotoi*) o la tilapia del Nilo (*Oreochromis niloticus*) no se detectó ningún efecto en el crecimiento tras la suplementación con MOS (Pryor *et al.*, 2003; Vendemiatti *et al.*, 2003). Respecto a la mejora de los parámetros inmunes, la adición de MOS a dietas de carpa común y trucha arcoíris produjo una mejora en éstos (Zhou y Li, 2004; Staykov *et al.*, 2005). En especies de agua marina (larvas de sargo común, *Diplodus sargus*) la suplementación con MOS a través de la *Artemia* produjo una mayor resistencia al estrés a la vez que mejoró la estructura de las microvellosidades intestinales (Dimitroglou *y* Davies, 2004). No obstante, hasta la fecha, pocos estudios han demostrado los efectos de la suplementación con MOS en la resistencia a enfermedades en peces.

Por lo tanto, la mayoría del trabajo actual sobre el efecto de este tipo de productos alternativos a los antibióticos, y particularmente MOS, se ha basado en la estimulación de los mecanismos de defensa de los peces. Por el contrario, existen muy pocos estudios sobre los mecanismos potenciales implicados en la mejora del crecimiento o de la tasa de conversión alimenticia derivada del uso de este tipo de productos, incluso en animales terrestres. La

capacidad de crecimiento de un pez puede estar condicionada por varios factores, entre ellos la digestión y la eficiencia frente a la conversión de los nutrientes o la pobre asimilación de estos.

La bibliografía referente al estudio de los efectos de la suplementación con prebióticos y/o inmunoestimulantes en general, y en concreto MOS, sobre los procesos de digestión/absorción de nutrientes es escasa, pero en peces concretamente son ampliamente desconocidos. La adición de una mezcla de enzimas-prebióticos en dietas de pollos basadas en harinas de la misma especie produjo un aumento en la eficiencia proteica y en la tasa de conversión alimenticia (Kirkpinar *et al.*, 2004). Iji *et al.* (2001) describieron un incremento en las actividades específicas de la leucina aminopeptidasa y la fosfatasa alcalina, a la vez que un aumento en la asimilación de L-triptófano en el intestino anterior de pollos tras la suplementación con MOS sin efecto detectable en la región posterior. Los autores relacionaron este incremento en la asimilación con una mejor funcionalidad de la membrana intestinal derivada del uso de MOS.

Tabla 10.5. Efectos de la suplementación dietética de MOS en algunas especies animales terrestres.

Especie	Parámetro	Referencia
Aves	↑FCR	Savage *et al.*, 1997 Sonmez *y* Eren, 1999 Spring, 1999 Spring *et al.*, 2000 Valancony *et al.*, 2001 Iji *et al.*, 2001 Fritts *y* Waldroup, 2003 Sims *et al.*, 2004
Aves	↑ Crecimiento	Savage *et al.*, 1997 Kumprecht *et al.*, 1997 Fairchild *et al.*, 2001 Spais *et al.*, 2003 Miguel *et al.*, 2004
Aves	↓ Mortalidad	Stanley *et al.*, 2000 Fritts *y* Waldroup, 2003 Hooge *et al.*, 2003
Aves	↑ Fagocitosis	Sisak, 1995
Aves	↓ Colonización intestinal de *Salmonella*	Oyofo *et al.*, 1989 Spring *et al.*, 2000
Cerdos	↑ Crecimiento	Pettigrew, 2000 Miguel *et al.*, 2002
Terneras	↑ Ingesta	Heinrichs *et al.*, 2001
Perros	↑ Ingesta	Grieshop *et al.*, 2004

La mayoría de efectos derivados del uso de estos productos en el metabolismo de lípidos están relacionados con cambios a nivel de absorción de colesterol o reducción de la deposición de grasas, aunque los mecanismos implicados en estos efectos no están todavía totalmente descritos. Un estudio en ratas diabéticas suplementadas con XOS reveló un menor incremento de colesterol y triglicéridos además de un aumento del nivel de triglicéridos en hígado cuando eran comparadas con ratas sanas (Imaizumi *et al.*, 1991). Otros estudios sobre la suplementación con FOS también han revelado una menor concentración de lípidos en sangre (Bornet *et al.*, 2002; Roberfroid, 2002), reducción que puede ser causada por una inhibición de las enzimas lipogénicas en hígado, hecho que puede ser desencadenado por la acción del propinato producido durante la fermentación de los prebióticos por la flora intestinal (Wolever *et al.*, 1991). En la misma línea Young *et al.* (2000) informaron de una reducción en la deposición de grasas en general y de colesterol en concreto en el hígado y huevos de gallinas ponedoras después de suplementarlas con MOS al 0,1% en la dieta.

Del mismo modo, se ha sugerido que la inclusión de otros prebióticos en la dietas para pollos podrían disminuir la actividad lipogénica mediante la reducción de la actividad del actilCoA carboxilasa (Santoso *et al.,* 1995; Kannan *et al.,* 2005). En cualquier caso, una reducción en la deposición grasa puede estar también relacionada con un aumento de la actividad lipolítica tal y como se describió en ratas (Gallaher *et al,* 2000). Pese a que algunos estudios han demostrado que determinados prebióticos tienen un efecto hipocolesterolémico en modelos animales (Sugano *et al.* 1978, 1980; LeHoux y Grondin, 1993, Razdan y Pettersson, 1994; Gallaher *et al.*, 2000), todavía no se ha descrito el modo de acción de estos compuestos tales como el MOS, sobre el metabolismo del colesterol. Algunos autores han argumentado que una posible reducción de las partículas de lipoproteína de muy baja densidad (VLDL) puede ser el desencadenante de esta menor cantidad de colesterol tras la suplementación con prebióticos ya que al aislar hepatocitos de ratas alimentadas con FOS se observó una menor incorporación de palmitato en los triglicéridos en comparación con los hepatocitos aislados de las ratas control (Gibson y Roberfroid, 1995).

Por otro lado, un incremento en la excreción de ácidos biliares derivado del uso de prebióticos podría también tener un efecto reductor en la absorción del colesterol ya que el colesterol plasmático o hepático sería utilizado para mantener la mezcla de ácidos biliares (Jie y Shu-Sheng, 1997; Gallaher *et al.*, 1999, 2000). En cualquier caso, la unión de los ácidos de la bilis dentro del

intestino delgado podría interrumpir la formación de micelas dando como resultado una menor capacidad de solubilizar el colesterol, y consecuentemente, reducir su absorción (Nauss *et al.*, 1983; Gallaher *et al.*, 2000). Han *et al.* (1999) describieron la inhibición de la lipasa pancreática *in vitro* en roedores después de una suplementación con una mezcla de quitina-chitosan, argumentando que esta inhibición puede venir dada por una acumulación de emulsiones lipídicas que derivaría en una mayor excreción de grasas. Otros autores han relacionado la menor absorción de colesterol con cambios en la viscosidad de los sobrenadantes digestivos después de ser suplementados con chitosan (Gallaher *et al.*, 1993a, b; Carr *et al.*, 1996) en este sentido, los efectos hipocolesterolémicos de los glucomananos (Shimizu *et al.,* 1991) en ratas parecen ser mediados por una reducción específica en la viscosidad asociada a la absorción de colesterol (Gallaher *et al.*, 2000).

En peces, Burr *et al.* (2008) informaron de una reducción en el coeficiente aparente de digestibilidad de lípidos después de alimentar al pargo con una dieta basada en harinas de soja y suplementada con MOS y GOS. Los autores argumentaron que puede darse la existencia de una interferencia entre los prebióticos y la absorción de lípidos, pero hay que realizar más estudios en este sentido para poder determinar si en peces, como ocurre en vertebrados terrestres, la excreción de ácidos biliares, la viscosidad de los sobrenadantes de la digesta o los posibles cambios en los perfiles de microbiota pueden estar afectuando la absorción de lípidos intestinales.

Los estudios del efecto de los prebióticos sobre la absorción/digestión de carbohidratos son escasos, especialmente en peces. Iji *et al.* (2001) describieron un aumento en la actividad específica de la maltasa en el intestino anterior de pollos alimentados con MOS. En cerdos alimentados con MOS se aumentó la absorción de glucosa después de suplementarlos con prebióticos (Breves *et al.,* 2001; Gatlin *et al.*, 2006). A su vez, el aumento en el co-transportador-1 sodio dependiente de D-Glucosa se relacionó con un aumento de la absorción de D-glucosa en humanos tras la suplementación de estos con prebióticos (Eberl, 2005). Muchos de los efectos de los prebióticos están relacionados con la promoción del crecimiento de determinadas bacterias beneficiosas en el intestino, estando este hecho relacionado a su vez con un incremento en la producción de ácidos grasos volátiles de cadena corta. De hecho Tappenden y McBurney (1988) describieron que un incremento en estos ácidos grasos volátiles de cadena

corta contribuye a un aumento del transportador de glucosa-2 plasmático así como de los niveles de GLP-2 y proglucagón mRNA en el intestino de roedores.

Entonces, y basado en estas evidencias, es necesario realizar estudios adicionales en este sentido con objetivo de aclarar los efectos y posibles mecanismos involucrados en el modo de acción del MOS respecto a las principales especies acuícolas marinas de peces, y en particular respecto aquellas que tienen una especial sensibilidad al manejo como puede ser la lubina.

10.1.4. OBJETIVOS

El principal objetivo de esta tesis fue investigar los efectos de la suplementación dietética de MOS en el cultivo de la lubina. Para ello, se plantearon los siguientes objetivos secundarios:

1. Investigar los efectos de la suplementación dietética de varios niveles de MOS en la producción de cultivo de la lubina, determinado la dosis óptima de inclusión para juveniles de esta especie en las épocas más críticas de cultivo en términos de aparición de patologías y situaciones de estrés (30-120g).

2. Determinar los efectos de la inclusión de MOS en dietas para lubina en la salud en términos de parámetros del sistema inmune, resistencia a enfermedades, así como la determinación de los posibles mecanismos implicados tales como los relacionados con la infección por patógenos potenciales, morfología intestinal, producción de mucus y tejido linfoide asociado al intestino o producción de prostaglandinas en el intestino.

3. Estudiar el efecto de la suplementación con MOS en el bienestar de la lubina, estudiando para ello su respuesta a paneles de estrés y su posible interacción con la resistencia a enfermedades.

4. Proporcionar un mejor entendimiento sobre los efectos de la suplementación con MOS en el crecimiento y el metabolismo de la lubina, así como los mecanismos potenciales implicados en este proceso como pueden ser la digestibilidad de nutrientes y el metabolismo hepático.

5. Estudiar los efectos de la suplementación con MOS a lo largo del ciclo de producción de la lubina sobre la calidad del filete producido.

10.2. MATERIALES Y MÉTODOS

10.2.1. DIETAS

En función del experimento realizado, se formularon dietas isolipídicas e isoproteícas con un nivel de inclusión de MOS de 0 $g \cdot kg^{-1}$ (Control), 2 $g \cdot kg^{-1}$ (BM2), 4 $g \cdot kg^{-1}$ (BM4) y 6 $g \cdot kg^{-1}$ (BM6) en forma de Bio-Mos© (Alltech Inc., USA) remplazando carbohidratos estándares y cubriendo los requerimientos nutricionales de la especie sometida a estudio (Izquierdo, 2005). En los experimentos I, II y III, las dietas fueron producidas por una empresa fabricante de piensos (Graneros de Tenerife S.A., Tenerife, España), mientras que las dietas utilizadas en los experimentos IV y V fueron producidas en la planta experimental de piensos del Grupo de Investigación en Acuicultura (GIA) tal y como se detalla en los siguientes apartados. En la Tabla 10.6 se especifica la formulación y composición proximal de las dietas.

