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EFFECTS OF DIETARY MANNAN OLIGISACCHARIDE (MOS) SUPPLEMENTATION IN RELATION TO INTESTINAL INTEGRITY MICROBIOTA, HEALTH AND PRODUCTION OF CULTURED FISH SPECIES

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DOCTOR OF PHILOSOPHY

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Effects of dietary mannan oligosaccharide (MOS) supplementation in relation to intestinal integrity, microbiota, health and production of cultured fish species

by

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ABSTRACT

A series of investigations were conducted in order to evaluate the effect of MOS supplementation in finfish aquaculture. Fish with great potential in aquaculture industry were tested with regards to effect of dietary MOS supplementation on intestinal histology and microbiology as well as overall animal health and production. Two levels of MOS supplementation were applied 0.2% and 0.4%. Experimental fish were Atlantic salmon (*Salmon salar*), rainbow trout (*Oncorhynchys mykiss*), sole (*Solea senegalensis*) and gilthead sea bream (*Sparus aurata*). The results from the sea bream studies revealed that MOS supplementation may have a beneficial effect on growth performance of fish greater than 100 g. Additionally, there is a systemic improvement of the intestinal histology for all species investigated, especially when using 0.4% of MOS supplementation level. Both light and electron microscopy revealed increased intestinal surface and improved intestinal integrity of MOS fed fish. MOS alters the intestinal microbiota, in the case of gilthead sea bream modulation was evident even when fish were fed 0.2% dietary MOS for as little as 2 weeks. Blood immune parameters were also affected by the MOS inclusion and total leukocytes counts were increased and leukocytes relative abundance was also changed. MOS induced intestinal microbial modulation was more evident in fish are reared in outdoor conditions. Feed utilization and digestibility were improved with the addition of 0.4% MOS supplementation in the Atlantic salmon. The sole experiment revealed that MOS could reduce fish mortalities induced by pasteurelliosis. These investigations, suggest a potential role for application of MOS in aquaculture. Future research should be conducted in order to evaluate other parameters that MOS may influence-and-ascertain optimum dosage for each fish species and developmental stage.

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ADC	Apparent digestibility coefficient
AU	Arbitrary units
CFU	Colony forming units
DAI	Daily appetite index
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
FCR	Feed conversion ratio
FI	Feed intake
FM	Fishmeal
FL	Final fish weight
FOS	Fructo-oligosaccharides
GOS	Gluco-oligosaccharides
HSI	Hepatosomatic index
K	Condition factor
LAB	Lactic acid bacteria
MD	Microvilli density
MOS	Mannan-oligosaccharides
MS222	Tricane methyl sulphonate
NFE	Nitrogen-free extract
NRC	National Research Council
NQC	Norwegian Quality Cut
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PER	Protein efficiency ratio
PR	Perimeter ratio
RNA	Ribonucleic acid
SBM	Soybean meal
SEM	Scanning electron microscopy
SGR	Specific growth rate

TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TOS	Transgalacto-oligosacharides
TSA	Tryptone soy agar
WG	Weight gained

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Merrifield, D. M., **Dimitroglou, A.**, Bradley, G., Baker, R. T. M., Davies, S. J., 2009. Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout (*Oncorhynchus mykiss* Walbaum). Journal of Fish diseases, 32: 755-766.

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Chapter 1 Introduction

1.1 Aquaculture

Nowadays, aquaculture represents one of the fastest growing food producing sectors of the world. World aquaculture has grown tremendously during the last fifty years from a production of less than a million tonnes in the early 1950s to more than 65.0 million tonnes in 2007 (food fish and aquatic plants). The aquaculture industry has effectively grown at an average annual rate of 8.8% since 1970 compared with 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period (FAO, 2008).

There are more than 110 aquatic species which are cultivated. The fish species that were selected for the present research play an important role in the world aquaculture industry as well as in the regional finfish production. Salmonids (i.e. salmons, trouts, etc.) represent the fourth biggest aquacultured fish group with 2 143 271 tonnes. Cyprinids (i.e. carps, barbells, etc.) represent the largest group by production followed by Cichilds (i.e. tilapias) and miscelanious fresh water fish. Atlantic salmon (*Salmo salar*) production since 1964 has continually increased reaching the 1 433 708 tonnes in 2007 with value of 7.6 billion USD (appr. 5.5 billion Euros). Similarly, rainbow trout (*Oncorhynchus mykiss*) production since 1950 has increased reaching 604 695 tones with value of 2.6 billion USD (appr. 1.9 billion Euros) in 2007. Over the last few decades, the Mediterranean region has experienced an exponential increase in gilthead sea bream (*Sparus aurata*) production. This increased production is due to the significant improvements in hatchery techniques and technology which lead to 125 355 tonnes in 2007 valued at 720.4 million USD (appr. 524.5 million Euros).

Sole (*Solea senegalensis*) is a newly introduced fish in aquaculture industry. Despite the progress in hatchery techniques the production of this species remains difficult. Since 2005 the production of sole has reached 36 tonnes in 2007 valued at 252 000 USD (appr. 183 456 Euros) (FAO, 2008).

Recent legislation and directives from the EU (regulation 1831/2003/EC) have resulted in the removal of antibiotic growth promoters from animal feeds, including those destined for the aquaculture sector. This has prompted major initiatives towards the development/appraisal of novel agents or functional dietary supplements in commercial feed production for fish and crustacean species. Such products include immunostimmulants (Raa, 2000; Cook et al., 2003; Skjermo et al., 2006), probiotics (Gatesoupe, 1999; Spanggaard et al., 2001; Balcázar et al., 2006) and prebiotics (Manning and Gibson 2004; Grisdale-Helland et al., 2008). In order to understand the effect on the host organism and the potential of such dietary supplements in the feed production industry we have to first analyze and understand a very complicated intestinal system which is related not only with the feed digestion and nutrient absorption but with other important function and processes such as the immune responses, mucosal tolerance and defense against enteric pathogens.

1.1 Fish immune system and mucosal immunity

The immune system is related with the protection of the organism against pathogenic organisms such as viruses, bacterial infections and parasites. In fish, like other vertebrates, the immune system consists of two parts: the innate immune system and the adaptive immune system. There are some differences comparing the immune system of fish to other vertebrates due to fish earliest

evolution. These differences are related to the immune organs where fish lack both bone marrow and lymph nodes, which are characteristic features of the immune systems of higher vertebrates. Thus, fish primary lymphoid tissues are the thymus, kidney, spleen and gut associated lymphoid tissues (GALT) (Rombout et al., 1993; Press and Evensen 1999). The function of these organs has been retained in higher vertebrates; with the only exception that the kidney is not included in lymphoid organs. The adaptive immune system is more specific compared to the innate immune system because its function is based on antigen recognition. Two types of cells are responsible for the function of the adaptive immune system: the B-lymphocytes and the T-lymphocytes. Their difference is that B-lymphocytes can produce and express specific immunoglobulin (Ig) on their cell surface membrane whereas T-lymphocytes cannot. When a pathogen is detected, T-lymphocytes respond to antigens presented by macrophages and once stimulated, they induce B-lymphocytes to produce specific antibodies against the presented antigen. On the other hand, the innate immune system is very different compared with the adaptive immune system and it is the earliest immune mechanism. In innate immune system the response is initiated through target molecules, including lipopolysaccharides and β-glucans, which are present on a range of pathogenic organisms. Hence, it is clear that the innate immune system is not focused on a particular antigen but in a range of pathogens (non-specific mode of action). The fish innate immune system involves a diversity of cellular defences such as white blood cells (granulocytes, macrophages and monocytes), the complement system which includes an enzyme and protein mixture that acts to destroy invading organisms by attacking their membrane complex and enzymes such as lysozyme. Lysozyme is produced and secreted by leukocytes i.e.

neutrophils, monocytes and a small amount in macrophages; and is present in numerous body fluids including blood, epidermal and intestinal mucus as well as internal organs i.e. kidney, spleen, gills and intestines. Additionally, one more important feature of fish innate immune system is that it exists before or just after hatching whereas the adaptive immune system takes a few weeks to months to develop during ontogeny (Saurabh and Sahoo, 2008). The fish innate system reacts within a very short time compared with the adaptive immune system because of its cellular defences.

In fish, the first line of defence against pathogens is the mucosal coating which can be found externally as epidermal mucus (covers externally the whole body of fish) and internally as intestinal mucus. Mucus acts as a physical barrier to infections by preventing pathogens from reaching the tissues by enclosing them within the mucus matrix and at the end discarding them. In addition to this, there are various components of the immune system within the mucus matrix such as B-lymphocytes, T-lymphocytes and Ig presenting macrophages (Rombout et al., 1993) and enzymes i.e. lysozyme (Saurabh and Sahoo, 2008), which make it a inhospitable environment for many bacterial pathogens.

1.2 Intestinal function and morphology

According to their feeding habits there are 4 types of fish: the detritivorous, herbivorous, omnivorous and carnivorous. Within these categories there are some subcategories depending on the range of different feeds i.e. euryphagous fish have a mixed diet; stenophagous fish eat a limited type of food and monophagous fish are strictly dedicated to one type of food (Moyle and Cech,

2000). Depending on the feeding types there are many differences in the gastrointestinal tract of fish. Fish which are fed with detritus or plants have long intestine and have to spend a lot of time feeding in order to cover their nutrient and energy requirements. On the other hand, omnivorous fish have shorter intestines and carnivorous fish have the shortest intestines (Bone et al., 1995). According to a rough rule the ratio between the lengths of the intestine to the whole body length in herbivorous fish is greater than 3; in omnivorous between 1 and 3; and in carnivorous less than 1. Additionally, fish feeding habits can influence the intestinal function and the existence of the stomach and pyloric caeca. This can be easily understood comparing herbivorous to carnivorous fish (Brix, 2002). In the carnivorous species, such as rainbow trout and Atlantic salmon, a stomach is present as well as pyloric caeca and the intestine can be divided in anterior and posterior regions. The stomach has either U-, Y- or J-shape and secretes hydrochloric acid which reduces the pH to 2 – 4 to aid protein digestion. In the anterior intestinal region the pH is raised again to 7 – 9 where the digestion of fat and carbohydrates takes place. The posterior intestinal region is mainly responsible for the absorption of nutrients. In many herbivorous species the stomach is absent and the intestine is responsible for the digestion as well. Usually herbivorous and detritivorous species are able to triturate the feed which increases the efficiency of feed digestion and fermentation by the intestinal microbiota. In omnivorous species, due to the variety of their feeding, they combine characteristics from both carnivorous and herbivorous species such as the presence of stomach and long intestines (i.e. gilthead sea bream and sole).

In fish, the main function of the intestinal mucosal folds, often called villi, is to increase the intestinal absorptive surface area (by ~10 times; Figure 1.1).

Enterocytes are the dominant cells which lay out across the surface of the villi. Enterocytes are joined with tight junctions in order to produce a barrier which protects the organism from large molecules such as the translocation of opportunistic microorganisms. Enterocytes are produced at the base of the villi and they migrate to the tip where they are constantly replaced (Buddington and Kuz'mina, 2000). Food is usually the reason that enterocytes are shed but bacterial diseases often affect the morphology of the intestine by altering its physiological condition, i.e. necrosis and shedding of the intestinal mucosa (Austin and Austin 1993; Diggles et al. 2000). Additionally, goblet cells, mucus secreting cells are located between the enterocytes. Mucus plays an important role in intestinal function and morphology by removing opportunistic pathogenic organisms and protecting the enterocytes. Hence, if the rate of enterocyte renewal is greater than the rate of enterocytes being shed the villi fold increases its length, and vice versa. Microvilli are formed on the apical membrane of enterocytes and they increase the absorptive area by ~200 times. Microvilli resemble finger-like filaments (brush border) and their length is affected by dietary habits, fish species, intestinal region and environmental conditions (Buddington and Kuz'mina, 2000).

Additionally, the intestine is related with the immune system. The lower intestinal tract (posterior intestinal region) has been associated with antigen uptake and processing (Powell, 2000). Hence, there are numerous studies where dietary compounds (i.e. probiotics) are able to affect the fish immune response (Panigrahi et al., 2004; Panigrahi et al., 2005; Salinas et al., 2005; Kim et al., 2006; Gómez and Balcázar, 2008; Ferguson et al., 2010).

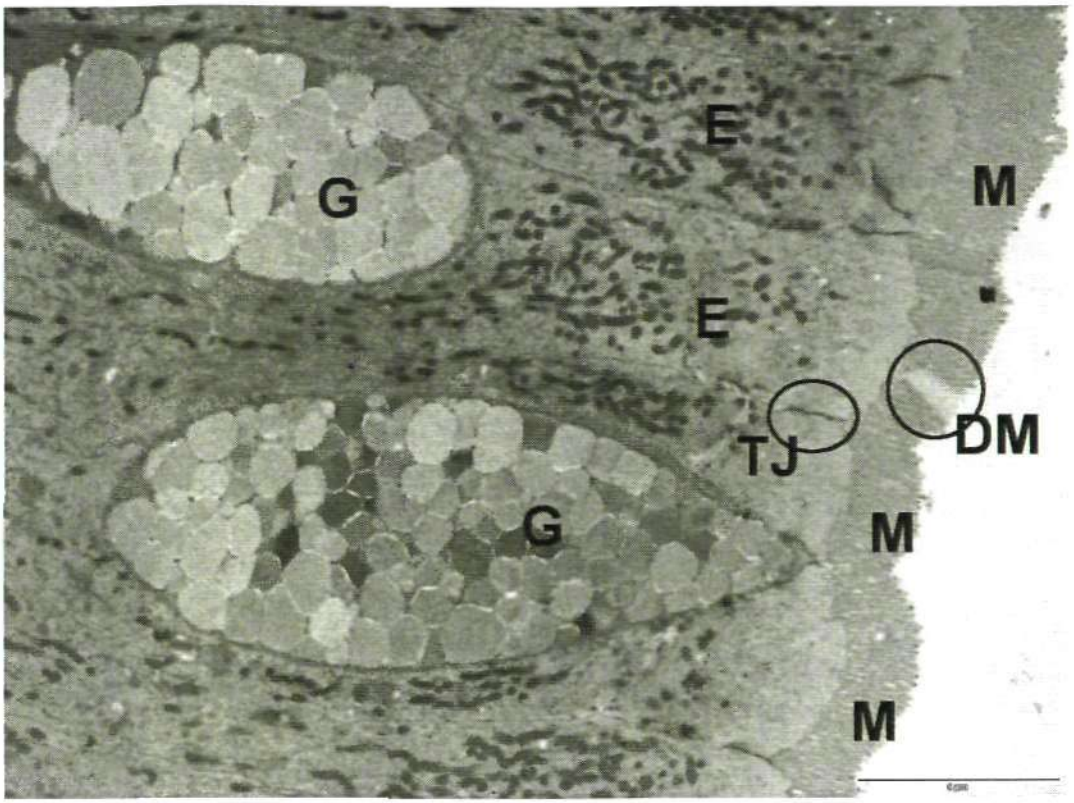

Figure 1.1 TEM micrograph presenting the villi folds from the posterior intestine of sea bream. Enterocytes (E) are the dominant cells across the villi and they are attached to each other with tight junctions (TJ). Goblet cells (G) are mucus secreting cells and they are between enterocytes. Microvilli (M) are on top of enterocytes and they are the smallest feature which is often damaged (DM) by mechanical reasons (i.e. feeding regime) and chemical reasons such as pathogens colonization. The scale bar is 5μm.

1.3 Intestinal microbiota

The intestinal microbiota can be classified into 2 categories: the autochthonous (or indigenous) where microbes are able to attach and colonize the intestinal surface and/or intestinal mucus layer; and the allochthonous (or

transient) where microbes are transient digesta associated. Many reviews have showed that aerobic Gram negative bacteria are the dominant bacteria in fish intestines in both marine and fresh water species (Ringø et al., 1995; Ringø and Gatesoupe, 1998; Ringø and Birkbeck, 1999). It is well documented that factors such as rearing environment, larval developmental stage, fish age, water chemistry, temperature, salinity, stress and dietary components can alter the intestinal microbiota of fish (Cahill, 1990; Ringø et al., 1995; Ringø and Gatesoupe, 1998; Ringø and Birkbeck, 1999; Gatesoupe et al., 1999; Ringø and Olsen, 1999; Merrifield et al., 2009a). Despite the numerous studies the intestinal microbiota plays an important role in the nutrition and health of the host organism. The intestine is an internal organ with high bacterial load and is a potential port of entry for pathogens (Brix, 2002; Birkbeck and Ringø, 2005). Potential pathogenic bacteria are part of the intestinal microbiota of every healthy organism (Ringø and Gatesoupe, 1998) and if the conditions within the intestine become favourable (i.e. the host becomes stressed or is subjected to poor nutrition etc) then there is potential for pathogenic proliferation, translocation and ultimately infection of the host organism. Additionally, the beneficial intestinal bacteria produces a range of compounds such as digestive enzymes and vitamins, which are likely contribute towards to improved host digestive function (Sugita et al., 1996; Ringø and Birkbeck, 1999; Birkbeck and Ringø, 2005; Ringø et al., 2007). The primary function of prebiotics as well as probiotics, is to modulate the intestinal microbiota in such way that reduces opportunistic pathogenic bacteria, elevates favourable bacteria and promotes microbial balance to improve host health.

1.5 Probiotics

A probiotic is defined as "viable microbial dietary supplement that beneficially affects the host through its effects in the intestinal tract" (Roberfoid, 2000). By definition in order for probiotics to be useful in the feed industry, they should have the following characteristics (Ewin and Cole, 1994):

- They should not be harmful to the animal (i.e. cause a disease) nor should be toxic for the host animal.
- Acid and bile resistant in order to survive through the stomach and reach the intestine alive.
- Ability to colonise the gut. The selected bacteria need to have appropriate capabilities which will allow them to colonise the gut i.e. colonisation and attachment factors.
- Ability to inhibit pathogen activities. The selected micro-organisms should produce materials (i.e. acid) which will inhibit pathogens.
- The selected micro-organisms should by stable and viable under manufacturing procedures and storage conditions in order to produce stable feeds ready to be used in the field.

Many studies have been investigating the effect of probiotics such as lactic acid bacteria (LAB), *Bacillus* spp., *Vibrio* spp. and *Aeromonas* spp. and other (Bogut et al., 2000; Spanggaard et al., 2001; Chang and Liu, 2002; Panigrahi et al., 2004, 2005, 2007; Salinas et al., 2005) for reviews see Ringø and Gatesoupe (1998), Gatesoupe (1999), Burr et al. (2005) and Balcázar et al. (2006).

1.6 Prebiotics

Today, the use of probiotics in many cases is difficult in the feed production industry because of the low viability of the bacteria after pelleting, during storage as well as problems with feed handling and preparation. Additionally, there is a possibility of environmental issues as probiotics enter into the aquatic ecosystem in non-recirculation facilities or in facilities without effective waste treatment. As an alternative, prebiotics have been assessed in an attempt to overcome issues associated with probiotic applications. Instead of introducing probiotic bacteria the aim of prebiotics is to stimulate selected indigenous microbiota. A prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of limited number of bacteria that can improve the host health" (Gibson and Roberfoid, 1995). Suggested properties of prebiotics were proposed by Collins and Gibson (1999) they should:

- Not be hydrolysed or absorbed in the gastrointestinal tract.
- Promote the beneficial bacterial colonisation of the gut.
- Be able to alter the gut microbiota producing favourable conditions for beneficial bacterial species.

The initial research with regards to the development of prebiotics dates back to the end of 1970s in Japan where several carbohydrates were tested in order to promote the growth of bifidobacteria in mammals (Yazawa et al., 1978). Prebiotics mainly consist of oligosaccharides which have proved to be able to promote beneficial bacterial growth within the GI tract (Gibson et al., 2003). Oligosaccharides are able to provide the necessary energy to bacteria. Such

oligosaccharides are mainly mannan oligosaccharides (MOS), fructooligosaccharides (FOS), glucooligosaccharides (GOS) and trans-galactooligasaccharides (TOS; galactooligosaccharides are also included). Inulin is a polysaccharide which is closely related to fructooligosaccharides. Not all of these oligosaccharides have been tested in aquaculture at this time. Table 1.1 shows a selection of published studies in fish.

In a more recent review for human intestinal microbiota the prebiotic definition was updated. Gibson et al. (2004) suggested that a prebiotic has to:

- Resist gastric acidity, hydrolysis by (mammalian) enzymes and gastrointestinal absorption.
- Be fermented by the intestinal microflora.
- Stimulate selectively the growth and/or activity of intestinal bacteria associated with health and wellbeing.

According to the authors of the latest review (Gibson et al., 2004), each one of these criteria is important but the third criterion, concerning the selective stimulation of growth and/or activity of beneficial bacteria, is the most important and the most difficult to achieve. This is especially true because this step requires anaerobic sampling and reliable quantitative microbial analysis needs to be undertaken for a wide range of bacterial species (i.e. total aerobic and anaerobic bacteria as well as total counts of Bifidobacteria, Enterobacteria, *Lactobacillus*, etc). For this purpose it is suggested that advanced molecular techniques should be needed such as fluorescence *in situ* hybridisation and DGGE (Gibson et al., 2004). Additionally, Gibson et al. (2004) evaluated 8 possible prebiotic candidates: inulin, TOS, lactulose, isomalto-oligosaccharides, lactosucrose, xylo-

oligosaccharides, soybean oligosaccharides and GOS. According to the results, only three oligosaccharides were classified as prebiotics: inulin, TOS and lactulose. Furthermore, it is mentioned that MOS it is still a candidate to be classified as a prebiotic but further research is required.

Table 1.1 Selected prebiotics studies with fish

Species	Prebiotic	Exp. Duration	Init. weight	Parameters investigated	References
Morone chrysops x *M. saxatilis*	GroBiotic A	7 and 4 weeks	~7 g for 7 weeks ~20 g for 4 weeks	Growth parameters after 7 weeks (+), immune response after 4 weeks (+)	Li and Gatlin III, 2004
Morone chrysops x *M. saxatilis*	GroBiotic A	21 weeks	~65 and ~118 g	Growth parameters (+), disease challenge (+)	Li and Gatlin III, 2005
Oncorhynchus mykiss	GroBiotic A	9 weeks	~14 g	Growth parameters (=), proximate analysis (+), immune response (=) and disease resistance (+)	Sealey et al., 2007
Psetta maxima	Raftilin, Raftilose and lactosucrose	26 days	~46 mg	Intestinal microbiota (+), growth performance (+), survivalability (=)	Mahious et al. 2006
Sciaenops ocellatus	GroBiotic A TOS, innulin	2 weeks	~500 g	Apparent nutrient digestibility (+)	Burr et al., 2008
Salmo salar	TOS, inulin	8 weeks	~200 g	Growth parameters (=), feed utilisation parameters (+), oxygen consumption (=), immune response (=), carcass proximate analysis (+) and apparent nutrient digestibility (=)	Grisdale-Helland et al., 2008
Salmon salar	Inulin	3 weeks	~172 g	Organosomatic indices (+), digestive enzymes activity (+), plasma chemistry (=) and intestinal histology (=)	Refstie et al., 2006a
Salmon salar	Inulin	3 weeks	~172 g	Organosomatic indices (+), intestinal microbiota (+) and histology (=)	Bakke-McKellep et al., 2007
Salvelinus alpinus	Inulin	4 weeks	~218 g	Intestinal microbiota (+)	Ringø et al., 2006a
Salvelinus alpinus	inulin	4 weeks	~218 g	Intestinal histology (-)	Olsen et al., 2001

(+) for positive effect, (=) for no effect and (-) for negative effect.

