UAlg
UNIVERSIDADE DO ALGARVE

UNIVERSITETET I BERGEN

UNIVERSITY OF THE ALGARVE

The effects of mannan-oligosaccharide supplementation on the skin and gut epithelium health status of European seabass (*Dicentrarchus labrax*)

by

Marco Freire Custódio

Dissertation for obtaining the master degree in:

Aquaculture and Fisheries – Specialization in Aquaculture

Resident supervisor: Dr. Maria Teresa Dinis (University of the Algarve, Portugal)

External supervisor: Dr. Karin Pittman (University of Bergen, Norway)

2014

Marco Freire Custódio

The effects of mannan-oligosaccharide supplementation on the skin and gut epithelium health status of European seabass (*Dicentrarchus labrax*).

Dissertation for obtaining the Master degree in Aquaculture and Fisheries – Specialization in Aquaculture, submitted to the Faculty of Science and Technology from the University of the Algarve.

Resident supervisor - Dr. Maria Teresa Dinis
Title – Professor Emeritus
Affiliation – Center of Marine Sciences, University of the Algarve

External supervisor - Dr. Karin Pittman
Title – Professor
Affiliation – Department of Biology, University of Bergen

Host institution - Department of Biology, University of Bergen

2014

The effects of mannan-oligosaccharide supplementation on the skin and gut epithelium health status of European seabass (*Dicentrarchus labrax*).

Declaration of authorship of this dissertation:

I declare myself to be the author of the present work, which is original and unpublished. Consulted authors and works are properly cited in the text and appear in the list of references included.

Acknowledgements

First of all, I would like to say thank you from all my heart to my supervisor Dr. Karin Pittman for giving me the opportunity to develop my master dissertation under her supervision at the University of Bergen, for all the guidance, help, encouragement and the precious knowledge that she shared with me. A special thanks to Aurora Campo, for helping me from the very beginning of my Norwegian adventure, all the advice, the patience and the priceless mentoring on every aspect of my thesis development. Thank you to Eivind Schartum for being a great lab mate, for sharing is knowledge and showing me some very interesting facts and places about Bergen.

Thank you to Dr. Maria Teresa Dinis for the counseling and advice during all the process, her valuable opinion and experience. A big thank you to my dear colleagues from the master course for walking along with me in this discovery journey of the incredible field of aquaculture, the companionship and the good moments in my beloved city of Faro.

The biggest thank you to my family for all the support and love. To my beautiful and lovely mother, Luzia Freire, for being the example that I try to follow every day, for the words of comfort, for the support, for the trust and for giving me so much. To my father, Manuel Custódio, because without his help I would never had been able to reach this far, a precious pillar in my life. To all my dear brothers and friends, for shaping me into the person I am today, for being my best advisors and for the sincere friendship.

I would also like to thank all the other people who crossed path with me at some point in my life. Every single one of you had an effect on who I turned to be, the way I think, the way I feel and the path that I chose.

And last but never the least, an honorable thanks to a great scientist and prodigious human being, Charles Robert Darwin, who had such a major impact on me since the day I first “met” him. An inspiration and a guiding light.

Abstract

In the present study we assessed the immune-modulatory response of the anterior and posterior gut epithelium and skin of European sea bass when fed two different commercial diets with soybean meal that differ exclusively in the oil type – fish oil and soybean oil - and the effects of their supplementation with MOS.

Fish were fed non-supplemented and supplemented diets with 4 $g.kg^{-1}$ MOS for 8 weeks. All diets resulted in similar weight gains. Microscopic analysis of the anterior gut revealed that the soybean-oil diet with MOS displayed increased ($P<0.05$) mucous cell area and density compared with its control and the fish-oil based diet with MOS. In the posterior gut no effects on cell density and area were detected in fish fed MOS relative to the controls, however there was an oil-type dependent effect, where fish fed fish-oil based diets had bigger cells ($P<0.05$) then fish fed soybean-oil based diets. In the skin, no differences on mucous cells parameters were observed between diets.

The mucous cells from the skin are larger ($P<0.01$) than gut's, and within the gut, the fish fed soybean-oil diets presented bigger ($P<0.05$) cells in the anterior gut compared with the posterior region. Comparing cell densities, the anterior gut has a higher ($P<0.01$) density than the posterior gut and the skin, regardless the diet.

MOS appears to modulate the innate immunity in the anterior gut. When the diet was soybean-oil based, MOS resulted on a greater storage capacity and density of the mucous cells. A potential effect is also suggested when added to the fish oil diets, with a possibly different mechanism of modulation.

This study shows that modulation of mucosal tissues is key to improve resistance against pathogens and that diet composition and prebiotics supplementation are fundamental in the ability of the tissue to exhibit that response.

Keywords: European seabass, mucous cells, mannan-oligosaccharides, gut health, soybean oil, microbiota.

Sumário

O robalo europeu (*Dicentrarchus labrax*) é uma espécie de grande importância para a aquacultura mediterrânica e é de grande interesse para os produtores poder estimular a saúde dos peixes de modo a melhorar a produção e proporcionar peixes saudáveis a consumidores cada vez mais conscientes e preocupados com o bem-estar animal.

O estado de saúde é definido pelo estado de equilíbrio entre o indivíduo, os patógenos e o ambiente. Um interveniente de grande peso na manutenção desse equilíbrio é o sistema imunitário. Fazendo parte deste, as barreiras epiteliais dos organismos são uma barreira de defesa de primeira linha que estão munidas de agentes imunológicos que compõem o sistema imunitário inato. As células produtoras de muco são um grupo de células especializadas que habitam os epitélios dos peixes e são intervenientes de grande relevância do sistema imunitário inato, prevenindo a entrada de microrganismos patogénicos no organismo através da síntese e secreção de muco na superfície do epitélio. A barreira de muco funciona como uma barreira física e é também constituída por compostos ativos com propriedades antibacterianas. O epitélio intestinal e a pele do peixe, que são os tecidos alvo deste estudo, estão em contato direto e constante com o meio externo, logo mais expostos a agentes patogénicos e é de maior importância que os seus componentes imunitários estejam totalmente funcionais.

As rações são um modulador chave do sistema imunitário dos peixes em aquacultura, visto que a maioria dos seus requerimentos nutricionais são obtidos através da alimentação. A generalidade dos peixes, incluindo o robalo, necessitam de uma alta percentagem de proteína de elevado valor biológico e de ácidos gordos essenciais (Ómega-3), que são obtidos em quantidades ótimas a partir de farinhas e óleos de peixe. No entanto, esta dependência tem vindo a contribuir para a imensa pressão colocada nos pesqueiros pelo sector das pescas, colocando em risco a sua sustentabilidade e aumentando os preços das matérias-primas. Por esse motivo, fontes alternativas desses nutrientes essenciais tem vindo a ser investigadas e certos vegetais, como a soja, apresentam-se como fontes adequadas com vantagens a nível económico e ambiental. No entanto, a utilização de fontes alternativas que não constituem uma fonte natural de alimento à qual o organismo de certos peixes marinhos (como o robalo) esteja adaptado pode resultar em efeitos secundários indesejáveis devido à introdução de anti nutrientes, que foram já identificados

na soja, e que interferem com o normal funcionamento do sistema gastrointestinal e induzem alterações no sistema imunitário.

Muito recentemente, outro foco bastante relevante tem sido colocado nos efeitos de imunoestimulantes, como os pré-bióticos, quando adicionados a rações com o propósito de estimular a capacidade e rapidez de reação do sistema imunitário contra bactérias patogénicas. Pensa-se que a inclusão destes em rações comerciais que incluem elementos de origem vegetal possa produzir efeitos positivos a nível do desenvolvimento e imunidade dos peixes. Alguns estudos reportam rácios de conversão de alimento mais baixos, melhorias nas taxas de crescimento, aumento da área do epitélio intestinal e das microvilosidades intestinais, diminuição de infeções bacterianas, modulação da flora intestinal e aumento do número de células de muco em várias espécies cultivadas em aquacultura.

Bio-Mos® é uma formulação comercial constituído essencialmente por mannan-oligossacáridos (MOS) e é um pré-biótico tradicionalmente utilizado em rações para gado com excelentes resultados na promoção da saúde intestinal. Portanto, experiências em peixes eram inevitáveis e resultados promissores foram já publicados para algumas espécies, incluindo para o robalo. Estudos prévios demonstraram que a inclusão de MOS na dieta do robalo resulta no aumento do número de células de muco e da densidade de leucócitos na *lamina propria* do intestino, bem como dobras intestinais mais largas, vilosidades intestinais mais compridas, maior crescimento, menor infeção por *Vibrio* spp., etc.

Neste estudo queremos determinar a resposta imuno-modulatória no epitélio do intestino anterior e posterior e na pele do robalo europeu quando alimentado com duas rações comerciais que já incluem farelo de soja na sua formulação e que diferem unicamente no tipo de óleo adicionado – óleo de peixe vs. óleo de soja – e os efeitos da adição de MOS a essas mesmas formulações.

Os peixes foram alimentados com rações não-complementadas e rações complementas com 4 $g.kg^2$ MOS (Bio-Mos®, Alltech Inc, USA). Todas a dietas resultaram em ganhos de massa semelhantes. A análise microscópica do intestino anterior revelou que a ração com óleo de soja complementada com MOS aumentou ($P<0.05$) a área das células de muco e a sua densidade no epitélio comparando com a ração controlo correspondente e a ração com óleo de peixe com MOS adicionado. No intestino posterior não foram observados efeitos significativos na dimensão e densidade das células nos peixes alimentados com MOS em relação aos controlos. No entanto, foi verificado um efeito

relacionado com o tipo de óleo usado, onde os peixes alimentados com ração à base de óleo de peixe apresentaram células de muco maiores ($P<0.05$) do que os peixes alimentados com ração à base de óleo de soja. Na pele não foram observadas quaisquer diferenças nos parâmetros celulares quantificados entre as várias dietas.

As células de muco da pele apresentam-se naturalmente maiores ($P<0.01$) do que às do intestino. Relativamente ao intestino, os peixes alimentados com rações com óleo de soja apresentaram células maiores ($P<0.05$) no intestino anterior comparativamente à região posterior. Em termos de densidades, o intestino anterior apresenta uma maior ($P<0.01$) densidade de células de muco em comparação com o intestino posterior e a pele, independentemente da dieta ingerida.

Os MOS aparentam estimular o sistema imunitário inato no intestino anterior quando utilizado como complemento em dietas que contenham óleos de soja, resultando numa maior capacidade de armazenamento das células de muco, sugerida pelo aumento de tamanho das células, e um aumento da sua densidade. Um possível efeito modulatório é também sugerido quando MOS é adicionado a rações com óleo de peixe, embora não tão evidente e por um mecanismo de modulação diferente.

Este estudo, portanto, demonstra que a modulação dos tecidos da mucosa é um ponto-chave no melhoramento da resistência contra microrganismo patogénicos e que o tipo de dieta e complementação com pré-bióticos são fundamentais na capacidade dos tecidos de exibirem essa resposta.

Index

List of Figures

List of Tables

1. Introduction

1.1. Aquaculture Production

Aquaculture is the fastest growing food producing sector in the world and is a major contributor to global food supply and economic growth in many countries. It is seen not anymore as an alternative to wild capture fisheries but more as mandatory activity in order to satisfy the increasing demand for seafood worldwide, since fisheries have reached the point of overexploitation. The Food and Agriculture Organization of the United Nations (FAO) estimated that in 2012, aquaculture production was around 66.5 million tonnes (not including aquatic algae), up by 6% from 62.7 million tonnes in 2011. This is based on preliminary data to be published on March 2014 (FAO, 2013a). World aquaculture production has increased steadily in the last two decades while capture fisheries has plateaued (Figure 1.1).

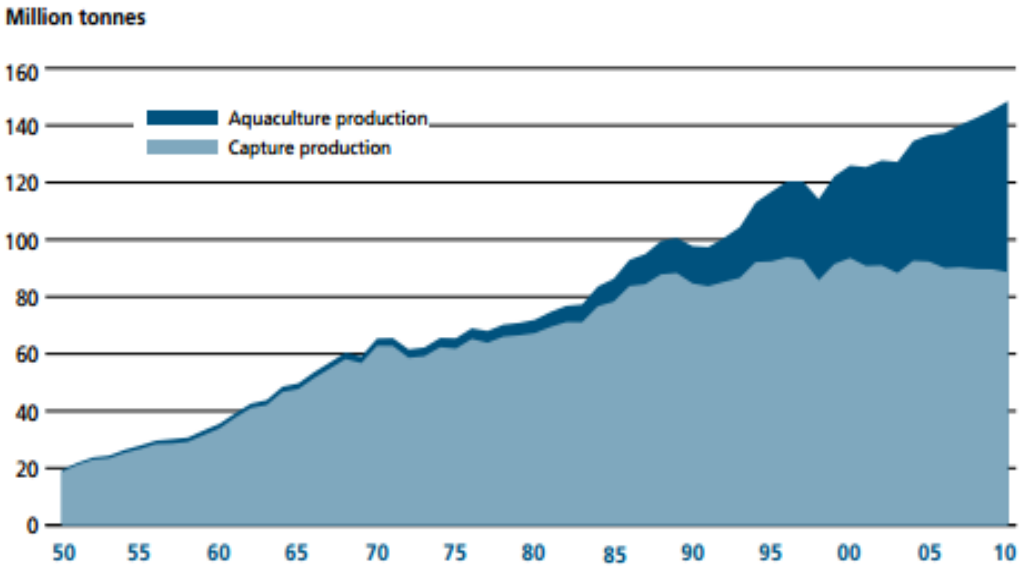

Figure 1.1: World capture fisheries and aquaculture production from 1950 to 2010 (FAO, 2012).

In 2010, the aquaculture production by the 27 European Union Member States reached 1.26 million tonnes and 3.1 billion Euros. It represents 1.6 % of the world production in volume but twice that (3.3 %) in value, for 2010. The EU production is mainly concentrated in France, Greece, Italy, Spain and United Kingdom. In terms of produced volume, Spain is the largest producer (20%), followed by France (18%), UK (16%), Italy (12%) and Greece

(9%), together yielding 75% of the total EU production in volume. In terms of value, France becomes the largest producer (21%), followed by the UK (19%), Spain (13%), Greece (12%) and Italy (11%), representing 76% of all the EU aquaculture value (Scientific, Technical and Economic Committee for Fisheries, 2013). In comparison, Portuguese aquaculture production in 2010 was approximately 8228 tonnes (0.7%) with a value of 47 million Euros (1.9%). In 2011, the production reached 9166 tonnes and a value of 58 million Euros, which represents an increase in quantity (+11.4%) and in value (+23.3%), comparing with the previous year (Instituto Nacional de Estatística, 2013). Nevertheless, the production increase is mainly due to a highly intensive production of turbot (*Psetta maxima*) which has compensated for the decrease of seabass and seabream production caused by the economic crisis in Portugal (Instituto Nacional de Estatística, 2013).

1.2. European Seabass

The European seabass (*Dicentrarchus labrax,* L. 1758; Moronidae; Perciformes) is a carnivorous marine fish species of great economic importance in Europe, particularly in the Mediterranean aquaculture. It is present all over the Mediterranean Sea, the Black Sea and the North Eastern Atlantic, from the south of Norway to Senegal. It is a eurythermic and euryhaline fish, therefore it can be found in coastal inshore waters to a depth of 100 m, as well as brackish waters, in estuarine areas and coastal lagoons. Occasionally, it can be found in freshwater rivers. It is a gonochoristic species with spawning occurring once a year, from December to March in the Mediterranean population, and up to June in the Atlantic populations. Seabass reaches sexual maturity, in the Mediterranean, at three years in males and at four years in females, whereas in the Atlantic, seabass males are mature at four years and females at seven years. There is high fecundity (an average of 200000 eggs kg^{-1} of female) of small pelagic eggs (1.02 - 1.39 mm) in waters with salinities between 30 ‰ and 35 ‰, close to river mouths, estuaries and littoral areas. *D. labrax* is a voracious predator, feeding on mollusks, crustaceans and small fish (FAO, 2013b).

In the wild, seabass can reach 1 m in length and weigh 12 kg, but farmed animals reach market size at around 300-500g, which takes from 1.5 to 2 years.

In 2012, the total aquaculture production of European seabass in Europe was estimated at 119466 tonnes (Figure 1.2). This represents an increase of 0.5% from 2011

(118825 tonnes). The main producing countries of seabass are Turkey (50000 tonnes), Greece (41500 tonnes) and Spain (14270 tonnes) (FEAP, 2013).

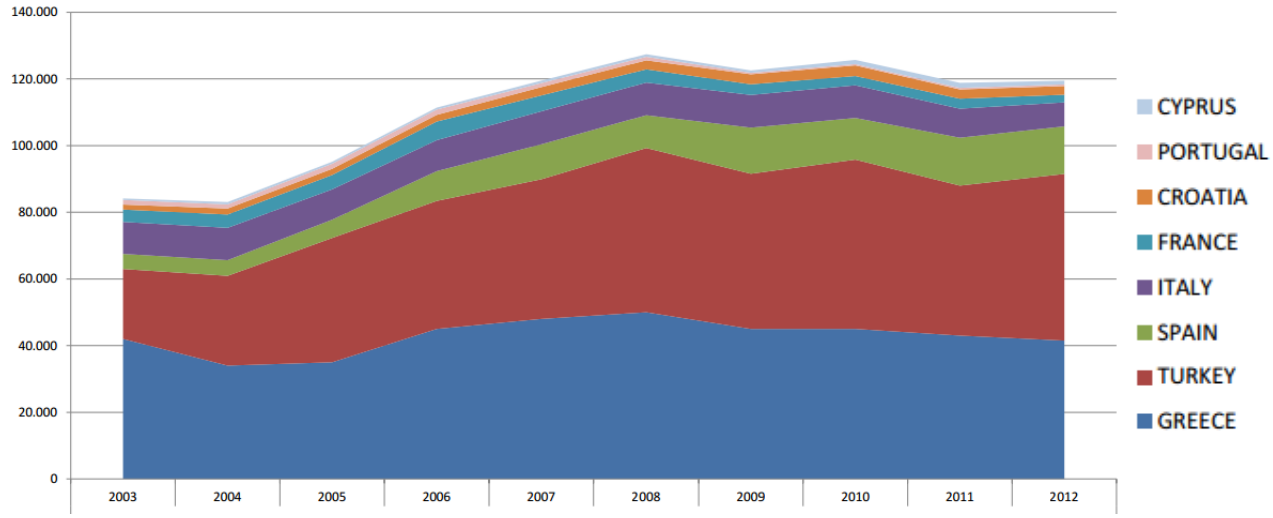

Figure 1.2: European seabass production (tonnes) from 2003-2012 by country (FEAP, 2013).

However, despite its important role in the Mediterranean aquaculture sector, the European seabass is a very sensitive species with regard to handling and vulnerable to infections under culture conditions. Additionally, the fact that it is a carnivorous species, the introduction of vegetables elements in the commercial feeds will produce adverse reactions on the gastrointestinal tract of the fish. Therefore, it is of special importance to address the effects of such elements in the guts of *D. labrax*.

1.2.1. Production constraints

Important economic losses in the seabass aquaculture were caused by disease outbreaks due to the specie's high vulnerability to stress and infections, mostly in the early development stages. Thus, both private and public organizations are exerting a concerted effort to find ways to maintain and enhance fish health in order to increase the production. Intensive production conditions can easily unbalance the equilibrium state between the triad host/pathogens/environment and lead to lower growth rates and high mortality rates. A key factor to overcoming the problem is to improve the innate immune system with the aim of preventing pathological outbreaks and, consequently, reducing the use of pharmaceutical interventions.

1.3. The Immune System

The immune system protects the body from harmful substances by recognizing and responding to antigens, which are molecular patterns typically found on pathological organisms. In fish the immune system is physiologically similar to that of higher vertebrates and is divided into two central components: the innate immune system and the adaptive immune system. The innate immune response plays a key role in fish, since they are free-living organisms from before hatching. This innate response comprises epithelial barriers, as well as cellular and humoral immune responses. The immunological agents include lytic enzymes, agglutinins and precipitins, growth inhibitors, antibodies, cytokines, chemokines and antibacterial peptides (Uribe *et al.,* 2011). Even though the innate immune system requires no previous experiences or "learning" in order to respond to a health challenge, several internal and external factors can influence its parameters, suppressing or enhancing the immune response (Magnadottir, 2006, 2010).

1.3.1. Epithelial Barriers

Physical and chemical barriers that are directly in contact with the external media are the first line of defense against pathological microorganisms. In fish they are mainly constituted by the dermis, epidermis, scales and mucous (Gómez and Balcázar, 2008). Mucous is produced by specific mucous cells, located in the epithelial tissue. It mainly comprises mucins, a type of glycoprotein secreted by the goblet cells (the characteristic mucous cells of the intestine), and water, but it also contains other active compounds, such as lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides, immunoglobulin-M and immunoglobulin-A, that, all together, prevent pathological agents from penetrating the barrier (Forstner et *al*., 1995; Nagashima et *al*., 2001; Hellio *et al*., 2002; Gómez and Balcázar, 2008).

The specific cases of the mucosal surface from the gastrointestinal tract and the skin are of special importance to introduce, as they are the focus of this study.

1.3.1.1. Intestinal Epithelium

The gastrointestinal tract is a complex structure comprising the epithelium, immune cells and resident microbiota which have co-evolved in such a way that each one relies on

the others in order to support the normal functions and homeostasis of the system (McCracken and Lorenz, 2001). Gut health depends, therefore, on the integrity of those components, which provide a first line of defense against harmful microorganisms and contribute to maintaining a stable state of the internal environment, a phenomenon named “homeostasis” (Canon, 1929). In fish, the intestine is usually divided into two different regions: the anterior gut, the foremost region connected to the stomach, and the posterior gut, the hindmost part connected to the rectum. Functionally, the anterior gut is the primary site for nutrient uptake (Nordrum *et al*., 2000), whereas the posterior region has less nutrient absorptive capacity, absorbing mostly remaining aminoacids and peptides, and more phagocytic activity (Ezeasor and Stokoe 1981; Sire and Vernier 1992; Buddington *et al.,* 1997).

The intestinal epithelium is composed of cells responsible for the absorption of nutrients, which takes place in the luminal side of the epithelial cells (ECs). To maximize this process and get the largest surface area, the small intestine consists of villi and crypts that greatly increase the quantity of ECs. Moreover, the luminal surface of the ECs presents microvilli that further increase the external surface area (Eri and Chieppa, 2013). The intestinal lumen is populated by several microorganisms, including bacteria, fungi, nematodes and viruses. The indigenous intestinal microbiota is composed of several bacterial groups, such as lactic acid bacteria (Ringo *et al.,* 1998; Gatesoupe, 2008), and they provide antagonism to potential pathogens through the production of a mixture of extracellular products (eg. lactic acid, hydrogen peroxide, carbon dioxide, siderophores, antibiotic peptides, organic acids, ammonia and diacetyl). They function to break down nutrients, produce vitamins and hormones and prevent harmful species from multiplying, all beneficial factors that represent an advantage to the host (Tremaroli and Backhed, 2012). Moreover, the autochthonous bacteria and the host interact in such an integrated way in order to mediate the development, preservation and effective functionality of the intestinal mucosal tissue. This was demonstrated with germ free and conventionally reared zebra fish (*Danio rerio*) larvae by comparing gut differentiation and gene expression (Rawls *et al.,* 2004; Bates *et al.,* 2006*;* Mulero *et al.,* 2007).