En el experimento I, los niveles de inclusión de MOS utilizados fueron: 0 $g \cdot kg^{-1}$ (Control), 2 $g \cdot kg^{-1}$ (BM2) y 4 $g \cdot kg^{-1}$ (BM4), mientras que en el segundo experimento además de estos niveles de inclusión se añadió un cuarto nivel con un contenido de 6 $g \cdot kg^{-1}$ (BM6). Los resultados obtenidos en estos dos experimentos determinaron el nivel de inclusión de MOS en los siguientes tres experimentos, en los que se escogió 4 $g \cdot kg^{-1}$ (BM4) como la dosis óptima a incluir en dietas para juveniles de lubina comprendidos en una talla entre 35 y 120 g (19-23.7°C).

10.2.1.1. FORMULACIÓN DE LAS DIETAS

Previo a la producción de las dietas usadas en los experimentos IV y V, los ingredientes fueron analizados con objeto de formular las dietas experimentales lo más similares posible a las dietas comerciales usadas anteriormente (Tabla 10.6). El índice de peróxidos de las dietas se analizó previo a su suplementación.

10.2.1.2. PREPARACIÓN DE LAS DIETAS

El primer paso en la preparación de las dietas fue mezclar cuidadosamente el ingrediente activo (MOS) con la harina de trigo. Una vez mezclado se añadieron una por una las

harinas vegetales y finalmente, se añadió la mezcla de vitaminas comercial. Por otro lado, la mezcla comercial de minerales se homogeneizó con la harina de pescado. Seguidamente, se combinaron las dos mezclas anteriores hasta conseguir una mezcla totalmente homogénea. Una vez llegados a este punto, se complementó la mezcla con mioinositol, fosfato de potasio, carbonato de calcio y cloruro de sodio. Independientemente a este proceso, se mezclaron cuidadosamente los aceites de pescado, vegetales y antioxidantes y se añadieron, en primer lugar manualmente y luego con ayuda de un mezclador horizontal DANAMIX BM-330 (Danamac, S.A., Guipúzcoa, España) a la mezcla de harinas, minerales, vitaminas y elementos traza. En este punto se disolvió el cloruro de colina en agua destilada y se combinó con el resto de la mezcla utilizandoo el mezclador horizontal mencionado anteriormente.

Una vez completada la hidratación de la mezcla se procedió a su peletización a través de un molde de 3mm de diámetro (MOBBA 2 HP, Mod. 8.3; Eriez Magnetics, UK). Los pellets obtenidos se secaron a 38°C durante aproximadamente 16h y se almacenaron a 4°C hasta su uso. Se tomaron muestras y se almacenaron a -80°C hasta su análisis.

10.2.2. CONDICIONES GENERALES DE CULTIVO: DISEÑO EXPERIMENTAL Y PROTOCOLOS DE MUESTREO

En esta sección se incluye la descripción metodológica general usada a lo largo de los cinco principales experimentos que comprenden esta tesis. Estos experimentos representan una investigación gradual y progresiva sobre los efectos sobre el crecimiento y la resistencia a enfermedades así como los posibles mecanismos implicados, derivados de la inclusión de MOS en dietas para juveniles de lubina.

Los juveniles de lubina empleados en los experimentos realizados (2005-2009) se obtuvieron de una granja local (ADSA, San Bartolomé de Tirajana, Islas Canarias, España) de la que fueron transportados al Instituto Canario de Ciencias Marinas (ICCM, Las Palmas, España) dónde fueron aclimatados a las nuevas condiciones de cultivo. Una vez aclimatados, se distribuyeron de forma aleatoria y homogénea en los tanques experimentales y fueron alimentados con las dietas correspondientes a cada uno de los experimentos.

A lo largo de toda la experimentación se siguieron condiciones de cultivo estandarizadas. El suministro de agua se realizó de forma continua a partir de agua de mar decantada procedente del tanque de sedimentación situado en la parte superior exterior de la planta de cultivo, llegando a los tanques por gravedad. La iluminación fue natural indirecta con un fotoperiodo natural de aproximadamente 12L:12O, correspondiente a la época del año. La temperatura y el oxígeno disuelto se midieron diariamente (Oxy Guard-Hyy beta; Zeigler Bros, Gardners, USA). En función del experimento, se utilizaron tanques de 500 y 1000 l de capacidad o de digestibilidad (120 l) (Figura 10.2a, 10.2b y 10.2c, respectivamente).

Los diseños experimentales y protocolos de muestreo seguidos durante los diferentes estudios se resumen en los esquemas representados en las figuras 10.3, 10.4, 10.5 y 10.6.

10.2.3. PARÁMETROS BIOLÓGICOS

10.2.3.1. CRECIMIENTO RELATIVO

Como primera medida para observar las diferencias entre la eficacia del alimento en las diferentes dietas se valoró el crecimiento en peso de cada lote experimental. Éste se define como la relación entre la biomasa ganada (g) respecto al peso inicial (g). Puede ser expresado en valores absolutos así como en valor porcentual, en este caso se expresó en porcentaje, corrigiéndolo respecto al peso individual del individuo, mediante la siguiente fórmula:

Crecimiento relativo = [(Peso final – Peso inicial) / Peso inicial] x 100

10.2.3.2. TASA DE CRECIMIENTO ESPECÍFICO O SGR

Como segunda medida a fin de observar las diferencias entre dietas se evaluó la tasa de crecimiento específico. Este factor relaciona el peso ganado respecto a la duración del experimento y se expresa en unidades porcentuales.

SGR= [(ln Peso final –ln Peso inicial) / n° días] x 100

10.2.3.3 TASA DE CONVERSIÓN DEL ALIMENTO O FCR

Se valoró el índice de conversión o FCR de cada dieta o lote experimental, con objeto de determinar la eficiencia del alimento ingerido. Se define como la relación entre el alimento ingerido (g) por biomasa generada (g peso húmedo).

FCR = Alimento ingerido / Biomasa generada

10.2.4. MÉTODOS ANALÍTICOS DE COMPOSICIÓN

A lo largo del estudio, la composición proximal de las dietas, heces y tejidos de los animales sometidos a estudio y se analizaron siguiendo protocolos estandarizados (AOAC, 1995). La totalidad de estos análisis se realizó por triplicado.

10.2.4.1. LÍPIDOS TOTALES

Los lípidos procedentes de las muestras sometidas a análisis fueron extraídos con una mezcla de cloroformo: metanol (2:1; v/v) siguiendo el método descrito por Folch *et al.* (1957). Una vez determinado el contenido en lípidos totales de la muestra, éstos se conservaron a -80°C disueltos en cloroformo y en atmósfera de nitrógeno, para evitar su oxidación.

10.2.4.2. PERFILES DE ÁCIDOS GRASOS

Para la determinación de los perfiles de ácidos grasos, los lípidos totales fueron sometidos a una transmetilación ácida-catalizada según el método descrito por Christie (1982). Los ésteres metílicos de los ácidos grasos obtenidos fueron separados y cuantificados por cromatografía de gases (Shimadzu C-R5A, columna de 30m x 0.32 m Sílice con Supelco-10) según las condiciones descritas por Izquierdo *et al.* (1992). Los ésteres metílicos individuales fueron identificados por comparación con estándares conocidos y datos publicados.

Figura 10.2. Tanques usados a lo largo de la experimentación. (a) 500 l; (b) 1000 l; (c) Tanques de digestibilidad con un sistema de recolección de heces descrito por por Cho *et al.* (1985) y modificado por Robaina *et al.* (1995).

Tabla 10.6. Principales ingredientes y composición de las dietas experimentales

Ingrediente	**Dieta (g·kg^{-1} peso seco)**										
	Experimento I			**Experimento II**				**Experimento III**		**Experimento IV y V**	
	C	**BM2**	**BM4**	**C**	**BM2**	**BM4**	**BM6**	**C**	**BM4**	**C**	**BM4**
Harina de pescado [1]	515,0	515,0	515,0	515,0	515,0	515,0	515,0	515,0	515,0	515,0	515,0
Harina de soja	97,8	97,8	97,8	97,8	97,8	97,8	97,8	97,8	97,8	100,0	100,0
Trigo	75,0	75,0	75,0	75,0	75,0	75,0	75,0	75,0	75,0	55,0	55,0
Gluten de trigo	75,0	75,0	75,0	75,0	75,0	75,0	75,0	75,0	75,0	86,0	86,0
Harina de maíz	65,3	63,3	61,3	65,3	63,3	61,3	59,3	65,3	61,3	64,0	60,0
Aceite de pescado[2]	126,9	126,9	126,9	126,9	126,9	126,9	126,9	126,9	126,9	135,0	135,0
Grasas y aceites	20,3	20,3	20,3	20,3	20,3	20,3	20,3	20,3	20,3	20,3	20,3
Mezcla minerales[3]	14,3	14,3	14,3	14,3	14,3	14,3	14,3	14,3	14,3	14,3	14,3
Mezcla vitaminas [4]	10,3	10,3	10,3	10,3	10,3	10,3	10,3	10,3	10,3	10,3	10,3
Antioxidante (BHT)	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1	0,1
MOS [5]	0	2	4	0	2	4	6	0	4	0	4
	Composición (% peso seco)										
Lipidos totales	28,62	27,38	28,89	24,07	24,82	24,04	23,43	24,07	24,04	20,37	20,32
Proteinas totales	44,57	46,81	47,82	48,71	48,77	48,33	48,37	48,71	48,33	51,62	52,07
Cenizas	8,85	9,48	9,28	9,98	9,81	9,86	9,69	9,98	9,86	9,74	9,82

[1]Harina de pescado peruana (65% proteina). [2] Aceite de pescado peruano. [3] TROUW Seabream/Seabass (1g) Carbonato de Calcio (0.2g), Monofosfato de potasio (0.19g) y NaCl 97% (0,04g). [4] TROUW Seabream/Seabass (0.8g), Colina (0.17g) e Inositol (0.06g). C= 0 g kg^{-1} MOS; BM2=2 g kg^{-1} MOS; BM4=4 g kg^{-1} MOS; BM6=6 g kg^{-1} MOS.

Figura 10.3. Representación esquemática del diseño experimental y protocolo de muestreo seguido en el Experimento I.