1.7 MOS

According to Spring (2003), the initial interest in using mannan oligosaccharide (MOS) as a feed additives for animal nutrition was adapted from studies at the end of 1980s by Oyofo et al. (1989ab). These studies evaluated the ability of mannose to inhibit the adherence/colonization of microbial pathogens, such as *Salmonella typhimurium,* to gastrointestinal epithelial cells of broiler chickens. Many studies in terrestrial animals have shown that MOS is able to reduce the bacterial load and pathogenic bacteria of the intestine (in chickens: Fernandez et al., 2002; in broiler chickens: Yang et al., 2008; in dogs: Grieshop et al., 2004; in pigs: Castillo et al., 2008). An essential step in the bacterial infection process is pathogenic attachment to epithelial cells (Swanson et al., 2002). Carbohydrate-binding proteins such as lectins are found on the exterior of cells and are associated with the antigen recognition and fimbrial adhesins (binding) of bacteria, especially of Gram negatives (Engering et al., 1997). Lectins bind to the epithelial cells of the gut by attaching to oligosaccharide components of glycoconjugate receptors. Type-1 fimbrial adhesins, which are common on numerous potentially pathogenic bacteria species such as Enterobacteriacecae, are specific for mannan residues (Oyofo et al., 1989a; Spring et al., 2000; Newman, 2006). It is suggested that MOS is able to interact with these receptors by acting as a receptor analogue for Type-1 fimbriae and thus preventing bacterial colonization of the gastrointestinal tract (Oyofo et al., 1989b; Spring, 2003; Newman, 2006). In other words, MOS binds to Type-1 fimbrial adhesins of potentially pathogenic bacteria and prevents them to colonize the gastrointestinal tract. Furthermore, MOS supplementation influences immune responses such as increased of antibody response against viral infection in broilers (Shashidhara and

Devegowda, 2003), decreased of peripheral lymphocyte concentrations in dogs (Grieshop et al., 2004) and increased serum immunoglobulin G (IgG) and immunoglobulin M (IgM) levels and decreased the peripheral blood T lymphocyte percentage in turkeys (Çetin et al., 2005). Later studies with MOS supplementation in terrestrial animals have been shown that MOS works in several different ways within the digestive tract. MOS can improve gut function and health, by increasing the villi height, uniformity and integrity (in broiler chickens: Iji et al., 2001 and Hooge, 2004; in pigs: Castillo et al., 2008). As a result, the feed within the digestive tract may be more efficiently digested leading to a superior nutrient absorption (Spais et al., 2003; Sims et al., 2004). The effect of MOS supplementation in the intestinal histology may explain the improved performance in terrestrial animals such as poultry (Shane, 2001; Fritts et al., 2003; Yang et al., 2008) and pigs (Spring and Privulesku, 1998; Miguel et al., 2004).

Currently there are a limited number of studies available with regards to MOS supplementation in aquatic animals as shown in Table 1.2. Pryor et al. (2003) appears to be the first study with MOS in fish. Pryor and co-authors fed Gulf sturgeon (*Acipencer oxyrinchus desotoi*) diets supplemented with 0.3% MOS for 4 weeks. The results showed that there were no significant effects on growth performance (i.e. condition factor (*K*), specific growth rate (SGR) and feed conversion ratio (FCR) remained unaffected). Additionally, light microscopy (LM) revealed that villi length, width and density were not affected by MOS supplementation.

Dimitroglou (2004) appears to be the first study in which white sea bream (*Diplodus sargus* larvae were fed 0.2% MOS supplementation (incorporated in *Artemia* enrichment). The results indicated that larval growth performance and

survivability were not affected by the MOS supplementation. However, light microscopy revealed that MOS supplementation significantly improved the intestinal morphology by increasing villi surface area by over 12%. Transmission electron microscopy (TEM) revealed that MOS supplementation increased the microvilli length by 26% compared to the control. Salinity challenge experiments showed that MOS significantly increased larval stamina and survival at both 0 mg L^{-1} and 60 mg L^{-1} salinity by 13% and 23% respectively. These improvements of larval quality at the early stages of fish development are important for the efficiency of the intensive hatchery production.

Zhou and Li (2004) evaluated the effect of different levels of MOS supplementation on growth performance, intestinal microbiota and immune parameters of Jian carp (*Cyprinus carpio* var. Jian). The results showed that when fish were fed 0.24% dietary MOS supplementation, growth parameters such as weight gain and FCR were improved. Furthermore, the presence of *Escherichia coli* was significantly reduced and *Bifidobacterium* spp. and *Lactobacillus* spp. were increased. It has been suggested that Bifidobacteria and lactic acid bacteria (LAB) are beneficial microbial species in the gastrointestinal (GI) tract because they produce bacteriocins, acetate, and lactate which decrease the luminal pH and create an unfavourable environment for several pathogens (Gibson et al., 2003). Zhou and Li (2004) also observed that serum lysozyme activity and antibody concentration 17 days after vaccination against *Aeromonas hydrophila* were increased with increasing levels of dietary MOS supplementation; the highest response was observed in fish fed 0.55% MOS.

The first MOS study in crustaceans was conducted by Daniels (2005) following the same protocol as the previous larval study of Dimitroglou et al.

(2004), in lobster (*Homarus gammarus*). The results showed that the addition of MOS supplementation to enrichment *Artemia* produced significantly decreased mortality rate of lobster larvae (i.e. a higher success rate to reach stage IV), with values up to 28.4% in MOS fed larvae compared to 5 - 13% for the control regime (larvae fed unenriched *Artemia*).

Culjak et al. (2006) evaluated the effect of 0.6% of MOS supplementation on juvenile carp with regards growth parameters, feed utilization and mortality. The results showed that MOS was able to significantly improve fish final weight, improved FCR and SGR and reduced mortality.

MOS supplementation was also tested on the European catfish (*Silurus glanis*) at a dietary level of 0.5% (Bogut et al., 2006). The results revealed that MOS supplementation was able to significantly improve weight gain by 8.8% and FCR by 11.7%. Furthermore, total mortalities were also significantly reduced by ~12%.

Genc et al. (2007a) published the first study of MOS supplementation in shrimp production using a similar protocol as Daniels (2005). *Penaeus semisulcatus* post-larvae were tested for 48 days receiving different levels of MOS supplementation (ranging between 0.15% and 0.45%). The results showed that 0.3% dietary MOS supplementation could enhance growth performance and feed utilization parameters (i.e. FCR, SGR) and reduce mortality. No significant differences were found on histological examination of the hepatopancreas tissue. In the same year, Genc et al. (2007b) evaluated the effect of MOS on hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) with different levels of supplementation as in previous study with shrimps. The results showed that there were no significant effect at any level of MOS supplementation on growth

performance and feed utilization parameters. However, the higher level of MOS supplementation elevated carcass protein composition and histological evaluation of the intestine revealed that villi length was increased.

Additionally, the effect of two levels of MOS supplementation (0.2% and 0.4%) was evaluated on European sea bass (*Dicentranchus labrax*) (Torrecillas et al., 2007). The results showed that MOS did not affect body proximate composition, fatty acid composition or growth parameters (except SGR which was increased). Blood serum alternative complement activity, lysozyme activity and intestinal histology were not affected by MOS supplementation. However, differences were found on liver lipid vacuolization and head kidney macrophage phagocytic activity. Furthermore, MOS supplementation showed that MOS was able to confer improved disease resistance against enteric *Vibrio alginolyticus* infection when fish were challenged by both cohabitation with infected fish and direct bacterial inoculation of the gut. In the cohabitation trials the presence of *V. alginolyticus* in sea bass head kidney was 33% for the control group and only 8% and 0% respectively for the 0.2% and 0.4% MOS fed groups. In the trials with direct gut inoculation with *Vibrio alginolyticus* the number of the infected fish in the control group was doubled within 48h in the control fish compared with the MOS group. Furthermore, the number of the infected fish in the MOS group remained unchanged from the start of the trial.

Salze et al. (2008) incorporated MOS in diets for cobia larvae (*Rachycentron canadum*) using a similar protocol as previous studies (Dimitroglou, 2004; Daniels, 2005). The results showed that MOS supplementation at 0.2% could significantly reduce mortalities and increase the larval stamina in salinity challenge trials. Histology of the intestinal ultrastructure

using TEM revealed that MOS supplementation could significantly increase microvilli length

A study with rainbow trout (*Oncorhynchus mykiss)* by Yilmaz et al. (2008) tested different levels of MOS supplementation between 0.15 – 0.45%. The results showed that only diets with the higher dose of MOS could increase final mean weight and carcass protein content of the fish. The villi length was increased only by 0.15% and 0.3% of MOS supplementation. However, FCR, PER and HSI were not affected by any dose of MOS supplementation.

A more detailed study with growth performance and immune parameters of rainbow trout (*Oncorhynchus mykiss)* raised either in net cages or raceways was conducted by Staykov et al. (2007) in fresh water. The study demonstrated that compared to the control groups, 0.2% dietary MOS supplementation could increase final body weight; reduce FCR and mortality in both net cage and raceway reared trout. MOS supplementation induced significant improvements of immune parameters compared to the control groups; however, variations in the benefits were observed between net cage reared trout and raceway reared trout. Serum antibody titre, bactericidal activity and lysozyme activity were significantly increased in the net cage reared trout fed MOS supplemented diets. In the raceway reared trout, MOS supplementation significantly increased only serum lysozyme activity, classical and alternative pathway complement activity.

Burr et al. (2008) examined the effect of diets supplemented with 1% of MOS on red drum (*Sciaenops ocellatus*). The results showed that in soybean meal (SBM) based diets, MOS supplementation could increase apparent protein and organic matter digestibility as well as energy coefficients. However, MOS reduced lipids apparent digestibility coefficients. Thus, this study suggested that

MOS supplementation could be used in SBM based diets in order to enhance the apparent nutrient and energy digestibility.

Grisdale-Helland et al. (2008) evaluated the effect of MOS supplementation on Atlantic salmon (*Salmon salar*). Dietary MOS was supplemented at 1% to sub adult salmon (initial weight ~200 g) for a period of 16 weeks. The results showed that apparent energy digestibility of the MOS supplemented diet was increased compared with the control treatment. Fish carcass composition analysis showed that gross energy was increased with the addition of MOS but crude protein was reduced. Furthermore, whole blood neutrophil oxidative radical production and serum lysozyme activity were reduced in fish fed the MOS supplemented diet. No significant effect was found in feed utilization parameters.

In coclussion, the available studies on fish suggest that dose level of MOS inclusion should be below 1% and should be range between 0.2 – 0.4%. Moreover, it is rather impossible to conclude on a clear patern of how dietary MOS supplementation acts in fish nutrition due to many differences within the current literature i.e. differences on fish species (marine and fresh water species), fish developmental stage (larva, fry, adult, etc.), feeding habits (omnivorous, carnivorous and herbivorous fish), experimental duration, rearing conditions (tanks, ponds, experimental facilities, feeding strategy, etc) and MOS supplementation dose. Hence, there is a clear need for further investigation in order to fully understand how MOS mediates benefits to the host, with specific emphasis on the effect on endogenous gut microbiota, the secondary bacterial-host interactions and the subsequent effects on the immune system and growth parameters.

Table 1.2 Summary of MOS studies in aquatic animals.

Species	Parameters investigated	Parameters affected	References
Acipencer oxyrinchus desotoi	Growth parameters, feed utilization parameters and intestinal histology	None	Pryor et al., 2003
Diplodus sargus	Growth parameters, mortality, intestinal histology and salinity tolerance challenge	Intestinal histology and salinity tolerance challenge	Dimitroglou 2004
Cyprinus carpio var. Jian	Growth parameters, intestinal microbiota and immune parameters	Growth parameters, intestinal microbiota and immune parameters	Zhou and Li, 2004
Homarus gammarus	Growth parameters and mortality	Mortality	Daniels, 2005
Cyprinus carpio	Growth parameters and mortality	Growth parameters and mortality	Culjac et al., 2006
Silurus glanis	Growth parameters and mortality	Growth parameters and mortality	Bogut et al., 2006
Penaeus semisulcatus	Growth parameters, mortality and hepatopancreas histology	Growth parameters and mortality	Genc et al., 2007
Oreochromis niloticus x *O. aureus*	Growth parameters, carcass composition, intestinal histology	Carcass composition and intestinal histology	Genc et al., 2007

Species	Parameters investigated	Parameters affected	References
Dicentranchus labrax	Growth parameters, liver and intestinal histology, carcass composition, immune parameters and disease challenge	Liver histology, immune parameters and disease challenge	Torrecillas et al., 2007
Oncorhynchus mykiss	Growth parameters, feed utilisation parameters, mortality and immune parameters	Growth parameters, feed utilisation parameters, mortality and immune parameters	Staykov et al. 2007
Rachycentron canadum	Growth parameters, intestinal histology and salinity tolerance challenge	Intestinal histology and salinity tolerance challenge	Salze et al., 2008
Oncorhynchus mykiss	Growth parameters, carcass composition and intestinal histology	Carcass composition and intestinal histology	Yilmaz et al. 2008
Sciaenops ocellatus	Apparent nutrient digestibility	Apparent nutrient digestibility	Burr et al., 2008
Salmon salar	Growth parameters, carcass proximate analysis, apparent nutrient digestibility and immune parameters	Carcass proximate analysis, apparent nutrient digestibility and immune parameters	Grisdale-Helland et al. 2008

1.8 Aims

The aims of the present research were to investigate the effect of MOS supplementation on:

- growth parameters
- feed utilization
- intestinal morphology
- intestinal microbiology
- immune responses

in important aquacultured finfish species.

Chapter 2 Effect of MOS supplementation in Mediterranean cultivated species

Chapter 2 describes 3 experiments with marine fish cultivated in the Mediterranean region. The first experiment, which lasted for two months, was with gilthead sea bream juveniles which were fed FM based diets with or without SBM inclusion and one of 3 levels of MOS supplementation (0%, 0.2% and 0.4%). In the second experiment, 3 levels of MOS supplementation (0%, 0.2% and 0.4%) were fed to sub-adult gilthead sea bream for 2 weeks. The purpose of the first two experiments was to investigate the effect of MOS supplementation in fish fed high quality diets (fish meal diets) and diets with soy bean mean inclusion as an alternative protein source. The third experiment was a field experiment where the synergistic effect of dietary MOS supplementation with vaccination was assessed on diseased sole for 10 weeks using commercially available diets. Due to external limitations (number of tanks, experimental cost, etc.) only the higher inclusion rate of MOS supplementation (0.4%) was tested in the sole experiment. Both fish species discribed in this chapter have similar feeding habits and both of them are omnivorous.

2.1 Effect of MOS supplementation on gilthead sea bream (*Sparus aurata*) growth performance, feed utilisation, intestinal histology and microbial diversity

2.1.1 Abstract

Two trials were conducted in order to investigate the effect of dietary mannan oligosaccharides (MOS) on gilthead sea bream (*Sparus aurata*). Trial I was designed to assess the effect of dietary MOS (0%, 0.2% and 0.4%) on fish fed diets containing fishmeal (FM) as the only protein source. Trial II was designed to assess the effect of MOS (0% and 0.4%) on fish fed soybean meal (SBM) as a partial replacement of FM (SBM inclusion at 31% of the diet). After 9 weeks feeding on the experimental diets the growth parameters, body composition, liver and intestinal histology and intestinal microbial diversity were assessed. The results showed that mean final weight, specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) remained unaffected by MOS supplementation of fish fed FM or SBM diets.

However, compared to the control group (FM0), condition factor (*K*) was and hepatosomatic index (HSI) were significantly lower in fish fed 0.2% MOS (FM02) and 0.4% MOS (FM04), respectively. These parameters were unaffected in SBM fed fish. Body proximate composition remained unaffected by MOS supplementation in fish fed either FM or SBM diets ($P > 0.05$). Histological evaluation revealed that MOS had no effect on glycogen deposition in liver and no effect on gross villi morphology in the anterior intestine in either Trial I or II. However, relative to the control groups (FM0) dietary MOS appeared to improve

gross morphological absorptive surface area in the posterior intestine in Trial I. Electron microscopy revealed that dietary MOS had a pronounced effect on the ultrastructural level in the both trials, as microvilli density and length were elevated in both intestinal regions in fish fed both the FM and SBM based diets. No significant histological differences were found between respective FM0 and SBM0 groups.

DGGE analysis revealed that both SBM and MOS affected the intestinal microbial species richness and diversity. However, the effect of dietary MOS on the gastrointestinal microbiota was more pronounced in FM based diets (Trial I) as was reflected by increased species richness and diversity and reduced similarity between microbial profiles of the different FM groups. The effect of MOS in Trial II on SBM fed fish was marginal, as species richness and diversity remained unaffected and similarity between microbial profiles of the SBM groups and replicates remained high (i.e. >80%). Dietary SBM exerted a greater effect on gut microbiota than dietary MOS.

Summarising the findings of the present study suggest that MOS supplementation alters the intestinal microbiota and morphology of gilthead sea bream and FM can be replaced up to 31% by SBM in diets for sea bream.

2.1.2 Introduction

Gilthead sea bream (*Sparus aurata*) is an important cultivated fish species with great economic interest. However, with the recent ban on the use of antibiotic growth promoters in aquafeeds within the EU (Regulation 1831/2003/EC) alternative nutraceutical products to enhance production and health status is a topic of concerted interest. Prebiotics, such as mannan

oligosaccharides (MOS) have proved to be effective at enhancing health and growth performance of fish (Staykov et al., 2007 where MOS supplementation was 0.2%; Torrecillas et al., 2007 where MOS supplementation was 0.4%; Burr et al., 2008 where MOS supplementation was 1%), improve gut morphology (Salze et al., 2008 where MOS supplementation was 0.2%). Despite the progress made with other species, the effect of MOS on Gilthead sea bream remains limited.

Soybean meal (SBM) is an important plant protein source for inclusion in aquafeeds due to its competitive price and relative availability (Gatlin et al. 2007). However, SBM has been demonstrated to induce histological and functional changes of the fish gastrointestinal tract which include enteritis (inflammation of the mucosal lining mainly of the small intestine), increased susceptibility to bacterial infection, increased presence of inflammatory cells, villi shortening and reduced microvilli density and length (Baeverfjord & Krogdahl, 1996; Krogdahl et al. 2000; Krogdahl et al. 2003; Sitjà-Bobadilla et al. 2005; Heikkinen et al. 2006; Bakke-McKellep et al. 2007; Merrifield et al. 2009a). Several studies have assessed the feasibility of including SBM in diets for gilthead sea bream (Robaina et al., 1995; Kissil et al., 2000; Bonaldo et al. 2008); however, to the author' knowledge only the study of Bonaldo et al. (2008) has provided a histological examination of the intestinal tract. Bonaldo and co-authors evaluated the effect of SBM on gut histology of sea bream using light microscopy following the criteria suggested by Krogdahl et al. (2003) on Atlantic salmon (*Salmo salar*). The results showed the sea bream can accept dietary levels of up to 300 g kg^{-1} SBM without significant effects on growth performance and morphology of the posterior intestinal region. SBM has been reported to affect the gut microbiota of Atlantic cod (Ringø et al. 2006b), Atlantic salmon (Bakke-McKellep et al. 2007; Ringø et

al. 2008) and rainbow trout (Heikkinen et al. 2006; Merrifield et al. 2009a). Additionally, the intestinal microbiota of fish responds both directly and indirectly to dietary changes (Ringø et al. 1995; Ringø & Gatesoupe 1998; Ringø & Birkbeck 1999). Given the importance of the gut microbiota in terms of host health and nutrition (Gomez & Balcazar 2008) the effect of SBM on the gut microbiota of sea bream is worthy of study.

As was recently highlighted by Gatlin et al. (2007), the approach of utilising prebiotics to improve utilisation of plant proteins, should be a topic of high priority. Therefore, the aim of the present research was to assess the effect of MOS (incorporated into diets with or without SBM) on gilthead sea bream growth performance, intestinal histology and intestinal microbiota.

2.1.3 Methodology

2.1.3.1 Dietary formulation

The trial was conducted in the research facilities of the University of Plymouth, U.K. Five diets (Table 2.1) were tested in fish held in 15 tanks (3 tanks per diet). Mannan oligosaccharide (MOS; Bio-Mos®, Alltech Inc. USA) was supplemented at levels of 0%, 0.2% and 0.4% (as suggested from the literature and agreed by Alltech Inc.) in Trial I. In Trial II, 31% FM was replaced with SBM and supplemented with either 0% or 0.4% MOS. Each diet was produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food Equipment, Australia). Warm water was added to produce a consistency suitable for cold extrusion to form 2-mm pellets (PTM Extruder system, Plymouth, UK). Diets were dried in a hot air oven at 45 °C for 48 h. The digestible protein was 40% and remained unchanged for all

experimental diets. All diets were analysed for moisture, energy, ash, crude protein and lipids using standard analytical procedures according to AOAC (1995). Sample dry matter (DM) was calculated from the weight of sample before and after drying in a fan assisted oven (Genlab ltd, UK) at 105 °C until a constant weight was achieved. Moisture was calculated as ((WW – DW) / WW) x 100, where WW is wet weight (g) and DW is dry weight (g). Crude protein analysis was performed using the Kjeldahl method (AOAC 976.05) to establish the total nitrogen content of the samples. This process has two steps (i) the digestion and (ii) the distillation. In digestion, 100 mg of sample was transferred into a Kjeldahl digestion tube along with catalyst tablet (3 g K_2SO_4, 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2; BDH Ltd UK) and 10 mL of concentrated H_2SO_4 (Sp.Gr. 1.84, BDH Ltd UK). Digestion was performed on a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 225 °C for 40 min and at 380 °C for 60 min. After digestion all samples were transferred to the distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany) where the ammonia formed after the addition of NaOH was steam distilled and trapped in boric acid solution. Back titration was finally performed with 0.1 M HCl solution. Crude protein is then determined as: (((ST – BT) x A x 14 x 6.25) / SW)*100, where A is the molarity of the acid, 14 the relative atomic mass of nitrogen, 6.25 is the constant relationship between N and animal protein, ST is sample titre (mL), BT is blank titre (mL) and SW is the initial sample weight (mg). Lipid content was determined using the Soxhlet extraction method. Approximately 3 g of sample was placed into a cellulose thimble, lightly plugged with cotton wool and inserted into the condensers (at the rinsing position) of a SoxTec extraction system (Tecator Systems, Höganäs, Sweden; model 1043 and service unit 1046).