The mucus layer, produced by mucous cells present in the gut epithelium (Figure 1.3), is the major factor preventing the adhesion of bacteria, both commensal and pathological, to the epithelial cells (Schenk and Mueller, 2008). The main structural components of the mucus are the mucins, which are heavily glycosylated proteins of high molecular weight. Mucins are a key component in several gel-like secretions, protecting

epithelial cells from infection, dehydration and physical/chemical injuries, as well as lubricating surfaces (Perez-Vilar and Hill, 1999). MUC2 is the major mucin component of the mucus layer in the small and large intestine in mammals, and mutations that involve MUC2 are related to chronic intestinal inflammation (Burger-van Paassen, 2011; Eri *et al.,* 2011). The mucus layer also concentrates the epithelial antimicrobial peptides (AMPs) (Figure 1.3) which are another fundamental mechanism to control and select commensal bacteria (Gallo and Hooper, 2012). Plasma cells, located in the lamina propria, secrete IgA molecules which are transcytosed through the epithelial cell layer to the mucous layer (Figure 1.3), limiting numbers of mucosa-associated bacteria and preventing bacterial penetration of host tissues (Hooper and Macpherson, 2010).

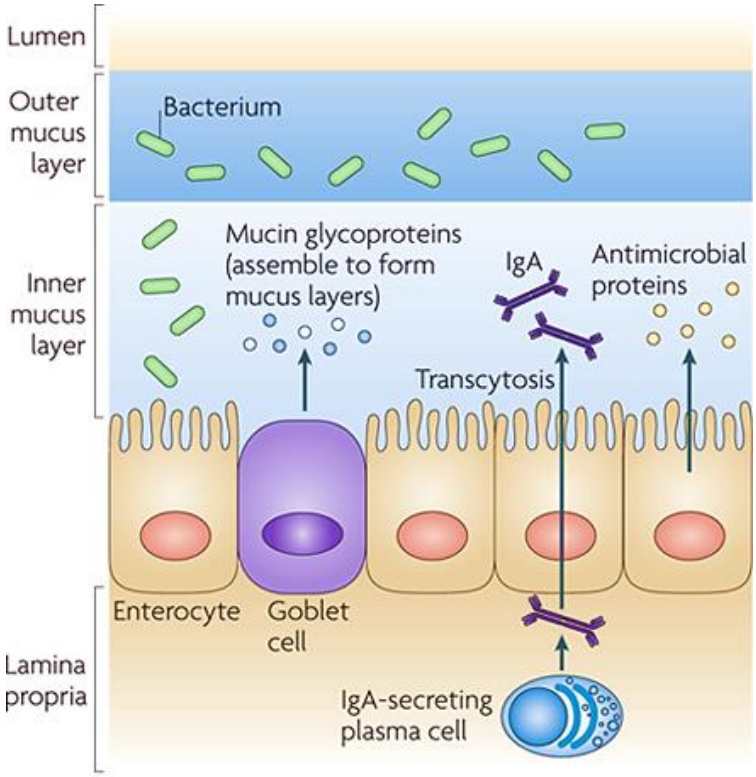

Figure 1.3: Intestinal epithelial surface. Goblet cells secrete mucins to build a stratified mucus layer. Bacteria are more abundant in the outer mucus layer than the inner layer, which concentrates more antimicrobial factors. Epithelial cells secrete AMPs. Plasma cells produce IgA that are secreted from the apical surface of epithelial cells (Hooper and Macpherson, 2010). Image licensed by Nature Publishing Group, license number: 3284690066429

It is possible to increase the secretion rate of mucins by appropriate stimulation of the goblet cells. Recently, dietary factors have been studied to assess their effects on improving gut health by stimulating goblet cells to produce mucus. Most of those studies were performed in humans and other mammals (Ouwehand *et al.,* 2005; Gaggìa *et al.,* 2010; Quigley, 2010). With the development of the aquaculture industry and the need to reduce the use of antibiotics and vaccines, probiotics and prebiotics have recently received considerable attention, especially with regard to strengthening the fish's innate immune system.

1.3.1.2. Skin

Skin in teleosts has unique characteristics and is histologically diverse (Fast *et al.,* 2002). Because of the direct contact of fish with the aquatic environment, which is rich in pathogenic microorganisms (Magnadottir, 2010), cutaneous diseases are very common and are one of the primary disease conditions that are presented to aquaculture producers (Groff, 2001). Fish integument is a large and multifunctional organ that acts as a mechanical barrier with a metabolically active tissue. Its components serve important roles in protection, locomotion, respiration, communication, sensory perception, ion regulation, excretion and thermal regulation (Elliott, 2011). In general, adult fish skin is divided into the mucous layer, epidermis and dermis (Figure 1.4). The epidermis is a squamous stratified epithelium composed of epithelial cells and mucous cells. It can itself be divided in three *strata:* the outermost *stratum superficiale,* the in-between *stratum spinosum*, and the innermost *stratum basale*. The dermis, which is separated from the epidermis by a basement membrane, is composed of two layers: the *stratum spongiosum* and the s*tractum compactum,* mainly composed of connective tissue, fibroblasts and chromatophores. The scales are transdermal and made of connective tissue with superficial mineralization (Hawkes, 1974a).

The mucosal layer is mainly produced by the goblet cells present in the epidermis, therefore their density in the skin is an important first line of immune response in fish. Many stressors may affect the density of those cells and, thus, affect the immune response. Vatsos *et al.* (2010) suggested evidence that the enumeration of skin mucous cells of fish can be used to monitor stress, although other authors prefer a combination of size of cells and their density to characterize the physical status of this innate immune system. Pittman *et al.* (2013) demonstrated that using that approach allied with systematic random sampling it was

possible to obtain highly significant differences in mean mucous cell area and mucous cell density at different body sites even with a small number of fish samples: Dorsolateral skin of 4 salmon had denser (≈ 8% of epithelium area) and larger (mean= 160µm^2) mucous cells, meanwhile the head had the lowest density (≈ 4% of epithelium area) and smallest mucous cell area (mean= 115µm^2). Therefore, such a method allows unbiased comparison of mucous cell dynamics in fish exposed to different treatments.

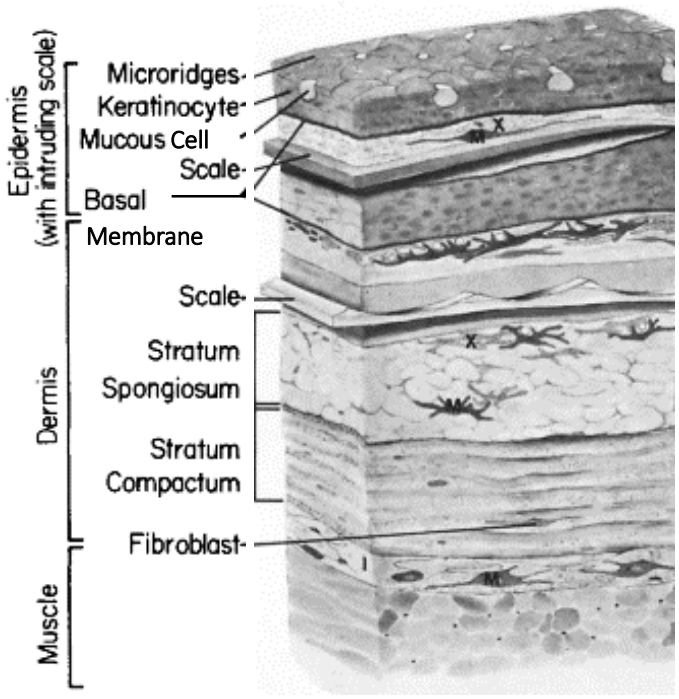

Figure 1.4: 3-D cross section of a teleost fish integument representing the main microscopic structures of the epidermis and dermis. X- Xanthophore, M- Melanophore (Hawkes, 1974b).

As mentioned above for the gut epithelium, dietary immunostimulants may also play an important role on improving skin mucosal immunity, showing that not only the mucosal system of the intestine is influenced (Pittman *et al.,* 2013; Sheikhzadeh *et al.,* 2012a; Van der Marel *et al.,* 2012; Xueqin *et al.,* 2012). Those observations highlight the interconnection of mucosal tissues in the body, underlining the importance of generating knowledge on the

application of functional feed additives to improve fish mucosal immunity. It also highlights the need to clarify if the mucosal immune system functions as one barrier or as several independently regulated systems.

1.3.1.3. Epithelial Tissue Processing – Stains

Staining is a technique utilized in microscopy to improve contrast on the microscopic images. Different dyes are used to highlight specific structures in biological tissues in order to facilitate its examination and study. In the specific case of the present study, we needed to produce histological images that highlight specifically the mucous cells. For the epithelial tissue embedded in Technovit®, Pittman *et al.* (2011, 2013) suggested that the "Periodic Acid Schiff (PAS) - Alcian Blue" was the one that gave better identification of the mucous cells than the Hemaetoxolin-Erythrosin Saffron (HES). PAS-Alcian Blue binds specifically to mucopolysaccharides, revealing clear and distinct mucous cells from the surrounding tissue, which is stained lighter, allowing epithelium quantification for cell density assessment, and that specificity allows the distinction of mucous cells from many types of artefacts, such as lipid droplets in the gut, that can be misidentified as mucous cells if using other types of stain. So, the combination of the Alcian Blue and the PAS techniques is used as a mean of staining both acid mucins and neutral mucins, in order that all mucins, regardless their charge, are stained (Yamabayashi, 1987).

However, tissue permeability might be important in order to stain the surrounding tissue sufficiently, which is more difficult in non-decalcified samples with thick scales (e.g. European seabass). Toluidine Blue is a viable option when epithelium is badly stained with PAS-Alcian Blue in skin samples with the scales present, since it has a more powerful penetration capacity and is a more general stain that still produces clear and distinct stained mucous cells. This possibility was tested in this experiment for the non-decalcified skin samples from European seabass, and results will be exposed in the 'Results' section.

1.4. Nutrition

Nutrition plays a critical role in aquaculture because it influences fish growth, health, waste production and, especially, the cost of production. It is necessary to meet the nutritional requirements of fish through balanced formulations and cost-effective diets (Gatlin

III DM, 2002). The carnivorous species, like the European seabass metabolize mostly proteins and lipids to produce energy and meet other physiological needs. Their metabolic capacity to use carbohydrates is, however, very restricted due to their natural feeding habits. These facts and the high requirement of amino acids constrain the capacity to utilize lower-cost carbohydrates and low protein diets (Buddington *et al.,* 1997).

Therefore, studies on diet composition for carnivorous species have been focusing mostly on protein, including plant proteins, and lipids requirements to optimize development, growth and health.

1.4.1. Proteins

Proteins are the most expensive part of a common diet and carnivorous fish require 40 to 50 percent crude protein in their diets. Fish, as other animals, will synthesize the body proteins from amino acids, but some of them are exclusively provided by the diet, the so called 'essential amino acids'. The 'non-essential amino acids' can be synthesized internally from other sources and do not depend solely on dietary protein sources. A balanced and optimal mixture of amino acids is crucial for adequate growth rates and healthy individuals, while avoiding unnecessary expense and negative environmental impacts from excessive excretion of nitrogenous wastes (Wilson, 2003; Gatlin III, 2010).

Fish meals derived from pelagic fisheries have been used as the main protein source for aquaculture feeds but concerns about sustainable marine fisheries and increasing prices of fish meals (Table I) led to a growing demand for alternative protein sources. Soybean meal is considered an interesting alternative and has been used as a partial substitute of fish meal (FM) due to its advantages of supply, price (Table I) and amino acid composition.

Table I: Comparison between prices of fish meal and soybean meal from May to October 2013. ROC – Rate of change (Index Mundi, 2013).

Month	Fishmeal Price (US Dollars per Metric Ton)	Soybean Meal Price (US Dollars per Metric Ton)	Fishmeal ROC	Soybean Meal ROC	Fishmeal / Soybean Meal Price Ratio
May 2013	1835,82	476,74	-	-	3,8508
Jun 2013	1743,89	503,56	-5,01%	5,63%	3,4631
Jul 2013	1598,54	528,34	-8,33%	4,92%	3,0256
Aug 2013	1621,63	470,99	1,44%	-10,85%	3,4430
Sep 2013	1525,27	490,19	-5,94%	4,08%	3,1116
Oct 2013	1520,09	460,83	-0,34%	-5,99%	3,2986

1.4.1.1. Soybean meal

Despite the economic advantages and protein content, soybean meals are rich on anti-nutritional factors which may have negative effects on fish, such as saponins and lectins, which disrupt the intestinal epithelium, triggering an inflammatory process (Chen *et al.,* 2011; Knudsen *et al.,* 2007; Krogdahl *et al.,* 2010). Feeds with soybean meal (SBM) inclusion have been reported to cause enteritis in salmonids (Knudsen *et al.,* 2007; Refstie *et al.,* 2000) and even a replacement of 50% of FM by SBM produced acute inflammation on the intestinal epithelium of rainbow trout (Merrifield *et al.,* 2009). Urán *et al.* (2008b) reported an up-regulation of the expression of pro-inflammatory genes (IL-1β and TNF-α1) in the intestinal intraepithelial lymphocytes of fish fed dietary SBM. By contrast, no morphological changes in gut histology were detected in gilthead seabream and European seabass fed dietary SBMs up to a level of 300g kg^{-1} (Bonaldo *et al.,* 2008). This may suggest an adaptation of the intestinal tissue to SBM, as has been demonstrated for the common carp (*Cyprinus carpio*) after the 4^{th} week of feeding with SBM (Urán *et al.,* 2008a). In this carp species, similar immunological reactions were observed during the enteritis process: invasion and degranulation of granulocytes, higher activity of T cells but also gene up-regulation of pro-inflammatory IL-1β and TNF-α1 and down-regulation of the anti-inflammatory IL-10. TGF-β seems to be up-regulated in carp in the 3^{rd} week after SBM feeding (Urán *et al.,* 2008a). In Atlantic salmon, TGF-β, IL-1β, interferon-γ-inducible lysosomal thiol reductase (GILT) but also CD3 and CD8-β (T-cells expression genes) were all down regulated in the 1^{st} week of SBM-induced enteritis (Lileeng *et al.,* 2009). These observations suggest that the SBM-induced enteritis in salmon might be correlated with the down-regulation of TGF-β. Therefore, the TGF-β up-regulation on carp after 3 weeks of feeding experiment in contrast to its down-regulation in the same period in Atlantic salmon, gives an important clue to the central role of TGF-β in the immune homeostasis and mucosal inflammation (Rombout *et al.,* 2011).

Moreover, SBM also influences the composition of fish gut microbiota. Hekkinen *et al.* (2006) developed one of the first studies to assess the effect of a diet with 45% SBM on the gut microbiota of the rainbow trout. After 2 months, the total culturable bacterial levels in the hindgut were at least one log scale lower in the fish fed SBM diet than fish fed the control FM diet. Also some genera were particularly affected, with a decrease of *Lactobacillus* spp. and *Sphingomonas* spp. and an increase of *Bacillus* spp. and *Chryseomonas* spp. in the group fed SBM. However, Merrifield *et al.* (2009) performed a similar dietary trial in the same

species using 50% SBM and after 16 weeks no significant differences were observed in total viable counts of culturable bacteria in the gut. Nonetheless, differences in genera composition were found: SBM lead to increased levels of *Psychobacter* spp. and yeast and reduction of *Aeromonas* spp. Comparable dietary trials were also performed on Atlantic salmon, where diet-dependent differences in bacterial diversity were also detected (Bakke-McKellep *et al.,* 2007, Ringo *et al.,* 2008). In gilthead seabream, allochthonous microbial communities were also affected by the inclusion of dietary soybean (at 31.3% inclusion) in feeds, however no phylotypes were sequenced for species identification (Dimitroglou *et al.,* 2010a).

More studies are necessary concerning this subject and regarding more species, since the aquaculture industry is relying more and more on plant-based diets to satisfy the demand for aquafeeds.

1.4.2. Lipids

Lipids are an important energy source due to their high specific energy value (9kcal/g), almost complete digestibility and necessity for maintaining cellular membrane integrity and function. In aquaculture feeds, the main sources of the lipid fraction have traditionally been fish oils and fish meals, derived from small pelagic fishes such as herring and anchovies which provide the required fatty acid profiles and result in good growth rates (Sargent *et al.,* 2003; Tacon *et al.,* 2006). Fish oils have high amounts of marine fatty acids with very long chain *n*-3 polyunsaturated fatty acids, of which the most important are eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA) (Ackman, 1982). Deficiency in these dietary essential fatty acids can cause problems such as poor feeding and swimming activities, lower growth rates, higher mortality, abnormal pigmentation, disaggregation of gill epithelia, immune-deficiency and higher stress levels (Izquierdo, 1996, 2005). Also, consumers equate consuming fish products with high levels of Omega-3 fatty acids which are beneficial to human health (Simopoulos, 2000).

It is estimated that aquaculture uses approximately 40% and 60% of the total global production of FM and fish oil, respectively (Nasopoulou and Zabetakis, 2012). In order to reduce the environmental impact and promote sustainable aquaculture, the industry has been replacing portions of the fish oils in feeds with vegetable oils. The most common vegetable oils used for fish feed production have been soybean, linseed, rapeseed,

sunflower, palm oil and olive oil (Turchini *et al.,* 2009; Nasopoulou and Zabetakis, 2012), which have low commodity prices (Table II). By comparison, the fish oil average price in the last quarter of 2012 was 2183 US Dollars per metric ton (Globefish, 2013), more than twice the price of an equal amount of soybean oil. The concerns with plant-for-fish replacement are the low n-3/n-6 ratio, due to high levels of linoleic acid (18:2 n-6, LA) and lower levels of n-3 PUFA (Izquierdo *et al.,* 2003; Izquierdo, 2005; Montero *et al.,* 2005).

Table II: Comparison between prices of four common vegetable oils from May to October 2013. (Index Mundi, 2013). The fish oil average price in the last quarter of 2012 was 2183 US Dollars per metric ton (Globefish, 2013).

Month	Price (US Dollars per Metric Ton)			
	Soybean oil	Sunflower oil	Rapeseed oil	Palm oil
May 2013	1082,78	1466,97	1117,66	763,38
Jun 2013	1058,59	1472,07	1115,86	763,04
Jul 2013	1000,84	1375,48	1003,37	729,86
Aug 2013	944,27	1152,39	991,21	722,84
Sep 2013	934,97	1158,38	985,02	725,80
Oct 2013	897,66	1187,13	1009,27	762,62

1.4.2.1. Vegetable Oils

Partial substitution of fish oil by vegetable oils is only desirable if the essential fatty acids are still obtained in sufficient quantities. Those requirements naturally differ between both plant and fish species. Some species are able to satisfy their requirements for fatty acids from vegetable oils by desaturating and elongating the linoleic and α-linoleic acids into arachidonic acid (20:4 n-6, ArA), EPA and DHA (Sargent *et al.,* 2003). Marine fish species have a very limited gene expression of Δ6 and Δ5 activity and thus have low capacity to synthesize polyunsaturated fatty acids from linoleic acid (Mourente and Tocher, 1993).

A couple of studies have been performed to evaluate the effects of different vegetable oils on European seabass and Gilthead seabream. Soybean oil and olive pomace oil (olive oil extracted from olive pulp, with solvents, after the first press) appear to be good substitutes for *S. aurata* regarding growth, but n-3 fatty acid profiles in the muscle of fish fed with these oils are significantly lower than in fish fed fish oil (Nasopoulou *et al.,* 2011; Wassef *et al.,* 2009). Olive, rapeseed and linseed oils could also be used as partial substitutes for fish oil

in *D. labrax* diet, maintaining an acceptable growth rate, despite decreases in EPA and DHA. These two fatty acids can be increased with a "finishing" diet of 100% fish oil (Mourente *et al.,* 2005; Nasopoulou *et al.,* 2011). However, high levels of substitution (up to 80%) may result in significant reductions of growth rates, feed conversion rates, as well as alterations in liver structure and immune system (Caballero *et al.,* 2004; Izquierdo *et al.,* 2005; Montero et al., 2003). Moreover, the inclusion of plant oils, including soybean oils, into feeds has been reported to promote a substantial accumulation of lipid droplets in enterocytes of species such as gilthead seabream (Caballero *et al.,* 2003), rainbow trout (Caballero *et al.,* 2002; Olsen *et al.,* 2003) and Arctic charr (Olsen *et al.,* 1999; 2000). This disorder appears to be connected to the impairment of lipoprotein synthesis in the enterocytes (Merrifield *et al.,* 2011).

The gut microbiota may also be affected by dietary soybean oil, however, to present date, only one study evaluated that hypothesis. Ringo *et al.,* (2002) observed that soybean oil modulates the gut autochthonous bacterial community of the Arctic charr by increasing the total culturable population and selecting for specific genera. Lower infection by *Aeromonas salmonicida* ssp. *salmonicida* was also reported when fish oil was replaced by soybean oil, which might be related with an increased production of mucus and an antibacterial effect from the autochthonous bacteria selected by the soybean oil diet, suggesting an improvement of immune defenses.

More studies are needed to assess levels of lipid and essential fatty acids requirements for optimum growth and proper immune function and the modulating effect of vegetable oils on the gut microbial community.

1.5. In-feed immune stimulants

Enteric bacteria and pathological bacteria co-exist in the intestines of animals in an uneasy truce. Control of the pathogens in crowded farm conditions is critical since they can cause illness and reduce animal performance, ultimately resulting in death. Colonization of the gut by any bacteria requires adhesion to the cells, a process mediated by interaction with carbohydrates present on cell surfaces (Bavington and Page, 2005). After anchoring to the surface of the GI tract, pathogens will multiply and produce toxins. They also damage the intestinal structures, resulting in less nutrient absorption, more gut inflammation and higher susceptibility to infections.

In-feed antibiotics of different classes, such as aminoglycosides, beta-lactans, nitrofurans, tetracyclines, sulphonamides, etc. (Defoirdt *et al.,* 2011) have been used by aquaculture producers to control pathogen numbers. This practice continues in some markets today but is highly regulated in others, particularly in Europe and the USA (Rodgers and Furones, 2009). However, antibiotics are not very selective and may also destroy beneficial bacteria. Bacteria also adapt to the environment upon continuous exposure to antibiotics, leading to the development of resistant strains (Schwarz and Chaslus-Dancla, 2001). This poses serious health concerns for the fish, the consumers and the environment (Romero *et al.,* 2012).

Good health management strategies and sanitary prevention methods such as vaccines (Thorarinsson and Powell, 2006) and immune stimulants (Dugenci, 2003; Rodríguez *et al.,* 2003; Dimitroglou *et al.,* 2010a; Torrecillas *et al.,* 2011, 2013) have been gradually replacing antibiotics and other therapeutic chemicals, becoming an area of intense research. An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases, especially those caused by pathogens (Bricknell and Dalmo, 2005). According to Sakai (1999) immunostimulants can be divided into different groups, depending on their sources: prebiotics such as bacterial derivatives and polysaccharides; animal and plant extracts; nutritional factors as vitamins C and E; and hormones and cytokines. Immunostimulants can be administered through intraperioneal injection, immersion or dietary inclusion (Sakai, 1999). The latter is the most promising option since it is naturally taken in through feeding behavior of the fish, is less stressful to the animal and can be used with all fish sizes. Its disadvantage is the inability to track the feed intake of the individuals, given that each fish may ingest different quantities of feeds.