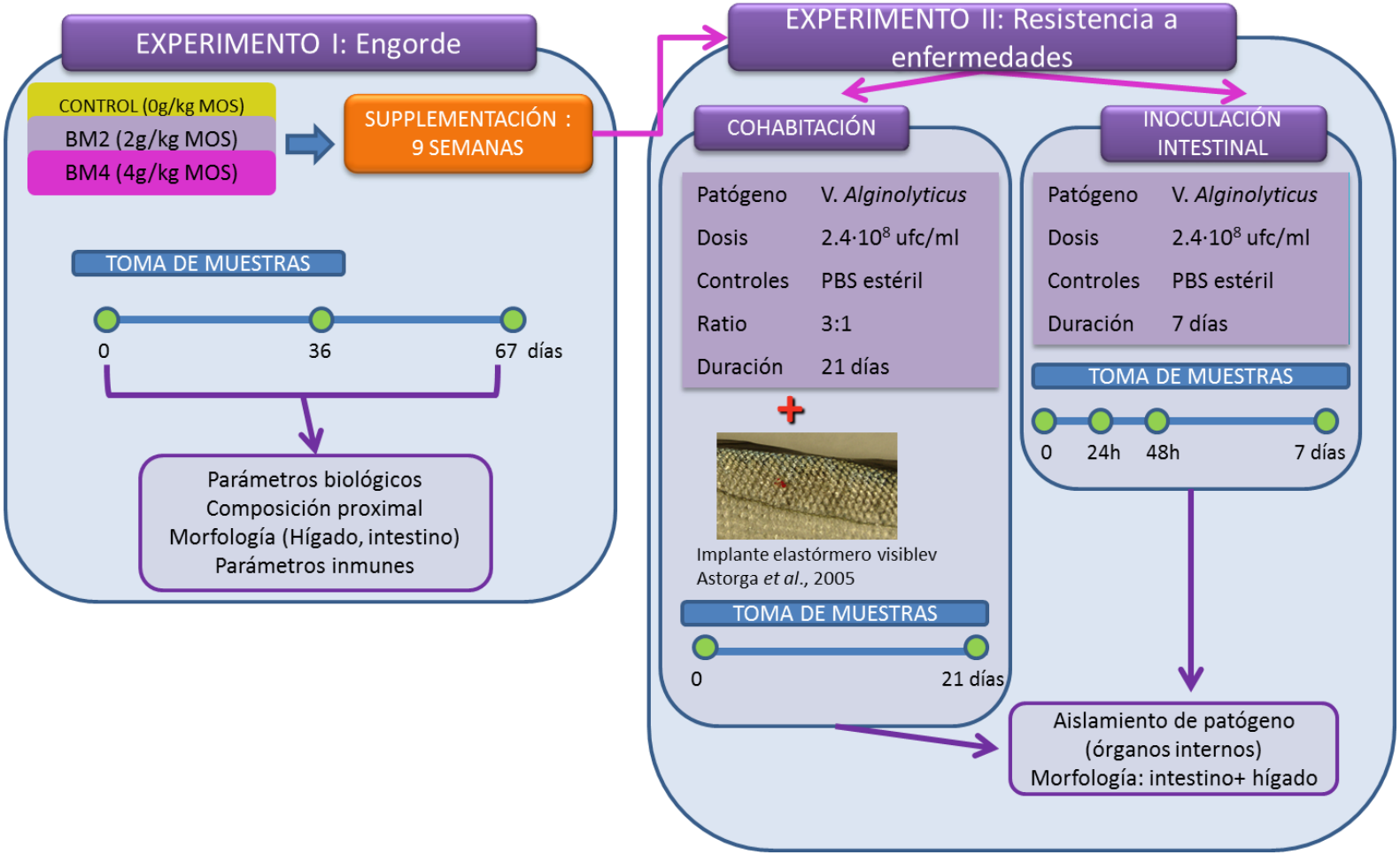


Figura 10.4. Representación esquemática del diseño experimental y protocolo de muestreo seguido en el Experimento II.

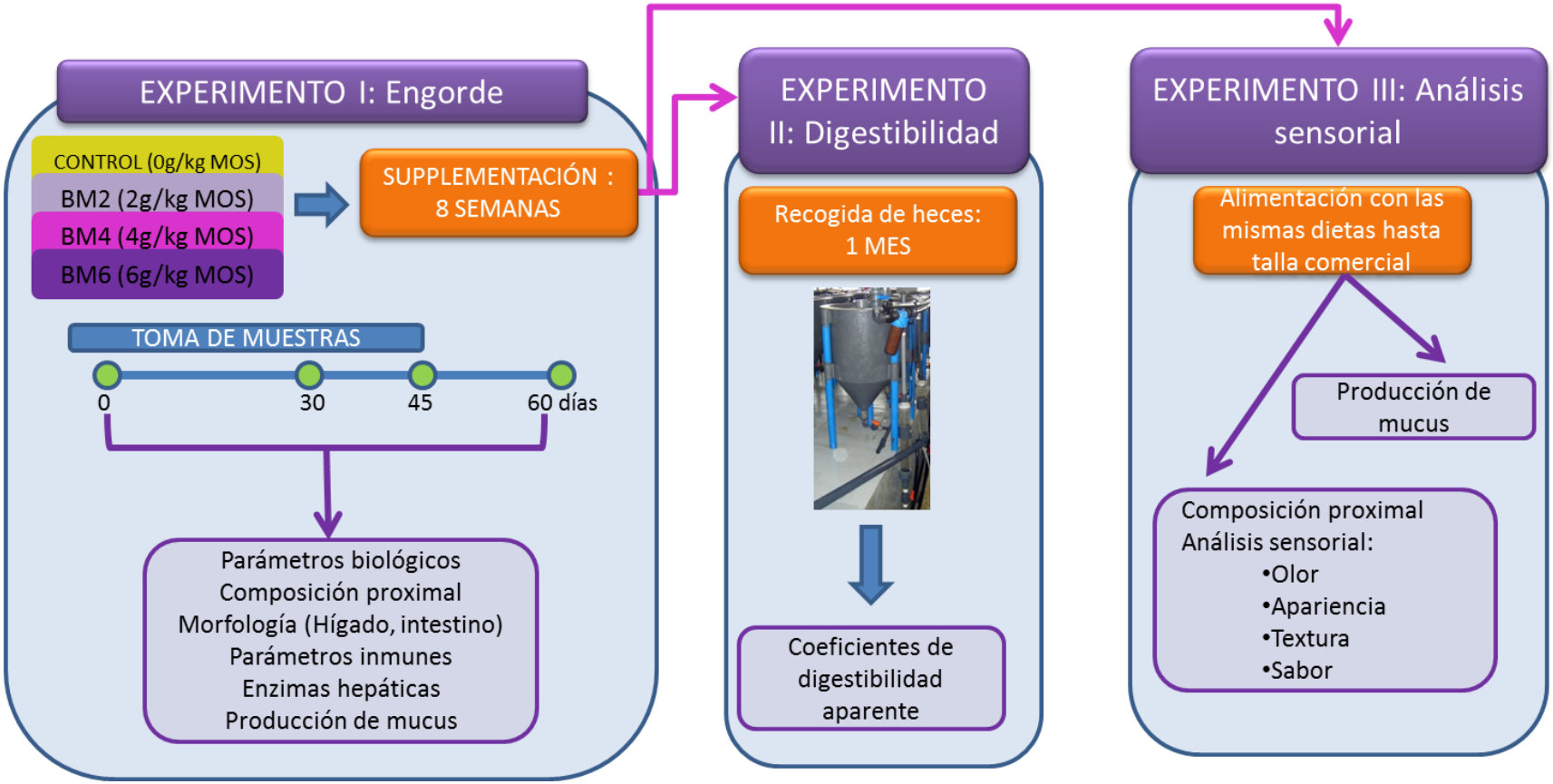


Figura 10.5. Representación esquemática del diseño experimental y protocolo de muestreo seguido en el Experimento III.

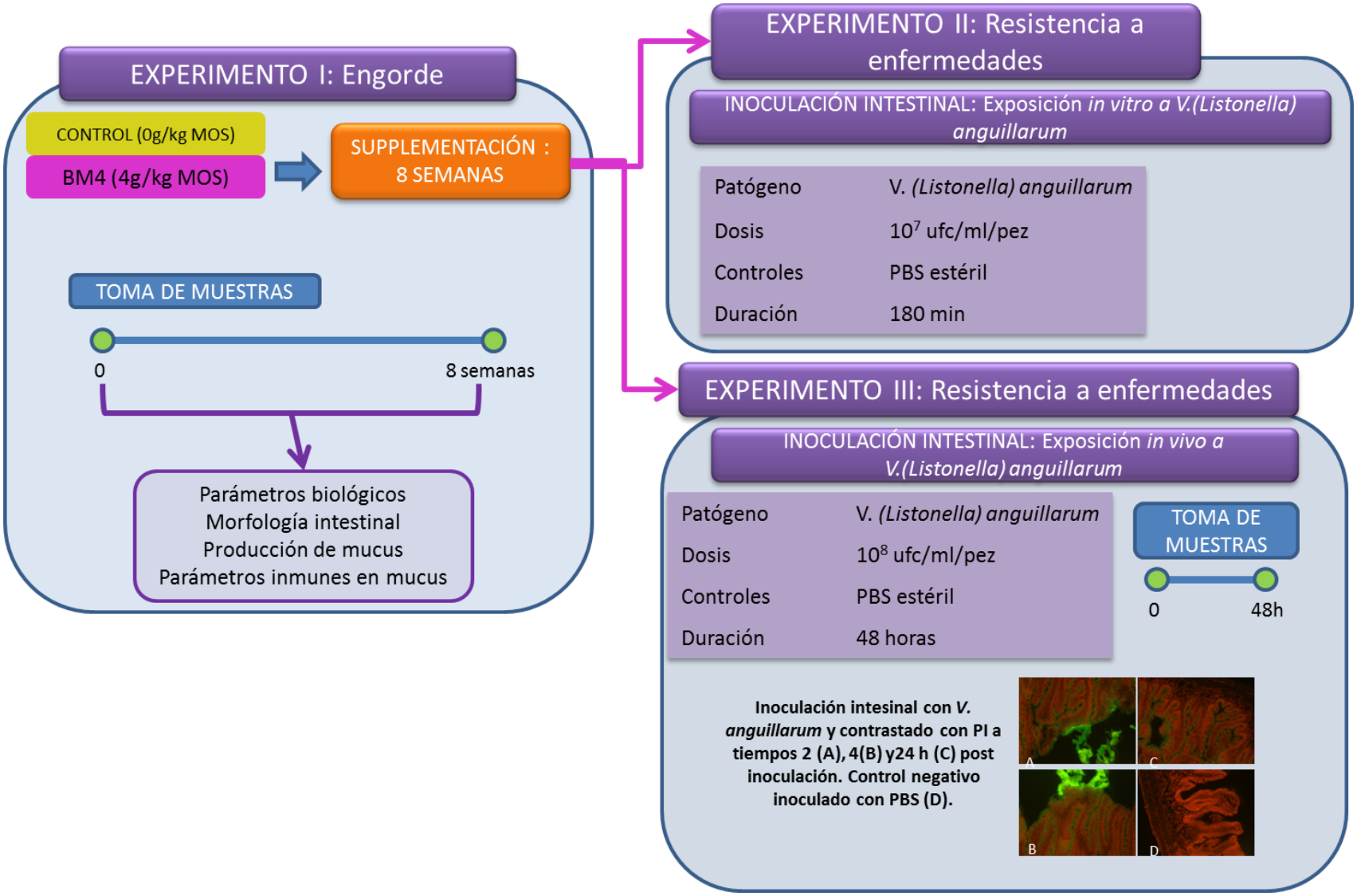
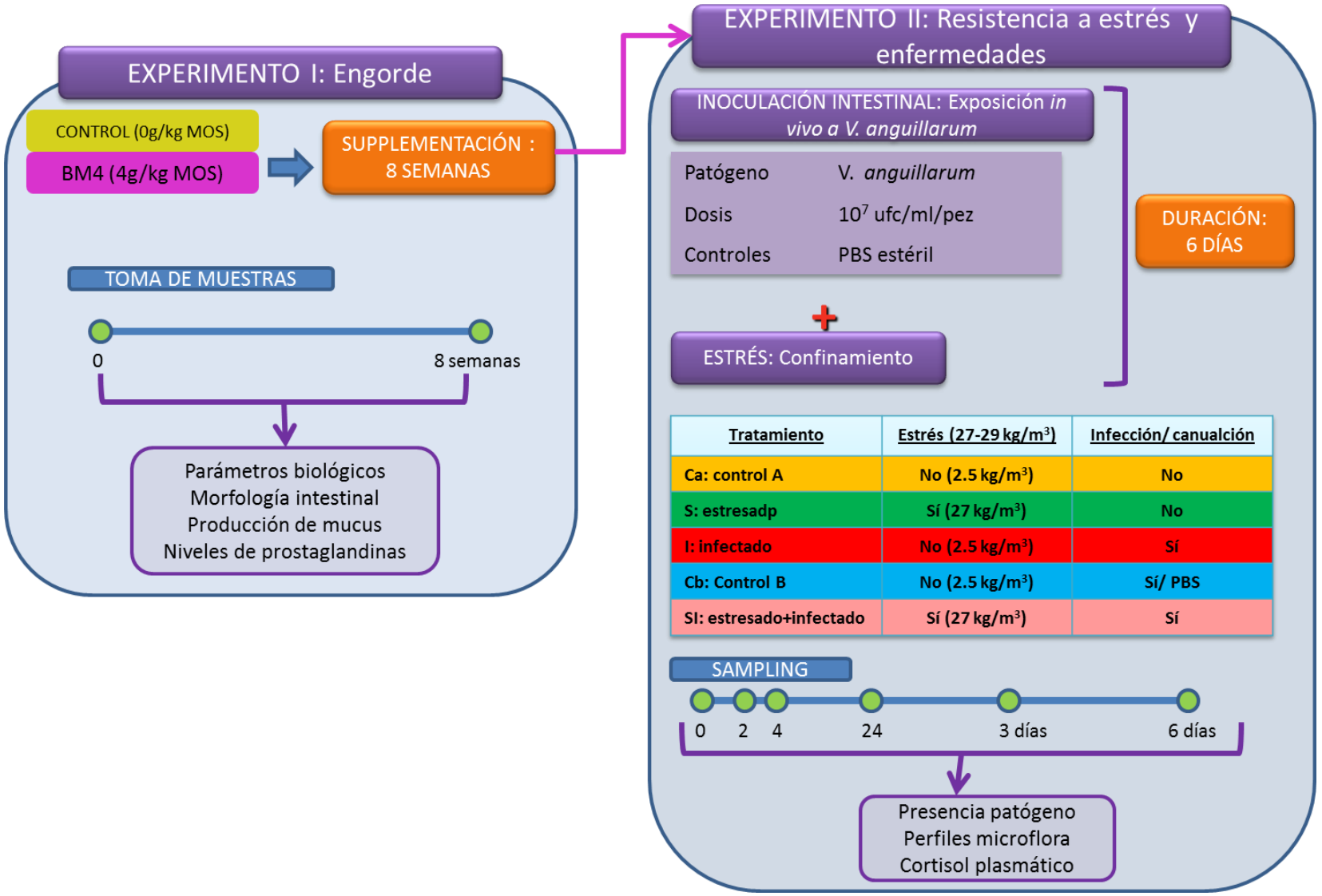