Pre-weighed cups containing 40 mL of petroleum ether were clamped into the condensers and the extraction knobs are moved to the boiling position for 30 min. The extraction knobs are then moved to the rinsing position for 45 min. The cups are then transferred to a fume cupboard for 30 min before weighing. Lipid content is then determined as: (LW / SW) x 100, where LW is lipid weight (determined from weight increase of cup, g) and SW is the initial sample weight (g). Sample total mineral or inorganic content (ash) was determined by adding a known weight of sample (~500 mg) to a pre-weighed china crucible. The crucible was then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550 °C for 12 hr (AOAC 942.05). Ash was calculated as follows: ((SR – CW) / SW)*100, where CW is crucible weight (g), SR is sample residue (including the crucible, g) and SW is the initial sample weight (g). Gross energy was determined using a Parr Adiabatic Bomb Calorimeter model 1356 (Parr 1045 Instrument Company, IL, USA). Firstly, ground and dried samples were pelleted using a tablet press and then weighed. The tablet obtained was placed in a nickel crucible which was afterwards positioned inside the bomb calorimeter. A piece of fuse wire was attached to each electrode and adjusted in such way that it makes contact with the sample. After having added 1 mL of distilled water to the bomb, it was then filled with oxygen to a pressure of 300 psi (20 bars). The bomb then was placed inside the calorimeter in a stainless steel water bath filled with distilled water. The run starts with a preliminary step necessary to equilibrate the water jacket temperature to the bucket temperature. When this was achieved the bomb fired and the temperature increase of the water bath was recorded until no further increase in the bucket temperature is detected. The resulting increase in temperature was used to calculate the energy content of the feed and carcass.

2.1.3.2 Feeding trial

Gilthead sea bream fry were imported from a commercial hatchery in France (Aquastream, Ploemeur) and acclimated for approximately 2 months prior the start of the trial. Thereafter, 49 fish per tank (~24 g) were randomly distributed into 15 x 120 L fibreglass tanks with internally rounded edges (Figure 2.1). Trials I and II were both conducted simultaneously in aerated re-circulated marine water at a rate of 360 L hr^{-1}. Fish were fed 2.7 – 3.0% biomass day^{-1}, provided in equal rations at 09.00 and 17.00 hr for a period of 9 weeks. Fish were weighted on weekly basis following a 24 hour starvation period and feeding rates were adjusted accordingly. Water temperature was maintained at 22 ± 1°C, salinity between 33 – 34 mg L^{-1} and a 12 hr light / 12 hr dark cycle was adapted.

Table 2.1 Diets composition (g kg^{-1}) and proximate composition analysis.

	Trial I			Trial II	
Regimes	FM0	FM02	FM04	SBM0	SBM04
Fishmeal (LT-94)	640	640	640	427	427
SBM[1]	0	0	0	313	313
Marine Fish Oil[2]	73.2	73.2	73.2	73.2	73.2
Starch[3]	110	110	110	110	110
Dextrin[4]	55	55	55	55	55
Vitamin mix[5]	10	10	10	10	10
Mineral mix[6]	5	5	5	5	5
αcellulose[7]	106.8	104.8	102.8	6.8	2.8
MOS[8]	0	2	4	0	4
Proximate analysis					
Dry matter (%)	92.95	93.05	93.24	93.26	93.42
Moisture (%)	7.05	6.95	6.76	6.74	6.58
Protein (%)	43.73	43.65	43.77	45.59	46.77
Lipids (%)	10.83	11.04	10.96	11.08	11.81
Ash (%)	12.24	12.19	12.17	10.58	10.53
NFE[9] (%)	26.16	26.15	26.34	26.01	24.31
Energy (MJ kg^{-1})	19.28	19.69	19.62	19.64	19.87

[1]: SBM solvent extracted (decortic); Skretting, U.K.

[2]: Interfish Ltd, U.K.

[3]: Starch from corn (Sigma S4126)

[4]: Dextrin type II from corn (Sigma D2130)

[5,6]: Skretting, U.K.

[7]: αcellulose (Sigma C8002)

[8]: Bio-Mos®, Alltech Inc.

[9]: Nitrogen Free Extracts (NFE) = Dry Matter – (crude lipid + crude ash + crude protein)

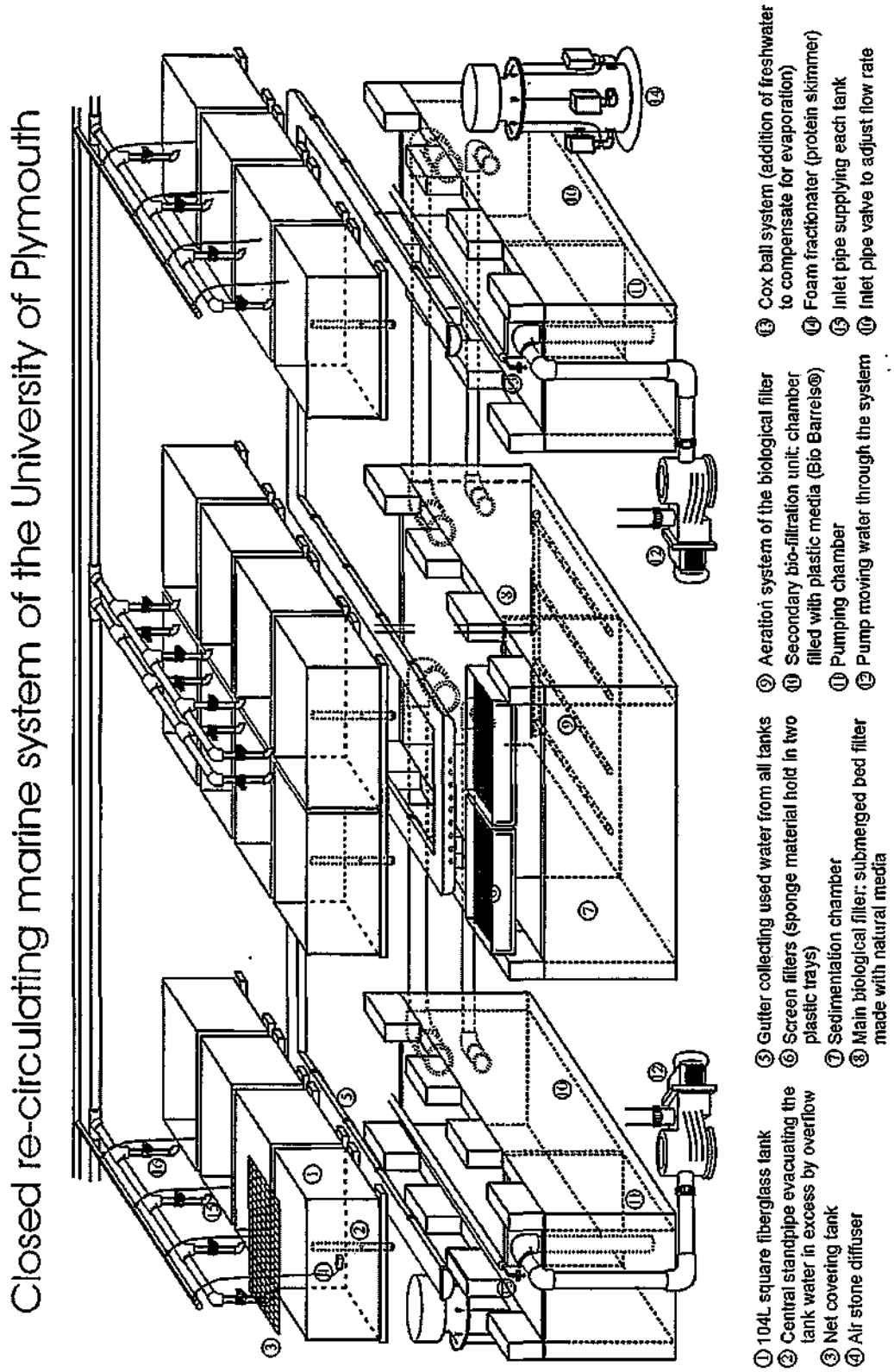

Figure 2.1 Schematic representation of the marine system that it was used (courtesy of J. Laporte 2007)

2.1.3.3 Growth parameters and body composition

Growth and feed utilization parameters were assessed by mean final weight, SGR, FCR, PER, condition factor (K) and hepatosomatic index (HSI). The parameters were calculated as: SGR = 100 x ($\ln W_{fin} - \ln W_{in}$) / d; FCR = FI / WG; PER = WG / PI; K = 100 x (W_{fin} / FL^3); HSI = 100 x (LW / W_{fin}). Where ln in the natural logarithm, W_{in} is the initial fish mean weight (g), W_{fin} is the final mean weight, d is the number of feeding days, WG is the weight (g) gained, FI is the feed intake (g), PI is the protein intake (g), FL is the final body length (cm) and LW is liver weight (g).

Additionally, the carcasses of 5 fish per tank were pooled at the end of the trial (n = 3) and analysed for body composition as previously mentioned for diets proximate analysis.

2.1.3.4 Histology

Fish were killed with an overdose of MS222, a hard blow in the head and cutting the spinal column. Liver and intestinal samples from 3 fish per tank (n = 9 per treatment) were retained for histological examination by electron and light microscopy. Intestinal sections from the middle of the small intestine (anterior region) and the middle of the large intestine (posterior region) were taken for both light and electron microscopy. Liver samples were analysed using light microscopy (LM).

Samples for LM were fixed in 4% saline formalin. The tissue samples were dehydrated in graded ethanol (30%, 50%, 70%, 90% and twice in 100%) for at least 15 min each before equilibration in xylene and embedding in paraffin wax (Refstie et al., 2006a). 8 μm transverse sections were cut and stained using either

alcian blue periodic acid-Schiff staining technique (AB-PAS) (Kiernan, 1981). Briefly, the AB-PAS staining protocol includes the following steps:

- take sections to water with graded ethanol and water solution,
- stain section with alcian blue at pH 2.5 for 20 min,
- wash in tap water and rinse with distilled water,
- 1% periodic acid for 10 min,
- rinse with distilled water,
- Schiff's reagent for 30 min,
- wash in tap water,
- 1% fast green for 20 min (counter stain),
- wash in tap water,
- dehydrate with graded ethanol and mount with cover slip and DPX.

Liver images were analysed for glycogen deposition in the hepatocytes by the ratio of a stained area by Schiff's reagent (magenta = glycogen) and the unstained area, producing arbitrary units (AU). Intestinal images from light microscopy were analysed to determine the perimeter ratio (PR) between the internal perimeter (IP) of the intestinal lumen and the external perimeter (EP) of the intestine (PR = IP/EP, arbitrary units AU; Figure 2.2; after Dimitroglou 2004). A high PR value indicates high absorptive surface area brought about by high villi length and/or increased mucosal folding. Additionally, intestinal LM samples were assessed for mucus pH (Alcian blue stains the acidic mucus blue and PAS stains the neutral to alkaline mucus magenta).

Samples for SEM were rinsed in 1% S-carboxymethyl-L-cysteine for 30 seconds prior to fixation in order to remove epithelial mucus. Then, samples were

fixed using 2.5% glutaraldehyde with 0.1 M cacodylate acid sodium salt (1:1) solution at pH 7.2 with additional 2.5% NaCl. Fixative was removed from samples by rinsing samples 3-times with 0.1 M sodium cacodylate buffer for 15 min each time. Afterwards, samples were dehydrated with graded alcohol solutions of 30%, 50%, 70%, 90% and twice in 100 % for at least 15 min each. Samples were dried using a K850 critical point drier (Emithech; Kent, UK) with ethanol as the intermediate fluid and CO_2 as the transition fluid. All samples were mounted on aluminium stubs and coated with gold using a K550 sputter coater (Emithech; Kent, UK), then screened with a Jeol JSM 5600 LV electron microscope at 15 kV (Jeol; Tokyo, Japan). High magnification (x 20,000) SEM images were analysed in order to measure the density of the microvilli (MD; Figure 2.3) of the enterocytes on top of the villi. Thus the ratio between the microvilli covered area (*M*, foreground) to the background (*B*, background) was calculated (MD = *M*/*B*, arbitrary units AU).

Samples for TEM were fixed using 2.5% glutaraldehyde with 0.1 M cacodylate acid sodium salt (1:1) solution at pH 7.2 with additional 2.5% NaCl. Samples were then rinsed twice with 0.1 M sodium cacodylate buffer for 15 min each and post-fixed in OsO_4 for 1 h. Afterwards, samples were rinsed again twice with 0.1 M sodium cacodylate buffer and dehydrated with graded alcohol solutions of 30%, 50%, 70%, 90% and twice in 100 % for at least 15 min each. Alcohol was removed by gradual replacement with low viscosity resin of 30%, 50%, 70% and 100% for at least 12 h in each solution followed by a second extra time in 100% resin for 24 h. Samples were placed in beem capsules and the resin was polymerised at 70 °C (overnight). Resin blocks were trimmed using a glass knife and then blocks were sectioned using a diamond knife (~90nm). Ultrathin

sections from each sample were placed in copper grids and stained with saturated uranyl acetate for 15 min, rinsed with distilled water and post stained with Reynolds lead citrate for 15 min (Lewis & Knight, 1977). Sections were screened with a Jeol JSM 1200EX transmission electron microscope at 120kV (Jeol; Tokyo, Japan). TEM images (magnification x 20,000) were analysed to measure the microvilli length (Hu *et al.* 2007).

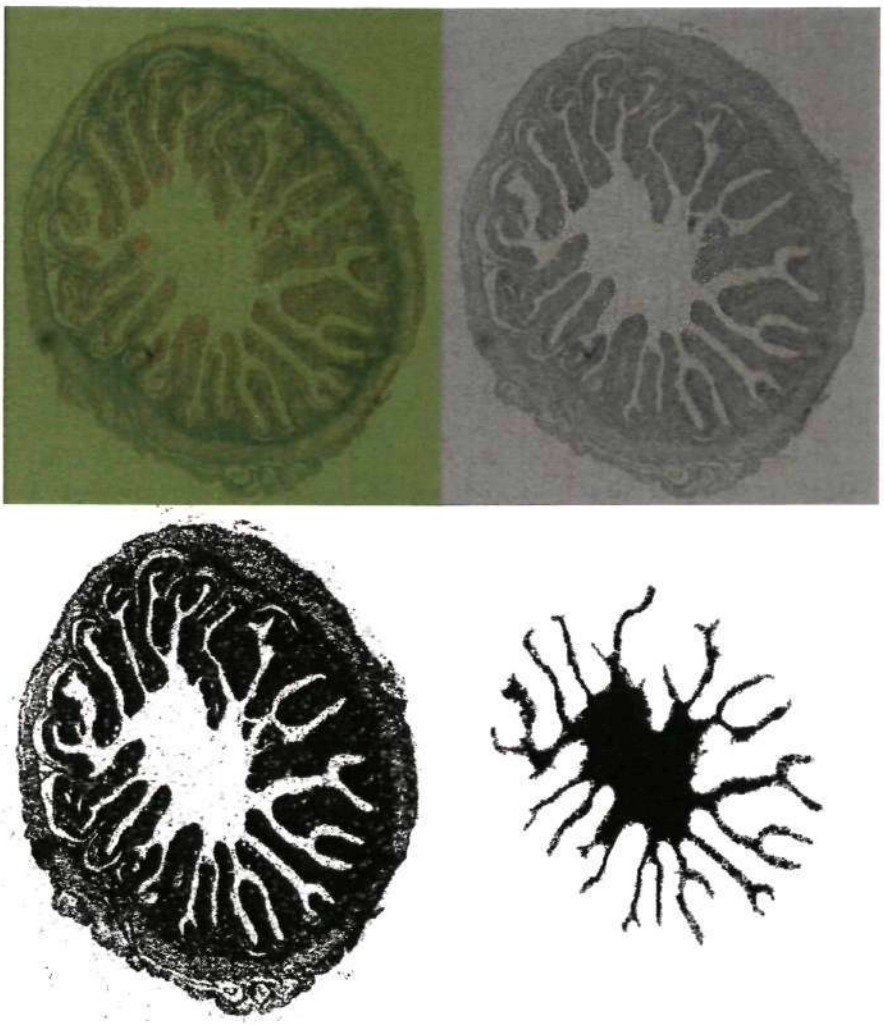

Figure 2.2 Steps for LM image analysis. Transverse section images were transformed to 8-bit and then threshold function (black and white) applied in order the perimeter ratio (PR) be calculated between the internal perimeter (*IP*, bottom right figure) of the gut lumen and the external perimeter (*EP*, bottom left figure) of the gut (PR = *IP*/*EP*, arbitrary units AU).

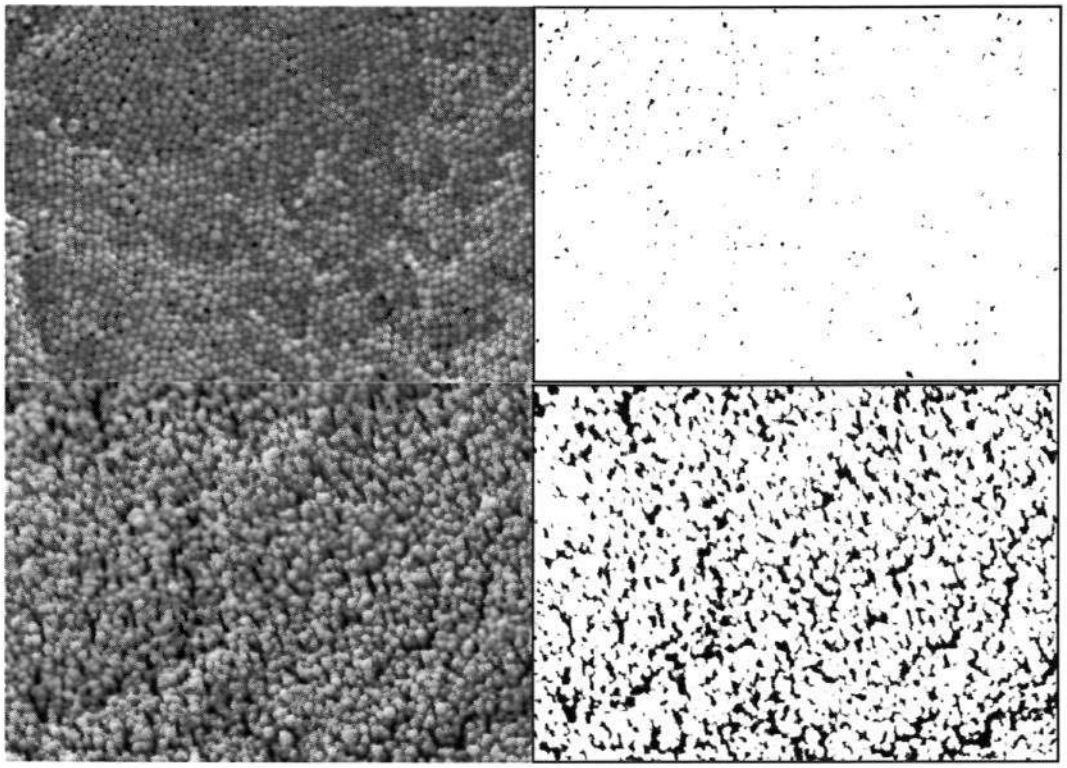

Figure 2.3 SEM micrographs were image transformed to 8-bit and then after applied the threshold function (changed to black and white) the MD ratio was calculated as microvilli covered area (*M*, foreground, white area) to the background (*B*, background, black area) was calculated (MD = *M*/*B*, arbitrary units AU).

2.1.3.5 Bacterial DNA extraction and 16S rRNA amplification and denaturing gradient gel electrophoresis (DGGE)

Time between termination and dissection did not exceed 2.5 h. After aseptic dissection, the entire digestive tract was removed. In order to avoid interfish variation, as previously reported (Spanggaard et al., 2000; Liu et al., 2008), the digesta from 3 individual fish per tank were pooled (Hovda et al., 2007; Merrifield et al., 2009a).

DNA was extracted from 2 replicates of the pooled digesta samples (from 9 fish per regime) using a modified protocol of the QIAamp® Stool Mini Kit (Qiagen). In details:

1. Incubate 200 mg of digesta/mucosa sample with 500 µL of lysozyme (50 mg mL^{-1} in TE) for 30 min at 37°C.
2. Add 700 µL of buffer ASL and vortex for 30 sec.
3. Heat for 5 min at 90 °C.
4. Vortex for 15 sec and centrifuge for 1 min
5. Place 700 µL of the supernatant into an Eppendorf and add half an Inhibitex tablet. Vortex for 30 sec or until suspended. Stand for 1 min.
6. Centrifuge for 4min and place 215 µL of the supernatant into a new tube.
7. Centrifuge for 4min.
8. Place 15 µl of Proteinase K into a fresh tube and add place 200 µL of the supernatant.
9. Add 200 µL of buffer AL and vortex for 15 sec.
10. Incubate at 70 °C for 10min.
11. Add 200 µL of ethanol and vortex.
12. Apply the entire sample to a QIAamp column and centrifuge for 1 min.
13. Place column into a new collection tube and add 500 µL buffer AW1. Centrifuge for 1 min.
14. Place column into a new collection tube and add 500 µL buffer AW2. Centrifuge for 3 min.
15. Place column into a new collection tube and add 200 µL Buffer AE. Stand for 3 min and centrifuge for 1 min.

All centrifugation is at 13,000rpm on a bench top microcentrifuge.

Concentration of DNA was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop™ 1000, DE, USA) and standardized. PCR amplification of the 16S rRNA genes was undertaken using the forward primer P3 with a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and the reverse primer P2

(5'-ATT ACC GCG GCT GCT GG-3'). The following reagents were included in each PCR tube: 1 μL each of primer P2 and P3 (50 pmol μL^{-1}; MWG-Biotech AG, Germany), 3μL DNA template, 25μL ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma) and PCR grade water to a final volume of 50μL. Giving a final concentration of 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs. The touchdown thermal cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min after Muyzer et al. (1993). The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at 55 °C for the remaining cycles. The PCR products were stored at 4 °C until used.