1.5.1. Prebiotics

Prebiotics are “non-digestible food ingredients, generally carbohydrates, which have beneficial effects to the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). These carbohydrates can be classified according to their molecular size or degree of polymerization (number of saccharide units) into monosaccharides, oligosaccharides or polysaccharides. The common prebiotics already incorporated in fish feeds to date include: inulin, fructooligosaccharides, short-chain fructooligosaccharides, mannanoligosaccharides (MOS),

galactooligosaccharides, xylooligo-saccharides, arabinoxylooligosaccharides, isomaltooligosaccharides, β-glucans and alginate. Studies carried out on fish and shellfish have looked at the effects on growth, feed conversion rate, cell damage and morphology, gut microbiota, resistance against pathogenic bacteria and innate immune parameters (Yousefian and Amiri, 2009; Ringo *et al*.,2010, Ringo *et al.,* 2012) but the results are still limited and variable in different species. The particular case of mannan-oligosaccharides is discussed next.

1.6. Mannan-oligosaccharides (MOS)

Bio-Mos® (commercial name) is a natural sugar derived from the outer cell wall of a select strain of the yeast *Saccharomyces cerevisiae*, mainly composed of mannan oligosaccharides (MOS) and produced by Alltech, Inc (Kentucky, USA). It is thought to act as a decoy in the intestine maintaining gut health by adsorption of pathogenic bacteria containing type-I fimbriae or by agglutinating different bacterial strains (Figure 1.5). The action is by attracting pathogens to attach to Bio-Mos® surface rather than in the gut villi surface (Newman, 1994; Spring *et al.,* 2000; Shane, 2001). Once immobilized, bacteria are removed by being flushed out from the intestine.

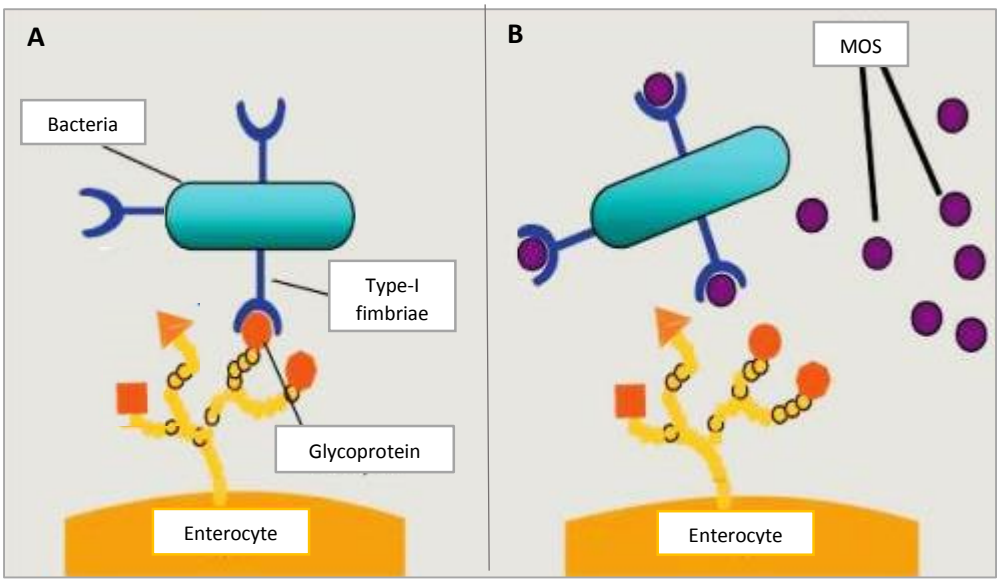

Figure 1.5: MOS decoy mechanism. A) Without MOS present in the lumen, the type-I fimbriae (mannose specific lectins) on the bacteria surface bind to specific glycoproteins (rich in mannose) on the enterocyte surface. B) MOS bind to type-I fimbriae of the bacteria, preventing it from binding on the enterocyte surface (Adapted from: Moran, 2009)

Bio-Mos® may also improve immune function by bundling pathogens and presenting them to dendritic cells. Dendritic cells respond to microbial antigens by activating a series of maturational processes involved in the innate antimicrobial and inflammatory responses (Figure 1.6). These cells reach out into the lumen from below the intestinal epithelium to capture floating agents. They digest the package and present pieces to the T-cells, activating them and initiating the adaptive immune response (Hooper *et al*., 2012; Reis e Sousa, 2004; Shane, 2001). T-cells that become active by contact with those antigens will send out signals called cytokines, which are then absorbed by B-cells, activating them in turn. The activated B-cells move back to the surrounding tissue and secret immunoglobulins. Therefore, Bio-Mos® has been suggested to increase the efficiency of the immune response by warning the immune system of the presence of specific pathogens. Immunoglobulins produced that way become concentrated in the villi, the mucous layer and the intestinal fluid, improving the immune response. Most of the studies with MOS on modulating the immune system and improving animal performance were performed on mammals (Spring *et al.,* 2000; Fairchild *et al.,* 2001; Iji *et al.,* 2001; Davis *et al.,* 2004; Grieshop *et al.,* 2004; Franklin *et al.,* 2005; Mourão *et al.,* 2006; Halas and Nochta, 2012). However, in the last decade, several studies have been developed on aquatic animals, with promising results.

Since the intensive nature of some cultures promotes the development of pathogens, studies on the incorporation of MOS in feeds have assessed the impact on microbial load, such as the *Vibrio spp*., an important pathogen for Mediterranean aquaculture and, in particular, the European seabass production. MOS supplementation decreased the infection by *Vibrio alginolyticus* (Torrecillas *et al.,* 2007) and *Vibrio anguillarum (*Torrecillas *et al.,* 2011a, 2011b). The inclusion of dietary mannan-oligosaccharides (MOS) also affected the microbiota when added to the FM diet, increasing the diversity. However no significant modulation effect was observed when MOS was added to the SBM diet, suggesting that any potential effect was masked by the greater general effect of dietary SBM on the gut microbiota (Dimitroglou *et al.,* 2010a).

Other papers reported improved performance, feed efficiency, increased leucocytes levels, etc. Some of those results are summarized in Table III.

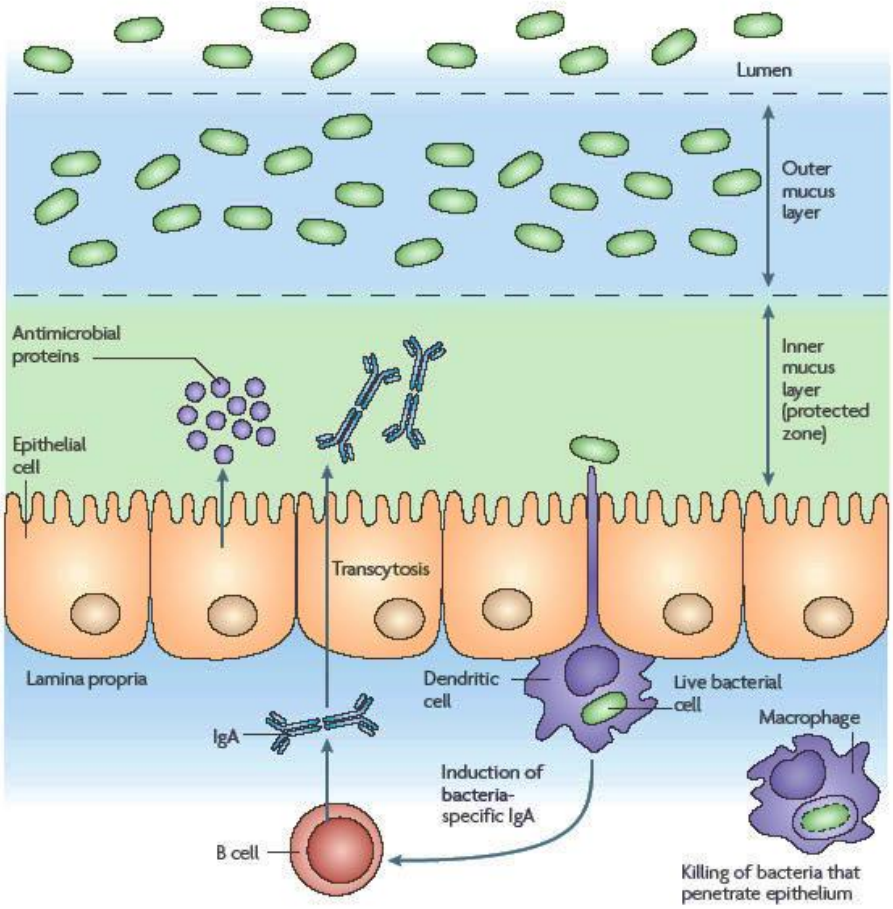

Figure 1.6: Immune system control of the gut microbiota. A) After capturing and digesting the pathogenic particles, the dendritic cells B) activate B and T cells that come in contact with the antigens of the pathogen. There is a recirculation of the induced B cells and T cells through the lymphatics and blood stream to mucosal sites, where B-cells differentiate into C) IgA-secreting plasma cells. (Adapted from: Hooper, 2009). Licensed by Nature Publishing Group, license number: 3286951496820.

Table III: Previous studies on the effects of mannan oligosaccharides (MOS) in aquatic animals.

Fish species	Dosage/Time/Mean fish size	Results	References
European seabass (*Dicentrarchus labrax*)	2 and 4 g kg^{-1} / 67 days / 35g	Increased growth; Lower lipid vacuolization, regular-shaped hepatocytes; Lower infection by *Vibrio alginolyticus*. Enhanced FCR;	Torrecillas *et al.* (2007)
	4 and 6 g kg^{-1} / 30, 45 and 60 / 60.64g	Lower lipid vacuolization, regular-shaped hepatocytes; Enhanced phagocytic activity of head kidney leukocytes; Increased mucous cells in the gut. No effects on sensorial parameters and biochemical composition of flesh.	Torrecillas *et al.* (2011a)
	4 g kg^{-1} / 8 weeks / 116g	Increased folds height, width and surface area of anterior gut; Increased surface area of posterior gut; Reduced fold length of rectum; Increased number of mucous cells; Higher density of eosinophilic granulocytes in the mucosa; Increased gut mucus lysozyme activity.	Torrecillas *et al.* (2011b)
	4 g kg^{-1} / 8 weeks / 45.95g	Higher weight gain, total length, specific and relative growth rates; Higher prostaglandins production on posterior gut; Decreased neutral lipids fraction from posterior gut; Increased polar lipids fraction; Increased number of goblet cells; Better preserved enterocytes, and healthier microvilli; Higher presence of lymphocytes and granulocytes.	Torrecillas *et al.* (2013)
Sharpsnout seabream (*Diplodus puntazzo*)	8 g kg^{-1} / 150 days / 100g (added to SBM diet)	No effects on final weight, SGR, FCR and PER; Higher moisture level; Lower lipid content; Lower polyunsaturated fatty acids.	Piccolo *et al.* (2013)
White seabream (*Diplodus sargus)*	Artemia enriched with 0.2% for 24h / 43 dph / larvae	No effects on growth and survivability; Increased villi surface area, microvilli length; Increased stamina and survival upon salinity challenge.	Dimitroglou *et al.* (2010b)
Gilthead eabream (*Sparus aurata*)	0.2 and 0.4 % / 9 weeks / 24g (added to FM and SBM diets)	No effects on final weight, SGR; FCR and PER; No effects on glycogen deposition in liver and villi morphology; Lower condition factor and HSI (FM diet) Improved absorptive area in posterior intestine; Increased microvilli density and length; Increased GI microbiota diversity (FM diet)	Dimitroglou *et al.* (2010a)
Atlantic salmon (*Salmo salar*)	10g kg^{-1} / 4 months / 200g	Less O_2 consumption and protein concentration in the body; More energy concentration in the body.	Grisdale-Helland *et al.* (2008)
	2000mg kg^{-1} in diet with 14% SFM + 14% SBM/ /11 weeks / 680g	Eliminated SBM-induce enteritis; Improved diarrheic condition; Faster growth; Higher protein retention.	Refstie *et al.* (2010)
Atlantic cod (*Gadus morhua*)	1 g kg^{-1} / 5 weeks / 90g	Higher expression of cytokines in posterior gut and rectum upon challenging with *Vibrio anguillarum.*	Lokesh *et al.* (2012)
Channel catfish (*Ictalarus punctatus*)	2g kg^{-1} / 4 weeks / 16g	No effects on: growth, hematology, immune functions, resistance to *Edwardsiella ictaluri*	Welker *et al.* (2007)
Cobia (*Rachycentron canadum*)	Artemia enriched with 0.2% for 24h / 13 dph / larvae	Increased larval survival; Enhanced height of microvilli; Reduced supranuclear vacuoles.	Salze *et al.* (2008)
Rainbow trout (*Oncorhynchus mykiss*)	2000 ppm / 42 days / 30g	Improved weight gain; Reduced FCR and mortality; Improved indicators of immune status.	Staykov *et al.* (2007)
	0.2% of diet formulation / 8 weeks / -	Increased absorptive surface of posterior gut; Increased microvilli length and density of posterior gut; Increased microvilli length of anterior gut;	Dimitroglou *et al.* (2008)
Red drum (*Sciaenops ocellatus*)	10g kg^{-1} / 3 weeks / 500g (added to SBM diet)	Increased protein, organic matter and energy ADC values; Decreased lipids ADC values.	Burr *et al.* (2008)
Nile tilapia (*Oreochromis niloticus*)	0, 2, 4, 6, 8, 10 g kg^{-1} / 45 days / 13.62g (added to commercial diet)	No effects on hematological parameters; Decreased daily feed consumption with increased MOS concentration;	Sado *et al.* (2008)
Gulf sturgeon (*Acipenser oxyrinchus desotoi)*	3 g kg^{-1} / 5 weeks / 130g	No effects on growth performance, GI morphology and spiral valve villi structure.	Pryor *et al.* (2003)
Common carp (*Cyprinus carpio*)	1, 2, 3 g kg^{-1} /45 days / 1.3g	No effects on growth and feeding parameters (highest for 1g $kg^{-1)}$; No effects on survival rate and body composition Increased hematocrit, lymphocyte, WBC, RBC, Hb and eosinophil (for 1g kg^{-1})	Akrami *et al.* (2012)
Pacific white shrimp (*Litopenaeus vannamei*)	2, 4, 6, 8 g kg^{-1} / 8 weeks / 2.52 g	Higher weight gain and SGR; Increased intestinal microvilli length; Higher survival rate after NH_3 stress;	Zhang *et al.* (2012)

Dph- days post-hatching, SBM- soybean meal, FM- fish meal, FCR- food conversion ratio, ADC- apparent digestibility coefficient, GI- gastro-intestinal, WBC- white blood corpuscles, RBC- red blood corpuscles, Hb- hemoglobin, SGR- specific growth rate, PER- protein efficiency ratio, HSI- hepatosomatic index

Refstie *et al.* (2010) demonstrated that soybean-induced enteritis in the distal intestine of Atlantic salmon was eliminated when 2000 mg kg^{-1} of MOS was added to a diet composed of 14% SBM. Nevertheless, no alteration on the severity of enteritis was detected when MOS was added to a diet with 32% of SBM, indicating that, at higher levels, the soybean components mask any potential effect of dietary MOS. The group fed the 14% SBM diet supplemented with MOS also displayed improved feed conversion efficiency, growth and nitrogen retention despite the similar quantity of feed intake to the group fed the same diet without the prebiotic, strongly indicating a positive effect on gut health by MOS. Studies in other species showed similar results, where MOS supplementation enhanced the length and density of microvilli and increased the surface area of the gut (Dimitroglou *et al.,* 2008, 2010a, 2010b; Torrecillas *et al.,* 2011b) along with an improvement of growth parameters (Salze *et al.,* 2008; Torrecillas *et al.,* 2013; Zhang *et al.,* 2012).

Torrecillas *et al.* (2011a, 2011b, and 2013) demonstrated an enhancement in the number of mucous cells per unit area in European seabass posterior gut fed dietary MOS at 4 $g.kg^{-1}$. This observation might be related to the lower infection level of seabass by post-inoculated *Vibrio* spp. due to increased mucus secretion. Such a level of MOS in the diet also resulted in a higher density of infiltrated eosinophil granulocytes in the lamina propria. This might be related to the higher presence of prostaglandins found in the posterior gut, which are produced during an inflammatory process to regulate homeostasis. These hormones are known to affect vascular permeability and stimulate mucin synthesis and release (Plaisancié *et al.,* 1998). Prostaglandin receptors are highly expressed in mucous cells of the gut of rats (Northey *et al.,* 2000). However, no significant modulation of innate immune functions was found in skin mucus of European seabass (Torrecillas *et al.,* 2011b). On the other hand, feeding fermented *Saccharomyces cerevisiae* to rainbow trout improved skin mucus innate immune parameters, such as enhanced enzyme activities, namely lysozyme, protease, alkaline phosphatase and esterase and a strong antibacterial activity against *Yersinia ruckeri* (Sheikhzadeh *et al.,* 2012a), which indicates a correlation between both gut and skin immune modulation. Thus, more studies are necessary to understand the effectiveness and specificity of immunostimulants at multiple mucosal sites.

1.7. Objective of the study

The production of mucus and the anti-adhesive properties of mucins in mucosal surfaces from the gut and skin of fish are important barrier mechanisms that prevent bacterial adhesion, therefore the improvement in mucus secretion can be directly related to reduced bacterial infection. In this study we aim to examine both tissues to assess the immune-modulatory response of the mucosal epithelia of European seabass (*D. labrax*) fed a commercial soybean meal feed mixed with either fish-oil or soybean-oil, and the effects of adding MOS to these commercial feeds.

Using the Mucosal Mapping™ technology described by Pittman *et al.* (2011, 2012), we applied an innovative objective method to measure mucous cell area and density. This method utilizes uniform and systematic random sampling and stereological procedures, producing unbiased and statistically reliable data.

2. Materials and methods

2.1. Dietary experiment

The dietary trial of this experiment was conducted at the aquaculture facilities from the 'Parque Científico Tecnológico de la Universidad de Las Palmas de Gran Canaria in Las Palmas de Gran Canaria, Spain.

Fifty-five European seabass with a mean weight of 36.25 ± 6.17g and mean length of 13.33 ± 1.67cm were equally distributed into 11 tanks (5 fish per tank) with a volume of 1000L and a natural photoperiod of 12L:12D. Tanks were supplied with filtered sea water. The period of dietary supplementation was 8 weeks. During the experiment, the animals from each tank were fed one of 4 different diets (Tables IV and V). Therefore, 3 diets were assigned to 3 tanks each and one ('Fish Oil + MOS') diet was assigned to 2 tanks. The two control diets were both 'Fish Oil' and 'Soybean Oil', which differ from each other only in the oil component. Two treatment diets were produced by adding to the control diets stated above 4g·kg^{-1} mannan oligosaccharides (Bio-Mos®, Alltech Inc, USA).

Table IV: Composition of experimental diets.

	Diet			
Ingredients (g kg-1 dry weight)	**Fish Oil**	**Vegetable Oil**	**Fish Oil + MOS**	**Vegetable Oil + MOS**
Fish meal [1]	515	515	515	515
Soybean meal	97,8	97,8	97,8	97,8
Wheat	85,3	85,3	85,3	85,3
Wheat gluten	85,3	85,3	85,3	85,3
Corn meal	65,3	65,3	61,3	61,3
Fish oil [2]	147,2	0	147,2	0
Soybean oil	0	147,2	0	147,2
Mineral+Vit mix 1	4	4	4	4
Antioxidant (BHT)	0,1	0,1	0,1	0,1
Bio-Mos®	0	0	4	4
Total weight	1000	1000	1000	1000

[1] Peruvian fish meal (65% protein). [2] Peruvian fish oil.

Table V: Treatment distribution and number of fish analyzed.

Treatment (Diet)	Tanks (Number of fish per tank: n=5)	Number of fish analyzed		
		Anterior gut	Posterior gut	Skin
Fish Oil	T4+T13+T18	2+3+4 = 9	1+1+1 = 3	2+3+4 = 9
Fish Oil + MOS	T7+T16	4+2 = 6	1+1 = 2	4+2 = 6
Soybean Oil	T1+T6+T15	3+3+3 = 9	1+1+1 = 3	3+3+3 = 9
Soybean Oil + MOS	T5+T14+T19	4+3+2 = 9	1+1+1= 3	4+3+2 = 9

2.2. Sampling

Sampling occurred at 21st March of 2013. Fish were caught by net from the tanks, anaesthetized with MS-222 and killed by a blow to the head before being transferred to the sampling room. The weight and length of each individual were measured (36.25±6.17g and 13.47±1.27cm), followed by sampling of intestine and skin. We used a subsample from the gut of the total number of fish, and the other subsample was taken for a series of biochemical and molecular analyses by Silvia Torrecillas, from Universidad de Las Palmas de Gran Canaria.

2.2.1. Intestine

The intestine was removed from the abdominal cavity of each specimen (n=55) and two parts were subsampled: anterior intestine and posterior intestine. Furthermore, a sub-sample of each part was sectioned for histological analysis. To ensure the correct sampling of the desired tissue, for the anterior gut, the sub-section was obtained from the uppermost part of the intestine and for the posterior gut, a sub-section from a region adjacent to the rectum (Figure 2.1). The samples were lightly rinsed with water to remove any content, put in labelled histocassettes and fixed in 4% phosphate-buffered formalin.

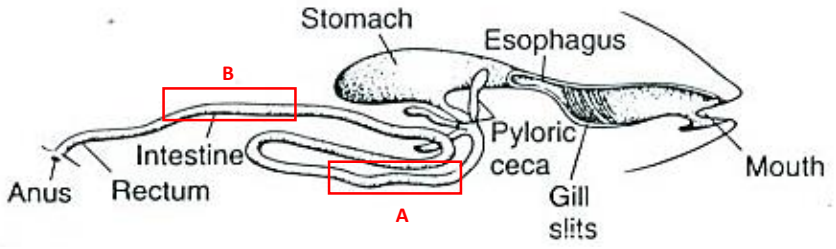

Figure 2.1: Generalized fish digestive tract scheme. A) Anterior intestine sampling region and B) posterior intestine sampling region (Image from: Kardong, 2012).

2.2.2. Skin

Skin samples were excised from the dorsolateral region of the fish. They were then processed according to Pittman *et al.* (2011, 2013), as described above. The step of decalcification in formic acid for calcified structures was not performed to ensure the epithelium integrity and avoid possible tissue shrinkage.

2.3. Processing Protocol

Following the Pittman *et al.* (2011, 2013) method for quantifying salmonid mucous cells, histological sections were prepared. The sub-samples fixed in formalin were dehydrated progressively in OH for 24h. They were then, embedded integrally in Technovit 7100 (Heraeus Kulzer GmbH & Co, KG) (Fig. 2.2), sectioned at 2µm with a rotary microtome (Leica®) (Fig. 2.3), stained with Periodic Acid Schiff (PAS)-Alcian Blue (only the intestine samples) and mounted with Mountex® (Histolab Products AB). Sectioning was performed at random orientation of the tissue. From both intestinal regions and the skin of each fish, 3 non-sequential sections were taken for mounting and posterior histological analysis.

The skin sample preparation differed from the above method in the stain that was applied, which was Toluidine Blue.