Figura 10.6. Representación esquemática del diseño experimental y protocolo de muestreo seguido en los Experimento s IV y V.



10.2.4.3. PROTEÍNA BRUTA

El contenido proteico de las muestras se determinó a partir de la composición en nitrógeno total de las muestras según la técnica Kjendahl (AOAC, 1995) para el análisis de piensos y peces con unidad de destilación Kjeltec Auto 1035/38. El procedimiento de análisis puede dividirse en tres fases: la primera en la cual se digiere de la muestra en ácido sulfúrico concentrado en presencia de un catalizador de Cobre, con objeto de convertir todo el N_2 presente en $(NH_4)_2SO_4$, en la segunda fase se libera el NH_3 de la muestra digerida añadiendo sosa en exceso, y destilando a vapor de este amoniaco en ácido bórico al 1%, y finalmente se determina el amoniaco liberado mediante valoración con ácido clorhídrico 0,1N. La conversión a porcentaje en proteína bruta de la muestra se calcula mediante conversión del valor de nitrógeno total obtenido, tal que: % Proteínas = [(ml HCl–ml HCl patrón) x N x M_w x F x100] / peso muestra (mg). Tal que: N= Normalidad del HCl utilizado en la valoración; M_w = Peso molecular N (14,007); F= Factor de conversión empírico (6,25).

10.2.4.4. HUMEDAD

La humedad contenida en las muestras se determinó siguiendo el método aceptado por la Asociación Química Analítica Oficial de Estados Unidos (AOAC, 1995) por desecación de una cantidad de muestra conocida (Pi) en una estufa a 110°C hasta conseguir peso constante (Pf). El porcentaje de humedad de la muestra se obtiene mediante la siguiente ecuación: % Humedad = [(Pi – Pf) / Pi] x 100.

10.2.4.5. CENIZAS

El contenido en cenizas de la muestra se determinó según el método aceptado por la AOAC (1995) mediante incineración de la muestra en Horno Mufla a 600°C hasta peso constante.

10.2.5. MÉTODOS HISTOLÓGICOS

Este tipo de estudios fueron llevados a cabo en tejido hepático e intestinal, en base al evidente papel de MOS como protector de la estructura intestinal (Sonmez y Eren, 1999; Spring, 1999; Iji *et al.,* 2001; Valancony *et al.,* 2001; Dimitroglou y Davies, 2004) además de que los cambios morfológicos a nivel hepático son un buen indicador del estado fisiológico del pez.

Las muestras de hígado fueron fijadas en formol tamponado al 10%, incluidas en parafina y teñidas con hematoxilina y eosina (H&E) (Martoja and Martoja-Pierson, 1970). Las imágenes fueron tomadas a una magnificación final de x400 con una microscopio Nikon Microphot-FXA equipado con una cámara Olympus DP50. Las áreas de los hepatocitos así como sus longitudes máximas y minímas tomando el nucleo como referencia se analizaron mediante un software de análisis (Image Pro Plus®).

Para cada segmento intestinal se cortaron de seis a ocho secciones transversales que fueron fijadas en formol tamponado al 10% e incluidas en parafina perpendicularmente a la base de los moldes. En función del experimento, para cada individuo se tiñeron 10 secciones seriadas (5µm) con tinciones de hematoxilina-eosina, Alcian Blue, Alcian Blue/PAS (pH=2,5) o May-Grünwald/Giemsa (MGG) (Martoja y Martoja-Pierson, 1970).

En el caso de los análisis morfométricos de intestino se utilizaron las secciones teñidas con hematoxilina-eosina o MGG. La longitud y anchura de las vellosidades intestinales se midieron con la herramienta de medida del programa de análisis de imagen Image Pro Plus®. Para obtener las medidas del área total de las vellosidades, las imágenes capturadas (MGG; X50) se procesaron con Adobe Photoshop CS 8.0.1 (Adobe Systems, San José, CA), tal que: primero se seleccionó la sección intestinal, se eliminó el fondo y se convirtió a formato binario en el cual se eliminó la luz intestinal. Entonces, la imagen resultante se convirtió a una escala de grises que facilitó la eliminación de la capa muscular mediante su selección. Finalmente, las vellosidades (incluyendo la lámina propia) se volvieron a convertir a formato binario y se midieron con el software anteriormente mencionado. Los comandos utilizados para estas conversiones fueron: Modo grises, relleno y umbral. Las imágenes obtenidas tanto en formato binario como en escala de grises se compararon con las originales para asegurar una conversión precisa.

Los niveles de infiltración de leucocitos en la lámina propria (principalmente granulocitos eosinófilos) fueron evaluados a nivel de microscopia óptica por dos evaluadores sin conocimiento de los tratamientos (MGG; X200; X400). Previo a la valoración se estableció un sistema de evaluación basado en una escala de presencia de leucocitos, tal que: 0, no se observan; 1, bajo; 2, moderado; 3, alto. El número de células secretoras de mucus por unidad de área se determinó mediante el software anteriormente mencionado.

La capacidad bacteriana de adhesión al epitelio intestinal se estudió con métodos de fluorescencia. Brevemente, los peces se inocularon mediante una canulación intestinal con 10^7 ufc de *V. anguillarum*·ml^{-1} marcado con fluorescencia. Tras 2, 4 y 24 horas postinoculación, los peces fueron sacrificados y se tomaron muestras del intestino anterior y posterior. Las muestras fueron incluidas en Tissue-Tek (O.C.T, Sakura Finetek, Torrance, CA) e inmediatamente congeladas a -80°C. Las criosecciones fueron teñidas con bromodesoxiuridina (5 µg·ml^{-1}), montadas con Fluoromont (Sigma, St.Louis, CA, USA) y evaluadas bajo el microscopio de fluorescencia (Olympus CX41, Olympus Optical, PA, USA).

10.2.6. MÉTODOS ENZIMÁTICOS

Las muestras de hígado fueron homogenizadas en 3 volúmenes de solución tampón (20 mM Tris-HCl, 0,25 M sacarosa, 2 mM EDTA, pH 7,4) y los homogeneizados se centrifugaron a 20000 g durante 40 min a 4°C. El contenido de proteína soluble de las muestras se determinó mediante el método Bradford (1976). Las actividades enzimáticas de la glucosa-6-fosfato dehidrogenasa (G6PD, EC 1.1.1.49) y la enzima málica (ME, EC 1.1.1.40) se analizaron siguiendo los métodos descritos por Dias *et al.* (1998). La actividad enzimática (UI), definidas como micromoles de sustrato convertido a producto a la temperatura ensayada por minuto, se expresaron por mg de proteína soluble (actividad específica). Todos los análisis se realizaron como mínimo por duplicado.

10.2.7. MÉTODOS IMMUNOLÓGICOS

10.2.7.1. OBTENCIÓN DE SANGRE Y PREPARACIÓN DE LAS MUESTRAS

La sangre se obtuvo por punción caudal con jeringuillas de 1 ml de capacidad. Con el objetivo de minimizar los efectos estresantes derivados del manejo, el tiempo de manipulación no superó el minuto. Previo a la obtención de suero por centrifugación, la sangre se transfirió a tubos Eppendorf donde se dejó coagular por 2h. Las muestras se almacenaron a -80°C hasta su análisis.