DGGE was performed using a DGGE-2001 system (C.B.S. scientific, CA, USA). 10 μL of standardized PCR products were run on 8% acrylamide gels (1 mm thick; 160 x 160 mm) with a denaturing gradient of 40-60% (where 100% denaturant is 7 M urea and 40% formamide). All samples from the same trial were run on the same gel to prevent issues of reproducibility and outside lanes were not used. The gel was run at 65 V for 17 h at 60 °C in 1 x TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA). Visualization of the DGGE bands was achieved by the optimized silver staining method of Benbouza et al., (2006). The Gel was scanned in a Bio-Rad universal hood II (Bio-Rad laboratories, Italy) and optimized for analyses by enhancing contrast and greyscale.

Additionally, agarose gel electrophoresis was used in order to evaluate the DNA product quality from the DNA extraction procedures and amplicon sizes from PCR amplification. Gels were prepared using 1.2% agarose (Cambrex Bio

Sciences, ME, USA) with 1 x Trisborate-EDTA (TBE) buffer. Ethidium bromide was added (EtBr 0.5 mg mL^{-1}) during the preparation of the gel to facilitate the visualization of DNA products. Gels run within standard gel electrophoresis tank (Pharmacia) with 1 x TBE buffer. Sample preparation for loading was 6 μL which consisted of 1 μL of loading buffer (Bioline) and 5 μL of DNA sample. Gels were run for 1.5 h at 65 V and were visualized in a Bio-Rad universal hood II (Bio-Rad laboratories, Italy).

2.1.3.6 Statistical analysis

One-way ANOVA and independent samples T-test were applied to growth and intestinal histological data for Trial I and Trial II respectively. Analyses were carried out on SPSS 15.0 (SPSS Inc., IL, USA) and significance was accepted at the $P < 0.05$ level. The results are presented as mean values followed by the standard deviation (M ± SD). DGGE banding patterns were transformed into presence/absence matrices for similarity assessment between treatments using Quantity one® version 4.6.3 analyses software (Bio-Rad laboratories, CA, USA) after Schauer et al., (2000). Band intensities were measured and analyzed using Primer v6 (Clarke and Gorley, 2006) and similarity percentages (SIMPER) were used for pairwise comparisons to determine differences between DGGE banding profiles. The diversity of the intestinal microbiota was assessed using the Shannon–Weaver index, calculated with the following formula: $H = -\Sigma(pi(\ln pi))$. Where *pi* is the proportion of the total number of individuals in the *i*th species.

2.1.4 Results

2.1.4.1 Growth analysis

Growth analysis data is presented on Table 2.2. All groups grew well and average fish weight increased by over 200%. In both Trials I and II final weight, specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) remained unaffected by MOS supplementation. In Trial I (FM based diets) condition factor (*K*) was significantly reduced ($P = 0.021$) from 2.01 ± 0.14 in control treatment to 1.89 ± 0.16 in FM02. FM04 had no effect on *K*. Hepatosomatic index (HSI) was significantly affected only by the higher dose of MOS supplementation in sea bream fed the FM based diet (Trial I). Both *K* and HSI were unaffected by dietary MOS in SBM fed fish (Trial II). Additionally, only the HSI was significantly decreased ($P < 0.001$) with the inclusion of dietary SBM (i.e. FM0 vs SBM0).

Analysis of body proximate composition (Table 2.3) revealed that moisture, crude protein, lipids, ash and energy were unaffected ($P > 0.05$) in both Trials I and II.

Table 2.2 Growth parameters and hepatosomatic indices of sea bream after 9 weeks feeding trial, n = 9 per treatment.

	Trial I			Trial II	
Treatments	FM0	FM02	FM04	SBM0	SBM04
Initial fish weight (g)	24.09 ± 0.45	23.44 ± 1.37	23.56 ± 1.77	23.61 ± 0.55	23.59 ± 1.04
Final fish weight (g)	74.91 ± 3.82	74.58 ± 3.15	73.59 ± 0.86	77.98 ± 4.78	79.34 ± 2.66
FCR	1.88 ± 0.05	1.86 ± 0.08	1.88 ± 0.05	1.84 ± 0.07	1.83 ± 0.05
SGR (% day^{-1})	1.80 ± 0.05	1.84 ± 0.04	1.82 ± 0.10	1.89 ± 0.06	1.93 ± 0.04
PER	1.22 ± 0.02	1.23 ± 0.05	1.22 ± 0.03	1.21 ± 0.02	1.18 ± 0.05
K (%)	2.01 ± 0.14 [a]	1.89 ± 0.16 [b]	1.96 ± 0.14 [a,b]	1.96 ± 0.15	2.02 ± 0.15
HSI (%)	1.74 ± 0.17 [a]	1.80 ± 0.23 [a]	1.47 ± 0.25 [b]	1.39 ± 0.26	1.48 ± 0.13

[a, b] Values within the same row, in the same Trial (I or II), with different superscripts are significantly different ($P<0.05$).

Table 2.3 Fish body proximate composition analysis. n = 9 fish per treatment.

	Trial I			Trial II	
Treatment	FM0	FM02	FM04	SBM0	SBM04
Moisture (%)	68.56 ± 0.48	68.96 ± 0.38	69.27 ± 0.52	68.23 ± 0.69	69.59 ± 1.37
Protein (%)	16.36 ± 0.38	16.29 ± 0.48	16.09 ± 0.08	16.64 ± 0.44	16.89 ± 0.40
Lipid (%)	10.90 ± 0.39	9.93 ± 0.64	9.70 ± 0.74	11.00 ± 0.82	9.13 ± 1.11
Ash (%)	3.23 ± 0.17	3.39 ± 0.37	3.24 ± 0.02	3.19 ± 0.23	3.34 ± 0.25
Energy (MJ kg^{-1})	23.76 ± 0.33	23.88 ± 0.76	23.52 ± 0.63	23.81 ± 0.21	23.07 ± 0.63

There were no significant differences between any groups.

2.1.4.2 Histological analysis

An overview of the histological analysis is presented in Table 2.4. Image analysis of PAS-Alcian Blue liver sections revealed that there were no significant effects of diet on glycogen deposition in either Trial I or II. Light microscopy revealed that the dietary treatments had no effect on villi surface area (i.e. perimeter ratio) or mucus pH in the anterior intestinal region. In the posterior intestinal region however the perimeter ratio was significantly increased from 3.92 ± 1.06 (AU: arbitrary units) in FM0 to 5.63 ± 1.67 AU to FM04 ($P = 0.046$) in Trial I. SEM analysis of the anterior and the posterior intestinal regions of fish from Trial I revealed that MOS supplementation significantly ($P < 0.050$) increased the microvilli density (Table 2.4, Figures 2.4 and 2.5). In the anterior intestinal region 0.2% MOS supplementation increased the microvilli density from 5.00 ± 1.97 AU in the control treatment (FM0) to >18 AU in MOS fed fish (FM02 an FM04) ($P < 0.001$). Similarly, the posterior intestinal microvilli density of the control fed group (FM0) increased from 6.69 ± 2.61 AU to >12 AU in MOS treatments (FM02 and FM04) ($P < 0.02$). TEM analysis demonstrated that in Trial I microvilli length was significantly increased with MOS supplementation in both intestinal regions (Table 2.4, Figures 2.6 and 2.7). The microvilli length in the anterior intestinal region increased from 1.76 ± 0.14 μm in the control group (FM0) to over 2 μm in the MOS fed groups (FM02 and FM04) ($P < 0.01$). However, in the posterior intestinal region, only high level MOS supplementation significantly increased the microvilli length: from 1.68 ± 0.29 μm in the control group (FM0) to 2.07 ± 0.22 μm in the 0.4% MOS fed fish (FM04) ($P < 0.006$).

In Trial II, LM analysis of the anterior and the posterior intestinal region revealed that MOS supplementation did not produce any significant effect on the

villi morphology or mucus pH. However, SEM analysis demonstrated that microvilli density was increased in both examined intestinal regions. In the anterior intestine microvilli density was increased from 6.13 ± 2.71 AU in SBM0 to 13.97 ± 8.54 AU in SBM04, $P = 0.018$. Similarly, in the posterior intestine density was increased from 6.63 ± 2.43 in SBM0 to 16.19 ± 9.15 in SBM04, $P = 0.014$ (Table 2.4, Figures 2.8 and 2.9). Microvilli length was only affected by MOS supplementation in the posterior intestine ($P = 0.008$; Figure 2.10). Additionally, no significant differences (independent samples T-test) of gut histological parameters were observed with the inclusion of dietary SBM (i.e. FM0 vs SBM0).

Table 2.4 Histological analysis of liver and intestinal samples of sea bream (n = 9).

		Trial I			Trial II	
	Treatments	FM0	FM02	FM04	SBM0	SBM04
	Liver (AU)	1.41 ± 0.23	1.49 ± 0.10	1.39 ± 0.22	1.35 ± 0.26	1.33 ± 0.35
Anterior intestine	Absorptive surface (PR)*	7.34 ± 1.27	6.81 ± 2.59	6.97 ± 1.64	6.12 ± 1.18	6.33 ± 0.94
	Microvilli density*	5.00 ± 1.97 ^{a}	20.41 ± 8.70 ^{b}	18.06 ± 8.79 ^{b}	6.13 ± 2.71 ^{c}	13.97 ± 8.54 ^{d}
	Microvilli length (µm)	1.76 ± 0.14 ^{a}	2.12 ± 0.23 ^{b}	2.02 ± 0.20 ^{b}	1.81 ± 0.36	1.93 ± 0.37
Posterior intestine	Absorptive surface (PR)*	3.92 ± 1.06 ^{a}	5.44 ± 1.47 ^{a,b}	5.63 ± 1.67 ^{b}	4.85 ± 0.98	5.36 ± 1.30
	Microvilli density*	6.69 ± 2.61 ^{a}	12.74 ± 5.71 ^{b}	13.81 ± 6.15 ^{b}	6.63 ± 2.43 ^{c}	16.19 ± 9.15 ^{d}
	Microvilli length (µm)	1.68 ± 0.29 ^{a}	1.82 ± 0.27 ^{a,b}	2.07 ± 0.22 ^{b}	1.72 ± 0.29 ^{c}	2.15 ± 0.30 ^{d}

*: Arbitrary units

$^{a, b, c, d}$ Values within the same row, the same Trial (I or II), with different superscripts are significantly different ($P<0.05$).

There were no significant differences between respective FM and SBM treatments (i.e. FM0 vs SBM0).

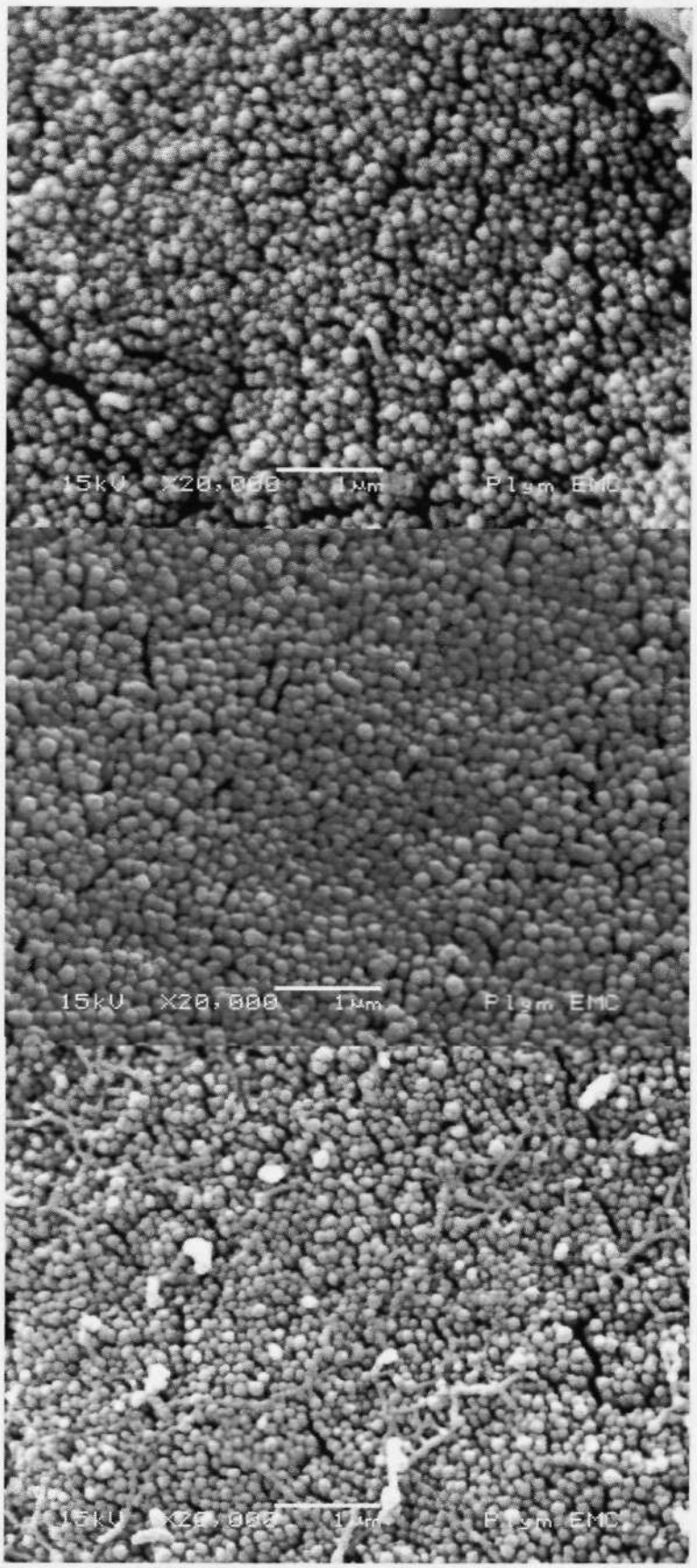

Figure 2.4 Comparative SEM micrographs from the anterior intestinal region. Top micrograph is from FM0 group, the middle from FM02 group and the bottom micrograph from group FM04, which are presenting the effect of MOS supplementation on FM based diets.

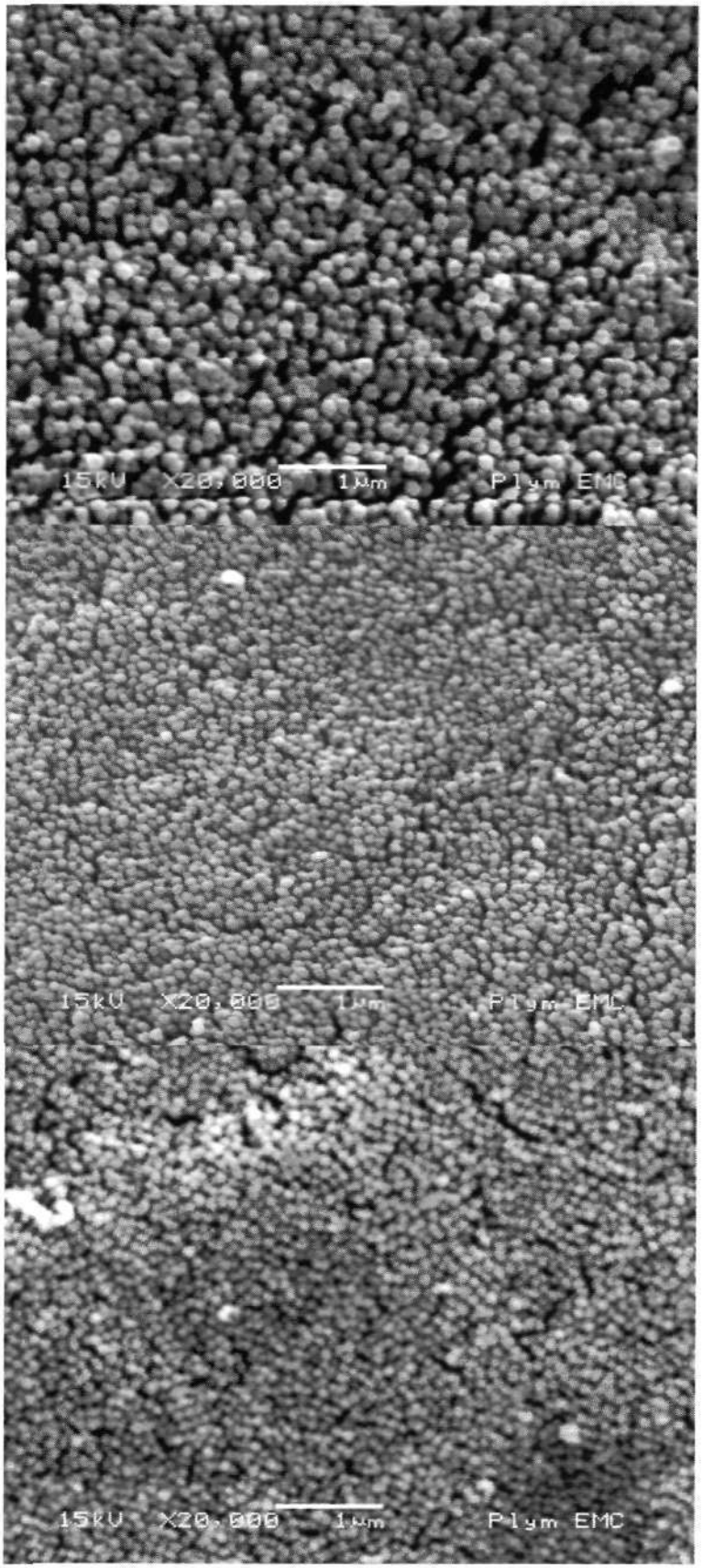

Figure 2.5 Comparative SEM micrographs from the posterior intestinal region. Top micrograph is from FM0 group, the middle from FM02 group and the bottom micrograph from group FM04, which are presenting the effect of MOS supplementation on FM based diets.

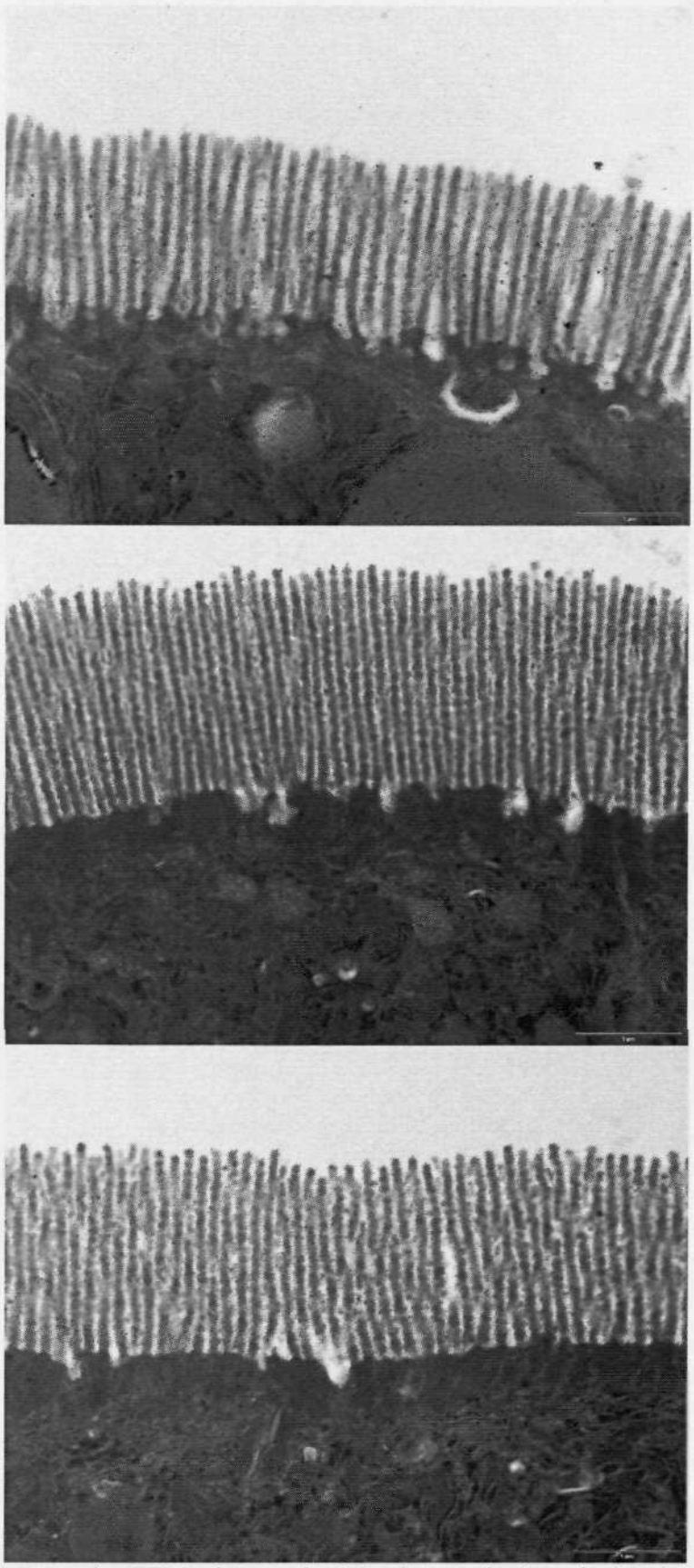

Figure 2.6 Comparative TEM micrographs from the anterior intestinal region. Top micrograph is from FM0 group, the middle from FM02 group and the bottom micrograph from group FM04, which are presenting the effect of MOS supplementation on FM based diets.