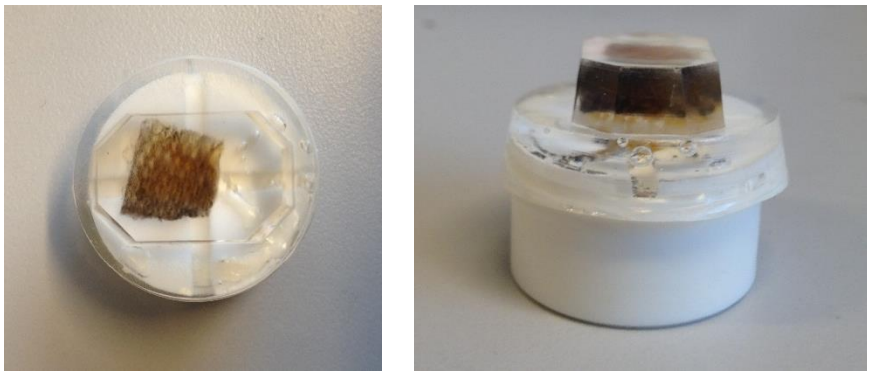

Figure 2.2: Embeded skin sub-samples in Technovit 7100. Blocks prepared for tissue sectioning with microtome.

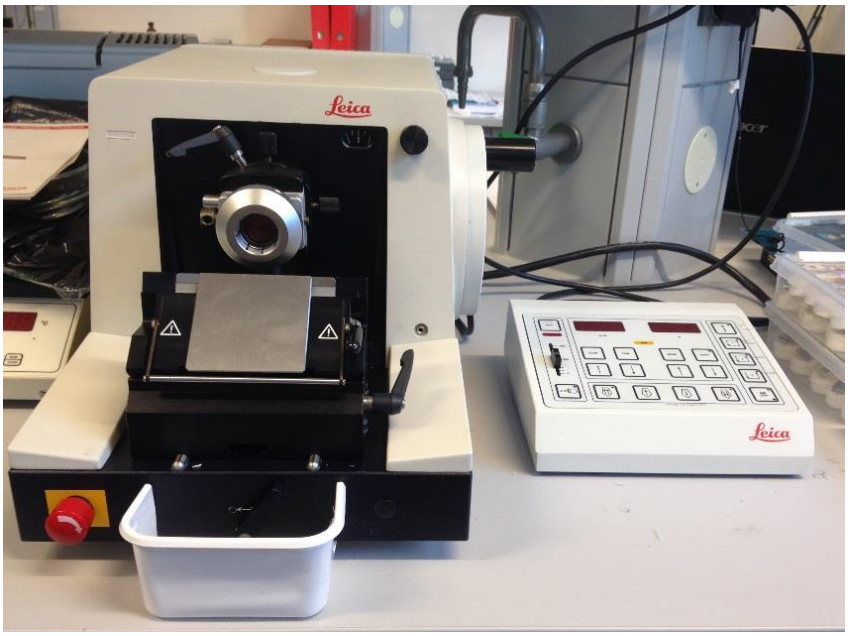

Figure 2.3: Rotary microtome (Leica®).

2.3.1. Stain optimization for skin samples

The PAS-Alcian Blue dye was substituted by Toluidine Blue upon confirmation that no statistically significant differences on mucous cells area and density were obtained by the different dyes. This new staining method for *D. labrax* skin is part of the results of this dissertation and is described and validated further on the '*Results'* section.

2.4. Histological Analysis

The sections were analyzed according to Pittman *et al. (*2013) using a Leica® Axioskop microscope combined with newCast® software (Visiopharm Integrator System, Version 3.6.5.0), which integrates image analysis and stereological tools, and a Prior Proscan digital stage, at a final magnification of 200x (Figs. 2.4 and 2.5). The mucous cells were counted in systematic random sections using counting frames and epithelial area and mucous cells area were measured using stereological probes (points and nucleator). Each section was delimited for regions of interest and the systematic uniform random sampling of those areas was executed to prevent observer bias. In accordance with stereological principles, the estimation of number and size using probes yield sufficient data to achieve significant precision in measurements (Howard and Reed, 2005).

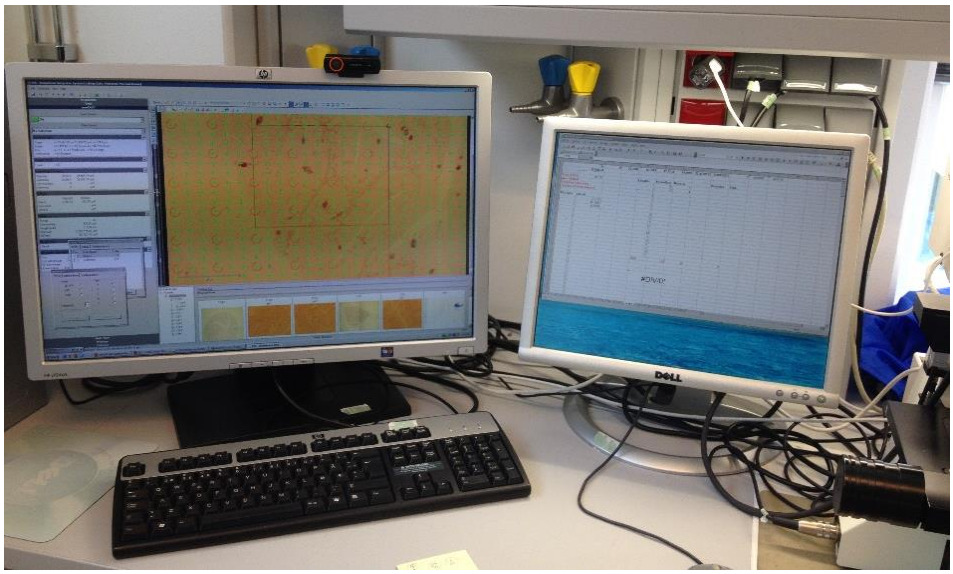

Figure 2.4: The computer used for image analysis with newCast® software (Visiopharm Integrator System, Version 3.6.5.0)

Figure 2.5: Leica® Axioskop microscope

2.5. Statistical Analysis

R (version 3.0.1) was used for the statistical analysis. Factorial ANOVA was performed to assess the effect of the factors 'diet' and 'prebiotic' and the interaction effect on the outcome variables. Kruskal-Walis test was used for non-parametric data. *Post-hoc* Tukey's HSD test for individual means comparison was performed when F-values indicated significance. Significant differences were considered for $P < 0.05$. All data were tested for normality and homogeneity of variance.

3. Results

3.1. Stain optimization for skin samples

PAS-Alcian Blue, previously validated for this methodology with *Salmo salar* skin samples and used to stain the *D. Labrax* gut samples, was not staining properly the epithelium from the skin of this species. Therefore, to stain the skin samples we needed to select a stronger dye that would better penetrate the tissue. In order to do that, we compared the cell area obtained from skin samples stained with the original staining technique against equivalent samples stained with Toluidine Blue 100% and 10%, to check if they would yield different cell areas or not.

PAS-Alcian Blue produces a clear identification of the mucous cells by specifically binding to mucins but the epithelium is barely identifiable (Figures A.3 – in Appendix), which might lead to a misquantification of the real epithelium area, which could impact the density results. Toluidine Blue 10% produced a clear identifiable epithelium and well distinguishable mucous cells (Fig. A.4). No significant differences were found between cell areas obtained by the different staining techniques (Figure 3.1). PAS - Alcian Blue produced cell areas with a mean value equal to 148.32 ± 78.07 μm^2, and with Toluidine Blue 100% and 10% we obtained mean values of 183.81 ± 93.21 μm^2 and 164.89 ± 72.65 μm^2, respectively. However, even though not significant, PAS-Alcian Blue yields a higher frequency of smaller cells (Figure 3.2) than Toluidine Blue (peak on 50-100 μm^2 for PAS-Alcian Blue and 150-200 μm^2 for Toluidine Blue 10%) which can have a potential impact on the results of mucus cells parameters from the skin by misidentification of smaller cells, which appear to be less visible and, therefore, are not counted.

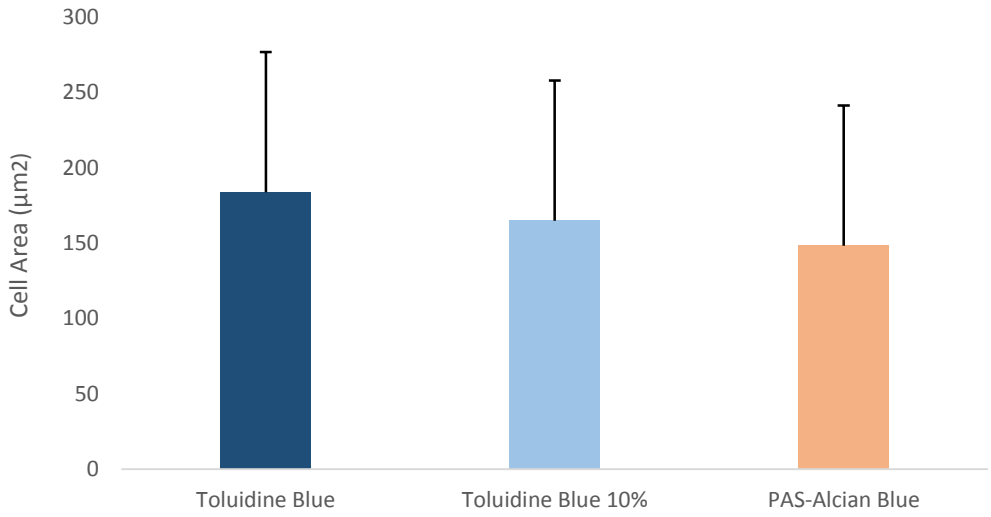

Figure 3.1: Mucous cell area from *D. labrax* skin samples per staining technique. One way ANOVA for significance testing (P<0.05). No significant differences observed. Number of cells counted per treatment: n=44; source: one fish.

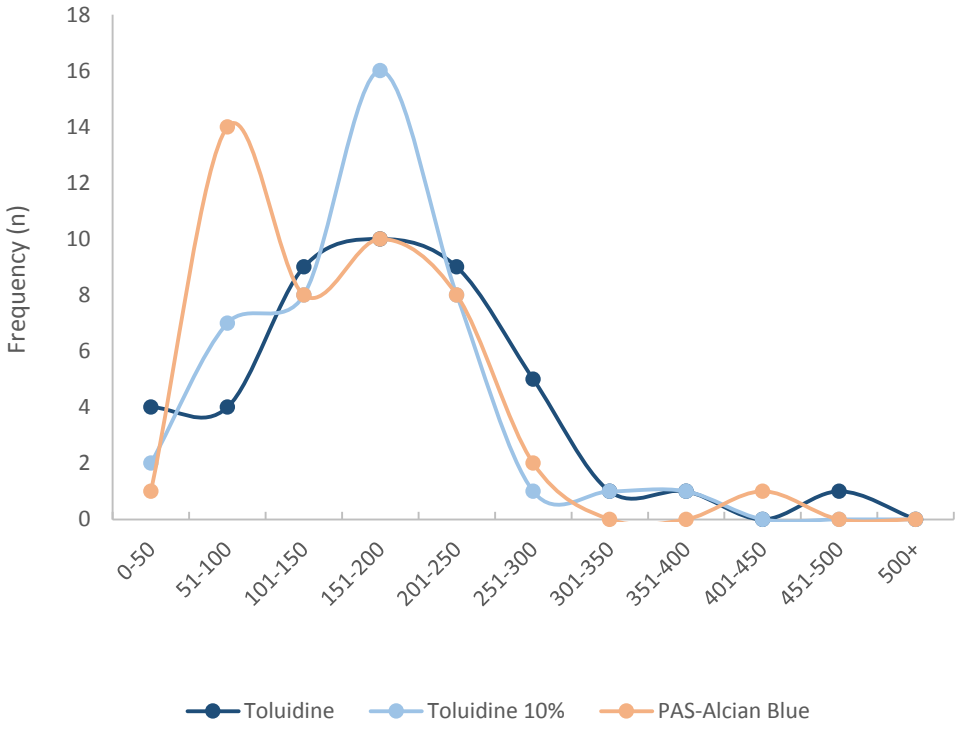

Figure 3.2: Frequency distribution of mucous cells area from *D. labrax* skin samples per staining technique. Number of cells counted per treatment: n=44; source: one fish.

3.2. Growth Parameters

No mortalities were registered in the tanks during the dietary treatment. After 8 weeks of feeding, the groups showed no significant differences in body weight and total length between each other. However, fish fed the control diets presented higher means in weight and length, compared with fish fed MOS-supplemented diets (Figures 3.3 and 3.4).

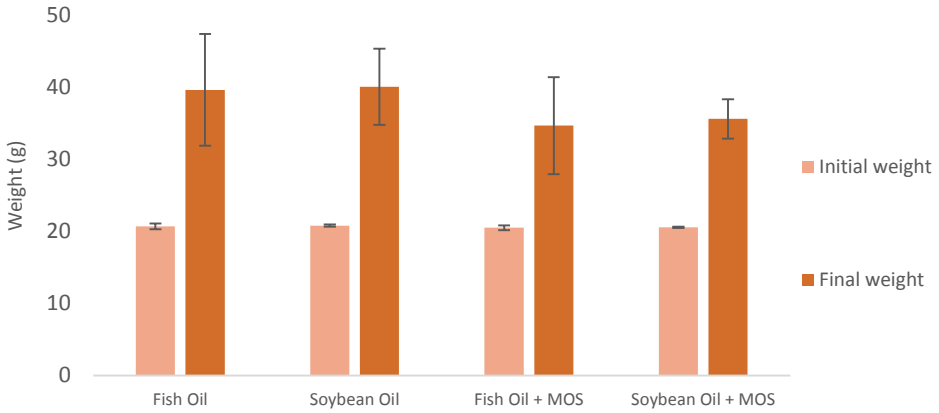

Figure 3.3: Initial (t=0) and final weight (g) of D. labrax (N=55, n=5) fed experimental diets 'Fish Oil', 'Soybean Oil', 'Fish Oil + MOS' and 'Soybean Oil + MOS' for 8 weeks. Factorial ANOVA for significance testing. No significant differences were observed between the different treatments (P<0.05) for final weights.

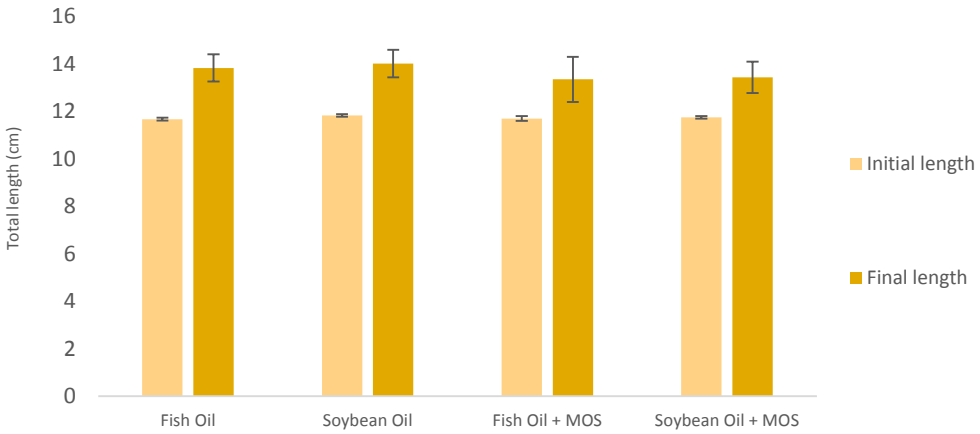

Figure 3.4: Initial (t=0) and final total length (cm) of D. labrax (N=55, n=5) fed experimental diets 'Fish Oil', 'Soybean Oil', 'Fish Oil + MOS' and 'Soybean Oil + MOS' for 8 weeks. Factorial ANOVA for significance testing. No significant differences were observed between the different treatments (P<0.05) for final weights.

3.3. Mucous Cells

3.3.1. Anterior Gut

Differences between mean mucous cell area and density from the anterior gut were evident in fish fed different diets (Figures 3.5, A.1). The mean mucous cell area in the anterior gut was 100.43 ± 15.48 μm^2 and 95.22 ± 9.35 μm^2 for fish fed the control diets 'Fish Oil' and 'Soybean Oil', respectively, and this difference was not significant. With the MOS-supplemented diets, mucous cell area decreased, non-significantly, to 78.33 ± 15.43 μm^2 for 'Fish Oil' and increased significantly ($P<0.01$) to 124.16 ± 14.13 μm^2 for 'Soybean Oil'. There were significantly bigger mucous cells in the mucosal tissue of fish fed the 'Soybean Oil' diet supplemented with MOS over the non-supplemented 'Soybean Oil' diet and the 'Fish Oil' diet supplemented with the prebiotic ($P<0.01$).

The mucous cell density, measured as a percentage of the total epithelial tissue, was at 10.25 ± 2.95 % and 7.90 ± 0.78 % for fish fed the control diets 'Fish Oil' and 'Soybean Oil', respectively, and the difference was significant ($P<0.05$). With the MOS-supplemented diets, mucous cell density was insignificantly reduced to 7.85 ± 2.07 % for 'Fish Oil+MOS' whereas it increased significantly ($P<0.05$) to 13.80 ± 6.01 % for 'Soybean Oil+MOS'. So, there was a significantly higher density ($P<0.05$) of mucous cell in the anterior gut of fish fed soybean oil diet with MOS than in fish fed the control 'Soybean Oil' diet and the fish oil diet supplemented with MOS. Likewise, the fish fed the control 'Fish Oil' diet showed a higher density ($P<0.05$) of mucous cells than the fish fed the control 'Soybean Oil' diet.

Looking at the ratio 'area:density' (Figure 14), we notice a large standard deviation and no significant differences are observed.

Note that a high density is not necessarily an indicator of higher number of mucous cells, but rather can reflect bigger mucous cells in the epithelium or reduced epithelium area. The surrounding epithelium can impact the density because its area can be affected by the type of diet and become decreased or enlarged.

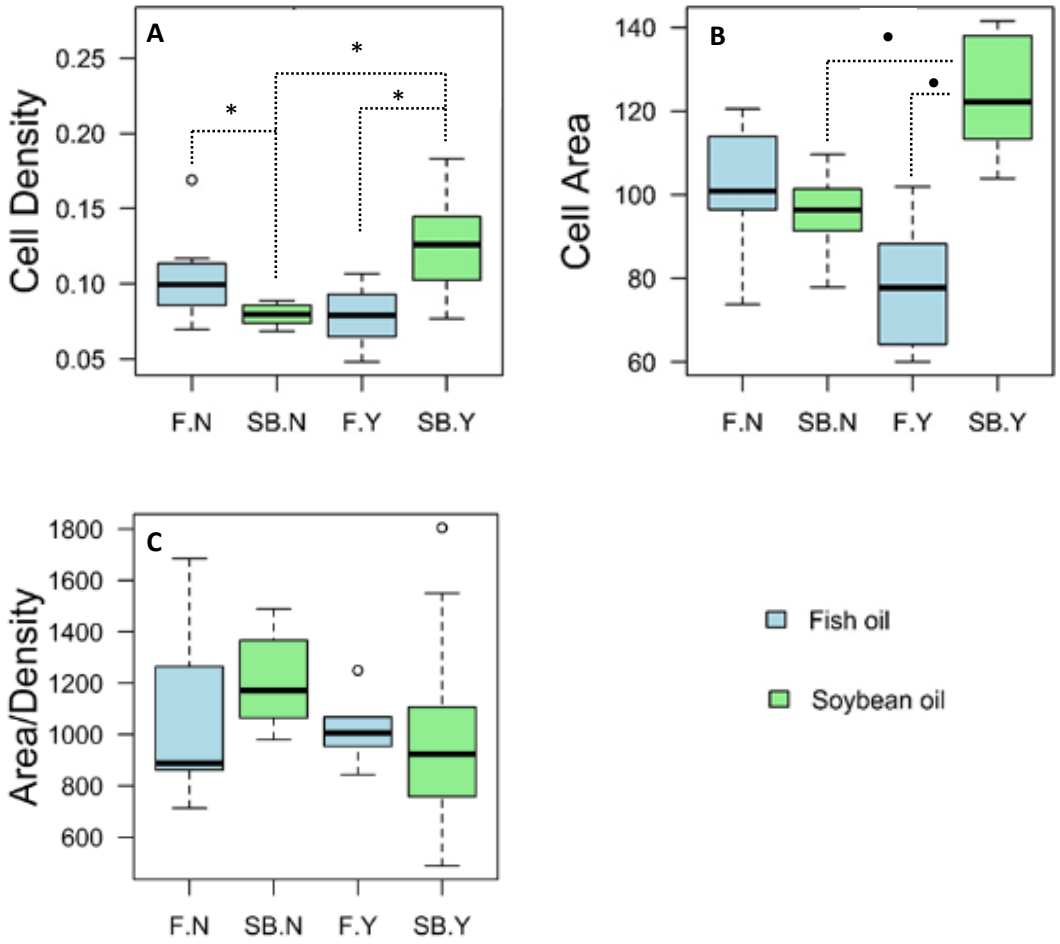

Figure 3.5: Effects of the diets in mucous cell density (A), mucous cell area (B) and area/density ratio (C) from the anterior gut of D. labrax (Total fish sampled = 33, number of counting frames (analyzed per fish/section)= 20). Factorial ANOVA for significance testing. Statistically significant differences are: * ($P<0.05$) and • ($P<0.01$). F.N- 'Fish Oil' , SB.N- 'Soybean Oil', F.Y-'Fish Oil' with MOS, SB.Y- 'Soybean Oil' with MOS.

3.2.2. Posterior Gut

In the posterior gut, significantly bigger mucous cells were observed on fish fed the fish oil diets over the soybean oil diets (Figures 3.6, A.2). For non-supplemented diets, 'Fish Oil' presented bigger ($P<0.05$) cells (88.35 ± 2.59 μm^2) than 'Soybean Oil' (78.69 ± 11.04 μm^2). For MOS supplemented diets, 'Fish Oil + MOS' resulted on bigger ($P<0.05$) mucous cells (88.46 ± 1.35 μm^2) than 'Soybean Oil + MOS' (75.30 ± 3.20 μm^2). No significant differences were observed in cell density, nonetheless, similarly to the observed for 'cell area', the Fish

Oil diets resulted on a higher density of cells in the tissue (3.40 ± 0.34 % for 'Fish Oil' and 4.02 ± 1.75 % for 'Fish Oil + MOS') then the Soybean Oil diets (2.88 ± 1.07 % for 'Soybean Oil' and 2.70 ± 0.24 % for 'Soybean Oil + MOS'). It is important to point out the diet effect, which suggests an important modulatory effect by the oil component in this region of the gut. On the other hand, MOS seems to have no modulation effect, suggesting a loss of its potential properties when it arrives to the hindmost region of the intestine.