10.2.7.2. OBTENCIÓN DE MUCUS Y PREPARACIÓN DE LAS MUESTRAS

El mucus utilizado en este estudio se obtuvo de piel e intestino. El mucus de piel se obtuvo de la superficie de los juveniles de lubina mediante el método descrito por Bordás *et al.* (1996). El mucus gastrointestinal se preparó mediante una modificación del método descrito por Chambrillón *et al.* (2005) y para ello los peces fueron mantenidos en ayuno las 48 h previas a la toma de muestras. Los intestinos fueron diseccionados, transferidos a una placa de Petri estéril y el mucus extraído mediante el raspado de la parte interior del intestino con una espátula de plástico estéril. Tras la extracción se homogenizó en PBS. Con objeto de eliminar el material particulado y celular, todas las preparaciones de mucus fueron centrifugadas a 20000 g durante 30 min a 4°C, esterilizadas bajo rayos UV y almacenadas a -80°C hasta su análisis.

10.2.7.3. POTENCIAL DEL BROTE RESPIRATORIO DE LOS NEUTRÓFILOS CIRCULANTES (NBT)

Se estimó el brote respiratorio de los neutrófilos circulantes mediante la técnica descrita por Siwicki *et al.* (1993). Esta técnica tiene como objetivo medir la producción de aniones superóxido producidos por los neutrófilos circulantes en sangre a través de la reducción del NBT (Nitroazul de tetrazolio) a formazán en presencia de radicales oxígeno.

10.2.7.4. VÍA ALTERNATIVA DEL COMPLEMENTO

La actividad de la vía alternativa del complemento se determinó según la técnica descrita por Sunyer y Tort (1995) para dorada usando células sanguíneas rojas de conejo (RBC). La dilución de suero causante de la lisis del 50% células sanguíneas se denomina ACH50 y los resultados se presentan como ACH50/ml.

10.2.7.5. LISOZIMA

La actividad de la lisozima se determinó mediante el método turbidimétrico descrito por Anderson and Siwiki (1994) que utiliza la lisis de *Micrococcus lysodeikticus* para la determinación enzimática usando lisozima de clara de huevo disuelto en solución de fosfatos tamponada con estándar. Los resultados se expresaron como unidades de lisozima/ml en muestras de suero y como µg de lisozima·mg^{-1} de proteína en muestras de mucus.

10.2.7.6. CAPACIDAD FAGOCÍTICA DE LOS LEUCOCITOS AISLADOS DEL RIÑÓN ANTERIOR

Se extrajeron los riñones anteriores de los peces, los cuales fueron macerados en un medio de cultivo (MEM) y filtrados a través de una membrana de nylon. La solución celular resultante se colocó sobre un gradiente de Percoll 34%/51% (v/v) con un 10% de solución balanceada de Hanks (HBSS) y fue centrifugada. Se recogieron células depositadas en la interfase, se lavaron por centrifugación y se determinó su concentración. Seguidamente, se ajustó la concentración de leucocitos a la deseada y se incubó durante una hora frente a la bacteria/bolas de látex siguiendo el protocolo descrito por Esteban y Messenger (1997) para *V. anguillarum*. Finalmente la actividad fagocítica se determinó según el método descrito por Blazer (1991).

10.2.7.7. ANÁLISIS DE LOS NIVELES DE PROSTANDINAS

10.2.7.7.1. ESTIMULACIÓN DEL TEJIDO

Una vez diseccionados los tejidos, se pesaron y se digirieron en 4 ml de HBSS sin Ca^{2+} y suplementada al 2% con colagenasa durante 30 min en agitación orbital continua ($100U \cdot min^{-1}$). Después de la digestión, las muestras fueron filtradas a través de una membrana de nylon y lavadas con 4 ml de HBSS sin Ca^{2+}. La suspensión celular obtenida se lavó por centrifugación y el pellet resultante se suspendió en 1 ml de HBSS sin Ca^{2+}. Las células fueron estimuladas durante 30 min con 50 µM Ca^{+} PMA (Phorbol 12-myristate 13-acetate, Sigma Chemicals, CA, USA) *y* 10 µM A321 (Sigma Chemicals, CA, USA) y la reacción se paró con 50 µl ácido fórmico 2M. Las muestras fueron almacenadas a -80°C hasta su purificación.

10.2.7.7.2. PURIFICACIÓN DE EICOSANOIDES

Para la purificación de las fracciones estimuladas (pool de 4 peces/tanque) en primer lugar se precipitaron los posibles restos/desechos por centrifugación. Los sobrenadantes fueron extraídos usando minicolumnas "Sep-pack" de sílice de octadecyl (C18) (Millipore, Watford, Reino Unido) según el método descrito por Powell (1982) y detallado por Bell *et al.* (1994). Se aplicó 1 ml del sobrenadante a la columna, la cual previamente fue prelavada con 5 ml de metanol y 10 ml de agua MiliQ. Seguidamente a la aplicación de la muestra la columna, ésta fue lavada sucesivamente con 10 ml de agua MiliQ, 5 ml de etanol al 15% (v/v) y 5 ml de hexano:cloroformo (65:35; v/v) antes de la elución de los prostanoides con 10 ml de acetato etílico. La solución resultante se evaporó a sequedad bajo nitrógeno, se suspendió de nuevo en 100 µl de metanol y se almacenó a -80°C hasta su uso.

10.2.7.7.3. IMMUNOENSAYO DE PROSTAGLANDINAS

Para este análisis se utilizó un kit específico de análisis de prostaglandinas (Prostaglandin E2 EIA Kit- Monoclonal; Cayman Chemical Co., MI, USA). Este análisis se basa en la competición entre la PGE_2 y un conjugado de PGE_2-acetilcolinaesterasa para una cantidad limitada de anticuerpo monoclonal PGE_2.

10.2.8. PARÁMETROS DE ESTRÉS

10.2.8.1. OBTENCIÓN DE SANGRE Y PREPARACIÓN DE LAS MUESTRAS

La sangre se obtuvo por punción caudal con jeringuillas de 1 ml de capacidad. Con el objetivo de minimizar los efectos estresantes derivados del manejo, el tiempo de manipulación no superó el minuto. La sangre se transfirió a tubos Eppendorf empapados con heparina como anticoagulante y se centrifugó inmediatamente para la obtención de la fracción plasmática. Las muestras se almacenaron a -80°C hasta su análisis.

10.2.8.2. ANÁLISIS DE CORTISOL

La concentración de cortisol en la muestras de plasma fue determinada por radioinmunoanálisis (RIA) (Rotllant *et al.*, 2001). El anticuerpo usyo en el ensayo (Biolink, S.L.; Costa Mesa, CA, USA) fue diluido a una dilución final de 1:6000. La reactividad cruzada de este anticuerpo con el cortisol es del 100%, mientras que con la desoxicorticosterona-21 es de 11,4%, con el desoxicortisol-11 de 8,90% y con la 17α-hydroxyprogesterona de 1,6%. La radiactividad fue cuantificada usyo un cuantificador de centelleo líquido. Los niveles de cortisol fueron expresados en $ng \cdot ml^{-1}$.

10.2.9. CALIDAD DEL FILETE

Con objeto de determinar la calidad del producto final en términos de calidad del filete, los peces fueron mantenidos en ayuno durante las 24 h previas a su sacrificio el cual fue llevado a cabo en hielo. Una vez sacrificados se eviscerаron, filetearon y se mantuvieron a 4°C hasta su análisis. La cocción de los filetes se llevó a cabo en recipientes de aluminio (3x4 cm) durante 10 min en un horno de vapor a 120°C. Inmediatamente después de ser cocinados, se ofrecieron aleatoriamente y en los recipientes cerrados y marcados con un código a un panel de 8 catadores entrenados (ISO 1985, ISO 1993). La evaluación se llevó a cabo en habitaciones aisladas, climatizadas y previstas de una luz estandarizada (ISO 1988). Los parámetros evaluados para las muestras de peces alimentados con las diferentes dietas experimentales se valoraron en una escala continua de 0 a 100 para cada uno de ellos y fueron los siguientes: Olor (marino, sin

olor y aceitoso), apariencia (jugosa, brillo y color), textura (elasticidad, firmeza, jugosidad) y sabor (marino, sin sabor y aceitoso) (Ginés *et al*., 2004)

10.2.10. INOCULACIÓN INTESTINAL in vivo/ex vivo CON PATÓGENOS DE ORIGEN BACTERIANO

10.2.10.1. INOCULACIÓN INTESTINAL ex vivo CON V. anguillarum

Para ello los peces fueron sacrificados en una sobredosis de anestésico, eviscerados y las fracciones anterior y posterior del intestino separadas y mantenidas en solución Ringer (140 mM NaCl, 2,5 mM KCl, 15 mM $NaHCO_3$, 1,5 mM $CaCl_2$, 1mM KH_2PO_4, 0,8 mM $MgSO_4$, 10 mM glucosa y 5 mM tampón HEPES; pH=7,8). En este punto los segmentos fueron invertidos y cerrados en sus terminaciones con fórceps formando un saco e introducidos en recipientes con 10 ml de solución Ringer en continua aireación y agitación por 60 min hasta la estabilización del tejido. Transcurrido este periodo de tiempo la solución fue sustituida por una solución Ringer que contenía 10^7 ufc *V. anguillarum* marcada con fluorescencia $\cdot ml^{-1}$ y la incubación se prolongó otros 60 min. Al final del periodo de exposición al patógeno, la cara exterior de los sacos intestinales (parte mucosa) se lavó tres veces con solución Ringer con objeto de eliminar las bacterias adheridas pero no translocadas. Posteriormente la cara interior de los sacos (parte serosa) intestinales se lavó tres veces para recoger las bacterias translocadas. Las muestras se centrifugaron inmediatamente a 6000g durante 10 min con el objetivo de concentrar las bacterias translocadas y eliminar la fluorescencia disuelta en el sobrenadante. La fluorescencia de la solución resultante se determinó mediante su medida flourimétrica durante 1 s a 480/535 nm (Victor 1420 Multilabel Counter; Wallac).

10.2.10.2. INOCULACIÓN INTESTINAL in vivo CON V. (Listonella) anguillarum/ V. alginolyticus

Los peces fueron sometidos a una infección intestinal con los patógenos *V. (Listonella) anguillarum* (10^8 ufc$\cdot ml^{-1}$ por pez) o *V. alginolyticus* ($2{,}4\cdot10^8$ ufc$\cdot ml^{-1}$ por pez) mediante canulación anal. Una vez transcurrido el tiempo de exposición y con el objetivo de determinar la capacidad del patógeno inoculado de traspasar el epitelio intestinal, se procedió a la caracterización e

identificación de las bacterias presentes en hígado, bazo y riñón anterior mediante su cultivo y posterior análisis. El grado de infección con el patógeno inoculado se determinó mediante la presencia/ausencia del mismo en órganos internos.

10.2.11. AISLAMIENTO Y CARACTERIZACIÓN DE BACTERIAS

Las muestras de hígado, bazo y riñón anterior de los peces sometidos a los paneles de infección se sembraron en TSA, agar infusión cerebro-corazón (BHI) o medio específico para el cultivo de *V. anguillarum* (VAM; Alsina *et al.,* 1994) y se incubaron a 25°C por 24-48 h. Las colonias aisladas se sometieron a un análisis taxonómico mediante pruebas morfológicas, fisiológicas y bioquímicas estándares (Smibert y Krieg, 1981) que fueron completadas con sistemas de análisis comerciales como API 20E.