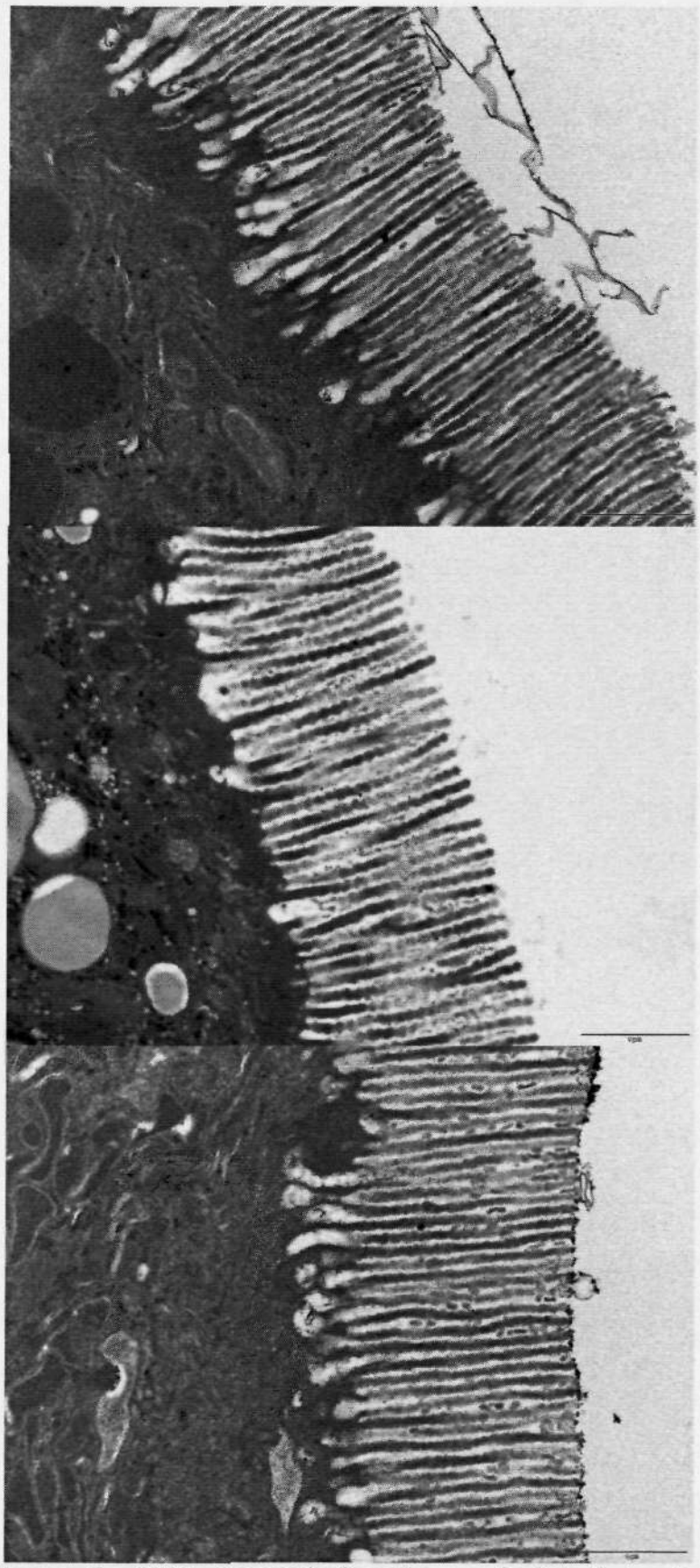

Figure 2.7 Comparative TEM micrographs from the posterior intestinal region. Top micrograph is from FM0 group, the middle from FM02 group and the bottom micrograph from group FM04, which are presenting the effect of MOS supplementation on FM based diets.

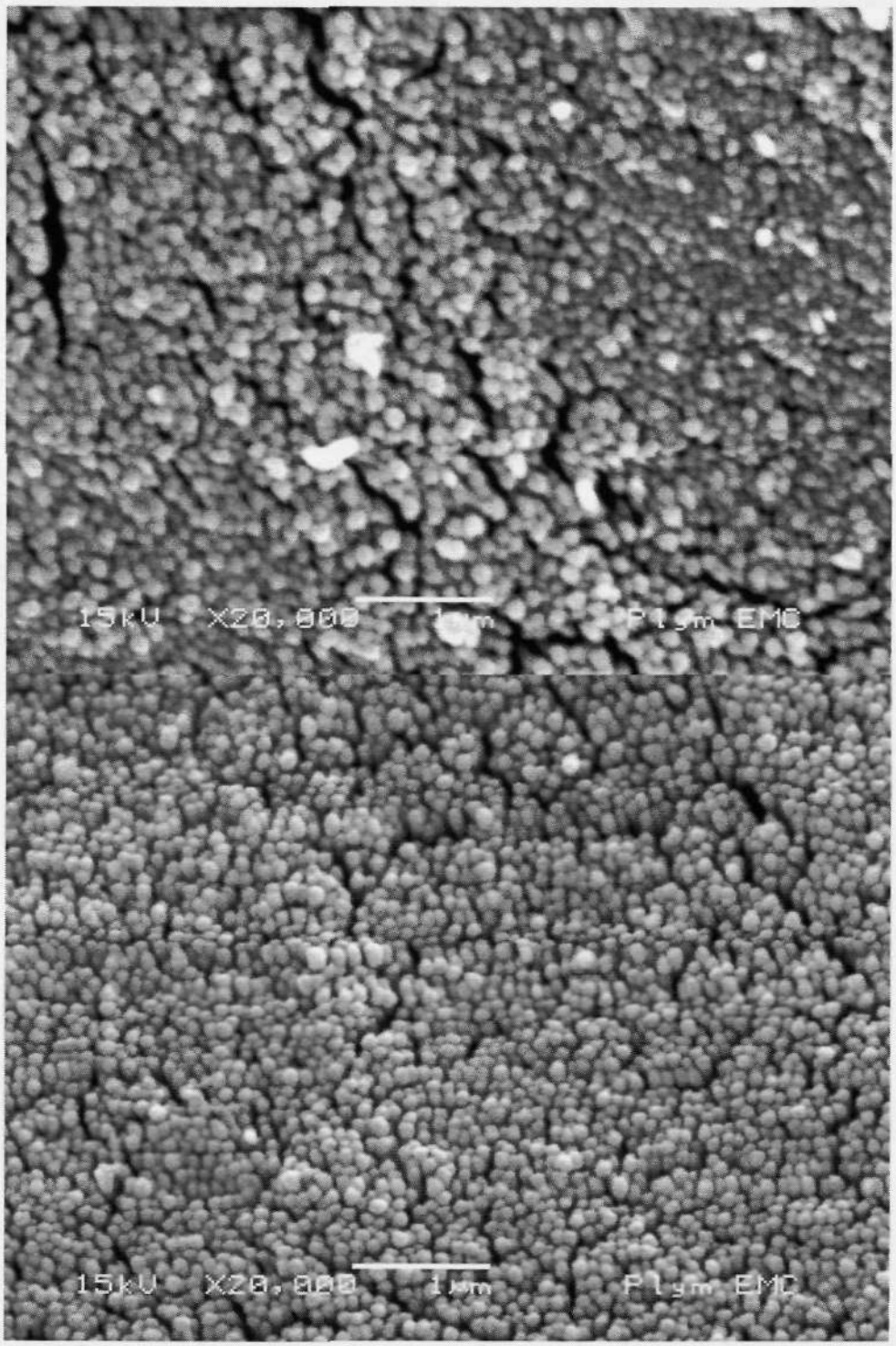

Figure 2.8 Comparative SEM micrographs from the anterior intestinal region. Top micrograph is from SBM0 group and the bottom micrograph from group SBM04, which present the effect of MOS supplementation on SBM based diets.

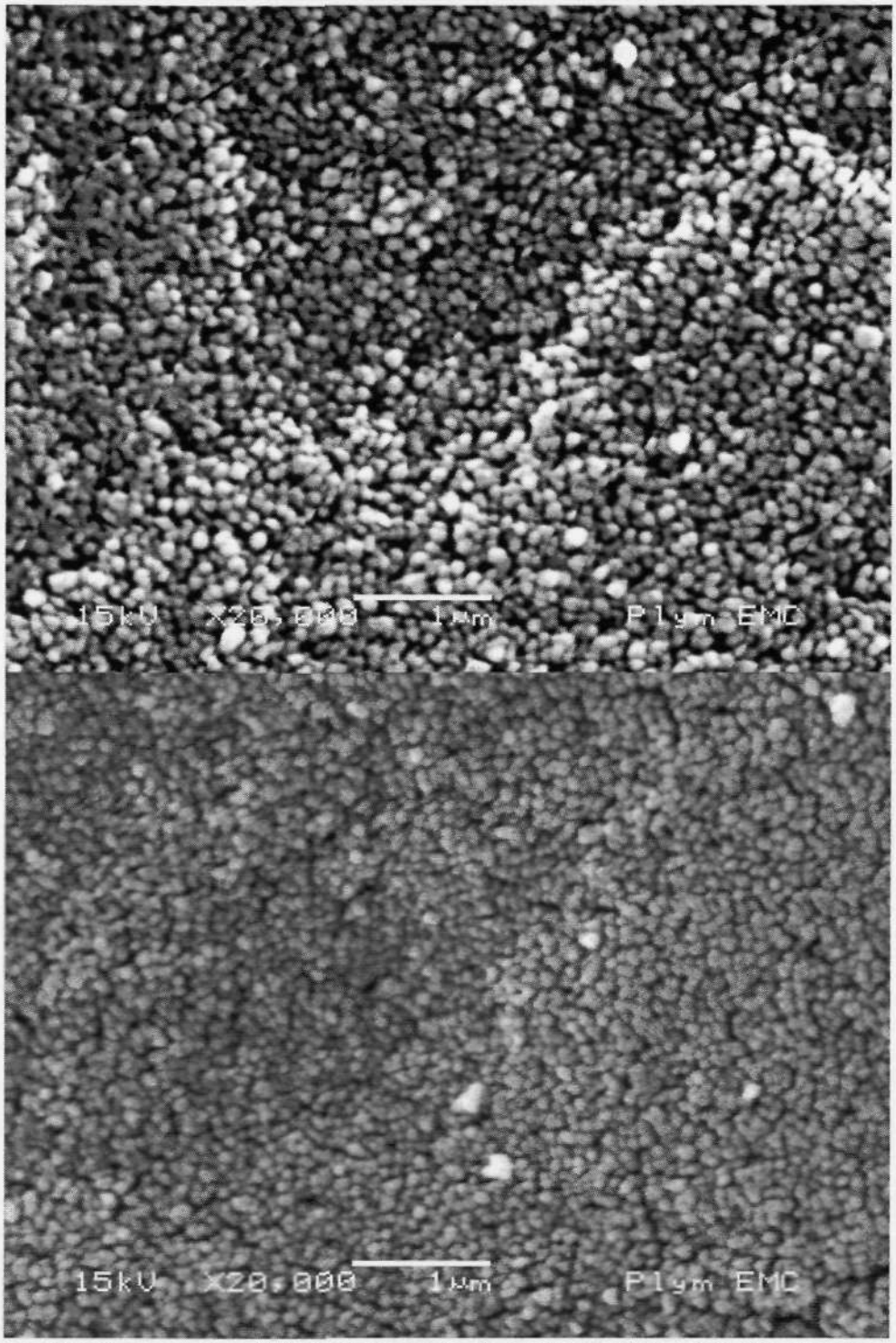

Figure 2.9 Comparative SEM micrographs from the posterior intestinal region. Top micrograph is from SBM0 group and the bottom micrograph from group SBM04, which present the effect of MOS supplementation on SBM based diets.

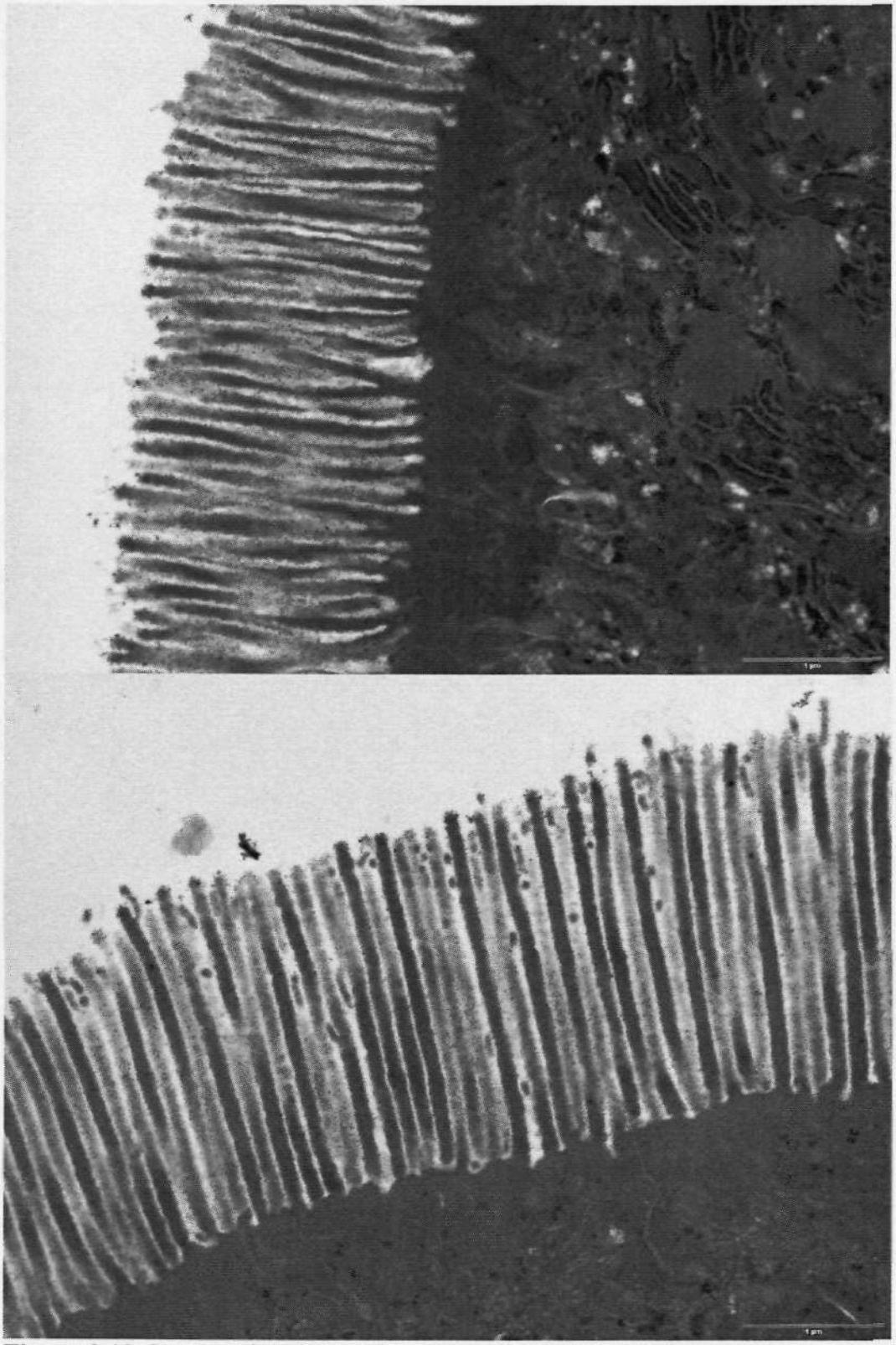

Figure 2.10 Comparative TEM micrographs from the posterior intestinal region. Top micrograph is from SBM0 group and the bottom micrograph from group SBM04, which present the effect of MOS supplementation on SBM based diets.

2.1.4.3 DGGE analysis

Table 2.5 presents the Bray-Curtis similarity half matrix of band intensities (weighted). The highest similarity was between groups SBM0 and SBM04 (86.98% ± 0.03) followed by the similarity between groups FM02 and FM04 (MOS supplemented FM diets, 79.24% ± 0.07). Similarity between groups FM04 and SBM04 was 42.99% ± 0.01 (0.4% MOS groups). Lowest similarity was observed between groups FM0 and SBM0 (control groups, 41.94% ± 0.04). Figure 2.11 displays the Bray-Curtis dendrogram and V3 16S rRNA DGGE fingerprints of bacterial communities from the intestine of sea bream from Trial 1 and Trial II. SBM fed fish (Trial II) displayed distinctly different profiles than fish fed FM (Trial I). Dietary MOS also affected the intestinal microbiota and subsequently the microbial profiles were clustered into 5 distinct groups. However, the effect was greater in Trial I than Trial II and no clear dose response effect by the MOS supplementation. In Trial I, dietary MOS increased species richness (from 15.5 ± 0.7 in FM0 to ca. 25 in FM02 and FM04) and diversity (Shannon Weaver Diversity Index increased from 2.65 ± 0.05 in FM0 to over 3 in FM02 and FM04) and reduced similarity between microbial profiles of FM fed groups (Table 2.6; Figure 2.11). However, MOS supplementation did not affect intestinal microbial species richness or diversity in fish fed SBM diets (Trial II).

Table 2.5 Similarity half matrix of Bray-Curtis analysis of DGGE bands of sea bream intestinal microbial populations.

		Trial I						Trial II			
Group		FM0		FM02		FM04		SBM0		SBM04	
	Replicate	1	2	1	2	1	2	1	2	1	2
FM0	1	100.00%	84.79%	54.50%	58.70%	54.09%	44.65%	45.25%	46.19%	47.19%	48.52%
	2		100.00%	46.24%	51.30%	46.63%	35.90%	37.63%	38.68%	38.79%	41.34%
FM02	1			100.00%	88.16%	85.16%	77.86%	46.24%	45.32%	44.34%	41.97%
	2				100.00%	83.49%	70.43%	41.31%	41.94%	42.24%	40.72%
FM04	1					100.00%	81.34%	45.46%	44.41%	44.36%	41.42%
	2						100.00%	46.06%	45.11%	44.15%	42.01%
SBM0	1							100.00%	92.25%	88.87%	85.85%
	2								100.00%	89.31%	83.91%
SBM04	1									100.00%	88.89%
	2										100.00%

Table 2.6 Summary of species richness, Shannon-Weaver diversity index, Jaccard indices, SIMPER and ANOSIM analysis of the intestinal microbiota of sea bream fed different experimental diets.

Groups	Species richness	Shannon-Weaver Diversity index	SIMPER Similarity	Jaccard Similarity	Jaccard Dissimilarity
FM0	15.5 ± 0.7	2.65 ± 0.05	84.79%	93.75%	
FM02	25 ± 0	3.06 ± 0.02	88.16%	92.31%	
FM04	24.5 ± 0.7	3.10 ± 0.05	81.34%	96.00%	
SBM0	25 ± 0	3.17 ± 0.01	92.25%	100.00%	
SBM04	25 ± 0	3.17 ± 0.02	88.89%	100.00%	
Pairwise comparison					
FM0 x FM02					44.23%
FM0 x FM04					43.12%
FM02 x FM04					7.73%
SBM0 x SBM04					0.00%

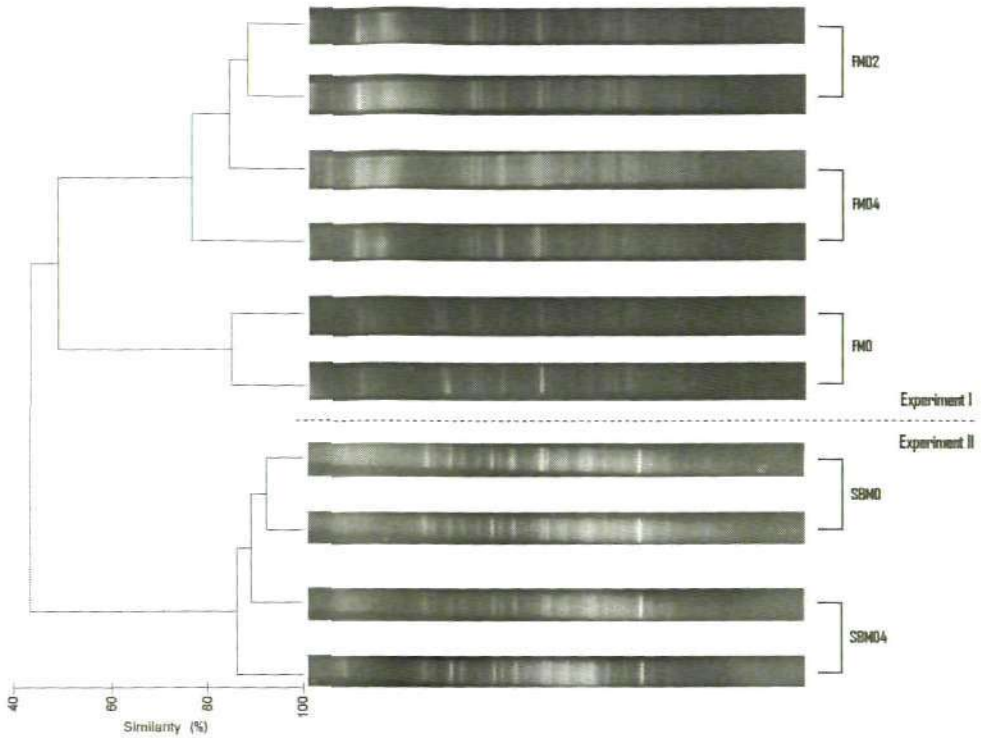

Figure 2.11 Dendrogram demonstrating the similarity analysis between the feeding groups.

2.1.5 Discussion

There is a great interest in order to understand and evaluate how prebiotics can be useful in fish production, especially when FM protein is being replaced by plant proteins. This may be particularly useful in sea bream aquaculture because sea bream is one of the major representatives of the Mediterranean aquaculture industry. From the limited previous studies it appears that the effects of MOS on growth performance of aquatic species are contradictory and depend on fish species, level of MOS supplementation, rearing conditions, experimental duration and maybe the initial fish weight. The present study demonstrated that dietary

MOS did not significantly affect the growth performance of fish fed either FM based diets or diets containing partial FM replacement with SBM. Similar to the results of the present study, previous investigations with Atlantic salmon (*Salmo salar*; Grisdale-Helland et al., 2008), cobia larvae (Salze et al., 2008), hybrid tilapia (*Oreochromis niloticus* x *O. aureus*; Genc et al., 2007a) and Gulf sturgeon (*Acipenser oxyrinchus desotoi*; Pryor et al., 2003) showed that MOS supplementation did not improve growth performance. In contrast, previous studies with rainbow trout (Staykov et al., 2007), European sea bass (*Dicentrarchus labrax*; Torrecillas et al., 2007) and green tiger prawn (*Penaeus semisulcatus*; Genc et al., 2007b) have shown that MOS can improve growth performance. Additionally, Burr et al. (2008) also demonstrated that dietary MOS improved red drum (*Sciaenops ocellatus*) protein, organic matter and energy ADC in SBM rich diets.

Body proximate analysis in the present study showed that none of the examined parameters were affected by MOS supplementation, whereas previous studies on rainbow trout (Yilmaz et al., 2007) and hybrid tilapia (Genc et al., 2007a) using 0.4% MOS supplementation reported increased body protein levels.

HSI is directly related with metabolism because glycogen and lipids can be stored in the liver (Robaina et al., 1995; Krogdahl et al., 2004). Additionally, the high level of MOS supplementation also reduced the HSI in fish fed FM based diets, while HSI remained unaffected by dietary MOS in SBM fed fish. Histological evaluation of the glycogen deposition in the liver tissue revealed that none of the dietary treatments appeared to affect hepatocyte glycogen deposition. Many factors such as high-energy feeds can cause steatosis in the livers of cultivated fish (Caballero et al., 2004), which is a morphological condition of

hepatocytes with excess lipid vacuoles. The finding that 0.4% MOS supplementation can reduce the HSI in the present study without affecting glycogen suggests that MOS supplementation may reduce lipids from liver, thus promoting fish welfare. This speculative hypothesis may be verified in future studies were liver lipids content will be measured using the appropriate technique.