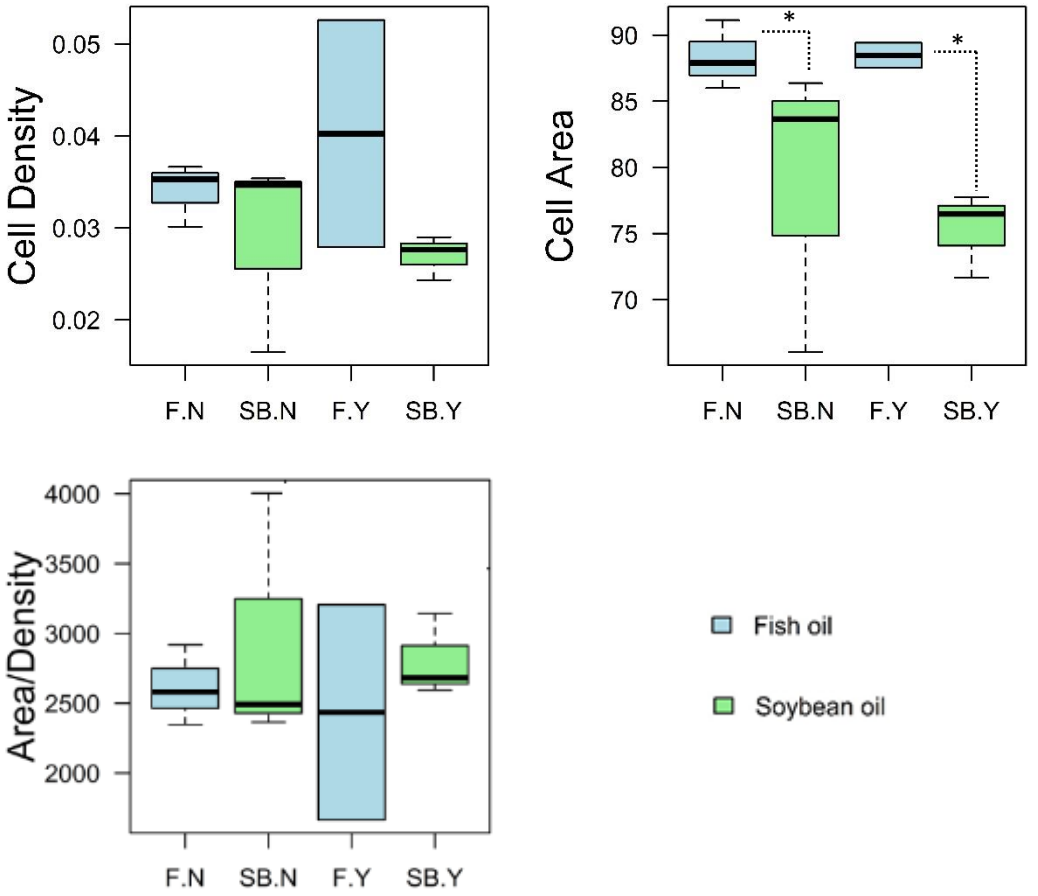

Figure 3.6: Effects of the diets in mucous cell density (A), mucous cell area (B) and area/density ratio (C) from the posterior gut of D. labrax (Total fish sampled = 11, number of counting frames (analyzed per fish/section)= 20). Factorial ANOVA for significance testing. Statistically significant differences are * ($P<0.05$). F.N- 'Fish Oil' , SB.N- 'Soybean Oil', F.Y-'Fish Oil' with MOS, SB.Y- 'Soybean Oil' with MOS.

3.2.3. Skin

In the skin epithelium, no significant differences in mucous cells area and density were observed (Figure 3.7). Nonetheless, the control diets 'Fish Oil' and 'Soybean Oil' gave rise to mucous cells with a mean area of 164.21 ± 27.13 μm^2 and 184.00 ± 18.71 μm^2, correspondingly. The MOS-supplemented diets resulted in mucous cells with 161.10 ± 15.76 μm^2 for 'Fish Oil+MOS' and 182.79 ± 30.35 μm^2 for 'Soybean Oil+MOS'.

Non-supplemented 'Fish Oil' diet resulted on a mean density of 2.38 ± 1.64 % whereas supplementation insignificantly increased density to 3.86 ± 1.54%. The non-supplemented 'Soybean Oil' diet had a density of 4.16 ± 1.71 % which was relatively unchanged by supplementation at 3.89 ± 2.42 %.

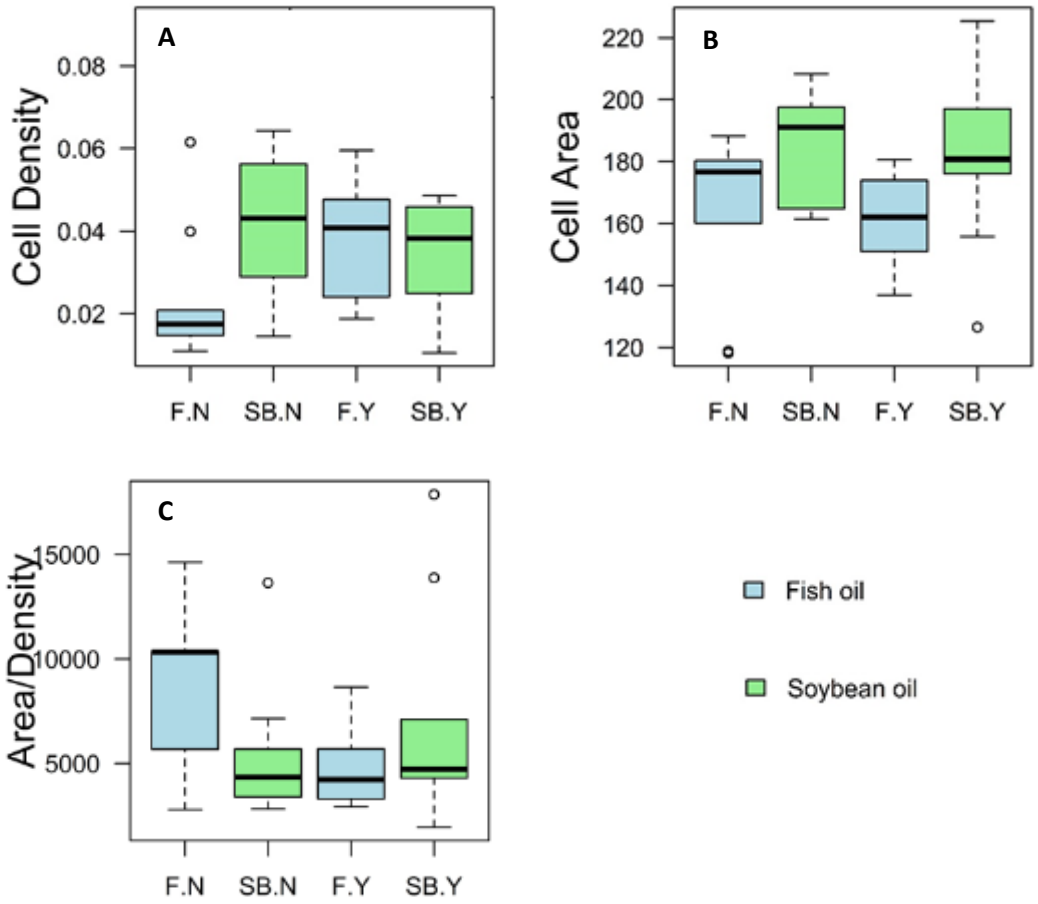

Figure 3.7: Effects of the diets in mucous cell density (A), mucous cell area (B) and area/density ratio (C) from the skin epithelium of D. labrax (Total fish sampled = 33, number of counting frames (analyzed per fish/section) = 20). Factorial ANOVA for significance testing (P<0.05). No significant differences observed.

3.2.4. Mucosal sites comparison

Comparison of the mucous cells parameters from the anterior gut, the posterior gut and the skin (Figures 3.8 - 3.10) reveals some differences between the mucosal tissues. The skin has much bigger mucous cells then the gut ($P<0.01$), regardless of diet. This indicates a natural adaptation towards that difference in terms of mucous cell morphology, since skin is more exposed to the external environment then the intestine, thus needs a continuous and faster synthesis and release of mucous and a bigger storage capacity. Cell area is significantly bigger ($P<0.01$) in the anterior gut of fish fed the both the supplemented and non-supplemented soybean oil based diets than in the posterior gut.

The anterior gut has a significantly higher density of cells than does the posterior gut and the skin, regardless of diet. The mucous cell density on the skin is about the same as that of the posterior gut but the cells are larger, as referred above. The highest density of mucous cells is observed in the anterior gut of fish fed the 'Soybean Oil + MOS' diet and in the posterior gut of the 'Fish-Oil + MOS' diet, although with a large standard deviation.

The area:density in the skin epithelium is about twice that of the posterior gut and about 5-8 times higher than the anterior gut, and only, but displays a big standard deviation in all treatments so significance is not observable in all treatments between skin and posterior gut (Figure 3.10). Area:density is significantly lower ($P<0.05$) in all treatments but for 'Fish-Oil+MOS', on the anterior gut compared with the posterior gut.

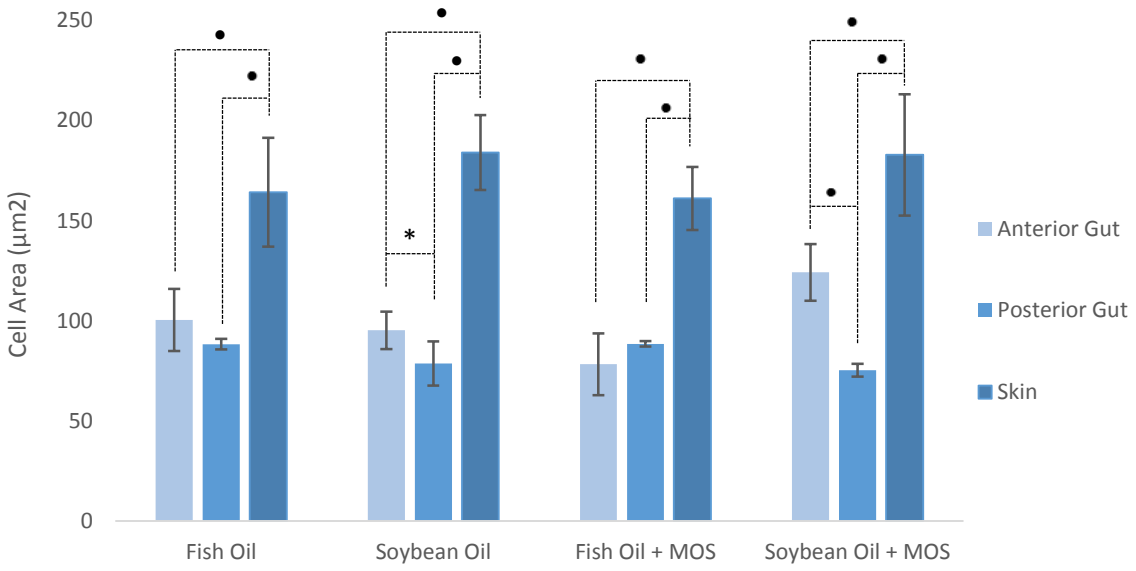

Figure 3.8: Mucous cells area from the different mucosal tissues by dietary treatment. T-test for significant difference testing between means. Statistically significant differences are: * ($P<0.05$) and • ($P<0.01$).

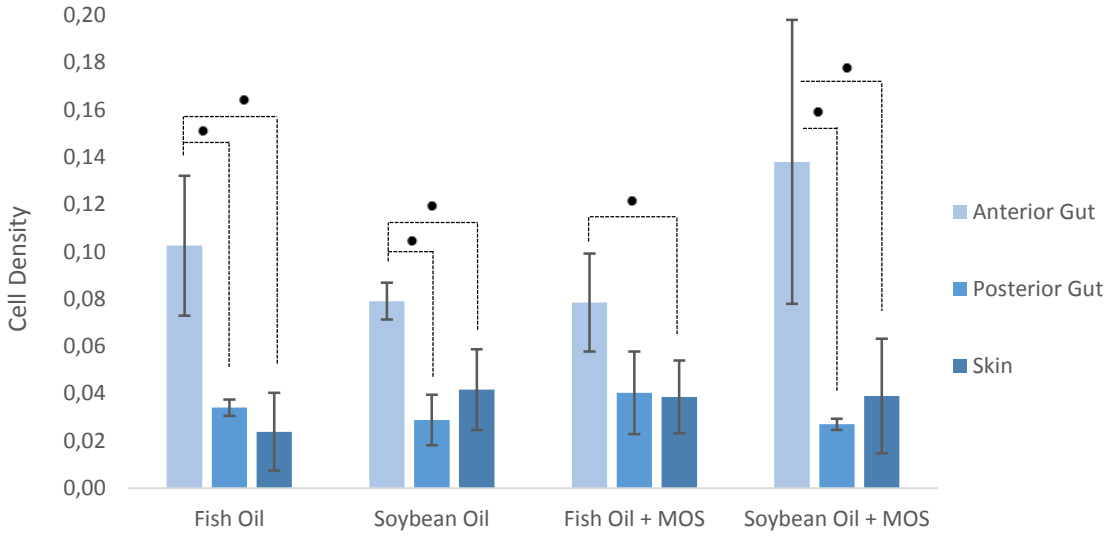

Figure 3.9: Mucous cells density from the different mucosal tissues by dietary treatment. T-test for significant difference testing between means. Statistically significant differences are: * (P<0.05) and • (P<0.01).

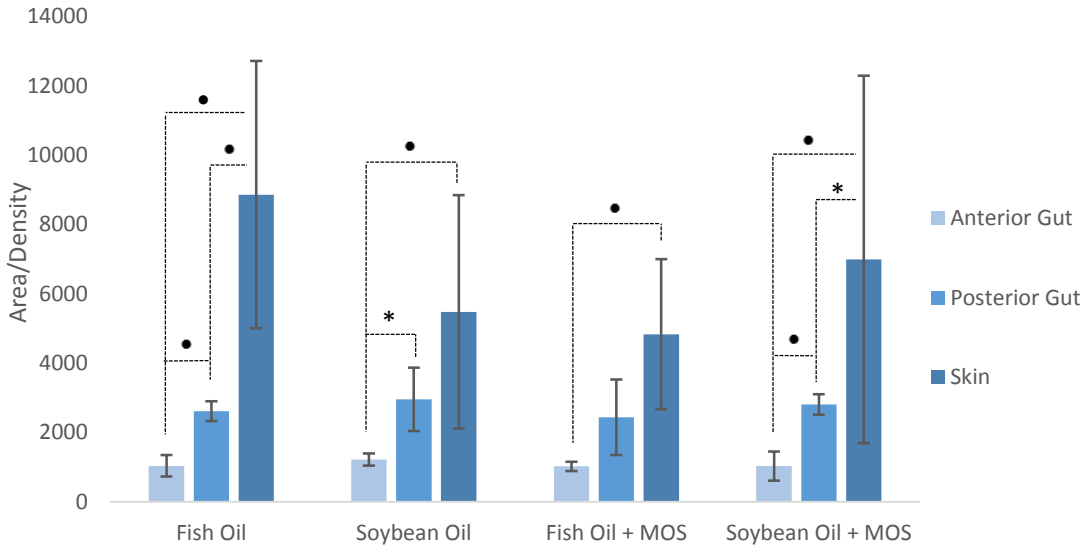

Figure 3.10: Mucous cells area/density ratio from the different mucosal tissues by dietary treatment. T-test for significant difference testing between means. Statistically significant differences are: * (P<0.05) and • (P<0.01).

4. Discussion

4.1. Stain optimization

The histological methodology developed by Pittman *et al.,* (2011, 2013) to quantify mucous cells was successfully applied to this study for the analysis of gut samples. However, for the histological study of the skin samples from the European seabass, PAS-Alcian Blue did not result in satisfactory staining of the epithelium, which is crucial for density assessment. Although, this dye stains specifically for mucopolysaccharides, binding to neutral and acidic glycoproteins (Yamabayashi, 1987), the epithelium was not clear and differentiable probably due to a low penetration of the dye through the embedding plastic medium and the calcified structures of the skin. We used Toluidine Blue in two different concentrations (100% and 10%) to optimize the staining protocol. Toluidine Blue is a basic thiazine metachromatic dye with high affinity for acidic tissue elements and is commonly used to highlight mucins as well (Sridharan and Shankar, 2012). It is a high penetrative dye, good to sharpen histology images and easy to prepare. No significant differences ($P<0.05$) were obtained for mean mucous cell area and the epithelium was totally differentiable, allowing for density measurements. These results allowed use to optimize the methodology to stain highly calcified samples for Mucosal Mapping™. Since both Toluidine Blue 100% and 10% yielded similar results, we decided to utilize the diluted version for reagent usage maximization purposes. The staining protocol was altered for skin sample as follows: dilution of 1 part of Toluidine Blue dye in 9 parts of distilled water, bathing the slides in the dye for 40 seconds and washing in water for 10 seconds.

It is, however, important to take into consideration the non-significant different peaks of mucous cell dimensions yielded by both dyes. Toluidine Blue seems to shift the mean mucous cell area to higher values, which means that cells with small dimensions were more difficult to distinguish on the skin, leading to a potential misidentification of smaller mucous cells. Therefore, an eventual increase of cell turnover might be masked by the use of Toluidine Blue.

Further research in staining procedures is highly recommended to optimize this methodology for any type of tissue resistant to PAS-Alcian Blue.

4.2. Mucosal tissue modulation

Epithelial mucosal surfaces have a number of defence mechanisms to avoid bacterial adhesion, which include mucus secretion (Ellis, 2001) and anti-adhesive action of mucins (Bavington et al., 2004). Consequently, increased mucus production could be responsible for enhanced gut health. The effect of MOS supplementation on improving the functional integrity of the intestine of fish species such as Gilthead seabream, White seabream, cobia and rainbow trout (see Table III), has been demonstrated in terms of higher microvilli density and length (Dimitroglou *et al.,* 2008, 2010a, 2010b; Salze *et al.,* 2008; Zhang *et al.,* 2012) as well as increased intestinal fold length (Torrecillas *et al.,* 2011b). Improved gut functionality could be directly related to an enhanced gut mucus production as reported in previous studies for *D. labrax* fed MOS (Torrecillas *et al.,* 2011a, 2011b).

An improved barrier better protects the enterocytes from damaging, leading to a better absorption of nutrients and can also reduce gut infection by harmful bacteria (Torrecillas *et al.* 2007). Generally, mucins are secreted by mucous cells at a baseline rate in order to maintain the mucus layer in the gut epithelium but, upon stimulation, these mucous cells might accelerate their release of mucous (Plaisancié *et al.*, 1998). In agreement with previous studies, we observed a modulation of the mucous cells area and density in the gut of European seabass for MOS supplemented diets, but the effects were mostly significant in the anterior region of the intestine. Remarkably, supplemented diets of 'Fish Oil' and 'Soybean Oil' produced opposite responses in the anterior gut, suggesting alternative mucosal modulation mechanisms depending on the oil source. Moreover, MOS added to the 'Soybean Oil' diet only produced significant effects in cell area and density in the anterior gut, meanwhile in the posterior gut no effects were detected. In the present study, all diets tested had in their composition an equal fraction of FM and SBM, but the 'Soybean Oil' diets add an additional soybean element, the oil, which substitutes the fish oil present on the 'Fish Oil' diets.

Soybean based feeds are rich in anti-nutrients, which may reduce feed intake, growth, nutrient digestibility and utilization, disturb the function of internal organs and affect disease resistance. Some important anti-nutritional factors are: fibers, which interfere with digestion, absorption and utilization of nutrients (van der Kamp *et al.,* 2004), enzyme inhibitors, which slow down digestion of nutrients (Krogdahl and Holm, 1979; Berg-Lea *et al.,* 1989); lectins, which bind to gut cell receptors, and are thought to be responsible for stimulating intestinal

tissue growth, turning it more permeable for macromolecules influx and bacteria, stimulate insulin production and modify the metabolism (Grant, 1991); saponins, which also increases the permeability of the gut mucosa, leading to tissue inflammation (Johnson *et al.,* 1986); phytoestrogens, which may deregulate the action of endogenous estrogen (Mazur and Adlercreutz, 1998); quinolizidine alkaloids, such as lupanin, which can cause nervous system conditions and gut disorders (Wink *et al.,* 1998); and oligosaccharides, which can modify gut microbiota (Cummings *et al.,* 1986).

Therefore, the introduction of extra soybean anti-nutritional factors with the addition of soya oils might possibly trigger a stronger inflammatory reaction and modify the gut microbiota. The interaction between the different gut microbiota arising from the different diets and the mucosal epithelium may be the mechanism by which the differential mucous cell stimulation occurred.

4.2.1. Anterior gut

In our dietary experiment, when fish were fed the control diets, the 'Soybean Oil' diet resulted on a significantly lower density of mucous cells in the anterior gut but roughly similar cell dimensions than the 'Fish Oil' diet. This observation can either mean less mucous cells on fish fed the 'Soybean Oil' control diet or more epithelium. Previous studies on Atlantic salmon reported an inflammation of the epithelial tissue upon feeding the fish with SBM based diets, causing enteritis (Baeverfjord and Krogdahl, 1996; Knudsen *et al.,* 2007, 2008; Urán *et al.,* 2008) and the severity was dependent on the quantity and strain of soya used (Urán *et al.,* 2009). The salmon displayed normal growth and feed intake, although they developed a strong inflammation in the gut, characterized by a great decrease of microvilli height, a swelling of the lamina propria and sub-epithelial mucosa, a higher number of mucous cells, an increased presence of eosinophilic granulocytes and ultimately a total tissue disruption (Urán *et al.,* 2009).

On previous studies, researchers have assessed the intestinal histology of fish fed SBM and have observed that various species can have different tolerance limits to the presence of anti-nutrients, which are thought to be the cause of enteritis (van den Ingh *et al.*, 1991; Heikkinen *et al.,* 2006; Bonaldo *et al.,* 2008). Soy saponins in particular, which increase intestinal permeability, combined with other feed components still to be identified,

are the main responsible agents for the inflammatory response on fish (Knudsen *et al.,* 2007, 2008). The severity of SBM-induced enteritis is also different among fish species (Urán *et al.,* 2008; Lilleeng et al., 2009) and, in most of the cases, histopathology was applied after 6 to 9 weeks of dietary experiment, the range where our trial of 8 weeks is inserted.

As referred earlier, the presence of an extra soybean component in the 'Soybean Oil' diet delivers, as expected, additional soybean anti-nutritional factors to the fish intestine. Therefore, it is very likely to assume that the lower density of mucous cells in the anterior gut of fish treated with the 'Soybean Oil' control diet compared with the 'Fish Oil' control diet is due to an increase of epithelial area because of the additional soybean oil component, which might trigger a more evident inflammatory reaction in the gut of European seabass. Since the epithelium is the reference volume for density measurements, then the change in density regards only the mucous cells, which must be proven to not be altered or reduced in undisputed cases of enteritis, but more research is needed to define the behavior of mucous cells in enteritis-affected tissues. Nonetheless, when MOS is added to the 'Soybean Oil' diet, the density and area of mucous cells is significantly increased in the anterior gut, comparing with the control. It also produces a higher density of mucous cells then the 'Fish Oil' based diets (both control and supplemented). The improved density of cells, thus, can be explained by both the increased cells dimension and the lower inflammatory response due to the presence of MOS.

On the other hand, the fish oil based diets, although including SBM, also yielded very interesting results in the anterior gut. Even though non-significant differences were found between both non-supplemented and supplemented diets, there was smaller cells in the anterior gut in the anterior gut of fish fed the 'Fish Oil + MOS' diet (78.33 ± 15.43 μm^2) when comparing with the control (100.43 ± 15.48 μm^2), even though the density was very similar (10.25 ± 2.95% for 'Fish Oil' and 7.85 ± 2.07 % for 'Fish Oil + MOS'). Therefore, since the addition of MOS resulted in a decreased mucous cell area but kept the density approximately the same, there was necessarily an increase of the number of mucous cells in the epithelium. So, an opposite modulation effect on the mucous cells population is produced by MOS when added to a soybean oil based diet and a fish oil based diet.

The 'Soybean Oil + MOS' produces bigger cells and a higher density relative to the 'Fish Oil + MOS' results, yet 'Fish-Oil+MOS' had a higher number of cells than the 'Fish Oil' control. A greater number of smaller mucous cells suggests an enhanced cell proliferation, therefore a faster cell turnover and, ultimately, a potential advantage in terms of mucous

production. Relatively to the large mucous cells observed for the 'Soybean Oil + MOS' diet, these are thought to be formed by a mechanism that increases the mucus storage within the cells.