10.2.12. PERFILES DE MICROFLORA BACTERIANA

10.2.12.1. EXTRACCIÓN DE ADN Y AMPLIFICACIÓN DE 16S RRNA

Previo a la extracción de ADN del intestino posterior (pool de 3peces/tanque; 3 tanques/tratamiento) con los kits de extracción Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA), las muestras fueron pre-tratadas con una solución de lisozima (50 mg/ml de tampón TE por 30 min a 37°C). Una vez extraído el DNA, se chequeó mediante electroforesis y se determinó su concentración con un kit comercial (Quant-it ds DNA HS assay kit; Invitrogen, CA, USA). La amplificación por PCR de los genes 16S rRNA se llevó a cabo el cebador inverso P1(5´-CCG TCA ATT C (AC) T TTG AGT TT-3´) y el cebador directo P2 con GC clamp (5´-CGC CCG CCG CGC CCC GCG CCC CTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG-3´). Los volúmenes finales en cada tubo de PCR (volumen final de 50 µl) fueron los siguientes: 2,0 µl BSA (10 $\mu g \cdot \mu l^{-1}$), 30µl of agua destilada estéril, 5µl 10X Tampón PCR sin $MgCl_2$ (Invitrogen, CA, USA), 1,75µl $MgCl_2$ (50mM, Invitrogen, CA, USA), 4µl dNTPs (2,5mM, Invitrogen, CA, USA), 0,25µl Taq DNA polymerase ($5U \cdot \mu l^{-1}$, Invitrogen, CA, USA), 4µl de cada cebador (10 $pmol \cdot \mu l^{-1}$; MWG-Biotech AG, Ebergberg, Alemania). Los ciclos térmicos aplicados fueron los descritos por Casamayor *et al.* (2000) modificado por Dhanasiri *et al.* (2010). Los productos

obtenidos se mantuvieron a -20°C hasta su análisis por electroforesis en gel con gradiente de desnaturalización (DGGE).

10.2.12.2. ELECTROFORESIS EN GEL CON GRADIENTE DE DESNATURALIZACIÓN

Los análisis por DGGE se llevaron a cabo en un sistema DGGE-Bio-Rad (Bio-Rad, CA, USA). Para ello los productos obtenidos tras la PCR se corrieron en un gel al 6% de acrilamida con un gradiente de 40-60% (dónde el 100% de desnaturalización es urea 7M y 40% formamida). El gel se corrió por 17h a 65V y 60°C en 1x tampón TAE (50X TAE Buffer, Merck, Darmstadt, Alemania). La visualización de las bandas se facilitó mediante su tinción con una dilución 1:10000 de SYBR green (Invitrogen-Molecular Probes, Eugene, USA) durante 45 min. El gel fue escaneado en una campana universal II de Bio-Rad (Bio-Rad, CA, USA) y se optimizó para su análisis con el software Quantity One 4.6.6 software (Bio-Rad, CA, USA) calculándose el coeficiente de Dice.

10.2.13. ANALISIS ESTADÍSTICOS

Todos los datos tratados cumplieron los criterios de normalidad y homogeneidad de la varianza. Se calcularon las medias y desviaciones estándar para cada parémetro evaluado. En función de los datos a analizar, la significación de la diferencia entre los tratamientos dietéticos ($P<0,05$-0,1) fue determinada mediante el análisis de la varianza unidireccional (ANOVA) o de dos vías seguido de pruebas de comparación múltiple (Duncan/Tukey/Scheffe test) (Sokal y Rolf, 1995). En los casos en los que las varianzas no cumplían una distribución normal, se aplicaron análisis no parámetricos (test de Kruskall–Wallis). Los análisis fueron llevados a cabo mediante el uso del software Statgraphics (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA).

10.3. RESUMENES DE LOS EXPERIMENTOS

10.3.1. CAPÍTULO 3: ESTIMULACIÓN DE LA RESPUESTA INMUNE Y MEJORA DE LA RESISTENCIA A ENFERMEDADES EN JUVENILES DE LUBINA (Dicentrarchus labrax) ALIMENTADOS CON MANANO OLIGOSACÁRIDOS (MOS)

El objetivo de este estudio fue determinar los efectos derivados de la suplementación dietética con MOS (Bio-Mos©, Alltech Inc., USA) en juveniles de lubina sobre el crecimiento, utilización del alimento, sistema inmune y resistencia a enfermedades. Para ello lubinas de 35 g (peso inicial) a una densidad de 3 $kg \cdot m^{-3}$ fueron alimentadas durante un periodo de 67 días con una dieta comercial suplementada con niveles de inclusión crecientes de MOS (0, 2 y 4 $g \cdot kg^{-1}$). Se evaluaron la tasa de conversión alimenticia, la tasa de crecimiento específica, la composición proximal de cuerpo entero, la capacidad fagocítica de leucocitos de riñón anterior, la producción de aniones superóxido por neutrófilos circulantes, la actividad de la lisozima y la vía alternativa del complemento en suero, así como, la morfología intestinal y hepática. Los resultados demostraron el efecto positivo del MOS sobre el crecimiento independientemente de la dosis suministrada. Se detectó un menor nivel de vacuolización en los hepatocitos así como una mejor ordenación de éstos alrededor de los cordones sinusoides indicando una mejor utilización de los nutrientes dietéticos. A nivel de microscopía óptica no se detectaron diferencias en la morfología intestinal. El nivel de inclusión de MOS al 4 $g \cdot kg^{-1}$ produjo una estimulación de la capacidad fagocítica de los leucocitos de riñón anterior. Se encontró una correlación positiva entre las actividades del lisozima y de la vía alternativa del complemento en suero y el nivel de inclusión de MOS en las dietas.

Finalizado el experimento de engorde, se llevaron a cabo ensayos de cohabitación (ratio 3:1) e inoculación intestinal con el patógeno *V. alginolyticus*. En el primer caso, tras 21 días de cohabitación con peces infectados, los peces alimentados con la dieta suplementada con 4$g \cdot kg^{-1}$ MOS no presentaron presencia del patógeno inoculado en los órganos internos frente al 33% de peces infectados en la dieta control. Finalmente, en el experimento de inoculación intestinal se detectó que a las 24 h post-inoculación la suplementación con MOS no causó ningún efecto mientras que a las 48 h post-inoculación el número de peces infectados en la dieta control doblaba el número detectado en las dietas suplementadas con MOS.

10.3.2. CAPÍTULO 4: MEJORA DE LA TASA DE CONVERSIÓN ALIMENTICIA, PRODUCCIÓN DE MUCUS Y ESTIMULACIÓN DE LA RESPUESTA INMUNE EN JUVENILES DE LUBINA (Dicentrarchus labrax) ALIMENTADOS CON MANANO OLIGOSACÁRIDOS (MOS)

El objetivo de este estudio fue determinar los efectos de la suplementación dietética con MOS (Bio-Mos©, Alltech Inc., USA) en juveniles de lubina sobre el crecimiento, digestibilidad, morfología hepática, enzimas relacionados con el metabolismo de carbohidratos y lípidos, propiedades organolépticas del filete, parámetros inmunes y producción de mucus a nivel intestinal. La suplementación con MOS redujo significativamente la actividad de las enzimas lipogénicas en hígado lo que resultó en una menor vacuolización lipídica de los hepatocitos así como una mejor ordenación de éstos alrededor de los espacios sinusiodes. Este hecho puede estar asociado a una mejor utilización energética y alimenticia ya que se detectó una mejor tasa de conversión alimenticia así como una reducción en los niveles de ingesta en los peces alimentados con MOS. La inclusión en las dietas de MOS a razón de 4 y 6 $g \cdot kg^{-1}$, estimuló la capacidad fagocítica de los leucocitos aislados del riñón anterior tras 30, 45 y 60 días de suplementación. Los estudios morfológicos cuantitativos en intestino mostraron un efecto potenciador dosis dependiente sobre el número de células secretoras de mucinas ácidas por unidad de área, hecho que podría ser relacionado con la mejora en la resistencia a enfermedades encontrada en previos experimentos. La inclusión de MOS en dietas para lubina no tuvo ningún efecto sobre la composición proximal o los parámetros sensoriales del producto final, en términos de calidad del filete.

10.3.3. CAPÍTULO 5: REDUCCIÓN DE LA TRANSLOCACIÓN INTESTINAL EN JUVENILES DE LUBINA (Dicentrarchus labrax) ALIMENTADOS CON MANANO OLIGOSACÁRIDOS (MOS)

El objetivo de este estudio fue determinar los efectos derivados de la suplementación dietética con MOS (Bio-Mos©, Alltech Inc., USA) en juveniles de lubina sobre la producción de mucus intestinal, la actividad de algunos parámetros inmunes seleccionados en mucus, así como sobre la translocación intestinal *in vivo* y *ex vivo* para juveniles de lubina. Para ello, los peces fueron suplementados con 4 $g \cdot kg^{-1}$ MOS durante un periodo de 8 semanas, tras la cuales se observó un aumento significativo ($P<0,05$; $n=240$) en la altura, anchura y área de la vellosidades del intestino anterior en los peces alimentados con MOS. El intestino posterior de los peces alimentados con MOS presentaron vellosidades más cortas pero más anchas, traduciéndose en un aumento de la superficie total de estas ($P<0,05$; $n=240$). El recto de los peces alimentados con MOS presentó vellosidades más cortas que los peces alimentados con la dieta control ($P<0,05$; $n=240$). Los análisis morfológicos mostraron un aumento significativo en el número de células secretoras de mucinas ácidas por unidad de área así como una mayor densidad de granulocitos eosinófilos (ECGs) infiltrados. En términos de parámetros inmunes en mucus intestinal, los peces alimentados con MOS presentaron una mayor actividad de la lisozima, hecho que puede estar relacionado con la reducción en las tasas de translocación bacteriana, tanto *in vivo* como *ex vivo*, encontradas en el presente experimento. No se encontraron efectos derivados de la suplementación con MOS en los parámetros inmunes del mucus de piel.

10.3.4. CAPÍTULO 6: RESPUESTA A ESTRÉS E INFECCION EN JUVENILES DE LUBINA (Dicentrarchus labrax) ALIMENTADOS CON MANANO OLIGOSACÁRIDOS (MOS)

El objetivo de este estudio fue determinar los efectos derivados de la suplementación dietética con manano oligosacáridos (MOS; Bio-Mos© Aquagrade, Alltech Inc., USA) en la respuesta a infección combinada con estrés, combinando una infección intestinal con *V. anguillarum* y un panel de estrés por confinamiento. A su vez, se incluyeron estudios sobre los niveles de cortisol plasmático y perfiles de microflora intestinal. Para ello, los peces fueron suplementados con 4 $g \cdot kg^{-1}$ MOS durante un periodo de 8 semanas. Tras este periodo los peces alimentados con MOS presentaron un mayor peso ($P<0,05$) y longitud total ($P<0,05$) que los peces alimentados con la dieta control. Los parámetros de crecimiento, en términos de tasa de crecimiento específica y crecimiento relativo, siguieron el mismo patrón aumentando significativamente ($P<0,05$) en los peces alimentados con MOS respecto a los alimentados con la dieta control.