The present study demonstrated that none of the dietary treatments appeared to affect gross villi structure i.e. intestinal PR in both exmined regions. Contrary to these findings, previous studies showed that MOS supplementation at similar levels (0.15% - 0.3%) increased villi length of rainbow trout (Yilmaz et al., 2007). Similarly, improvements of gut morphology have been reported in poultry and swine (Iji et al., 2001; Hooge, 2004; Peet-Schwering et al., 2007; Castillo et al., 2008) using low level MOS supplementation (0.1 – 0.2%). However, similar to the present study, Torrecillas et al. (2007) reported that 0.4% MOS supplementation did not increase European sea bass intestinal villi length. The differences between the reported effects of MOS on villi structure to date might be due to the different doses used, different species assessed, different gut microbiota within these species, different rearing conditions or different methodological approaches used (i.e. villi length measurement vs perimeter ratio method). Future studies should help to reveal the complex mechanisms underpinning the potential improvements induced by prebiotics in the gut of fish. Dietary SBM incorporation of ~30% did not produce any gross histological alteration at the level of villi structures in the present study, which supports the findings of Bonaldo et al. (2008).

SEM analysis in the present study revealed that MOS supplementation increased microvilli density in both the anterior and posterior intestinal regions.

SEM analysis suggests that MOS supplementation can improve microvilli structures which should provide the potential to improve nutrient capture. TEM analysis of the intestine revealed that MOS supplementation could increase the microvilli length, which is in accord with Salze and co-authors (2008) who observed that 0.2% dietary MOS supplementation increased the microvilli length of cobia larvae. Despite the clear increase of both microvilli density and length observed in the study, these improvements did not translate to improved nutrient utilisation or growth performance in the present study. Despite this, the improvement of microvilli morphology may lead to improved apical brush border integrity which should be assessed by pathogenic challenge studies in future. Previous studies with salmonids have demonstrated that dietary SBM decreased microvilli length and density (Bakke-McKellep et al., 2007; Merrifield et al., 2009a) which is in contrast to the findings of the present study, where SBM had no effect. This suggests that gilthead sea bream is not as susceptible to SBM induced enteritis as salmonids. The specific reason for this is currently unclear but may be due to the physiology and the different dietary habits of this omnivorous fish species (i.e. gilthead sea bream) compared to other carnivorous fish species which often face great difficulties in accepting plant proteins (i.e. salmonids).

PCR-DGGE analysis in the present study suggests that both MOS and SBM can influence the intestinal microbiota in terms of microbial abundance and species richness. Shannon-Weaver diversity indices and species richness were increased with MOS supplementation in the FM groups. Additionally, cluster analysis demonstrated a clear shift of intestinal microbial profiles with each dietary treatment clustered into distinct groups. SBM has been shown to effect the gut microbiota of fish previously (Heikinnen et al., 2006; Ringø et al., 2006;

Bakke-McKellep et al., 2007; Ringø et al., 2008; Merrifield et al., 2009a) and the present study indicates that the gut microbiota of sea bream are also sensitive to dietary SBM. In fact our findings suggest that dietary SBM exerted a greater effect on gut microbiota than dietary MOS as the clear differences in microbial profiles and diversity induced by dietary MOS in FM fed fish appeared to be reduced or eliminated in diets containing SBM. The reason for this is not at present clear, however, this might be due to fermentable carbohydrates provided by SBM which typically comprise approx. 4-5% oligosaccharides on dry weight basis (Obendorf et al., 1998). Indeed, SBM oligosaccharides have been discussed in terms of prebiotic potential and microbial fermentability previously (Gibson 2004) and thus these components of SBM may have exerted some effect on gut microbial populations. Future studies should incorporate 16S rRNA sequence analysis and quantitative culture independent techniques such as FISH to fully elucidate these changes.

The findings of the present study suggest that MOS supplementation alters the intestinal microbiota and morphology of gilthead sea bream. Furthermore, gilthead sea bream can accept up to 31% dietary SBM without suffering from SBM induced enteritis. According to the previous research with MOS, it is appears that the fish size, species, rearing conditions and level of MOS supplementation are the factors which determine the effect of MOS in fish. Hence, further research is required in order to evaluate and optimise MOS applications especially with regards to the intestinal microbiota and intestinal histology.

2.2 Effect of short term dietary MOS supplementation on gilthead sea bream (*Sparus aurata*) growth parameters, appetite, intestinal microbiota and haematological immune parameters

2.2.1 Abstract

A study was conducted in order to evaluate the effect of short term dietary MOS supplementation on growth performance, appetite response, intestinal microbiota and haematological immune parameters of sub adult (approx. 105g) gilthead sea bream (*Sparus aurata*). The experimental design consisted of 3 feeding regimes with the same basal diet of which the first was the control (0% MOS), the second included 0.2% MOS supplementation and the third included 0.4% MOS supplementation. After feeding on the experimental diets for 2 weeks, the results showed that compared to the control group, MOS supplementation improved FCR from ~1.5 to ~1.1; SGR from ~1.3% day^{-1} to ~1.8% day^{-1} and PER from ~1.5 to ~1.9. However, daily appetite index was not affected. Haematological immune parameters such as serum lysozyme and alternative complement activity were not affected by MOS supplementation. Total leukocyte counts increased with MOS supplementation and alterations of leukocytes types were observed. Intestinal microbial analysis revealed that MOS supplementation reduced the culturable intestinal microbial load but did not influence the relative abundance of the indentified bacterial species. No dose response effect of MOS supplementation was found in the present study. The findings suggests that MOS supplementation can positively affect growth performance and health parameters of gilthead sea bream receiving MOS supplementation for 2 weeks.

2.2.2 Introduction

Functional dietary supplements are developed in order to protect fish health and improve fish performance. There are only three published studies which evaluate the effect of MOS on fish health with regards immune parameters. Zhou and Li (2004), Toreccillas et al. (2007) and Staykov et al. (2007) all fed MOS supplementation for at least 6 weeks before sampling their fish. The previous chapter describes the only study which has assessed the effect of MOS supplementation on the intestinal microbiota of fish. The results suggested that MOS supplementation for 10 weeks could modulate the microbiota, however it is not clear what duration of MOS supplementation is required in order to modulate the intestinal microbiota. Furthermore, it has been stated that MOS can modulate the intestinal and systemic immune systems, by acting as a non-pathogenic microbial type antigen with an adjuvant like effect (Fischer et al. 2001). However, there is a lack of published data regarding the effect of short term dietary MOS supplementation on fish and the effect of MOS on dietary palatability. The aim of the present study was to evaluate the effect of short term dietary MOS on growth, appetite, intestinal microbiota and immune parameters of sea bream fed commercial diets supplemented with MOS.

2.2.3 Methodology

The trial was conducted in the research facilities of the University of Plymouth, U.K.. Fish were imported from a commercial hatchery in France (Aquastream, Ploemeur) and were acclimatised for approximately 2 months prior to the start of the trial and 180 fish were distributed in 9 tanks with 20 fish on each

(3 replicate tanks per feeding regime). The first feeding regime was the control in which fish were fed a basal diet. The second feeding regime consisted of the basal diet supplemented with 0.2 % MOS and the third feeding regime consisted of the basal diet with 0.4 % MOS supplementation as the previous study (Chapter 2.1). All diets were soy free and manufactured in commercial facilities in Greece by Astrea SA, Korithos. Proximate analysis of the diets for ash, moisture, lipid, protein and NFE was undertaken according to AOAC (1995) as explained in Chapter 2.1.3.1 (Table 2.7). Briefly sample dry matter (DM) was calculated from the weight of sample before and after drying in a fan assisted oven (Genlab ltd, UK) at 105 °C until a constant weight. Moisture was calculated as the difference between sample wet weight (g) dry weight (g). Crude protein analysis was performed using the Kjeldahl method. Lipid content was determined using the Soxhlet extraction method. Sample total mineral or inorganic content (ash) was determined by incineration in a muffle furnace (Carbolite, Sheffield, UK). Gross energy was calculated according to Brett and Groves, 1979). Due to industrial confidentiality the precise formulation of feed cannot be provided. Diet ingredients were fish meal, wheat meal, fish oil, wheat gluten, lecithin GMO-free, shell meal, vitamin and mineral premix. All ingredients were provided by the Astrea SA suppliers. Fibreglass tanks with internally rounded edges and 120 L volume were used. Each tank was provided with re-circulated aerated marine water at a rate of 360 L hr^{-1}. Fish were fed to apparent satiation (until the first feed refusal was visually observed) twice a day for 14 days (2 weeks), at 09.00 and 17.00 hr.

Water pH, ammonia, nitrite, nitrate, dissolved oxygen salinity and temperature were monitored regularly. Water temperature was maintained at 22 ±

1°C and salinity between 33 – 34 mg L^{-1} with a 12 hr light / 12 hr dark photoperiod.

Table 2.7 Proximate analysis of experimental diets.

	Control	0.2% MOS	0.4% MOS
Dry matter	92.68	92.76	92.64
Moisture (%)	7.32	7.24	7.36
Protein (%)	47.42 ± 0.64	47.74 ± 0.21	47.80 ± 0.63
Lipids (%)	14.91 ± 0.40	15.28 ± 0.22	15.16 ± 0.30
Ash (%)	10.99 ± 0.13	10.72 ± 0.09	10.88 ± 0.11
NFE[1] (%)	19.36	19.02	18.81
Energy[2] (MJ kg^{-1})	20.37	20.53	20.47
Vitamin A (IU kg^{-1})	6000	6000	6000
Vitamin D3 (IU kg^{-1})	2000	2000	2000
Vitamin E (mg kg^{-1})	400	400	400
Vitamin C (mg kg^{-1})	350	350	350
Cu (mg kg^{-1})	10	10	10
Se (mg kg^{-1})	0.3	0.3	0.3
MOS[3] (g kg^{-1})	0	20	40

[1]: Nitrogen Free Extracts (NFE) = Dry Matter – (crude lipid + crude ash + crude protein).

[2]: Gross energy (MJ kg^{-1}) calculated according to 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.0 kJ g^{-1} fro NFE.

[3]: Bio-Mos®, Alltech Inc.

2.2.3.1 Growth parameters and appetite

Growth performance was assessed in terms of specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (K) and hepatosomatic index (HSI) were calculated as SGR = 100 x ($\ln W_{fin} - \ln W_{in}$) / d; FCR = FI / WG; PER = WG / PI; K = 100 x (W_{fin} / FL^3); HSI = 100 x (LW / W_{fin}). Where ln in the natural logarithm, W_{in} is the initial fish mean weight (g), W_{fin} is the final mean weight, d is the number of feeding days, WG is the weight gain (g), FI is the feed intake (g), PI is the protein intake (g), FL is the final body length (cm) and LW is liver weight (g). Fish mean weight was calculated as fish bulk weight divided by the number of fish. Additionally, feeding duration was recorded and the daily appetite index (DAI) was calculated as daily feed intake (g) / feeding duration (min). A higher value indicates greater fish appetite.

2.2.3.2 Culture based microbial analysis

At the end of the trial the intestinal digesta from 6 fish per tank (12 fish per treatment) were aseptically removed and were pooled into 2 samples in order to avoid inter-fish variation (Spanggaard et al., 2000; Liu et al., 2008) and the time between termination and dissection did not exceed 1 hour. All samples were serially diluted to 10^{-7} with phosphate buffered saline (PBS) and 100 µL was spread onto duplicate tryptone soy agar plates (TSA) after Huber et al. (2004) and Merrifield et al. (2009a) with the addition of 2.5% of NaCl. Colony forming units (CFU) g^{-1} for aerobic heterotrophic populations were calculated after 7 days aerobic incubation at 22 °C. 25 random colonies from plates containing 30 – 300 CFU were taken and sub-cultured on TSA until pure cultures were achieved. A total of 450 isolates were then tentatively placed into groups or genera based on

the colony morphology, cell morphology, Gram stain, production of catalase, oxidase, glucose fermentation, motility and endospore formation (Cowan and Steel, 1993; Holt and Bergey, 1994).

2.2.3.3 Haematology

Blood samples were collected on the final day of the trial. Fish (3 fish per tank, 9 fish per treatment) were euthanized by overdose of Tricaine methanesulfonate (MS222; Pharmaq, Fordingbridge, UK; 300 mg L^{-1} water) and blood was removed from the caudal vein into Eppendorf tubes. Blood smears were prepared immediately following blood removal. Blood smears were air-dried, fixed in methanol for 5 min and stained with Giemsa stain (BDH, Poole, UK), rinsed with water, air-dried and examined under a light microscope. A minimum of 200 cells per slide were counted to generate a statistically significant sample. Neutrophils, monocytes, thrombocytes and lymphocytes were identified following the classifications of (Hibiya, 1982) and the total number of each leukocyte class was expressed as a percentage of the total leukocyte population. Total leukocytes were counted per 1000 blood cells.

The rest of the blood was allowed to clot for at least 3h at room temperature prior centrifuging for 5 min in 3600 *g*. Serum was collected and stored at -80 °C until analysis of lysozyme and alternative complement activity. Lysozyme activity was assessed using a turbidometric assay where 50 µL of serum was added to 950 µL of *Micrococcus lysodeikticus* at a concentration of 0.2 mg mL^{-1} in 0.05 M Na_2HPO_4 (pH 6.2). After mixing, the reduction in turbidity was measured between 0.5 and 4.5 min at 530 nm at 22 °C. 1 unit of lysozyme activity was defined as a decrease in absorbance of 0.001 units min^{-1} (Ellis 1990).

Alternative complement activity was assayed using sheep red blood cells (SRBC; TCS Biosciences, Claydon, UK) following the procedure of Ortuno et al. (2001). Briefly, SRBC were washed 3 times at 1000 g for 10 minutes in complement fixation test (CFT) buffer (Oxoid, UK) and were adjusted to 3% solution using CFT buffer. Test serum in 500 µL aliquots was diluted in CFT buffer and added to 500 µL of SRBCS to give final serum concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.1565 and 0.078%. Serum and SRBCS were incubated for 1 h at 25 °C and the samples were centrifuged at 1000 g for 7 min to remove un-lysed SRBCS. The relative haemoglobin concentration of the supernatants was assessed at 540 nm where 100 % lysis was achieved using 500 µL of distilled water and minimum lysis was achieved using 500 µL CFT buffer. The degree of haemolysis was calculated and the lysis curve obtained by plotting haemolysis against volume of serum according to Yano (1992). The volume of serum yielding 50% haemolysis was determined and used to calculate the complement activity of the sample (ACHA50 units mL^{-1}).

2.2.3.4 Statistical analysis

A one-way ANOVA was applied on SPSS 15.0 (SPSS Inc., IL, USA) with significance level of $P < 0.05$. The results are presented as mean values followed by the standard deviation (M ± SD).

2.2.4 Results

2.2.4.1 Growth parameters and appetite analysis

Growth data is presented in Table 2.8. Feed utilization parameters (FCR, SGR and PER) were significantly affected by MOS supplementation. Specifically, compared to the control, FCR was significantly ($P = 0.012$) reduced in fish fed 0.2% of MOS supplementation from 1.47 ± 0.20 to 1.01 ± 0.08. Similarly, PER was significantly ($P = 0.005$) affected only in the lower level of MOS supplementation, increasing from 1.45 ± 0.23 in control group to 2.06 ± 0.27. SGR was also significantly increased with MOS supplementation from 1.33 ± 0.23% in control group to 1.90 ± 0.10% in 0.2% MOS ($P = 0.003$) and 1.67 ± 0.56% in 0.4% MOS ($P = 0.034$) groups. Final weight was not affected but despite this K was increased in the high dose of MOS supplementation from 1.99 ± 0.14 in the control group to 2.13 ± 0.13 ($P = 0.019$). Daily feeding time and DAI were not affected by diet in any of the experimental groups ($P > 0.05$).

Table 2.8 Growth performance, feed utilization parameters and feed appetite index of sea bream fed the experimental diets for 2 weeks (n = 3).

	Control	0.2% MOS	0.4% MOS
Initial fish weight (g)	105.13 ± 8.57	102.97 ± 0.23	109.07 ± 3.11
Final fish weight (g)	126.57 ± 10.52	134.90 ± 1.71	140.60 ± 6.22
SGR (% day^{-1})	1.33 ± 0.23^{a}	1.90 ± 0.10^{b}	1.67 ± 0.56^{b}
FCR	1.47 ± 0.2^{a}	1.01 ± 0.08^{b}	$1.17 \pm 0.12^{a, b}$
PER	1.45 ± 0.23^{a}	2.06 ± 0.27^{b}	$1.78 \pm 0.20^{a, b}$
K	1.99 ± 0.14^{a}	2.02 ± 0.17^{a}	2.13 ± 0.13^{b}
Daily feed consumed per tank (g)	44.41 ± 5.20	46.09 ± 2.69	49.20 ± 5.04
Daily tank feeding time (min)	9.62 ± 1.27	9.59 ± 0.89	9.83 ± 0.94
DAI (g min^{-1})*	11.17 ± 2.58	11.23 ± 2.89	10.64 ± 2.62

$^{a, b}$: Values within the same row with different superscripts are significantly different ($P < 0.05$).

*: DAI = feed intake (g) / feeding duration (min) where higher value indicates greater appetite.

2.2.4.2 Culture based microbial analysis

Table 2.9 presents the culturable aerobic heterotrophic intestinal microbial composition of each group. Viable intestinal populations ranged between 10^4 and 10^5 CFU g^{-1}. Viable populations in MOS supplemented groups were significantly lower than the control groups; by ca 1 log scale (down from 10^5 to 10^4 CFU g^{-1}; $P < 0.05$). No clear changes in the relative abundance of the bacterial microbiotã were observed. *Micrococcus* spp. and *Pseudomonas* spp. were the dominant indentified species within the groups (30-40%). Yeast were identified as minor components of the microbiota of fish fed MOS supplemented diets but were not detected in the control fed fish. Additionally, Shannon-Weaver diversity index demonstrated that there was no significant difference in microbial biodiversity between the experimental groups.

Table 2.9 Composition of viable gut microbiota of sea bream fed a control or MOS supplemented diets, expressed as percentages.

	Control	0.2% MOS	0.4% MOS
Viable population (CFU g^{-1})	2.86×10^{5a}	2.65×10^{4b}	3.12×10^{4b}
Composition			
Micrococcus spp.	39%	33%	40%
Acinetobacter spp.	9%	9%	14%
Enterococcus spp.	-	-	2%
Pseudomonas spp.	41%	35%	32%
Bacillus spp.	4%	3%	8%
Kurthia spp.	6%	5%	2%
Unidentified Gram-negative spp.	-	-	4%
Unidentified Gram-positive spp.	7%	13%	8%
Yeast	-	8%	2%
Shannon-Weaver diversity index	0.97 ± 0.49	1.41 ± 0.23	1.36 ± 0.42

[a, b]: Values within the same row, in the same age group, with different superscripts are significantly different ($P < 0.05$).

n = 6 (pooled from 12 fish into 2 replicates)

2.2.4.3 Haematology

Serum lysozyme and alternative complement activity were not affected by MOS supplementation ($P > 0.05$; Table 2.10). Total leukocytes numbers significantly ($P < 0.001$) increased in both MOS fed groups compared with the control; from 7.39% ± 1.99 in control fish to 12.26% ± 1.94 for 0.2% MOS and 13.07% ± 2.11 for 0.4% MOS. MOS supplementation significantly reduced the percentage of monocytes while lymphocytes and granulocytes were increased. Specifically, monocytes were reduced from 7.68% ± 3.17 in control to 2.73% ± 1.25 and 3.02% ± 1.15 in 0.2% and 0.4% MOS supplementation, respectively ($P = 0.001$). Lymphocytes were increased from 69.34% ± 3.79 in the control group to 75.86% ± 5.56 and 74.84% ± 4.16 in 0.2% and 0.4% MOS groups, respectively ($P = 0.006$ and $P = 0.017$ respectively). Similarly, granulocytes were increased from 7.67% ± 2.11 in the control group to 9.76% ± 1.96 and 10.49% ± 2.29 in 0.2% and 0.4% MOS groups, respectively ($P = 0.048$ and $P = 0.010$ respectively). Thrombocytes remained unaffected by the MOS supplementation ($P > 0.05$).

Table 2.10 Blood immune parameters and leukocytes counts of sea bream fed the experimental diets (n = 9).

	Control	0.2% MOS	0.4% MOS
Lysozyme activity (units mL^{-1})	341.32 ± 151.22	297.69 ± 141.01	340.51 ± 158.56
ACHA50 (units mL^{-1})	94.38 ± 61.47	51.17 ± 34.52	72.38 ± 47.65
Total leukocytes (%)	7.39 ± 1.99^{a}	12.26 ± 1.94^{b}	13.07 ± 2.11^{b}
Lymphocytes (%)	69.34 ± 3.79^{a}	75.86 ± 5.56^{b}	74.84 ± 4.16^{b}
Monocytes (%)	7.68 ± 3.17^{a}	2.73 ± 1.25^{b}	3.02 ± 1.15^{b}
Granulocytes (%)	7.67 ± 2.11^{a}	9.76 ± 1.96^{b}	10.49 ± 2.29^{b}
Thrombocytes (%)	15.31 ± 3.45	11.65 ± 5.54	11.44 ± 3.47

$^{a, b}$: Values within the same row with different superscripts are significantly different ($P < 0.05$).

2.2.5 Discussion

In the present study MOS supplementation improved the growth parameters (i.e. growth rate) of sea bream fed to satiation for 2 weeks. The literature regarding the effect of MOS supplementation on growth performance of aquatic species is inconclusive. Previous studies on Atlantic salmon (*Salmo salar*; Grisdale-Helland et al., 2008), cobia larvae (Salze et al., 2008), hybrid tilapia (*Oreochromis niloticus* x *O. aureus*; Genc et al., 2007b), Gulf sturgeon (*Acipenser oxyrinchus desotoi*; Pryor et al., 2003) as well as Chapter 2.1 showed that MOS supplementation did not affect growth parameters (i.e. SGR, FCR, *K* and weight gained). However, in accordance with the present study, previous studies on rainbow trout (Staykov et al., 2007; Yilmaz et al., 2007), European sea bass (*Dicentrarchus labrax*; Torrecillas et al., 2007) and green tiger prawn (*Penaeus semisulcatus*; Genc et al., 2007a) have shown that MOS can improve growth performance. Additionally, the feeding strategy appears to influence the growth parameters for example in the studies of Staykov et al. (2007), Yilmaz et al. (2007), Torrecillas et al. (2007) and the present trial fish were fed to satiation (MOS supplementation levels were ranged between 0.15 – 0.45%) which resulted in improved growth performance. There are two more studies which support the results of the present trial such as Genc et al. (2007b), in which MOS supplementation level ranged between 0.15 – 0.45% and Grisdale-Helland et al. (2008) in which MOS supplementation level was 1% where both of them fed fish a fixed percentage of body weight. These studies as well as the previous Chapter 2.1 with the sea bream indicate that the effect of MOS supplementation on fish growth performance is related with the feeding strategy. Thus possibly suggesting that when fish are fed to satiation there is a significant improvement in growth

parameters but when fish fed by a fixed percentage of body weight then there were no significant effects on growth performance. Another suggestion for the different effect of MOS supplementation in fish growth is the fish age difference between the studies, which is very speculative at this point. In the present trial, *K* factor was increased only when fish were fed the diet supplemented with 0.4% of MOS.