As stated before, mucus is known to function as a protective barrier against pathogens, however, nutrients need to cross that barrier in order to reach the enterocytes and be absorbed. If the mucus layer is excessively thick it can act as an obstacle against that process. But in the present study no adverse effects were observed in terms of growth performance, suggesting no changes in the gut mucus layer against nutrient uptake. This does not mean the findings would not affect growth on the long term. The enlargement of the mucous cells was also previously observed in the small intestine of chicks after a period of starvation, without affecting food conversion rate (Smirnov *et al.,* 2004, 2005) and goblet cell hyperplasia and hypertrophy with consequent enhanced mucous production was also observed in mammalian intestines (Miller, 1987; Marzouk *et al.,* 2002) and fish intestines (Bosi *et al.,* 2005) after parasite infection without affecting growth performance as well. This indicates an improved capacity of the gastro-intestinal tract to respond to pathogenic attacks through increased flexibility of mucous cell size and storage capacity without deleteriously increasing secretion to reduce growth performance.

4.2.2. Posterior gut

In the posterior gut no significant differences between dietary treatments were observed in terms of cell density. It is, however, remarkable to see that the overall cell density in the posterior gut is lower for all diets when compared with the anterior gut. The 'Fish Oil + MOS' diet resulted on both the highest mean cell density observed in the posterior gut (4.02 ± 1.75 %) and the lowest mean cell density in the anterior gut (7.85 ± 2.07 %). This is an expected outcome since the posterior gut has a naturally larger epithelial area than the anterior gut where structures are less for nutrient absorption (generally amino acids and proteins) and mostly for the adaptive immunity with high quantities of lymphoid cells and a thicker lamina propria, resulting on a lower mucous cell density (Ezeasor and Stokoe 1981; Sire and Vernier 1992; Buddington *et al.,* 1997). Therefore, significant effects from the different treatments on mucous cell dynamics are more difficult to notice in that region of the gut.

In terms of cell area, which does not depend on the epithelium dimension, the addition of MOS did not result in any significant difference in size of gut mucous cells over the controls diets. However, the fish-oil based diets resulted in significantly bigger ($P<0.05$) cells than the soybean-oil based diets, meanwhile no significant differences were found when MOS was added. The reason for such effect must be based on the oil-type added to the diet, thus the substitution of fish oil with soybean oil induced a reduction in size on the mucous cells in the posterior gut and the effect of MOS was undetected.

By contrast, Torrecillas *et al.* (2011a, 2011b, and 2013) verified an effect of adding MOS 4 $g.kg^{-1}$ to a 'Fish Oil' diet, which improved mucous cells number in the posterior gut of European seabass. However, numerical density doesn't give any information about the cell size so the increasing number of cells might not be, by itself, an indication of enhanced mucosal immunity. An unbiased estimation of cell density and cell area given by this methodology gives a more precise look into the dynamics of the tissue.

A possible explanation for the lack of effects of MOS on the posterior gut might be its low bioavailability. Hence, the oil-type is the variable that is affecting the mucous cell area and the substitution of fish-oil with soybean oil in the diet is driving the mucous cells to become smaller in size.

4.2.2.1. Comparing both regions of the intestine.

For fish fed the fish oil based diets, with or without MOS, the cells are very similar in size ('Fish Oil': 100.43 ± 15.48 μm^2 in anterior gut and 88.35 ± 2.59 μm^2 in posterior gut; 'Fish Oil+MOS': 78.33 ± 15.43 μm^2 in anterior gut and 88.46 ± 1.35 μm^2 in the posterior gut). By contrast, the control 'Soybean Oil' diet resulted on a slight, but perceptible difference in the size of cells between each region: the anterior gut had cells with 95.22 ± 9.35 μm^2 and the posterior gut, 78.68 ± 11.04 μm^2, significantly smaller ($P<0.05$) than the control 'Fish Oil' diet. The soybean oil based diet supplemented with MOS resulted in an even more obvious difference in sizes of mucous cells between the regions of the intestine: 124.16 ± 14.13 μm^2 in the anterior gut and 75.30 ± 3.20 μm^2 in the posterior gut. Therefore, MOS added to a soybean oil based diet significantly increased the mean mucous cell area in the anterior gut, but had no effect in the posterior gut. This suggests that MOS has a greater effect at the

foremost part of the intestine, maybe due to a lowering of availability along the intestinal tract, so the effects are less detectable in posterior gut.

Since this posterior region has a lower density of mucous cells due to its larger epithelium area, it is consequently more difficult to detect an eventual modulatory effect of statistical importance. The anterior gut presents the highest density in all the treatments, where Mucosal Mapping™ takes into consideration both the size and the number in a reference volume of epithelium. Other studies use numerical density which does not consider size. The presented results are in conflict with other two studies that detected a higher numerical presence of mucous cells on the posterior region than in the anterior region of *D. labrax* intestine (Torrecillas *et al.,* 2011a, 2011b). However, the present study shows larger cells at higher densities in the anterior gut of all treatments relative to the posterior gut. It is therefore the size of the cells which explain the differing conclusions of the studies. Nonetheless, the lack of studies on this subject make it difficult to draw a conclusion, and further studies are needed to confirm or disprove such observations.

4.2.3. Skin

In the skin epithelial tissue, no evidence of significant effects from the diet nor the prebiotic were observed. This suggests that the ability of MOS to modulate the innate immunity seems to be more evident in the gut than in the skin. A previous study (Torrecillas *et al.,* 2011b) where MOS was also given to European seabass through diet, skin mucus innate functions were not significantly modulated, according with our results. Nonetheless, another prebiotic, Ergosan, and fermented *Saccharomyces cerevisiae* (a common probiotic) fed to rainbow trout successfully enhanced skin mucus immune parameters (Sheikhzadeh *et al.*, 2012a, 2012b). These studies together indicate an underlying communication between both gut and skin mucosal tissues in rainbow trout. Therefore, there seems to be a species-dependent response to dietary components. More studies addressing the effects of dietary prebiotics on the skin are necessary and future dietary experiments on this topic should include the analysis of the suite of mucosal epithelia: the gut, the skin and the gills.

A reference about the methodology should be made in this sub-chapter since the results are a direct outcome of the methodology adaptation for skin samples. It is, therefore, important to take that into account when interpreting the results. The data from the ‘stain

optimization for skin samples' chapter evidenced a higher frequency of bigger mucous cells when skin samples are stained with Toluidine Blue, comparing with PAS-Alcian Blue. Since the presence of smaller cells is an important indicator of cell turnover, their misidentification can be a problem when we aim for alteration on mucosal cells dynamics. Therefore, the possible misidentification of the smaller mucous cells might have masked the potential effects of the diet and the prebiotic in the skin. Regarding the exposed, it is not possible to completely exclude an effect of the diet and the prebiotic.

To ensure the accuracy of future experiments, more trials on staining optimization for this specific type of highly calcified tissue are needed.

4.3. Considerations of potential MOS effects in the gut

Mucosal Mapping of mucous cell quantification gave no significant differences in either mucous cell density or mean mucous cell area in the posterior region of the gut. Nonetheless, when MOS is added to soybean oil diets, the density and area of the mucous cells in the anterior gut increases significantly, confirming the potential of MOS to modulate innate immunity. Likewise, in the anterior gut, the cell density and area were not affected by adding MOS to the fish oil diet, through our observations. Torrecillas *et al.* (2011a) demonstrated an enhancement in the total number of mucous cells on posterior gut of European seabass fed fish oil diets supplemented with MOS. However, it is important to refer that the methodology used by Torrecillas *et al.* (2007, 2011a) to quantify mucous cells was based on number per unit area rather than a percentage (Table VI).

Torrecillas *et al.* (2007, 2011b) also demonstrated that fish fed MOS added to fish oil based diets with SBM were less infected by *V. alginolyticus* and *V. anguillarum* than non-supplemented fish, and correlated this observation with the higher number of cells secreting mucins (Torrecillas *et al.,* 2011a). Nonetheless, we did not find any evidence of significant differences on cell area and density by testing MOS on fish oil based diets. This means that significant variations in cell number were unlikely in our experiment. Hence, the lower infection rate might not be directly caused by a higher presence of mucous cells but rather related with the improvement of other immune parameters, such as head kidney macrophages phagocytic activity, eosinophilic granulocytes in the mucosa and mucous lysozyme activity (Torrecillas *et al.,* 2011a, 2011b). Nonetheless, the mentioned papers lack

a description of the method for a better comparison of both methodologies and correspondent results.

Table VI: Previous studies on the effects of MOS in *D. labrax* with gut mucous cell density measurements.

Fish species and **MOS (dose/time)**	**Gut site**	**Mucous cell density**		**Units**	**References**
		Control	**MOS**		
European seabass (*Dicentrarchus labrax*) 4 g kg^{-1} / 8 weeks	Anterior gut	≈ 615 ± 210	≈ 700 ± 300	Mucous cell/10^6 unit of area	Torrecillas *et al.* (2011a)
	Posterior gut	≈ 950 ± 275	≈1125 ± 455		
	Anterior gut	406.09 ± 125.36	480.84 ± 167.55	Mucous cell/10^6 unit of area	Torrecillas *et al.* (2011b)
	Posterior gut	697.46 ± 355.50	869.29 ± 321.76		
	Posterior gut	2821.58 ± 283.94	3230.54 ± 538.87	Mucous cell/10^6 unit of area	Torrecillas *et al.* (2013)

Studies about the effects of soybean oils in fish gut and immunity are scarce, and the presented experiment intends to shed a light into the subject. Lower infection by *Aeromonas salmonicida* spp. *salmonicida* was observed in Arctic charr when fed a diet with soybean oil (Ringo *et al.,* 2002). Yet, the antibacterial effect detected is probably due to the autochthonous microbiota selected by the diet, which themselves are thought to able to modulate mucus secretion through liberation of modulatory substances (Kandori *et al.,* 1996; Comelli *et al.,* 2008; Wrzosek *et al.,* 2013) The microbiota produces a wide range of carbohydrate-degrading enzymes which process otherwise indigestible dietary compounds and mucus polysaccharides (Flint *et al.,* 2012; Koropatkin *et al.,* 2012). Therefore, different diet compositions are able to select for specific bacteria and, thus, modify gut microbiota diversity according to the ability of individuals to metabolize those compounds. For example, Wrzosek *et al.* (2013) showed that *Bacterioides thetaiotaomicron* enhances goblet cells differentiation leading to an increase of goblet cells number and mucin gene expression in the colon of gnotobiotic rats.

The addition of prebiotics also modulates the fish gut microbiota as it introduces new molecules that will interact with the bacterial population. A previous study with rainbow trout assessed the effects of MOS on the gut microbiota and intestinal morphology (Dimitroglou

et al., 2009) where the control diet had FM and SBM and the oil component was from a fish source. MOS was added to this diet at 0.2%. Its addition lead to an increased gut absorptive surface area (measured as the ratio between internal perimeter of the gut lumen [villi and mucosal folds length] and external perimeter of the gut, and high values indicate augmented absorptive surface) from both anterior and posterior regions in sub adult groups as well as increased microvilli length and density. The cultured microbiota was significantly reduced by MOS. The levels of *Aeromonas/Vibrio* spp. were significantly reduced in juvenile individuals. It also reduced species diversity and increased resemblance of bacterial populations found within the groups. Juvenile individuals showed a significant reduction of *Micrococcus* spp. (22 to 7 % of total microbiota), *Aeromonas/Vibrio* spp. (37 to 9 %) and unidentified gram-positive rods (25 to 6%). On the other hand, it increased the density of *Enterococcus* spp. (3 to 19 %) and *Enterobacteriaceae* (5 to 39 %). Sub adult individuals showed a decrease in *Micrococcus* spp. (27 to 6 %) and *Enterobacteriaceae* (22 to 5 %) and increased *Pseudomonas* spp. (7 to 26 %). MOS is able to bind to certain gram-negative bacteria (like *Aeromonas/Vibrio, Enterobacteriaceae* and other gram-negative strains), inhibiting intestinal colonization, resulting in a removal mechanism of bacteria from the gut (Spring *et al.,* 2000). This may explain the changes of viable populations observed in rainbow trout, with large reduction of gram-negative populations.

Another study on rainbow trout (Rodriguez-Estrada *et al.*, 2009) assessed the effect of MOS on growth performance and immune response when added to a commercial diet without SBM, but the oil component was soybean based. After 12 weeks of feeding, fish fed with MOS recorded significantly higher ($P<0.05$) weight gain and SGR values. Also significantly higher hematocrit values were recorded when compared with the control, as well as phagocytic activity. A higher quantity of skin mucus was produced on fish fed MOS diet, indicated by a significantly higher mucus weight (skin mucus scrapped with a glass slide - 10cm line from the base of the operculum). A lower infection of *Vibrio anguillarum* was recorded by a lower presence of this pathogen on head kidney of fish fed MOS. Therefore, innate immune function was improved, suggesting that this supplement stimulates immune function on rainbow trout. Peterson *et al.* (2009) and Sang *et al.* (2009) also verified that MOS has an immune stimulant capacity, conferring protection against pathogens. It was suggested that MOS may stimulate the mannose receptors (Engering *et al*. 1997) and the mannose binding lectin by liver secretion, activating a cascade that stimulates the non-specific immune system (Janeway, 1993).

In Gilthead seabream, which is also a carnivorous Mediterranean species as the European seabass, feeding 0.2 and 0.4 % MOS for 14 days gave an increase in total leucocyte levels and reduction in the culturable microbial load without influencing relative abundance of identified bacterial species (Dimitroglou *et al.,* 2010a). The effects of MOS in the microbiota was more pronounced in FM based diets than in SBM based diets (higher species diversity, richness and reduced similarity between FM groups). It was suggested that the contrasting effects of MOS on the gut microbiota of fish fed diets with or without the inclusion of SBM might be due to the large numbers of oligosaccharides present in SBM, which may themselves affect gut microbiota and mask or overpower the effects of MOS (Dimitroglou *et al.,* 2010a). Actually, SBM oligosaccharides have been considered a potential prebiotic as they are fermented and metabolized by some species of bacteria and, therefore, can modulate the gut microbiota (Gibson *et al.,* 2004).

Since our study included only commercial feeds with SBM included, it is important to consider a combined effect of soybean components with MOS, which are most likely to produce results that are hard to be compared with previous studies that looked into the effects of MOS added to exclusively FM based commercial feeds. Unfortunately, the mechanism by which MOS regulates intestinal microbiota has not been well described and the data that exist about bacterial populations in the fish gut are still limited and variable, therefore further studies in relation with this subject are required.

According to these Results and Discussion, it is clear that there are advantages of supplementing the diet of European seabass with MOS when soybean derivatives are used as substitutes of fish derivatives, as shown by the mucosal tissue modulation in the anterior gut. This is particularly interesting in commercial diets with a fraction of SBM already included where soybean oil is used as a substitute for fish oil. This extra vegetable ingredient might be adding additional anti-nutritional factors that can disturb the gut microbiota and epithelial integrity. The addition of MOS will improve the innate immune system awareness by increasing mucous storage capacity of the mucous cells and their density in the gut epithelium, which will result on better response upon potentially pathogenic bacteria selected by the soybean based diets.

4.4. Mechanisms of mucosal modulation in the gut

The opposite effects produced by MOS in the anterior gut when added to a fish oil based diet and a soybean oil based diet might be explained by different mechanisms of mucosal modulation.

With the 'Fish Oil + MOS' diet, the number of mucous cells was increased and the mean cell size was decreased, which indicates a faster turnover of mucous cells in the tissue. The role of MOS on the modification of mucous cells dynamics might be explained by the following: the gut of *D. labrax* fed the control 'Fish Oil' diet has an assumed normal population of autochthonous bacteria. These indigenous bacteria are able to metabolize the mucus layer by enzyme degradation (Hoskins and Boulding, 1981; Corfield *et al.,* 1992). The presence of allochthonous bacteria is also important in maintaining a good equilibrium of both bacteria populations. However, when MOS is added to the diet, it binds to non-enteric bacteria and aggregates them, which give a window of opportunity for the enteric bacteria to multiply. It was suggested that mucus secretion is typically enhanced in response to intestinal microbes (Mack *et al.,* 1999; Deplancke and Gaskins, 2001), thus, increased presence of enteric bacteria stimulates the production of more mucus. Therefore, mucous cell turnover is also amplified to keep the faster rate of mucus production and more quantity of small cells will be available in the tissue. The increased mucus production also increases the flushing of the bundled bacteria which increases their elimination from the gut and decreases pathological infections. Thus, MOS stimulates mucus production indirectly by aggregating and inactivating allochthnous bacteria and allowing the autochthonous to multiply and chemically stimulate mucous production.

The presence of MOS might also promote a better cohesion of the enterocytes (Campo *et al.,* 2014), which characterizes a healthy and strong epithelial barrier, and the higher pressure that results from it can prevent the mucous cells to grow, reducing the mean cell area. In this case, as well, a recruitment of more mucous cells is necessary to keep the optimal mucous cell density and normal production of mucous.

With the 'Soybean Oil + MOS' diet the effects were different, as we verified an increment of mucous cells sizes in the tissue. A possible explanation is related with an increased capacity of the cells to store mucus and, thus, become bigger. The changes in microbial population in the gut of fish fed MOS with soybean based diets are low relative to the controls, as opposed to the clear modulation from fish based diets (Dimitroglou *et al.,*

2010a). A possible reason is that the soybean components of the diet (the protein and the oil) add additional molecules, as oligosaccharides, that are used as substrate by some bacteria and allow specific allochthonous species to develop in the gut. MOS might not be enough to eliminate those allochthonous bacteria since those vegetable elements are feeding them and letting them multiply and strive. Thus the potential effects of MOS are masked by the soya components which select for those bacteria. Therefore, another mechanism of protection is in motion. The bundled bacteria by MOS molecules are not being flushed out so fast since the mucous is not being released at a fast rate. So, those are more prone to be detected by dendritic cells which initiate a cascade of immunological changes against the presence of a high number of potentially pathogenic bacteria. The stimulation of the mucous cells storage capacity might be one of the results of that immune response, has a preventive measure for upcoming bacterial attacks. This allows a higher protection capacity in case the homeostasis is disrupted by increased pathogenic assaults due to a higher population of allochthnous bacteria. Thus, MOS stimulates innate immunity by presenting potentially pathogenic bacterial strains to the cellular immune agents, ultimately resulting in the increased storage capacity of the mucous cells as adaptive response.

5. Conclusion

The modulation of the mucosal tissues by dietary supplementation with immunostimulants has been receiving a lot of attention and has become a very important method for controlling pathological infections in aquaculture production. It mitigates the need of vaccination, promotes a healthy gut environment and, therefore, reduces the need for therapeutic procedures for pathogen control. MOS is a prebiotic that has been tested in several fish species in the last decade with promising results. It has shown positive effects on improving growth parameters, enhancing gut morphological features and the innate immune system (Dimitroglou *et al.,* 2008, 2009, 2010a, 2010b; Refstie *et al.,* 2010; Rodriguez-Estrada *et al.*, 2009; Salze *et al.,* 2008; Staykov *et al.,* 2007; Torrecillas *et al.,* 2007, 2011a, 2011b, 2013; Zhang *et al.,* 2012).

In the present study we tested the modulatory effects of MOS on gut and skin mucosa when added to commercial SBM+FM feed formulations with different oil components: fish based and soybean based. This was determined by mucous cell analysis in the target tissues using a novel stereology-based image analysis methodology (Pittman *et al.,* 2011, 2013).

MOS appear to improve innate immunity in the anterior gut when added to commercial diets. However the mechanism of improvement is different when the oil component is fish based or soybean based. 'Fish-Oil + MOS' resulted on a faster turnover of mucous cells, evidenced by the higher number of smaller cells when compared with the control, which might also indicate an improved cohesion of the enterocytes. In the other hand, 'Soybean + MOS' resulted on a greater storage capacity of the mucous cells, demonstrated by the higher number of larger cells and consequent increased density of cells in the tissue.

It is important in the future to analyze the microbe population from the gut of *D. labrax* for the different dietary treatments to see in what extent does the addition of an extra soybean component to the diet, in this case the oil, can modify the diversity and type of bacteria by selecting specific strains and how MOS is capable to modulate that effect in both regions of the gut.

The use of prebiotics is important not only for improving aquaculture production by growing healthier fish, less prone to disease, but also for welfare purposes, which is an important subject to take into consideration when breeding live animals as it is also directly linked with the mentioned higher productivity. Good welfare is the result of the capacity to

maintain homeostasis and the normal biological functions of an individual which ultimately reflect on the absence of disease (Segner *et al.,* 2012). This methodology is, therefore, a very useful and statistically robust way to assess mucosal health status and, thus, animal welfare.

The observations of the present study give strength to the hypotheses that the ability to change the gut mucosal tissue's cellular response is a key element to improve the resistance to pathological infection in the gut. It also points out that diet composition is fundamental in the ability of the tissue to exhibit that response, probably by providing crucial elements for mucosal cell turnover and increased mucous storage. Indeed, the present dissertation could recommend a minimum time of 8 weeks of MOS supplementation ($4g.kg^{-1}$) with both commercial diets, which seems to be necessary to result on a positive effect on enhanced health status, shown by an improvement of the gut innate immunity. Nevertheless, the raw materials used to produce the feeds are determinant in the potential effects of MOS in the gut.

For future goals, it is important to further understand the mechanisms underlying the modes of action of MOS in fish gut, and the possible interconnection of the mucosal tissues. Furthermore, microbiota populations should be identified and mapped and microbiota modulation by MOS should also be addressed in future studies and respective mechanisms of selection. Relative to the methodology, more dietary experiments should be performed with different species and tissue types in order to optimize the method and create a quick and reliable technique to assess mucosal modulation through image analysis.

Appendix

Histological images

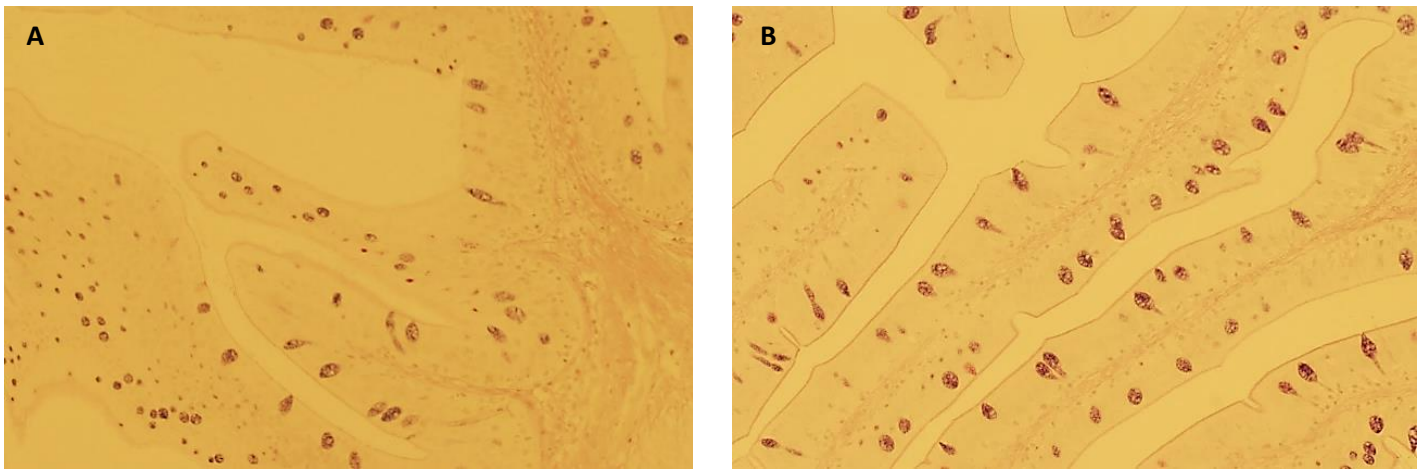

Figure A.1: Anterior gut samples from D. labrax stained with PAS-Alcian Blue. A) Sample from fish fed the 'Fish Oil + MOS'. B) Sample from fish fed the 'Soybean Oil + MOS' diet. It is possible to identify numerous smaller mucous cells on A) and bigger cells on B), which illustrates the results obtained on cell area measurements.