Finalizado el experimento de engorde, se expuso a los peces a una inoculación intestinal con el patógeno *V. anguillarum* combinado con un panel de estrés por confinamiento. La inclusión de MOS en la dieta redujo la mortalidad acumulada causada por *V. anguillarum* de un 66% a un 12,5% y de un 54,1% a un 25% en los grupos sometidos a estrés + infección y a infección, respectivamente. Los resultados revelaron el efecto positivo de la suplementación con 4$g \cdot kg^{-1}$ MOS en juveniles de lubina en relación a la protección frente a *V. anguillarum* además de una reducción del efecto del estrés sobre los perfiles de microflora intestinal. Ambos factores, junto con la mayor producción de mucus intestinal y presencia de granulocitos eosinófilos infiltrados en la mucosa intestinal encontrados en previos experimentos, sugieren el refuerzo de la eficiencia de la barrera intestinal como primera línea de defensa frente a microorganismos patógenos en juveniles de lubina alimentados con MOS.

10.3.5. CAPÍTULO 7: REGULACIÓN DE LA PRODUCCIÓN DE MUCUS INTESTINAL EN JUVENILES DE LUBINA (Dicentrarchus labrax) ALIMENTADOS CON MANANO OLIGOSACÁRIDOS (MOS)

El objetivo de este estudio fue tener una visión más clara sobre la regulación de la secreción de mucus intestinal causada por la inclusión de manano oligosacáridos (MOS) en dietas para lubina (*Dicentrarchus labrax*), y si este proceso está influenciado por el potencial de producción de prostaglandinas (PGs), en base a que estos eicosanoides son moduladores importantes de la secreción y síntesis de mucus en otros vertebrados. Para ello, los peces fueron suplementados con 4 $g \cdot kg^{-1}$ MOS durante un periodo de 8 semanas. Tras este periodo, los peces alimentados con MOS presentaron un mayor peso y longitud total ($P<0,05$) que los peces alimentados con la dieta control. Los parámetros de crecimiento, en términos de tasa de crecimiento específica y crecimiento relativo, siguieron el mismo patrón aumentando significativamente ($P<0,05$) en los peces alimentados con MOS respecto a los alimentados con la dieta control. Además, los peces alimentados con MOS presentaron un mayor número de células secretoras de mucus en intestino anterior y posterior. Finalmente, el intestino posterior estimulado de los peces alimentados con MOS presentaron mayor ($P<0,05$) producción de PGs que los de los peces alimentados con dieta control. Mientras que no se encontraron diferencias en intestino anterior y riñón anterior. Este estudio muestra que la suplementación con MOS mejora el potencial de producción de PGs en intestino posterior en lubina en relación a una mayor presencia de células secretoras de mucus y leucocitos en este tejido.

10.4. CONCLUSIONES

1. La inclusión de MOS a razón de 4 g·kg^{-1} en dietas para juveniles de lubina potenció el crecimiento (Capítulo 3, 6 y 7) de esta especie cuando los peces fueron cultivados a densidades de experimentación bajas (3-4 kg·m^{-3}), en relación a una mayor tasa de ingesta. Por otro lado, cuando los peces fueron cultivados a densidades experimentales superiores (7-10 kg·m^{-3}) la incorporación de MOS en dietas para lubina a niveles de 4 y 6 g kg^{-1} mejoraron la tasa de conversión alimenticia (Capítulo 4).

2. La suplementación dietética con MOS en dietas para juveniles de lubina tuvo como resultado una menor vacuolización lipídica en hígado y tamaño de los hepatocitos de acuerdo con la reducción en la actividad de las enzimas lipogénicas G6PD y ME encontradas en este tejido y con la tendencia hacia la disminución del índice viscerosomático y al aumento de peso eviscerado de los peces alimentados con MOS (Capítulo 4).

3. Los parámetros sensoriales así como la composición proximal y perfil de ácidos grasos del filete de los peces alimentados con MOS no se vieron afectados a lo largo de la totalidad del ciclo de producción, independientemente del nivel incluido en la dieta.

4. La incorporación de MOS a razón de 4 y 6 g·kg^{-1} en dietas para juveniles de lubina activó la respuesta immune celular y humoral no específica en términos de capacidad fagocítica de leucocitos de riñón anterior (Capítulos 3 y 4) así como en términos de producción de eicosanoides en intestino posterior (Capítulo 7). A su vez, la inclusion de MOS (4 g·kg^{-1}) en dietas para esta especie produjo un efecto dosis dependiente sobre los niveles de lisozima y actividad de la vía alternativa del complemento (Capítulo 3).

5. La inclusión de MOS (4 g·kg^{-1}) en dietas para juveniles de lubina redujo la translocación *in vivo* de *V. alginolyticus* (Capítulo 3), así como la translocación *ex vivo* e *in vivo* del patógeno *V. anguillarum* (Capítulo 5). Cuando los peces alimentados con MOS se expusieron a un sistema combinado de infección intestinal *in vivo* con el patógeno *V. anguillarum* y un panel de estrés por confinamiento, la mortalidad y los porcentajes de infección se vieron reducidos en peces alimentados con MOS (Capítulo 6).

6. La inclusión de MOS (4 $g \cdot kg^{-1}$) en dietas para juveniles de lubina durante dos meses potenció algunos mecanismos implicados en la primera línea de defensa intestinal frente a organismos patógenos, tales como, la infiltración de granulocitos eosinófilos, la producción de mucus, la actividad lisozímica del mucus y la reducción de los efectos derivados del estrés sobre las comunidades bacterianas que componen la microflora intestinal. Todo esto sugiere que la potenciación de los mecanismos implicados en la primera línea de defensa del tracto gastrointestinal es la principal causa de la protección frente a organismos patógenos de los peces alimentados con MOS (Capítulo 5).