There was a clear reduction in total culturable intestinal bacteria in fish fed MOS supplemented diets. In the present study, MOS did not change the species relative abundance as was seen in the previous experiment with the sea bream (Chapter 2.1). According to a previous study on Jian carp with regards to intestinal microbiota demonstrated that MOS supplementation can alter the intestinal microbiota by increasing the numbers of Bifidobacteria and Lactobacillus species and at the same time reducing the numbers of *E. coli* (Zhou and Li, 2004). Additionally, the duration of Zhou and LI (2004) study was 10 weeks suggesting that the duration of the trial is related with the effect of MOS supplementation on the intestinal microbiota and the present study suggests that 2 weeks is a short period of time in order MOS is able to effect the bacterial relative abundance.

Serum lysozyme and alternative complement activity were not affected by MOS supplementation, which agrees with the previous study in European sea bass (Torrecillas et al., 2007) for more than 9 weeks experimental period. However, a previous study with rainbow trout reared in fresh water demonstrated that MOS supplementation increased both serum lysozyme and alternative and classical complement activity after 6 weeks of feeding MOS supplemented diets (Staykov et al., 2007). This may be related with the physiology of species (i.e. marine water

fish and freshwater fish) given that the average weight of the fish samples from the studies of Torrecillas et al., 2007 and Staykov et al., 2007, was always > 100 g. In the present study there was a reduction in the relative abundance of monocytes by the addition of MOS supplementation, thus the relative proportion of the rest of the leukocytes such as lymphocytes and granulocytes abundances were increased. Additionally, the total leukocytes counts were increased which suggests that despite the lack of changes in the humoral parameters measured (complement and lysozyme activity) the cellular innate immune system of sea bream appears to be more responsive.

Due to the great interest in order to understand and evaluate how prebiotics can be useful in the animal production, the present study evaluated the effect of MOS supplementation in one of the most important species in Mediterranean aquaculture industry. MOS supplementation improved the growth parameters when fish fed to apparent satiation for 2 weeks. The findings of the present study suggest that MOS supplementation can improve growth performance and feed utilization and quantitatively alter the intestinal microbiota. Total and differential leukocytes counts were also affected by the MOS supplementation which suggests that MOS interacts with the innate immune system of the fish (adjuvant-like effect).

2.3 Effect of MOS on mortality and gut integrity of sole (*Solea senegalensis*, Kaup) infected by *Photobacterium damselae* subsp. *piscicida*

2.3.1 Abstract

A study was conducted in order to investigate the effect of MOS supplementation on the gut morphology of sole (*Solea senegalensis*) with an initial mean weight of 55 g. The dietary inclusion rate was 0.4% which was used either alone or in combination with a vaccination regime against bacterial diseases (*Pasteurella spp.* and *Vibrio spp.*). From the start of the experimental period, all fish were infected with *Photobacterium damselae* subsp. *piscicida* due to a naturally occurring disease outbreak. A two-way ANOVA showed that only MOS supplementation reduced fish mortality by ca. 13% (P = 0.050). Additionally, light microscopy examination of both the anterior and posterior intestinal regions revealed a significant increase of the mucosal folding (+29% vs the control; P = 0.016) in the anterior intestine (+33% vs the control; P = 0.002) in the posterior intestine in fish fed MOS supplemented diets. Scanning electron microscopy demonstrated that both MOS supplementation (+13%; P = 0.028) and vaccination (+30%; P = 0.001) could significantly increase the microvilli density of microvilli structures in the anterior intestinal region. In the posterior intestinal region neither MOS supplementation nor vaccination could significantly affect the microvilli density (P = 0.050). The present study suggests that MOS can protect the intestinal morphology of infected sole and promote recovery from disease.

2.3.2 Introduction

Improving and protecting fish health from production associated stressors is a major factor in applied aquaculture practices. Vaccination is a stressor that is routinely applied in aquaculture for the protection of the fish stock (Horne and Ellis, 1988). Furthermore, many diseases caused by bacteria such as *Yersirinia ruckeri* and *Vibrio alginolyticus* have been associated with abnormal intestinal condition and function (Austin and Austin, 1993; Diggles et al., 2000). As previously discussed in the introduction MOS binds to certain bacterial species (especially Gram negative bacteria) including several strains of *E. coli* and *Salmonella* spp. and prevents them from attaching to and colonizing the digestive tract (Spring et al., 2000; Hooge, 2004). Additionally, it has been stated that it can modulate the intestinal and systemic immune systems, by acting as a non-pathogenic microbial type antigen with an adjuvant like effect (Fischer et al., 2001).

The aim of the investigation was to evaluate the prophylactic effect of MOS in the intestinal morphology and fish mortality against the effect of a common Gram negative bacterial disease such as Pasteurellosis, which is caused by *Photobacterium damselae* subsp. *piscicida* (formerly known as *Pasteurella piscicida*).

2.3.3 Methodology

The experiment was conducted in Spain under commercial conditions. Round plastic tanks with 3 m diameter and 2 m^3 water containing 850 fish (~55g) each were used. Fish were subjected to one of four different treatments, in triplicate, for a period of 10 weeks. The first treatment (control) consisted of

feeding with a standard commercial diet (control diet; 2 mm, "LE Europa 18%" from Skretting). The second treatment (vaccinated) consisted of vaccinated fish fed with the control diet. The third treatment (MOS-vaccinated) consisted of vaccinated fish fed the control diet supplemented with MOS (at 0.4%). The fourth treatment (MOS) consisted of unvaccinated fish which fed the diet supplemented with MOS (0.4%). MOS was provided by Alltech Inc., USA, and the level of supplementation was based on the previous experiments in sea bream (Chapters 2.1 and 2.2). The fish in all treatments were fed the same basal (control) diet which includes fishmeal, soy bean meal, corn gluten meal, wheat, fish oil, vitamins and minerals premix (Table 2.11). The experimental vaccine used was manufactured by HIPRA (AMER, Spain) and protects against *Pasteurella spp.* and *Vibrio spp.* The dose was injected (endoperitoneal) according to manufacturer instructions. Since the start of the trial, as the water temperature elevated, pasteurellosis, caused by *Photobacterium damselae* subsp. *Piscicida,* was confirmed and verified in a representative number of fish and water samples across the trial facilities by the veterinary laboratory of Skretting Spain (Trouw España, Cojobar, 09620 Burgos). It was verified that the disease outbreak was caused by bad water quality with temperature range of 19 – 21 ^{0}C.

Table 2.11 Proximate compositional analysis and vitamin profile of the basal (control) diet.

Basal Diet	
Protein	57.0%
Fat	18.0%
Ash	11.5%
Energy	19.9 MJ kg^{-1}
Vitamin A	5,000 U.I. Kg^{-1}
Vitamin D_3	750 U.I. Kg^{-1}
Vitamin E*	250 mg Kg^{-1}

*Vitamin E was supplied as alpha-tocoferol acetate

During the trial mortalities were recorded daily and all fish weighted weekly. Feeding rate was reduced from 1.5% of body weight at the start of the trial to 0.8% when fish lost their appetite due to disease. Mean water temperature was 18 °C at the start of the trial the and gradually increased to 26 °C at the end of the trial. Water supply was the same for all tanks and water was renewed hourly; the photoperiod was natural throughout the investigation period (approximately 14 hr light: 10 hr dark at the start and 15 hr light: 9 hr dark at the end of the trial).

2.3.3.1 Histological assessment

At the end of the trial period, intestinal samples (4 fish per experimental tank, 12 fish per treatment) were retained for histological examination by light and scanning electron microscopy. Anterior and posterior intestinal sections were examined. Anterior intestinal samples were excised approximately 1 cm after the stomach and the posterior samples were excised approximately 1 cm before the anus. Material for light microscopy was fixed in 4% saline formalin, embedded in

paraffin wax and cut into 8 μm thickness sections. The sections were stained using Mallory's Trichrome staining technique as described by Handy et al. (1999) and screened under low power magnification microscope (Olympus SZH-ILLD) with an attached digital camera (Nikon CoolPix 990). Briefly the Mallory's Trichrome staining technique include the following steps:

- take sections to water, haematoxylin for 30 min,
- wash in tap water, flush sections (1 – 2 sec) with blue $LiCO_3$,
- rinse with distilled water,
- flush in 1% acidic alcohol (this helps to differentiate the structures inside the section),
- wash with distilled water,
- flush again with blue $LiCO_3$,
- wash with distilled water,
- acid fuchsin for 8 – 10 min,
- wash with distilled water,
- phosphomolybdic acid for 90 seconds,
- wash with distilled water, Mallory's stain for 5 sec,
- wash with distilled water and examine under a microscope in order to ascertain whether the connective tissue has blue colour. If not, this step must be repeated,
- dehydrate with graded ethanol and mount with cover slip and DPX

Samples for the SEM were rinsed in 1% S-carboxymethyl-L-cysteine for 30 s in order to remove epithelial mucus and then fixed in 2.5% glutaraldehyde with 0.1 M cacodylate buffer and 2.5% NaCl, and adjusted to pH 7.2 (as described previously 2.1.3.4). The samples were treated using an Emitech K850 critical

point drier, with ethanol as the intermediate fluid and CO_2 as the transition fluid. All samples were covered with gold in Emitech K550 sputter coater and were observed with a Jeol JSM 5600 LV electron microscope at 15 kV.

All digital images were analyzed using Image J version 1.36 (National Institutes of Health, USA). Images from light microscopy were analyzed to determine the perimeter ratio (PR) between the internal perimeter (*IP*) of the gut lumen (villi and mucosal folding length) and the external perimeter (*EP*) of the gut (PR = *IP*/*EP*). A high PR value indicates high villi length and and/or increase mucosal folding. High magnification (x 14,000) SEM images were analyzed in order to measure the density of the microvilli (arbitrary units).

2.3.3.2 Statistical analysis

Data analysis was carried out using a two-way ANOVA (SPSS 15.0, Chicago, IL, USA). Significance was accepted at $P < 0.05$ level. Results are presented as mean ± standard (M ± SD) deviations unless otherwise is stated.

2.3.4 Results

2.3.4.1 Mortality

Statistical analysis showed that there was a significant effect ($P = 0.050$) of MOS supplementation on total mortality which tended to be lower (by ca. 13%) in MOS treated fish compared to the control. Vaccination effect and interaction effect between MOS supplementation and vaccination had no significant ($P = 0.120$ and $P = 0.494$, respectively) effects on fish mortalities. MOS treated fish without vaccination (MOS group) had the lowest mortality at 47.74%, followed

by MOS-vaccinated group of fish with 53.37%, 56.02% in control group of fish and 61.71% in vaccinated group of fish (Table 2.12).

Table 2.12 Stocking numbers and cumulative mortality for each group of sole (mean ± SD).

Treatment	Control	Vaccinated	MOS-Vaccinated	MOS
Initial stocking	2581	2488	2625	2482
Final stocking	1137	956	1228	1296
Total mortalities	56.02 ± 0.82% [a]	61.71 ± 4.75% [a]	53.37 ± 3.31% [b]	47.74 ± 7.29% [b]

[a, b]: Values within the same column with different superscripts are significantly different ($P < 0.05$, n = 12).

2.3.4.2 Histological assessment

In the anterior and the posterior intestinal region only fish fed diets with MOS supplementation alone or in combination with vaccination (groups MOS and MOS-vaccinated) displayed a significant increase of PR ($P = 0.016$ and $P = 0.002$ respectively; Table 2.13, Figures 2.12 and 2.13) i.e. longer mucosal foldings. The effect of vaccination and interaction effect between MOS supplementation and vaccination on PR was not significant for the anterior intestine ($P = 0.493$ and $P = 0.242$ respectively) and the posterior intestine ($P = 0.095$ and $P = 0.101$ respectively). In the anterior intestinal region PR increased from 3.44 ± 1.01 AU in control group to 4.16 ± 0.68 AU in vaccinated group, 4.80 ± 0.25 AU in MOS-vaccinated group and 5.00 ± 0.17 AU in MOS group.

Similarly, on the posterior intestinal region PR increased from 3.16 ± 0.42 AU in control group to 4.14 ± 0.62 AU in vaccinated group, 4.87 ± 0.30 AU in MOS-vaccinated group and 4.86 ± 0.42 AU in MOS group. Additionally, MOS treated fish displayed an increased number of vacuoles (Figure 2.13) in the posterior intestinal region.

SEM analysis showed that in the anterior region of the intestine MOS supplementation and vaccination had significant effects on microvilli density (P = 0.028 and P = 0.001 respectively). There was no significant interaction effect between the MOS supplementation and the vaccination on the anterior intestinal region (P = 0.960). Microvilli density increased from 2.11 ± 0.60 AU in control group (Figure 2.14) to 2.71 ± 0.57 AU in MOS group 4, 3.03 ± 0.69 AU in vaccinated group and 3.66 ± 1.11 AU in MOS-vaccinated group of fish. In the posterior intestinal region neither MOS supplementation nor vaccination significantly (P = 0.188 and P = 0.371 respectively) improved the condition of the microvilli under these conditions.

Table 2.13 Gut lumen perimeter ratio (PR) and microvilli density (MD) (mean ± SD) of sole fed the experimental diets with or without the vaccination.

	PR*		MD*	
Treatment	**Anterior**	**Posterior**	**Anterior**	**Posterior**
Control	3.44 ± 1.01[a]	3.16 ± 0.42[a]	2.11 ± 0.60[a]	3.21 ± 0.49
Vaccinated	4.16 ± 0.68[a]	4.14 ± 0.62[a]	3.03 ± 0.69[b]	3.19 ± 0.73
MOS-Vaccinated	4.80 ± 0.25[b]	4.87 ± 0.30[b]	3.66 ± 1.11[b]	3.12 ± 0.56
MOS	5.00 ± 0.17[b]	4.86 ± 0.42[b]	2.71 ± 0.57[b]	2.72 ± 0.50

* Arbitrary units

[a, b]: Values within the same column with different superscripts are significantly different ($P < 0.05$, n = 12).

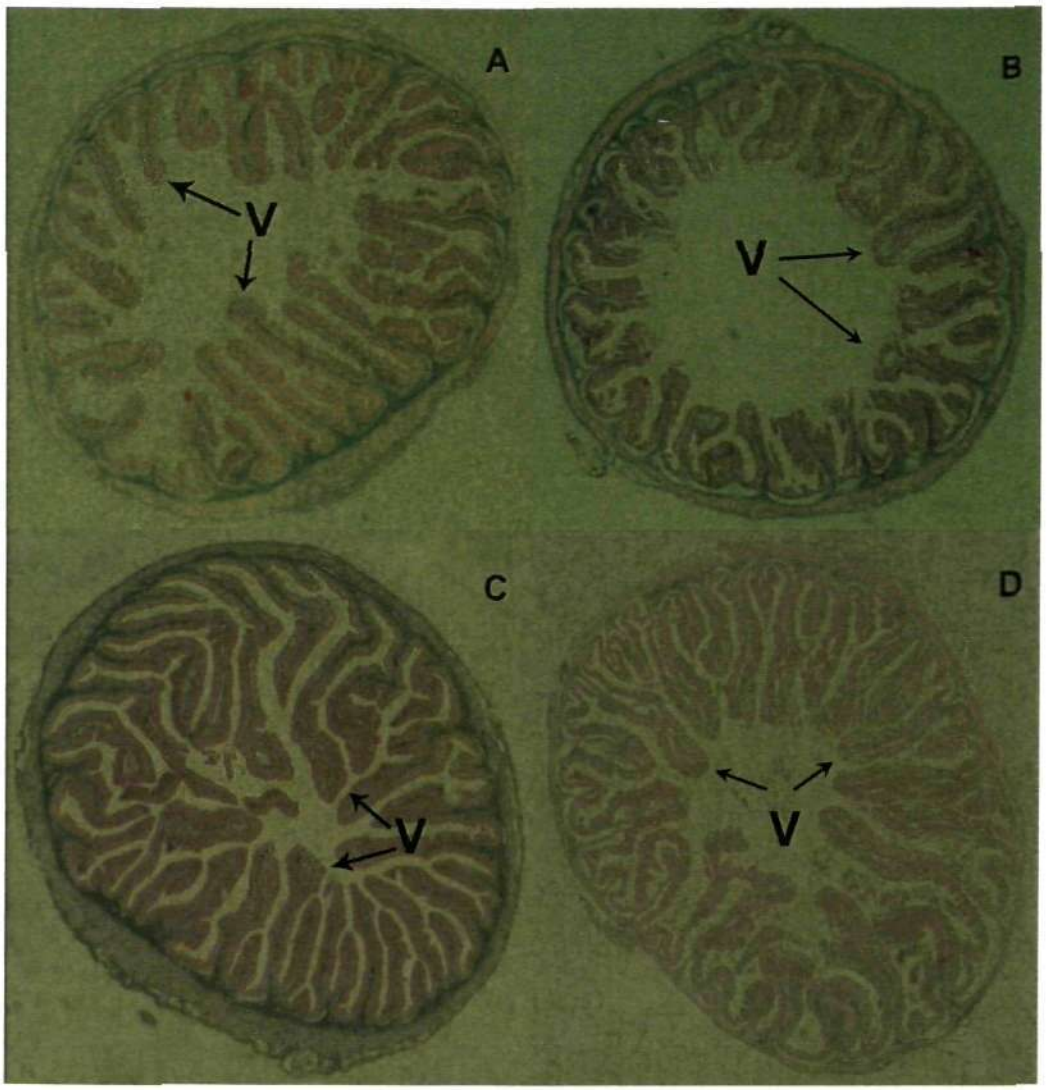

Figure 2.12 Comparative light microscopy sections of the anterior part of the intestine. A: fish from control group; B: vaccinated fish; C: MOS treated vaccinated fish and D: MOS treated fish. Arrows indicate an apparent improved condition of the villi (V) (longer villi structure) compared with the control group.

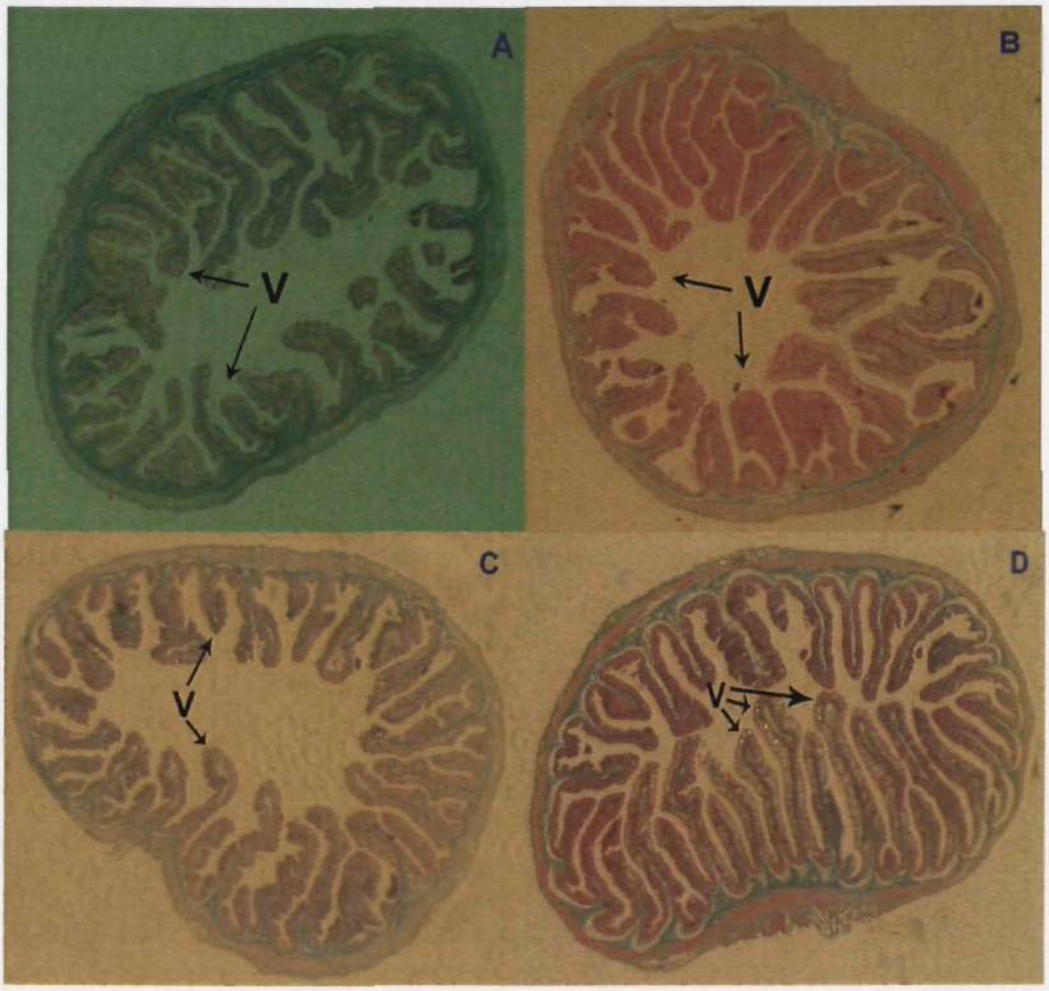

Figure 2.13 Comparative light microscopy sections of the posterior intestinal region. A: fish from control group; B: vaccinated fish; C: MOS treated vaccinated fish and D: MOS treated fish. The arrow indicates the improved villi (V) structure and the increased number of vacuoles (some of them have been painted as yellow dots in figure D close to the tips of the arrows).