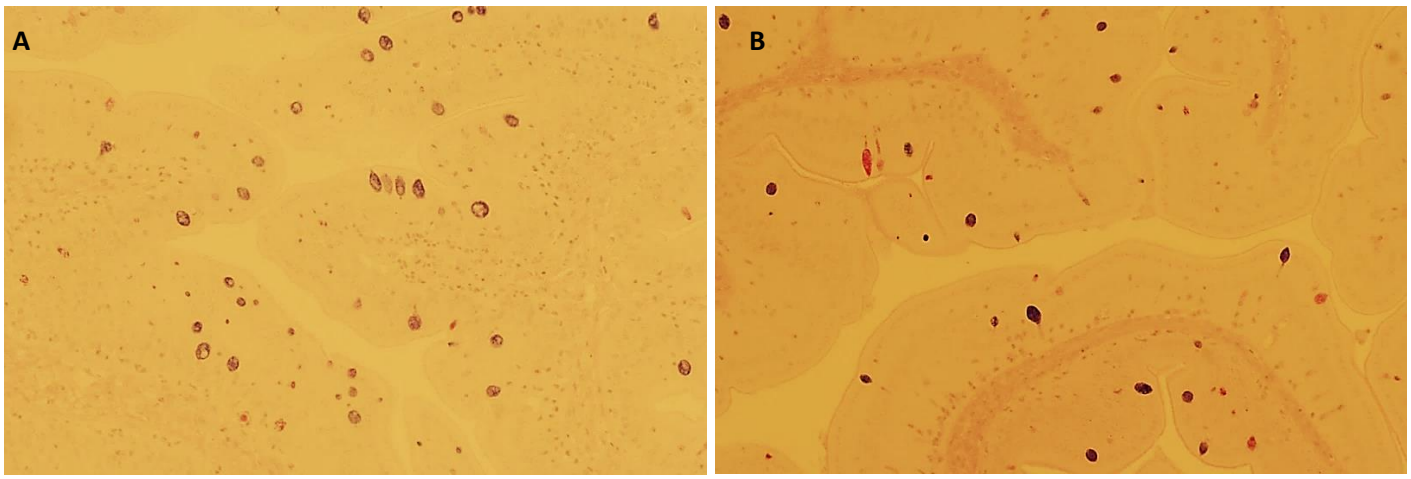

Figure A.2: Posterior gut samples from D. labrax stained with PAS-Alcian Blue. A) Sample from fish fed the 'Fish Oil + MOS'. B) Sample from fish fed the 'Soybean Oil + MOS' diet. It is possible to identify slightly bigger cells and more numerous on A) and smaller and less cells on B), which illustrates the results.

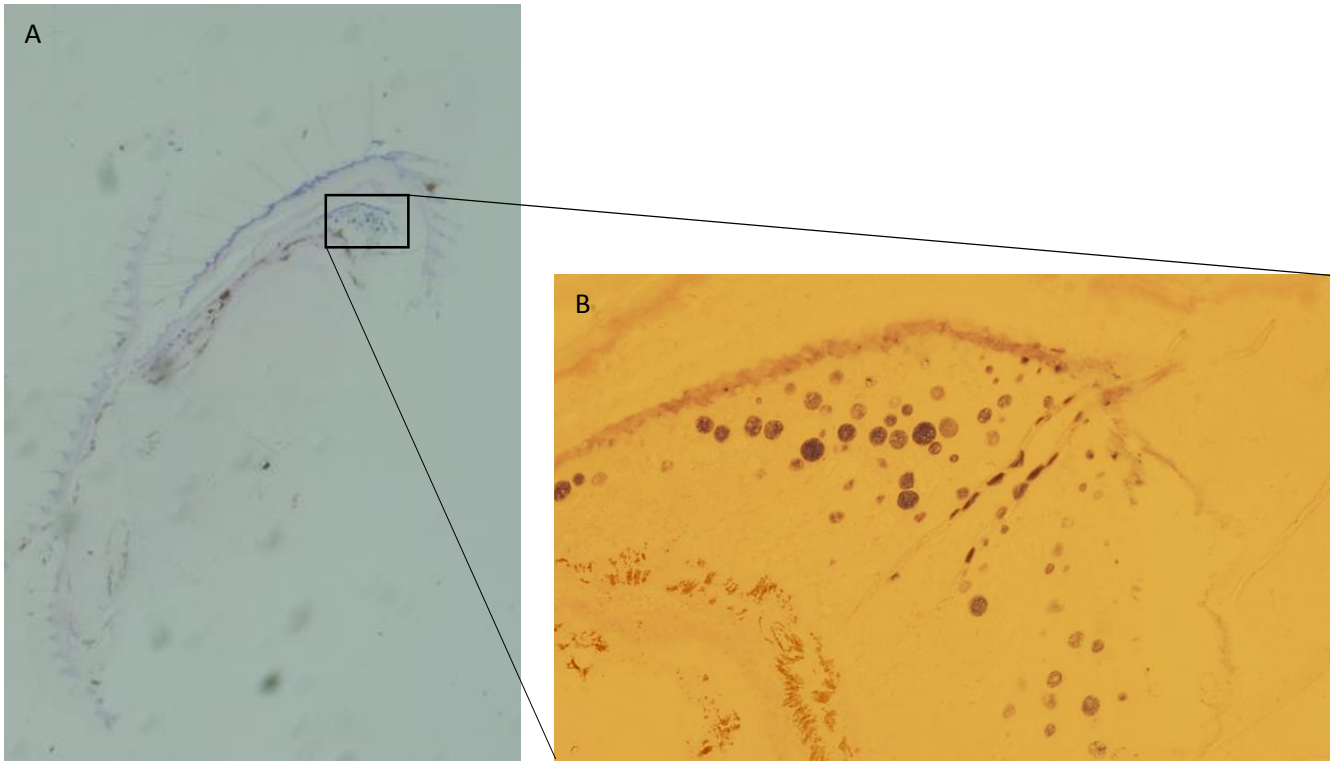

Figure A.3: Tissue section from D. labrax skin stained with PAS-Alcian Blue.

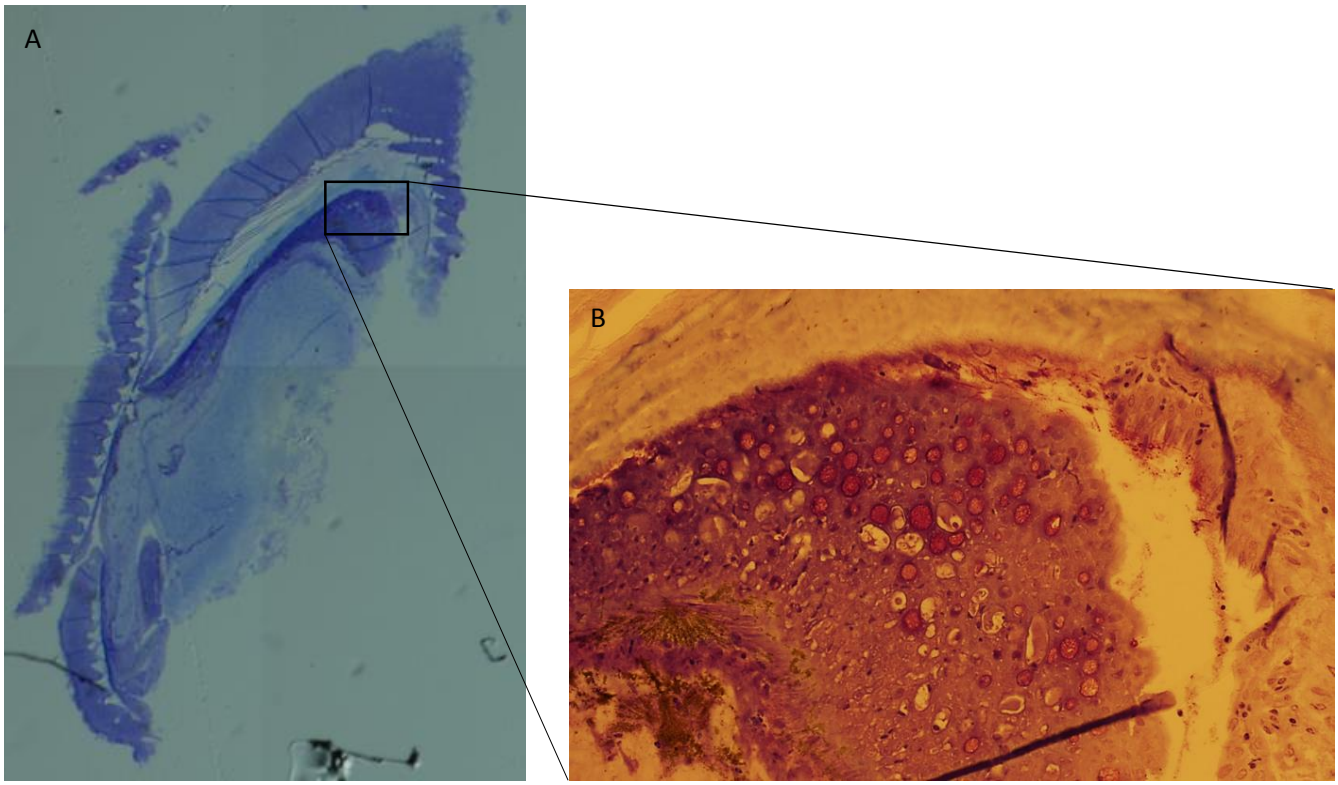

Figure A.4: Consecutive tissue section of the same skin sample from Figure A.3, stained with Toluidine Blue 100%.

References

Ackman RG. 1982. Fatty acids composition of fish oils. In: Barlow SM, Stansby ME (Editors). Nutritional evaluation of long-chain fatty acids in fish oil. London, Academic Press, pp. 25-88;

Akrami R, Razeghi-Mansour M, Chitsaz H, Ziaee R, Ahmadi Z. 2012. Effect of dietary mannan oligosaccharide on growth performance, survival, body composition and some hematological parameters of carp juvenile (Cyprinus carpio). J Anim Sci Adv 2(11):879-885;

Baeverfjord G, Krogdahl Å. 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, Salmo salar L.,distal intestine: a comparison with the intestines of fasted fish. J Fish Dis 19:375-387;

Bakke-McKellep AM, Penn MH, Salas PM, Refstie S, Sperstad S, Landsverk T, Ringo E, Krogdahl A. 2007. Effects of dietary soybean meal, inulin and oxytetracycline on gastrointestinal histological characteristics, distal intestine cell proliferation and intestinal microbiota in Atlantic salmon (Salmo salar L.). British Journal of Nutrition 97:699-713;

Bates JM, Mittge E, Kuhlman J, Baden KN, Cheesman SE, Guillemin K. 2006. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev Biol 297:374–86;

Bavington CD, Lever R, Mulloy B, Grundy MM, Page CP, Richardson NV. 2004. Anti-adhesive glycoproteins in echinoderm mucus secretions. Comp Biochem Phys B 139: 607-617;

Bavington CD, Page CP. 2005. Stopping bacterial adhesion: a novel approach to treating infections. Respiration 72:335-344;

Berg-Lea T, Brattas LE, Krogdahl A. 1989. Soybean proteinase inhibitors affect nutrient digestion in rainbow trout. In: Recent Advances of Research in Antinutritional Factors in Legume Seeds (ed. Huisman J, van der Pool TFB and Liener I), pp.99-102. Pudoc, Wageningen;

Bonaldo A, Roem AJ, Fagioli P, Pecchini A, Cipollini I, Gatta PP. 2008. Influence of dietary levels of soybean meal on the performance and gut histology of gilthead sea bream (Sparus aurata L.) and European sea bass (Dicentrarchus labrax L.). Aquaculture Research 39:970–978;

Bosi G, Arrighi S, Di Giancamillo A, Domeneghini C. 2005. Histochemistry of glycoconjugates in mucous cells of Salmo trutta unifected and naturally parasitized with intestinal helminthes. Dis Aquat Org 64:45-51;

Bricknell I, Dalmo RA. 2005. The use of immunostimulants in fish larval aquaculture. Fish and Shellfish Immunology 19(5):457-472;

Buddington RK, Krogdahl A, Bakke McKellep AM. 1997. The intestines of carnivorous fish: structure and functions and the relations with diet. Acta Physiol Scand 161(638):67-80;

Burger-van Paassen N, van der Sluis M, Bouma J, Korteland-van Male AM, Lu P, Van Seuningen I, Boehm G, van Goudoever JB, Renes IB. 2011. Colitis development during the suckling-weaning transition in mucin Muc2-deficient mice. Am J Physiol Gastrointest Liver Physiol 301(4):G667–678;

Burr G, Hume M, Neill WM, Gatlin DM III. 2008. Effects of prebiotics on nutrient digestibility of a soybean meal-based diet by red drum, Sciaenops ocellatus (Linnaeus). Aquacult. Res. 39:1680–1686;
Caballero MJ, Izquierdo MS, Kjorsvik E, Fernández AJ, Rosenlund G. 2004. Histological alterations in the liver of sea bream, Sparus aurata L., caused by short- or long-term feeding with vegetable oils. Recovery of normal morphology after feeding fish oil as the sole lipid source. Journal of Fish Diseases 27(9):531-541;
Caballero MJ, Izquierdo MS, Kjorsvik E, Montero D, Socorro J, Fernandez AJ, Rosenlund G. 2003. Morphological aspects of intestinal cells from gilthead seabream (Sparus aurata) fed diets containing different lipid sources. Aquaculture 225:325-340;
Caballero MJ, Obach A, Rosenlund G, Montero D, Gisvold M, Izquierdo MS. 2002. Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, Oncorhynchus mykiss. Aquaculture 214:253-271;
Campo A, Pittman K, Schartum E, Karlson S, Sweetman E, Sourd P, Johansen J, Ravnoy B, Zanna A. 2014. Mucosal mapping finds three distinct populations of mucus cells in salmon gills and skin. Manuscript submitted for publication;
Cannon WB. 1929. Organization for physiological homeostasis. Physiol Rev 9(3):399–431;
Chen W, Ai Q, Mai K, Xu W, Liufu Z, Zhang W, Cai Y. 2011. Effects of dietary soybean saponins on feed intake, growth performance, digestibility and intestinal structure in juvenile Japanese flounder (Paralichthys olivaceus). Aquaculture 318:95-100;
Comelli EM, Simmering R, Faure M, Donnicola D, Mansourian R, Rochat F, Corthesy-Theulaz I, Cherbut C. 2008. Multifaceted transcriptional regulation of the murine intestinal mucus layer by endogenous microbiota. Genomics 91:70–77;
Corfield AP, Wagner SA, Clamp JR, Kriaris MS, Hoskins LC. 1992. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infect Immun 60:3971–3978;
Cummings JH, Englyst HN, Wiggins HS. 1986. The role of carbohydrates in lower gut function. Nutrition Reviews 44:50-54;
Davis ME, Maxwell CV, Erf GF, Brown DC, Wistuba TJ. 2004. Dietary supplementation with phosphorylated mannas improves growth response and modulates immune function of weanling pihs. J Anim Sci 82:1882-1891;
Defoirdt T, Sorgeloos P, Bossier P. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology 14:251-258;
Deplancke B, Gaskins HR. 2001. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutr June 73(6):1131-1141;
Dimitroglou A, Davies S, Sweetman J. 2008. The effects of dietary mannan oligosaccharides on the intestinal histology of rainbow trout (Oncorhynchus mykiss). Abstracts/Comparative Biochemistry and Physiology, Part A 150:S56–S63;
Dimitroglou A, Davies SJ, Sweetman J; Divanach P, Chatzifotis S. 2010b. Dietary supplementation of mannan oligosaccharide on white sea bream (Diplodus sargus L.) larvae: effects on development, gut morphology and salinity tolerance. Aquaculture Research 41:e245-e251;
Dimitroglou A, Merrifield DL, Moate R, Davies SJ, Spring P, Sweetman J, Bradley G. 2009. Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Anim Sci 87:3226-3234;
Dimitroglou A, Merrifield DL, Spring P, Sweetman J, Moate R, Davies SJ. 2010a. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed

utilisation, intestinal histology and gut microbiota of gilthead sea bream (Sparus aurata). Aquaculture 300:182-188;
Dugenci SK. 2003. Some medical plants as immunostimulant for fish. Journal of Ethnopharmacology, 88:99-106;
Elliott DG. 2011. THE SKIN | The Many Functions of Fish Integument. In: Farrell AP (Editor) Encyclopedia of Fish Physiology. Academic Press, San Diego, pp 471-475;
Ellis AE. 2001. Innate host defence mechanism of fish against viruses and bacteria. Dev Comp Immunol 25:827-839 ;
Engering AJ, Cella M, Fluitsma, Brockhaus EC, Hoefsmit M, Lanzavecchia A. 1997. The mannose receptors function as a high capacity and broad specificity antigen receptor in human dendritic cells. Eur J Immun 27:2417-2425;
Eri R, Chieppa M. 2013. Messages from the inside. The dynamic environment that favor intestinal homeostasis. Frontiers in Immunology 4:1-7;
Eri RD, Adams RJ, Tran TV, Tong H, Das I, Roche DK, OanceaI, PNG CW, Jeffery PL, Radford-Cmith GL, Cook MC, Florin TH, McGuckin. 2011. An intestinal epithelial defect conferring ER stress results in inflammation involving both innate and adaptive immunity. Mucosal Immunol 4:354–364;
Ezeasor DN, Stokoe WM. 1981. Light and electron microscopic studies on the absorptive cells of the intestine, caeca and rectum of the adult rainbow trout, Salmo gairdneri, Rich. J Fish Biol 18(5):527-544;
Fairchild AS, Grimes JL, Jones FT, Wineland MJ, Edens FW, Sefton AE. 2001. Effects of hen age, Bio-Mos® and Flavomycin® on poult susceptibility to oral Escherichia coli challenge. Poultry Science 80:562–571;
FAO Fisheries and Aquaculture Department. 2013a. Global Aquaculture Production Statistics for the year 2011 [online]: ftp://ftp.fao.org/FI/news/GlobalAquacultureProductionStatistics2011.pdf
FAO Fisheries and Aquaculture Department. 2013b. Cultured Aquatic Species Information Programme. Dicentrarchus labrax. Text by Bagni M. In: FAO, Fisheries and Aquaculture Department [online]. Rome. Retrieved from: http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en. Accessed at: 2 October 2013;
FAO. 2012. The State of World Fisheries and Aquaculture 2012. Rome. 209 pp. [online]: http://www.fao.org/docrep/016/i2727e/i2727e.pdf
Fast MD, Sims DE, Burka JF, Mustafa A, Ross NW. 2002. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp Biochem Physiol A Mol Integr Physiol 132(3):645-657;
FEAP Secretariat. 2013. European Aquaculture Production Report 2003-2012. Liege, Belgium;
Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. 2012. Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3:289–306;
Forstner JF, Oliver MG, Sylvester FA. 1995. Production, structure and biologic relevance of gastrointestinal mucins. In: Blaser MJ, Smith PD, Ravlin JI, Greenberg HB, Guerrant RL. (Editors). Infections of the gastrointestinal tract. New York, Raven Press, pp 71-88;
Franklin ST, Newman MC, Newman KE, Meek KI. 2005. Immune parameters of dry cows fed mannan oligosaccharide and subsequent transfer of immunity to calves. J Dairy Sci 88(2):766-775;
Gaggìa F, Mattarelli P, Bivati B. 2010. Probiotics and prebiotics in animal feeding for safe food production. International Journal of Food Microbiology 141:515-528;

Gallo RL, Hooper LV. 2012. Epithelial antimicrobial defence of the skin and intestine. Nature Reviews 12:503-516;
Gatesoupe FJ. 2008. Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. J Mol Microbiol Biotechnol 14:107–114;
Gatlin III DM. 2002. Nutrition and fish health. In: Halver JE and Hardy RW (Editors). Fish Nutrition, 3rd Edition. London, Academic Press, pp 671-702;
Gatlin III DM. 2010. Principles of fish nutrition. SRAC Publication 5003. Texas, USA;
Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutr Res Rev 17:259–275;
Gibson GR, Roberfroid MB. 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. Nutr. 125:1401-1412;
Globefish. 2013. Fish oil – June 2013. Retrieved from: http://www.globefish.org/fish-oil-june-2013.html. Accessed at: 21 November 2013;
Gómez GD, Balcázar JL. 2008. A review on the interaction between gut microbiota and the innate immunity of fish. FEMS Immunol Med Microbiol 52:145-154;
Grant G. 1991. Lectins. In: Toxic Substances in Crop Plants (ed. D' Mello JPF, Duffus CM and Duffus JH), pp. 49-67. The Royal Society for Chemistry, Cambridge, UK;
Grieshop CM, Flickinger EA, Bruce KJ, Patil AR, Czarnecki-Maulden GL, Fahey GC Jr. 2004. Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. Arch Anim Nutr 58(6):483-493;
Grisdale-Helland, B, Helland SJ, Gatlin DM III. 2008. The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galsctooligosaccharide on the growth and feed utilization of Atlantic salmon (Salmo salar L.). Aquaculture 283:163–167;
Groff JM. 2001. Cutaneous biology and diseases of fish. Veterinary Clinics of North America 4(2):321–411;
Halas V, Nochta I. 2012. Mannan oligosaccharides in nursery pig nutrition and their potential mode of action. Animals 2:261-274;
Hawkes JW. 1974a.The structure of fish skin I. General organization. Cell and Tissue Research 149:147–158;
Hawkes JW. 1974b.The structure of fish skin II. The Chromatophore Unit. Cell and Tissue Research 149:159–172;
Heikkinen J, Vielma J, Kemilainen O, Tiirola M, Eskelinen P, Kiuru T, Navia-Paldanius D, von Wright A. 2006. Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (Oncorhynchus mykiss). Aquaculture 261:259-268;
Hellio C, Pons AM, Beaupoil C, Bourgougnon N, Le Gal Y. 2002. Antibacterial, antifungal and cytotoxic activities of extracts from fish epidermis and epidermal mucus. Int J Antimicrob Agents 20:214-219;
Hooper LV, Liitman DR, Macpherson AJ. 2012. Interactions between the microbiota and the immune system. Science 336(6086):1268-1273;
Hooper LV, Macpherson AJ. 2010. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nature Reviews Immunology 10:159-169;
Hooper LV. 2009. Do symbiotic bacteria subvert host immunity? Nature Reviews Microbiology 7:367-374;
Hoskins LC, Boulding ET. 1981. Mucin degradation in human colon ecosystems. Evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. J Clin Invest 67:163–172;