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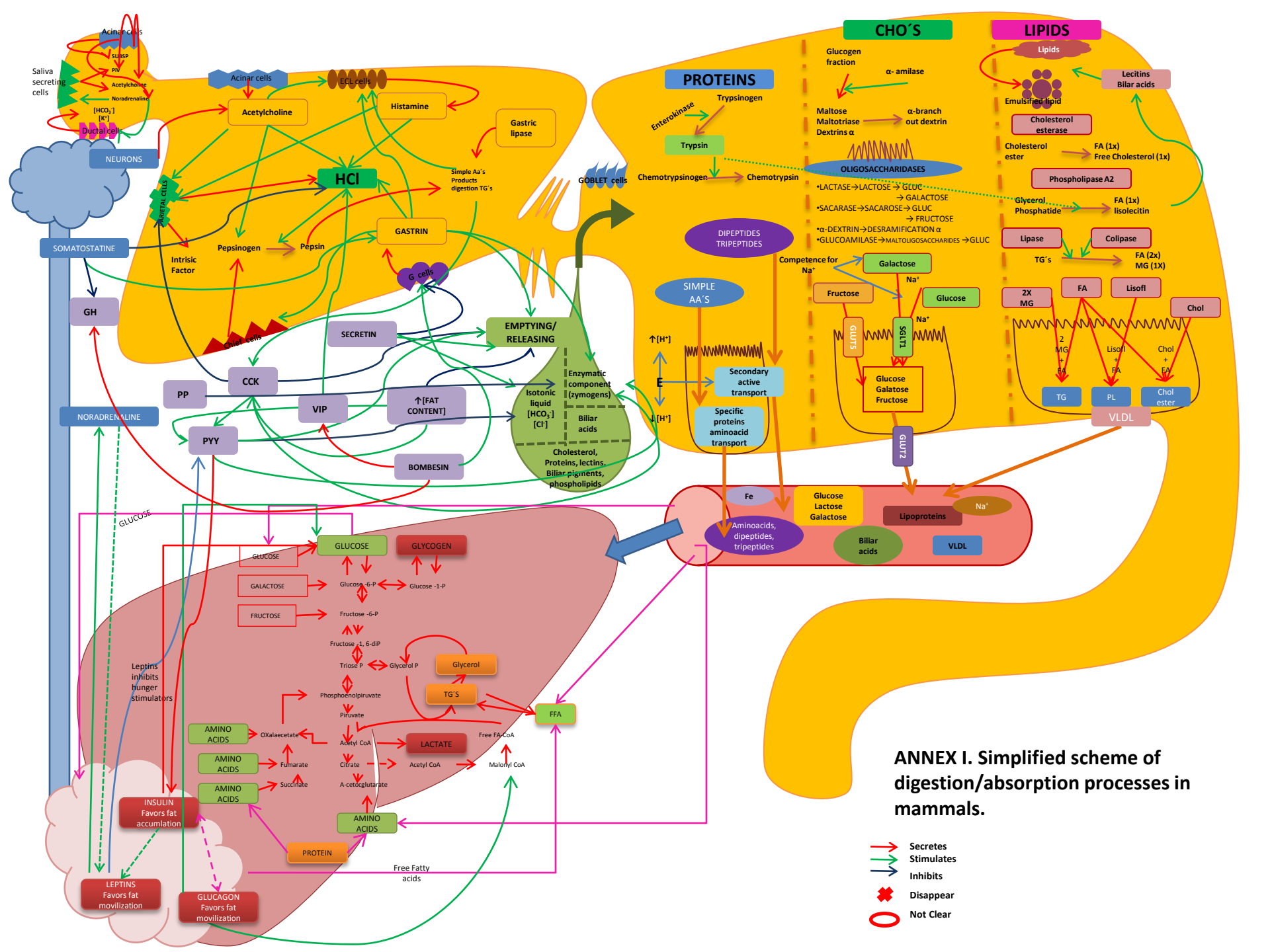
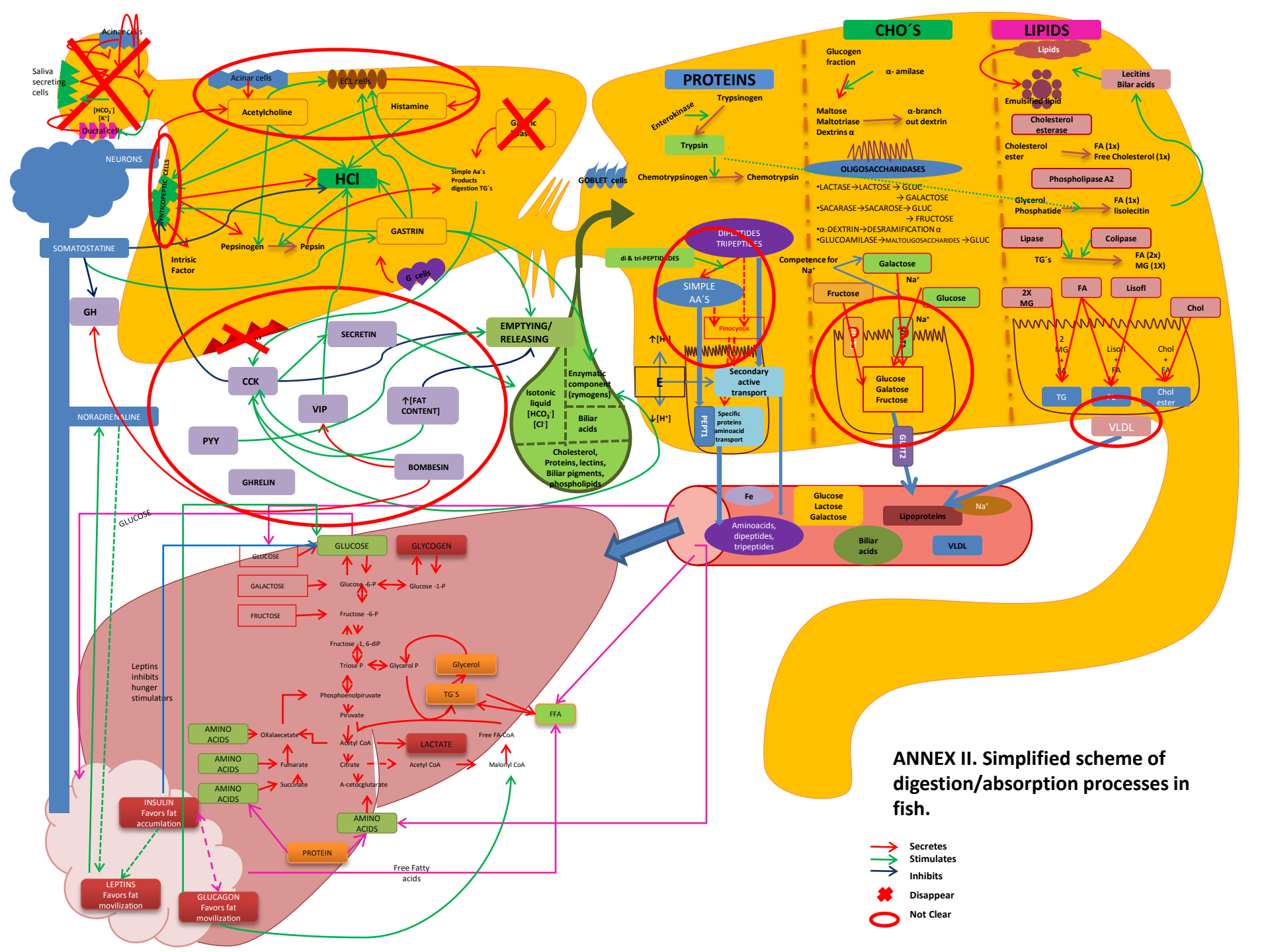
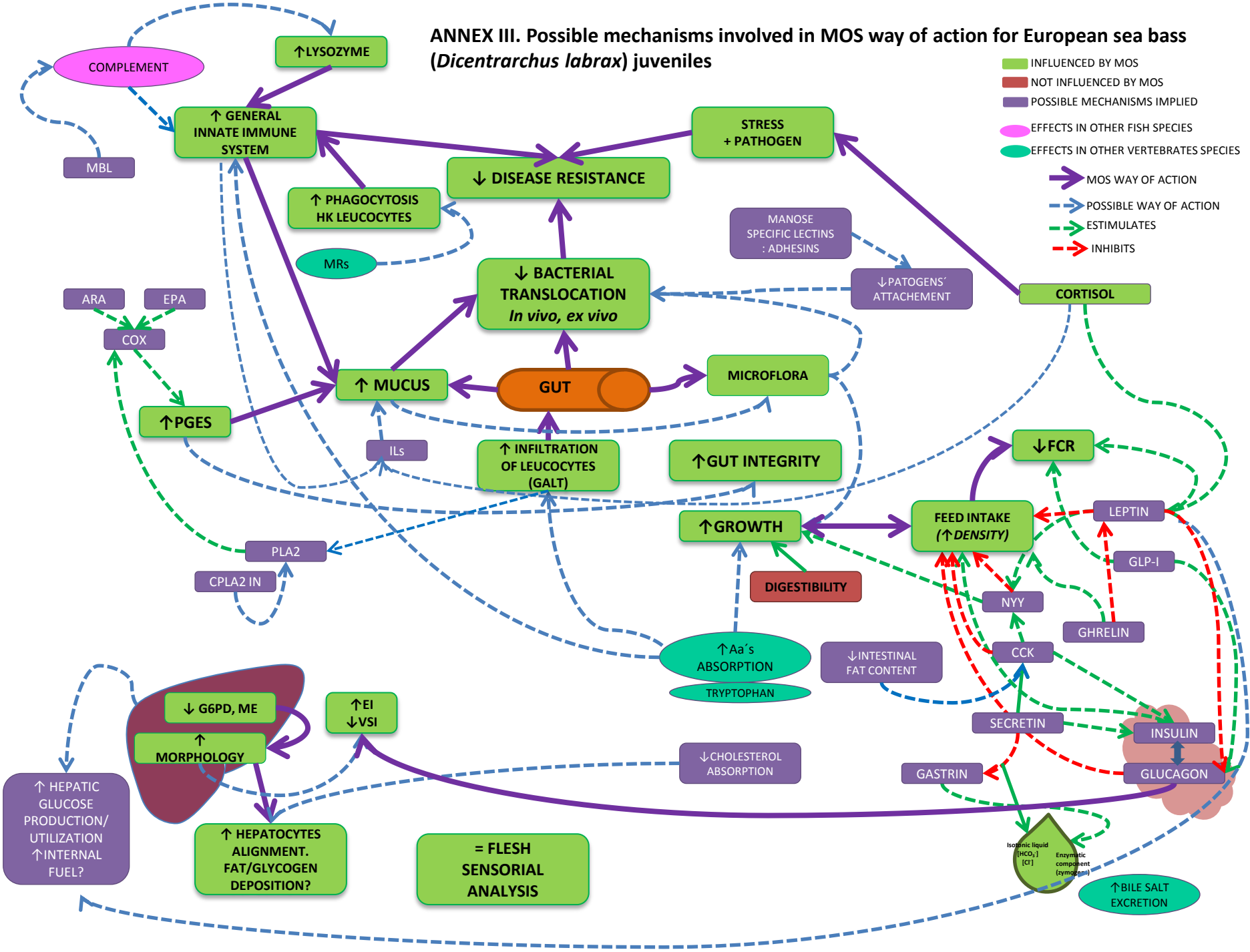


ANNEX I. Simplified scheme of digestion/absorption processes in mammals.

- Secretes
- Stimulates
- Inhibits
- Disappear
- Not Clear


Acinar cells
Saliva secreting cells
[HCO3-] [K+]
Ductal cells
NEURONS
Acinar cells
ECL cells
Acetylcholine
Histamine
PARIETAL CELLS
HCl
Simple Aa´s Products digestion TG´s
GOBLET cells
SOMATOSTATINE
Intrisic Factor
Pepsinogen
Pepsin
GASTRIN
G cells
GH
SECRETIN
CCK
VIP
↑[FAT CONTENT]
PYY
BOMBESIN
GHRELIN
NORADRENALINE
EMPTYING/ RELEASING
Enzymatic component (zymogens)
Isotonic liquid [HCO3-] [Cl-]
Biliar acids
Cholesterol, Proteins, lectins, Biliar pigments, phospholipids
PROTEINS
Trypsinogen
Enterokinase
Trypsin
Chemotrypsinogen
Chemotrypsin
DIPEPTIDES TRIPEPTIDES
di & tri-PEPTIDES
SIMPLE AA´S
Pinocytosis
↑[H+]
↓[H+]
E
Secondary active transport
PEPT1
Specific proteins aminoacid transport
CHO´S
Glucogen fraction
α- amilase
Maltose Maltotriase Dextrins α
α-branch out dextrin
OLIGOSACCHARIDASES
•LACTASE→LACTOSE→ GLUC → GALACTOSE
•SACARASE→SACAROSE→ GLUC → FRUCTOSE
•α-DEXTRIN→DESRAMIFICATION α
•GLUCOAMILASE→MALTOLIGOSACCHARIDES →GLUC
Competence for Na+
Galactose
Fructose
Glucose
Na+
SGLT1
GLUT2
Glucose Galatose Fructose
LIPIDS
Lipids
Emulsified lipid
Lecitins Bilar acids
Cholesterol esterase
Cholesterol ester
FA (1x) Free Cholesterol (1x)
Phospholipase A2
Glycerol Phosphatide
FA (1x) lisolecitin
Lipase
Colipase
TG´s
FA (2x) MG (1X)
2X MG
FA
Lisofl
Chol
2 MG + FA
Lisofl + FA
Chol + FA
TG
Chol ester
VLDL
Fe
Glucose Lactose Galactose
Aminoacids, dipeptides, tripeptides
Biliar acids
Lipoproteins
VLDL
Na+
GLUCOSE
GLUCOSE
GALACTOSE
FRUCTOSE
GLYCOGEN
Glucose -6-P
Glucose -1-P
Fructose -6-P
Fructose -1, 6-diP
Triose P
Glycerol P
Glycerol
TG´S
FFA
Phosphoenolpiruvate
Piruvate
AMINO ACIDS
OXalaecetate
Acetyl CoA
LACTATE
Free FA-CoA
Fumarate
Citrate
Acetyl CoA
Malonyl CoA
Succinate
A-cetocglutarate
AMINO ACIDS
Leptins inhibits hunger stimulators
INSULIN Favors fat accumulation
LEPTINS Favors fat movilization
GLUCAGON Favors fat movilization
PROTEIN
Free Fatty acids
ANNEX II. Simplified scheme of digestion/absorption processes in fish.
Secretes
Stimulates
Inhibits
Disappear
Not Clear


ANNEX III. Possible mechanisms involved in MOS way of action for European sea bass (*Dicentrarchus labrax*) juveniles
INFLUENCED BY MOS
NOT INFLUENCED BY MOS
POSSIBLE MECHANISMS IMPLIED
EFFECTS IN OTHER FISH SPECIES
EFFECTS IN OTHER VERTEBRATES SPECIES
MOS WAY OF ACTION
POSSIBLE WAY OF ACTION
ESTIMULATES
INHIBITS
COMPLEMENT
↑LYSOZYME
↑ GENERAL INNATE IMMUNE SYSTEM
MBL
STRESS + PATHOGEN
↓ DISEASE RESISTANCE
↑ PHAGOCYTOSIS HK LEUCOCYTES
MRs
MANOSE SPECIFIC LECTINS : ADHESINS
↓ BACTERIAL TRANSLOCATION *In vivo, ex vivo*
↓PATOGENS´ ATTACHEMENT
CORTISOL
ARA
EPA
COX
↑ MUCUS
GUT
MICROFLORA
↑PGES
ILs
↑ INFILTRATION OF LEUCOCYTES (GALT)
↑GUT INTEGRITY
↓FCR
↑GROWTH
FEED INTAKE (↑*DENSITY*)
LEPTIN
GLP-I
PLA2
CPLA2 IN
DIGESTIBILITY
NYY
GHRELIN
↑Aa´s ABSORPTION
TRYPTOPHAN
↓INTESTINAL FAT CONTENT
CCK
↓ G6PD, ME
↑ MORPHOLOGY
↑EI ↓VSI
SECRETIN
INSULIN
GLUCAGON
↓CHOLESTEROL ABSORPTION
GASTRIN
↑ HEPATIC GLUCOSE PRODUCTION/ UTILIZATION ↑INTERNAL FUEL?
↑ HEPATOCYTES ALIGNMENT. FAT/GLYCOGEN DEPOSITION?
= FLESH SENSORIAL ANALYSIS
Isotonic liquid [HCO_3^-] [Cl^-]
Enzymatic component (zymogens)
↑BILE SALT EXCRETION