Howard V, Reed M. 2005. Unbiased Stereology (2nd Edition). BIOS Scientific Publishers, Oxford;
Iji PA, Saki AA, Tivey DR. 2001. Intestinal structure and function of broiler chickens on diets supplemented with a mannan oligosaccharide. J Sci Food Agric 81:1186–1192;
Index mundi. 2013. Commodity Prices. Retrieved from: http://www.indexmundi.com/commodities. Accessed at: 21 November 2013;
Instituto Nacional de Estatística, IP. 2013. Estatistica da Pesca 2012. Lisbon, Portugal;
Izquierdo MS, Montero D, Robaina L, Caballero M J, Rosenlund G, Ginés R. 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (Sparus aurata) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture 250(1, 2):431-444;
Izquierdo MS, Obach A, Arantzamendi L, Montero D, Robaina L, Rosenlund G. 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquaculture Nutrition 9(6):397-407;
Izquierdo MS. 1996. Review article: essential fatty acid requirements of cultured marine fish larvae. Aquac Nutr 2:183-191;
Izquierdo MS. 2005. Essential fatty acid requirements in Mediterranean fish species. Cah Options Mediterr 63:91-102;
Janeway CA. 1993. How the immune system recognizes invaders. Sci Am 269:73-79;
Johnson IT, Gee JM, Price K, Curl C, Fenwick GR. 1986. Influence of saponins on gut permeability and active nutrient transport in vitro. J Nutr 116(11): 2270-2277;
Kandori H, Hirayama K, Takeda M, Doi K. 1996. Histochemical, lectinhistochemical and morphometrical characteristics of intestinal goblet cells of germfree and conventional mice. Exp Anim 45:155–160;
Kardong KV. 2012. Vertebrates: comparative anatomy, function, evolution (6th Edition). McGraw Hill. New York, NY, USA. pp 527;
Knudsen D, Jutfelt F, Sundh H, Sundell K, Koppe W, Frøkiær H. 2008. Dietary soya saponins increase gut permeability and play a key role in the onset of soybean-induced enteritis in Atlantic salmon (Salmo salar L.). Br J Nutr 100:120-129;
Knudsen D, Urán P, Arnous A, Koppe W, Frøkiær H. 2007. Saponin containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon. Journal of Agricultural and Food Chemistry 55:2261-2267;
Koropatkin NM, Cameron EA, Martens EC. 2012. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol 10:323–335;
Krogdahl A, Holm H. 1979. Inhibition of human and rat pancreatic proteinases by crude and purified soybean proteinase inhibitors. Journal of Nutrition 109:551-558;
Krogdahl A, Penn M, Thorsen J, Refstie S, Bakke AM. 2010. Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. Aquaculture Research 41:333-344;
Lilleeng E, Penn MH, Haugland Ø Xu C, Bakke AM, Krogdahl Å, Landsverk T, Froystad-Saugen MK. 2009. Decreased expression of TGFb, GILT and T cell markers in the early stages of soybean enteropathy in Atlantic salmon (Salmo salar L. Fish Shellfish Immunool 27:65-72;
Lokesh J, Fernandes JMO, Korsnes K, Bergh O, Brinchmann MF, Kiron V. 2012. Transcriptional regulation of cytokines in the intestine of Atlantic cod fed yeast derived mannan oligossacharide or β-Glucan and challenged with Vibrio anguillarum. Fish & Shellfish Immunology 33(3):626-631;
Mack DR, Michail S, Wei S, McDougall L, Hollingsworth MA. 1999. Probiotics inhibit enteropathogenic E. coli adherence in vitro by inducing intestinal mucin gene expression. Am J Physiol 276:G941–950;

Magnadottir B. 2006. Innate immunity of fish (overview). Fish and Shellfish Immunology 20:137-151;
Magnadottir B. 2010. Immunological control of fish diseases. Journal of Marine Biotechnology 12:361-379;
Marzouk MSM, Ezz El-Din DN, Mohamed ME, Mahmoud AM, Moahmed RS. 2002. Epizootiological and pathological investigations on fishes of Lake Manzala infested with some gill and intestinal parasites. Egyptian J Agric Res 80:901-913;
Mazur W, Adlercreutz H. 1998. Naturally occurring estrogens in food. Pure and Applied Chemistry 70:1759-1776;
McCracken VJ, Lorenz RG. 2001. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity, and microbiota. Cell Microbiol 3:1-11;
Merrifield DL, Bradley G, Baker RTM, Dimitroglou A, Davies SJ. 2009. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout (Oncorhynchus mykiss Walbaum). Journal Fish Diseases 32:755-766;
Merrifield DL, Olsen RE, Myklebust, Ringo E. 2011. Dietary Effect of Soybean (Glycine max) Products on Gut Histology and Microbiota of Fish In: El-Shemy H (Editor), Soybean and Nutrition. InTech, Rijeka, Croatia, pp 231-250;
Miller HRP. 1987. Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. Parasitology 94:S77-S100;
Montero D, Kalinowski T, Obach A, Robaina L, Tort L, Caballero M J, Izquierdo MS. 2003. Vegetable lipid sources for gilthead seabream (Sparus aurata): effects on fish health. Aquaculture 225(1-4):353-370;
Montero D, Robaina L, Caballero MJ, Ginés R, Izquierdo MS. 2005. Growth, feed utilization and flesh quality of European sea bass (Dicentrarchus labrax) fed diets containing vegetable oils: a time-course study on the effect of a re-feeding period with a 100% fish oil diet. Aquaculture 248(1-4):121-134;
Moran CA. 2009. Nutritional approaches to controlling Salmonella. Pig progress 25(2). Retrieved from: http://www.pigprogress.net/Health-Diseases/Health/2009/4/Nutritional-approaches-to-controlling-Salmonella-PP005919W/;
Mourão JL, Pinheiro V, Alves A, Guedes CM, Pinto L, Saavedra MJ, Spring P, Kocher A: 2006. Effect of mannan oligosaccharides on the performance, intestinal morphology and cecal fermentation of fattening rabbits. Animal Feed Science and Technology 126:107–120;
Mourente G, Good JE, Bell JG. 2005. Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass (Dicentrarchus labrax L.): effects on flesh fatty acid composition, plasma prostaglandins E2 and E2α immune function and effectiveness of a fish oil finishing diet. Aquaculture Nutrition 11:25-40;
Mourente G, Tocher DR. 1993. Incorporation and metabolism of 14C-labelled polyunsaturated fatty acids in juvenile sea bream Sparus aurata L.in vivo. Fish Physiol. Biochem., 21:443–453;
Mulero I, Garcia-Ayala A, Meseguer J, Mulero V. 2007. Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: a mini review. Aquaculture 268:244–250;
Nagashima Y, Sendo A, Shimakura K, Shiomi K, Kobayashi T, Kimura B, Fujii T. 2001. Antibacterial factors in skin mucus of rabbit fishes. J Fish Biol 58:1761-1765;
Nasopoulou C, Stamatakis G, Demopoulos CA, Zabetakis I. 2011. Effects of olive pomace and olive pomace oil on growth performance, fatty acid composition and cardio

protective properties of gilthead sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax). Food Chemistry 129(3):1108-1113;
Nasopoulou C, Zabetakis I. 2012. Benefits of fish oil replacement by plant originated oils in compounded fish feeds. A review. LWT – Food Science and Technology 47:217-224;
Newman K. 1994. Mannan-oligosaccharides: Natural polymers with significant impact on the gastrointestinal microflora and the immune system. In: Lyons TP, Jacques KA (Editors). Biotechnology in the Feed Industry: Proceedings of Alltech's 10th Annual Symposium. Nottingham, UK, Nottingham University Press. pp 167–174;
Nordrum S, Bakke-McKellep AM, Krogdahl A, Buddington RK. 2000. Effects of soybean meal and salinity on intestinal transport of nutrients in Atlantic salmon (Salmo salar L.) and rainbow trout (Oncorhynchus mykiss). Comp Biochem Physiol B Biochem Mol Biol 125, 317-335;
Northey A, Denis D, Cirino M, Metters KM, Nantel F. 2000. Cellular distribution of prostanoid EP receptors mRNA in the rat gastrointestinal tract. Prostaglandins Other Lipid Mediat 62:145-156;
Olsen RE, Dragnes BT, Myklebust R, Ringo E. 2003. Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, Oncorhynchus mykiss Walbaum. Fish Physiology and Biochemistry 29:181-192;
Olsen RE, Myklebust R, Kaino T, Ringo E. 1999. Lipid digestibility and ultrastructural changes in the enterocytes of Arctic char (Salvelinus alpinus L.) fed linseed oil and soybean lecithin. Fish Physiology and Biochemistry 21:35-44;
Olsen RE, Myklebust R, Ringo E, Mayhew TM. 2000. The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic char (Salvelinus alpinus L.): a quantitative ultrastructural study. Fish Physiology and Biochemistry 22:207-216;
Ouwehand AC, Derrien M, de Vos W, Tiihonen K, Rautonen N. 2005. Prebiotics and other microbial substrates for gut functionality. Current Opinion in Biotechnology 16:212-217;
Perez-Vilar J, Hill RI. 1999. The structure and assembly of secreted mucins. Journal of Biological Chemistry 274 (45):31751-31754;
Peterson B, Booth T, Bramble T, Manning B. 2009. Improved survival in channel catfish fed mannaoligosaccharides in an extruded diet. J World Aquac Soc 40: 310-315;
Piccolo G, Centoducati G, Bover F, Marrone R, Nizza A. 2013. Effects of mannan oligosaccharide and inulin on snarpshout seabream (Siplodus puntazzo) in context of partial fish meal substitution by soybean meal. Italian Journal of Animal Science 12:133-138;
Pittman K, Pittman A, Karlson S, Cieplinska T, Sourd P, Redmond K, Ravnoy B, Sweetman E. 2013. Body site matters: an evaluation and application of a novel histological methodology on the quantification of mucous cells in the skin of Atlantic salmon Salmo salar L. Journal of Fish Diseases 36:115-127;
Pittman K, Sourd P, Ravnoy B, Espedal O, Fiksdal I, Oen T, Pittman A, Redmond K, Sweetman J. 2011. A novel method for quantifying salmonid mucous cells. Journal of Fish Diseases 34:931-936;
Plaisancié P, Barceló A, Moro F, Claustre J, Chayvialle JA, Cuber JC. 1998. Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon. Am J Physiol Gastrointest Liver Physiol 275:1073-1084;
Pryor GS, Royes JB, Chapman FA, Miles RD. 2003. Mannanoligosaccharides in fish nutrition: effects of dietary supplementation on growth and gastrointestinal villi structure in Gulf of Mexico sturgeon. N. Am. J. Aquac. 65:106–111;
Quigley EMM. 2010. Prebiotics and probiotics: modifying and mining the microbiota. Pharmacological Research 61:213-218;

Rawls JF, Samuel BS, Gordon JI. 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc Natl Acad Sci USA 101:4596–4601;
Refstie S, Baeverfjord G, Seim RR, Elvebo O. 2010. Effects of dietary yeast cell wall β-glucan and MOS on performance, gut health and salmon lice resistance in Atlantic salmon (Salmo salar) fed sunflower and soybean meal. Aquaculture 305:109-116;
Refstie S, Korsoen O, Storebkken T, Baeverfjord G, Lein I, Roem AJ. 2000. Differing nutritional responses to dietary soybean meal in rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (Salmo salar). Aquaculture 190:49-63;
Reis e Sousa C. 2004. Activation of dendritic cells: translating innate into adaptive immunity. Curr Opin Immunol 16:21-25;
Ringo E, Gatesoupe FJ. 1998. Lactic acid bacteria in fish: a review. Aquaculture 160:177–203;
Ringo E, Lodemel JB, Mykelbust R, Jensen L, Lund V, Mayhew TM, Olsen RE. 2002. The effects of soybean, linseed and marine oils on aerobic gut microbiota of Arctic charr Salvelinus alpinus L. before and after challenge with Aeromonas salmonicida ssp. salmonicida. Aquaculture Research 33:591-606;
Ringo E, Lodemel JB, Myklebust R, Jensen L, Lund V, Mayhew TM, Olsen RE. 2002. The effects of soybean, linseed and marine oils on aerobic gut microbiota of Arctic charr Salvelinus alpinus L. before and after challenge with Aeromonas salmonicida ssp. salmonicida. Aquaculture Research 33(8): 591-606;
Ringo E, Olsen RE, Gifstad TO, Dalmo RA, Amlund H, Hemre GI, Bakke AM. 2010. Prebiotics in aquaculture—a review. Aquac Nutr 16(2):117–136;
Ringo E, Olsen RE, Vecino JLG, Wadsworth S, Song SK. 2012. Use of immunostimulants and nucleotides in aquaculture: a review. J Marine Sci Res Development 2 (1);
Ringo E, Sperstad S, Kraugerud OF, Krogdahl A. 2008. Use of 16S rRNA gene sequencing analysis to characterize culturable intestinal bacteria in Atlantic salmon (Salmo salar) fed diets with cellulose or non-starch polysaccharides from soy. Aquaculture Research 39:1087-1100;
Rodgers CJ, Furones MD. 2009. Antimicrobial agents in aquaculture: practice, needs and issues. In: Rogers C, Basurco B (Editors). The use of veterinary drugs and vaccines in Mediterranean aquaculture. Zaragoza, CIHEAM, pp 41-59;
Rodríguez A, Cuesta A, Ortuno J, Esteban MA, Meseguer J. 2003. Immunostimulant properties of cell wall-modified whole Saccharomyces cerevisiae strain administered by diet to seabream (Sparus aurata L.). Veterinary Immunology and Immunopathology 96:183-192;
Rodriguez-Estrada U, Satoh S, Haga Y, Fushimi H, Sweetman J. 2009. Effects of single and combined supplementation of Enterococcus faecalis, mannan oligosaccharide and polyhydroxybutyrate acid on growth performance and immune response of rainbow trout Oncorhynchus mykiss. Aquaculture Sci. 57(4): 609-617;
Rombout JHWM, Abelli L, Picchietti S, Scapigliati G, Kiron V. 2011. Teleost intestinal immunology. Fish & Shellfish Immunology 31:616-626;
Romero J, Feijoo GF, Navarrete P. 2012. Antibiotics in aquaculture – use, abuse and alternatives. In: Carvalho ED, David GS, da Silva RJ (Editors). Health and Environment in Aquaculture. InTech, Rijeka, Croatia, pp 159-198;
Sado RY, Almeida Bicudo AJD, Cyrino JEP. 2008. Feeding dietary mannan oligosaccharides to juvenile Nile tilapia, Oreochromis niloticus, has no effect on hematological parameters and showed decreased feed consumption. J. World Aquac. Soc. 39:821–826;
Sakai M. 1999. Current research status of fish immunostimulants. Aquaculture 172:63-92;

Salze G, McLean E, Schwarz MH, Craig SR. 2008. Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia. Aquaculture 274:148–152;
Sang HM, Ky LT, Fotedar R. 2009. Dietary supplementation of mannan oligosaccharide improves the immune response and survival of marron, Cherax tenuimanus (Smith, 1912) when challenged with different stressors. Fish Shellfish Immunol 27(2): 341-342;
Sargent J, Tocher D, Bell G. 2003. The lipids. In: Halver JE, Hardy RW (Editors). Fish Nutrition. (3rd Edition). Elsevier Science, San Diego, CA, USA. pp 181-257;
Schenk M, Mueller C. 2008. The mucosal immune system at the gastrointestinal barrier. Best Parct Res Clin Gatroenterol 22(3):391-409;
Schwarz S, Chaslus-Dancla E. 2001. Use of antimicrobials in veterinary medicine and mechanisms of resistance. Veterinary Research 32(3-4):201–225;
Scientific, Technical and Economic Committee for Fisheries (STECF). 2013. The Economic Performance of the EU Aquaculture Sector - 2012 exercise (STECF-13-03). Publications Office of the European Union, Luxembourg, EUR 25975 EN, JRC 81620, 237 pp;
Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, Ruane N, Jutfelt F, Toften H, Vaughan L. 2012. Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish Physiol Biochem 38:85-105;
Shane MS. 2001. Mannan oligosaccharides in Poultry nutrition: mechanism and benefits. In: Lyons TP, Jacques KA (Editors). Biotechnology in the Feed Industry: Proceedings of Alltech's 17th Annual Symposium. Nottingham, UK, Nottingham University Press, pp 65-77;
Sheikhzadeh N, Heidarieh M, Pashaki AK, Nofouzi K, Farshbafi MA, Akbari M. 2012a. Hilyses®, fermented Saccharomyces cerevisiae, enhances the growth performance and skin non-specific immune parameters in rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol 32:1083-1087;
Sheikhzadeh N, Pashaki AK, Nofouzi K, Heidarieh M, Tayefi-Nasrabadi H. 2012b. Effects of dietary Ergosan on cutaneous mucosal immune response in rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol 32:407-410;
Simopoulos AP. 2000. Human requirement for n-3 polyunsaturated fatty acids. Poultry Science 79:961-970;
Sire MF, Vernier JM. 1992. Intestinal absorption of protein in teleost fish. Comp Biochem Physiol 103A: 771-781;
Smirnov A, Perez R, Amit-Romach E, Sklan D, Uni Z. 2005. Mucin Dynamics and microbial populations in the chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J Nutr 135: 187-192;
Smirnov A, Sklan D, Uni Z. 2004. Mucin dynamics in the chick small intestines are altered by starvation. J Nutr 134:736-742;
Spring P, Wenk C, Dawson KA, Newman KE. 2000. The effects of dietary mannaoligosaccharides on cecal parameters and the concentrations of enteric bacteria in the ceca of salmonella-challenged broiler chicks. Poult Sci 79(2):205-11;
Sridharan G, Shankar AA. 2012. Toluidine blue: a review of its chemistry and clinical utility. J Oral Maxillofac Pathol. 16(2):251-255;
Staykov Y, Spring P, Denev, S, Sweetman J. 2007. Effect of a mannan oligosaccharide on the growth performance and immune status of rainbow trout (Oncorhynchus mykiss). Aquacult. Int. 15:153–161;
Tacon AG, Hasan MR, Subasinghe RP. 2006. Use of fishery resources as feed inputs to aquaculture development: trends and policy implications. FAO Fisheries Circular No. 1018. Rome;

Thorarinsson R, Powell DB. 2006. Effects of disease risk, vaccine efficacy, and market price on the economics of fish vaccination. Aquaculture 256:42-49;
Torrecillas S, Makol A, Benítez-Santana T, Caballero MJ, Montero D, Sweetman J, Izquierdo M. 2011b. Reduced gut bacterial translocation in European sea bass (Dicentrarchus labrax) fed mannan oligosaccharides (MOS). Fish & Shellfish Immunology 30:674-681;
Torrecillas S, Makol A, Betancor MB, Montero D, Caballero MJ, Sweetman J, Izquierdo M. 2013. Enhanced intestinal epithelial barrier health status on European sea bass (Dicentrarchus labrax) fed mannan oligosaccharides. Fish and Shellfish Immunology 34: 1485-1495;
Torrecillas S, Makol A, Caballero MJ, Montero D, Ginés R, Sweetman J, Izquierdo M. 2011a. Improved feed utilization, intestinal mucus production and immune parameters in sea bass (Dicentrarchus labrax) fed mannan oligosaccharides (MOS). Aquaculture Nutrition 17:223-233;
Torrecillas S, Makol A, Caballero MJ, Montero D, Robaina L, Real F, Sweetman J. 2007. Immune stimulation and improved infection resistance in European sea bass (Dicentrarchus labrax) fed mannan oligosaccharides. Fish Shellfish Immunol. 23:969–981;
Tremaroli V, Backhed F. 2012. Functional interactions between the gut microbiota and host metabolism. Nature 489:242–249;
Turchini GM, Torstensen BE, Wing-Keong NG. 2009. Fish oil replacement in finfish nutrition. Review in Aquaculture 1:10-57.
Urán PA, Gonçalves A, Taverne-Thiele JJ, Schrama JW, Verreth JAJ, Rombout JHWM. 2008a. Soybean meal-induced enteritis in common carp (Cyprinus carpio L.) and the gene expression of inflammatory mediators in intestinal leukocytes. Fish & Shellfish Immunology 25:751-760;
Urán PA, Jaafari S, Baardsen G, Schrama JW, Rombout JHWM, Koppe W, Verreth JAJ . 2009. Variation between commercial sources of soybean meal influence the severity of enteritis in Atlantic salmon (Salmo salar L.). Aq Nutr 15:492-499;
Urán PA, Schrama JW, Rombout JHWM, Obach A, Jensen L, Koppe W, Verreth JAJ. 2008b. Soy bean meal-induced enteritis in Atlantic salmon (Salmo salar L.) at different temperatures. Aq Nutr 14:324-330;
Uribe C, Folch H, Enriquez R, Moran G. 2011. Innate and adaptive immunity in teleost fish: a review. Vetrinarni Medicina 56:486-503;
Van den Ingh TSGAM, Krogdahl A, Olli JJ, Hendriks HGCJM, Koninkx JGJF. 1991. Effects of soybean containing diets on the proximal and distal intestine in Atlantic salmon (Salmo salar): a morphological study. Aquaculture 94:297-305;
Van der Kamp JW, Asp NG, Miller Jones J, Schrama JW. 2004. Dietary fiber: bioactive carbohydrates for food and feed. Wageningen Academic Publishers, Wageningen;
Van der Marel M, Adamek M, Gonzalez SF, Frost P, Rombout JH; Wiegertjes GF, Savelkoul HF, Steinhagen D. 2012. Molecular cloning and expression of two β-defensin and two mucin genes in common carp (Cyprinus carpio L.) and their up-regulation after β-glucan feeding, Fish and Shellfish Immunology 32(3):494–501;
Vatsos IN, Kotzamanis Y, Henry M, Angelidis P, Alexis M. 2010. Monitoring stress in fish by applying image analysis to their skin mucous cells. European Journal of Histochemistry 54:107-111;
Wassef EA, Saleh NE, El-Hady HAE. 2009. Vegetable oil blend as alternative lipid resources in diets for gilthead seabream, Sparus aurata. Aquaculture International, 17(5), 421-435;

Welker TL, Lim C, Yildirim-Aksoy M, Shelby R, Klesius PH. 2007. Immune response and resistance to stress and Edwardsiella ictaluri challenge in channel catfish, Ictalurus punctatus, fed diets containing commercial whole-cell yeast or yeast subcomponents. J. World Aquac. Soc. 38:24–35;

Wilson RP. 2003. Aminoacids and proteins requirements. In: Halver JE,Hardy RW (Editors). Fish nutrition (3rd Edition). Elsevier Science, San Diego, CA, USA, p 143-179;

Wink M, Schmeller T, Latz-Bruning B. 1998. Modes of action of allelochemical alkaloids: interaction with neuroreceptors, DNA, and other molecular targets. Journal of Chemical Ecology 24:1881-1937;

Wrzosek L, Miquel S, Noordine M, Bouet S, Chevalier.Curt MJ, Robert V, Philippe C, Bridonneau, Cherbuy C, Robbe-Masselot C, Langella P, Thomas M. 2013. Bacteroides thetaiotaomicron and Faecalibacterium prausnitzii influence the production of mucus glycans and the development of goblet cells in colonic epithelium of gnotobiotic model rodent. BMC Biology 11:61;

Xueqin J, Kania PW, Buchmann K. 2012. Comparative effects of four feed types on white spot disease susceptibility and skin immune parameters in rainbow trout, Oncorhynchus mykiss (Walbaum). Journal of Fish Diseases 35(2):127–135;

Yamabayashi S. 1987. Periodic acid – Schiff – Alcian Blue: a method for the differential staining of glycoproteins. Histochemical Journal 19:565-571;

Yousefian M, Amiri MS. 2009. A review of the use of prebiotic in aquaculture for fish and shrimp. African Journal of Biotechnology 8(25):7313-7318;

Zhang J, Liu Y, Tian L, Yang H, Liang G, Xu D. 2012. Effects of dietary mannan oligosaccharide on growth performance, gut morphology and stress tolerance of juvenile Pacific white shrimp, Litopenaeus vannamei. Fish & Shellfish Immunology 33(4):1027-1032.