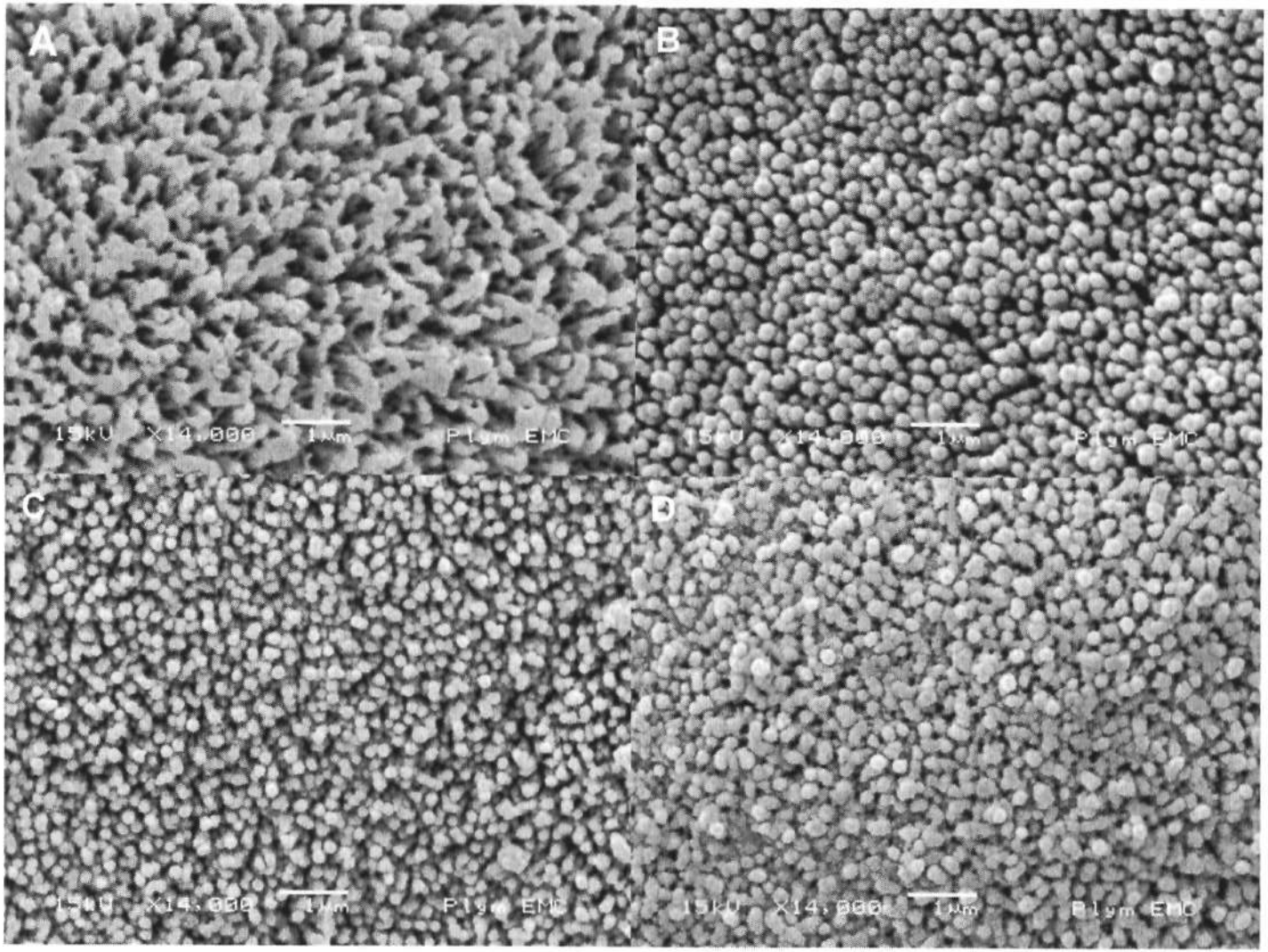

Figure 2.14 Comparative scanning electron micrograph of the anterior intestinal region, A: fish from control group; B: vaccinated fish; C: MOS treated vaccinated fish and D: MOS treated.

2.3.5 Discussion

Endoperitoneal vaccination is a stressful procedure and it is suggested that it should be avoided in cases of diseased fish (Horne and Ellis, 1988; Midtlyng et al., 1996). This is especially true with juvenile flatfish, such as sole, as it is an exceptionally difficult husbandry procedure due to the fish morphology. This stress is likely partially responsible of the increased mortalities observed in the vaccinated groups and especially in the group without MOS supplementation. Indeed, in the present study, it is appears that MOS supplementation reduced the positive effect that vaccination had on the mortality, which agrees with previous studies by Culjak et al. (2006), Bogut et al. (2006), Genc et al. (2007a) and Staykov et al. (2007) where MOS enhanced survival of common carp, European catfish. shrimp and trout respectively.

During the present study fish were infected naturally by pasteurellosis which is caused by Gram negative bacteria and according to Austin and Austin (1993) is related with the gastrointestinal tract. Fish internal organs such as liver, kidney and spleen in sole (*Solea senegallensis*), sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) have previously been examined histologically for the effect of pasteurellosis (Toranzo et al., 1991; Zorrilla et al., 1999) but there is a lack of information with regards of the effects on the gastrointestinal tract. Bacterial diseases often affect the morphology of the gut by altering its physiological condition, i.e. causing necrosis and sloughing of the intestinal mucosa (Diggles et al., 2000). However, during the healing process, the gut usually returns to its normal condition and structure.

A recent study by Naka et al. (2005) showed that *Photobacterium damselae* subsp. *piscicida* has strong adherence to the fish intestine, possibly due to the mannose sensitive hemaglutinin (*msh*A) on the bacterial cell. It is believed that MOS is involved in this antigen recognition and binding process so the bacteria become MOS bound (Engering et al., 1997). These studies, as well as the findings of Torrecillas et al. (2007) suggest that MOS can work as a non-pathogenic microbial antigen and stimulate the immune system through the b-glucans and mannose binding lectins (MBL). This may also partially explain the reduction in mortality observed in MOS treated fish.

The observed histological changes from SEM micrographs of the anterior intestine suggest that MOS can work therapeutically to positively affect the recovery process. The exact mechanism for the improved intestinal recovery is not known yet but a suggestion could be due to MOS interaction with the intestinal microbial load as seen in the previous chapters (both sea bream experiments). It should be noted that microvilli are the smallest formation on lumen exposed surface of enterocytes and are responsible for the capture and absorption of nutrient molecules (McLean et al. 1999; Salze et al. 2008). Additionally, the increase of the supranuclear vacuoles can be positively explained as these vacuoles are related with the absorption of lipids, proteins and polypeptides in the gut, leading to a superior nutrient utilization (McLean et al. 1999).

Overall, evidence from this study indicates that MOS affects both gut regions of sole under these conditions, where fish were diseased and stressed. It seems that the anterior part of the intestine is more responsive to MOS supplementation than the posterior region with respect to morphological features. Furthermore, MOS appears to confer a positive modulatory effect on stressed and

diseased sole and either may possesses recovery properties (i.e. improving the intestinal morphology) or reduces damages on enterocytes against Gram negative bacterial infections. This needs to be further investigated for other species of aquaculture importance as the cost of losses from bacterial diseases in aquaculture industry is great.

Chapter 3 Effect of MOS supplementation on Salmonids

Chapter 3 describes two experiments comprise where the effect of MOS was investigated on the major representatives of the Salmonids, trout and salmon. Both of these species are omnivorous and are major contributors to aquaculture output both within the EU and globally. The first experiment investigated the effect of 0.2% dietary MOS supplementation on juvenile and sub-adult rainbow trout at a fresh water fish farm. In the second experiment, using Atlantic salmon in marine experimental facilities, the MOS supplementation level was double to 0.4%.

3.1 Dietary MOS supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* W.

3.1.1 Abstract

A study was conducted to investigate the effect of MOS on the gut microbiota and intestinal morphology of rainbow trout under commercial farming conditions. Juvenile (mean initial weight 38.2 ± 1.7 g) and sub-adult (111.7 ± 11.6 g) trout were subjected to two dietary treatments for 111 and 58 days, respectively. The control treatment consisted of a standard commercial diet and the MOS treatment consisted of the control diet supplemented with 0.2% MOS. The morphology of the anterior and the posterior intestine was examined using both light microscopy (LM) and electron microscopy (EM). LM demonstrated increased gut absorptive surface area in the sub-adult MOS group. Additionally, EM revealed a significant increase in microvilli length and density in the sub-adult MOS group compared to the control ($P < 0.05$). However, no significant histological improvements were found in the juvenile group. Culture-based evaluation of the intestinal microbiota showed that the MOS diet significantly reduced the viable gastrointestinal bacterial populations (by approximately 2 log scales in all cases). Levels of *Aeromonas/Vibrio* spp. were significantly lower in the juvenile MOS group (9% of the total microbiota) than the juvenile control group (37%). Additionally, analysis of microbial communities was conducted using denaturing gradient gel electrophoresis (DGGE) of PCR amplified 16S

rDNA. DGGE fingerprinting revealed an alteration of bacterial populations; ANOSIM, SIMPER and nMDS analysis showed that MOS reduced species richness and increased similarity of bacterial populations found within both the sub-adult and juvenile groups. The current study shows that 0.2% MOS supplementation modulates intestinal microbial communities in both examined fish groups and improved intestinal morphology and epithelial brush border condition in the sub-adult trout.

3.1.2 Introduction

The documented benefits of MOS in commercial production of terrestrial animals have led to initiatives to evaluate the potential of MOS with regards to finfish aquaculture. The use of functional feed additives, such as MOS to improve growth and health performance in the aquaculture industry is becoming increasingly important as today's consumers demand eco-friendly production practices. There is a clear lack of information regarding the effect of MOS on the economically important species, particularly rainbow trout (*Oncorhynchus mykiss*). The aim of the present study was to evaluate the prebiotic effects of MOS on gut structure, morphology and the intestinal microbial ecology of rainbow trout under commercial rearing conditions.

3.1.3 Methodology

3.1.3.1 Fish

The present study was conducted under commercial rearing conditions at Test Valley Trout farm, Romsey, UK. During the experimental period two separate batches of fish were monitored; batch 1, initial mean weight of 111.7 ± 11.6 g (referred to as sub-adult group hereafter) and batch 2, mean weight of 38.2 ± 1.7 g (referred to as juvenile group hereafter). Both sub-adult and juvenile trout were subjected to two dietary treatments: (i) control group, fed a standard Skretting commercial feed and (ii) MOS group, fed a standard Skretting feed supplemented with MOS (Bio-MOS, Alltech USA). A supplementation level of 0.2% was chosen based on previous studies (Dimitroglou, 2004; Staykov et al., 2007; Salze et al., 2008) and previous experiments with gilthead sea bream (Chapter 2). Excel 30 diet (48% protein/19% lipid) was used as the basal diet (with or without MOS addition in the relevant groups) until all fish reached approx. 100 g and crystal 45 + astaxanthin (45% protein/26% lipid) was used thereafter (Skretting, UK). Approximately 20,000 fish were distributed into 4 outdoor earth ponds. Sub-adult groups were fed experimental diets for 58 days (until approx. market weight) and the juvenile groups fed for 111 days. Trout were fed approximately 1.5% of body weight day^{-1}. The farm ponds were fed by a local river and so temperature (11-16 °C) and pH (~7 – 8) altered naturally with seasonality.

3.1.3.2 Sampling

At the end of the experiment 12 fish from each experimental group were euthanized by a hard blow to the head followed by destruction of the brain. Mean final body weight of fish sampled were 232.2 ± 18.5 g for sub-adult control group, 267.9 ± 30.9 g for sub-adult MOS group, 132.8 ± 23.4 g for the juvenile control group and 153.5 ± 32.1 g for juvenile MOS group.

3.1.3.3 Histological examination

Intestinal samples from 6 fish per experimental group were retained for histological examination by LM, SEM and TEM. Sections from the intestine distal to the pyloric cecae (anterior region) and the intestinal section prior to the anus (posterior region) were taken. Samples were analyzed according to the previously explained methodology (Chapter 2.1.3.4). Briefly, samples for light microscopy were fixed in 4% saline formalin. The tissue samples were dehydrated in graded ethanol before equilibration in xylene and embedding in paraffin wax. 8 µm transverse sections were cut and stained using alcian blue periodic acid-Schiff staining technique (AB-PAS; Kiernan, 1981). Samples for scanning and transmission electron microscopy (SEM & TEM) were fixed in 2.5% glutaraldehyde with 0.1 M cacodylate acid sodium salt solution (1:1 vol.), pH 7.2. Prior to fixation, SEM samples were rinsed in 1% S-carboxymethyl-L-cysteine (Sigma) for 30 sec in order to remove epithelial mucus. SEM samples were dried using a K850 critical point drier (Emithech; Kent, UK) with ethanol as the intermediate fluid and CO_2 as the transition fluid. All samples were coated with gold using K550 sputter coater (Emithech; Kent, UK) and screened with a Jeol JSM 5600 LV electron microscope at 15 kV (Jeol; Tokyo, Japan). Samples for

TEM were post-fixed in OsO_4 for 1 h and embedded with the standard resin procedure. Resin blocks were sectioned using a diamond knife (~90 nm). Ultrathin sections from each sample were placed in copper grids and stained with uranyl acetate, post stained with lead citrate and screened with a Jeol JSM 1200 transmission electron microscope at 120kV (Jeol; Tokyo, Japan).

All digital images were analyzed using Image J version 1.36 (National Institutes of Health, USA). Images from light microscopy were analyzed to determine the perimeter ratio (PR) between the internal perimeter (*IP*) of the gut lumen (villi and mucosal folding length) and the external perimeter (*EP*) of the gut (PR = *IP*/*EP*). A high PR value indicates high villi length and and/or increase mucosal folding. High magnification (x 20,000) SEM images were analyzed in order to measure the density of the microvilli (arbitrary units). TEM images (magnification x 20,000) were analyzed to measure the microvilli length.

3.1.3.4 Culture based microbial analysis

Time between termination and dissection did not exceed 2.5 h. After aseptic dissection, the entire digestive tract was removed and the digesta form 6 fish per treatment were pooled into 2 samples. Water samples from the main supply of the farm were also taken. All samples were processed according to the methodology described in Chapter 2.2.3.2. Briefly, samples were serially diluted to 10^{-7} with phosphate buffered saline (PBS: Oxoid) and 100 µL was spread onto duplicate tryptone soy agar plates (TSA; Oxoid). Colony forming units (CFU) g^{-1} for aerobic heterotrophic populations were then calculated after 7 days aerobic incubation at 20 °C. 25 random colonies from plates containing 30 – 300 CFU were taken and sub-cultured on TSA until pure cultures were achieved. A total of

450 isolates were then tentatively placed into groups or genera, as described by Cowan and Steel (1993) and Holt and Bergey (1994), based on the colony morphology, cell morphology, Gram stain, production of catalase, oxidase, glucose fermentation, motility and endospore formation.

3.1.3.5 Bacterial DNA extraction and 16S rRNA amplification and denaturing gradient gel electrophoresis (DGGE)

DNA was extracted from 3 intestinal samples (pooled from 9 of the 12 fish sampled) per treatment using QIAamp® Stool Mini Kit (Qiagen) with a lysozyme pre-treatment (50 mg mL^{-1} TE buffer for 30 min at 37 °C) and PCR amplification of the 16S rRNA genes was conducted as previously explained in Chapter 2.1.3.5). PCR amplification of the 16S rRNA genes was undertaken using the forward primer P3 with a GC clamp and the reverse primer P2 after Muyzer et al. (1993). The following reagents were included in each PCR tube: 1 µL of both primer P2 and P3 (50 pmol μL^{-1}; MWG-Biotech AG, Germany), 3µL DNA template, 25µL ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ (Sigma) and PCR grade water to a final volume of 50µL. Giving a final concentration of 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 0.2 mM dNTPs. The touchdown thermal cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min. The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at 55 °C for the remaining cycles. The PCR products were stored at 4 °C until use.

The DGGE was performed using a DGGE-2001 system (C.B.S. scientific, CA, USA). Briefly: 10 µL of standardized PCR products were run on 8% acrylamide gels with a denaturing gradient of 40-60% (where 100% denaturant is 7 M urea and 40% formamide). All samples were run on the same gel to prevent issues of reproducibility and outside lanes were not used. The gel was run at 65 V for 17 h at 60 °C in 1 x TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA). Visualization of the DGGE bands was achieved by the optimized silver staining method of Benbouza et al. (2006). The Gel was scanned in a Bio-Rad universal hood II (Bio-Rad laboratories, Italy) and optimized for analyses by enhancing contrast and grayscale.

3.1.3.6 Statistical analysis

An independent samples two-tailed T-test was applied in order to evaluate the effect of MOS on intestinal histology and culture based microbiota on each of the two batches of fish (juvenile and sub-adult trout). Analyses were carried out on SPSS 15.0 (SPSS Inc., IL, USA) and significance was accepted at the $P < 0.05$ level. The results are presented as mean values followed by the standard deviation (M ± SD). DGGE banding patterns were transformed into presence/absence matrices for similarity assessment between treatments using Quantity one® version 4.6.3 analyses software (Bio-Rad laboratories, CA, USA) after Schauer et al. (2000). Band intensities were measured and analyzed using Primer v6 (Clarke and Gorley, 2006). Non-metric multidimensional scaling analysis (nMDS) was used to represent the relative similarities between treatments (Powell et al., 2003). Stress level was low (0.04) and cluster analysis was used to check the groupings by the nMDS procedure. A one-way analysis of similarity (ANOSIM) was used to

compare groups and similarity percentages (SIMPER) for banding contributions (Abell and Bowman, 2005).

3.1.4 Results

3.1.4.1 Histological examination

The results of the histological examination of the absorptive surface area and microvilli morphology are presented in Table 3.1. The LM assessment of the gastrointestinal tract from trout demonstrated that MOS had a beneficial effect on the gut absorptive area in the sub-adult fish groups. The anterior region of the sub-adult MOS group (PR = 4.50 ± 0.30) displayed a significantly higher absorptive surface area (P = 0.032) than the control group (PR = 3.46 ± 0.48) (Figure 3.1). The absorptive surface area of the posterior intestine of the sub-adult MOS group (PR = 5.29 ± 0.39 AU) was also significantly higher (P = 0.041) than the control group (PR = 3.03 ± 0.19 AU). However, there were no significant differences in PR between the juvenile fish groups. The analysis of SEM images showed that the microvilli density of the posterior intestinal region in the sub-adult MOS treated fish (MD = 6.54 ± 1.08 AU) was significantly increased (P = 0.015) compared to the control treated fish (MD = 2.53 ± 0.75 AU). Comparative examples are presented in Figure 3.2. However, there were no other significant differences in microvilli density in the anterior region of the sub-adult group or in the anterior and posterior intestinal region of the juvenile group. TEM revealed that MOS significantly increased the microvilli length in sub-adult fish for both anterior and posterior intestinal regions. Microvilli length from the anterior region increased from 1.33 ± 0.72 μm in the control fish to 1.90 ± 0.15 μm in the MOS fish (P =

0.002). Similarly, microvilli length in the posterior intestine increased from 1.22 ± 0.01 μm in the control group to 1.53 ± 0.11 μm in the MOS fed fish ($P = 0.007$). Comparative examples are shown in Figures 3.3 and 3.4. However, no significant differences were found in the juvenile fish groups in any of the parameters investigated.

Table 3.1 Anterior and posterior intestinal morphology of sub-adult and juvenile rainbow trout fed either a control diet or MOS supplemented diet.

Parameter	Intestinal region	Juvenile Trout		Sub-adult Trout	
		Control	MOS	Control	MOS
Intestinal absorptive surface (PR)	Anterior	3.68 ± 0.22	3.75 ± 0.21	3.46 ± 0.48[a]	4.50 ± 0.30[b]
	Posterior	4.25 ± 0.65	3.00 ± 1.09	3.03 ± 0.19[a]	5.29 ± 0.39[b]
Microvilli density*	Anterior	4.54 ± 1.43	4.74 ± 0.86	3.51 ± 0.23	3.13 ± 0.52
	Posterior	2.45 ± 0.12	4.53 ± 1.15	2.53 ± 0.75[a]	6.54 ± 1.08[b]
Microvilli length (μm)	Anterior	1.29 ± 0.17	1.65 ± 0.21	1.33 ± 0.72[a]	1.90 ± 0.15[b]
	Posterior	1.21 ± 0.37	1.37 ± 0.05	1.22 ± 0.01[a]	1.53 ± 0.11[b]

*: Arbitrary units AU

[a, b]: Values within the same age group, in the same row, with different superscripts are significantly different ($P < 0.05$).

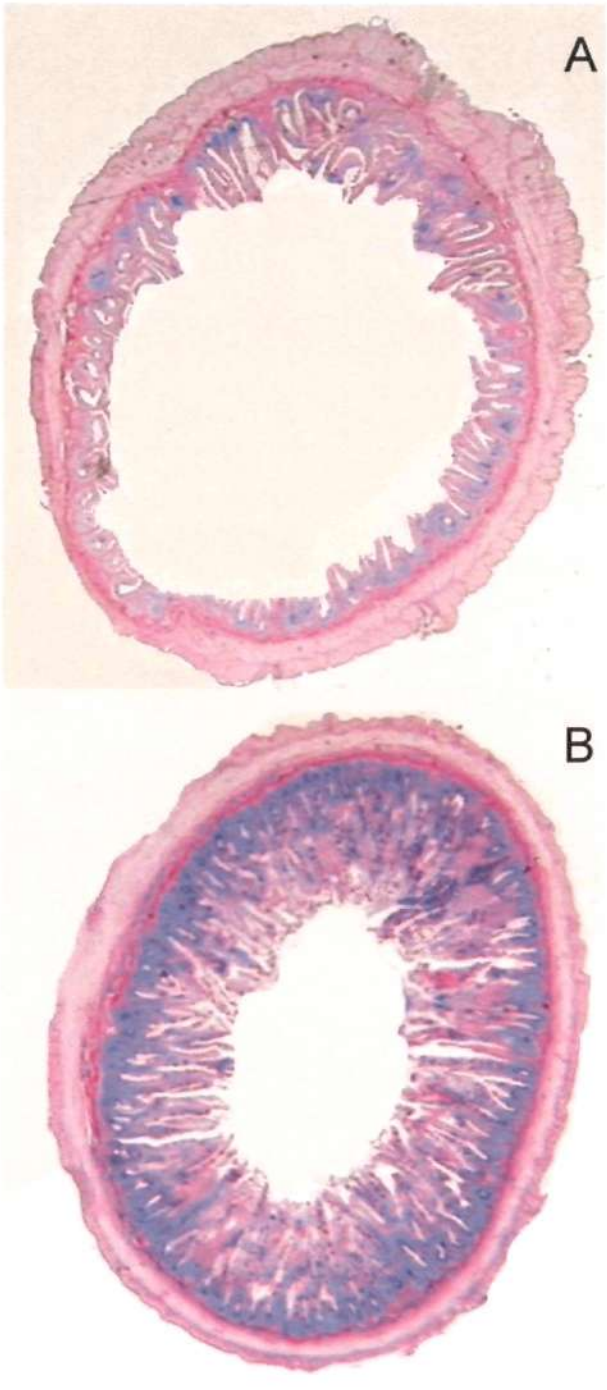

Figure 3.1 Comparative LM images of anterior region of sub- adult rainbow trout fed either control diet (A) or MOS supplemented diet (B). Villi structures appear much longer in the MOS fed fish ($P = 0.032$).

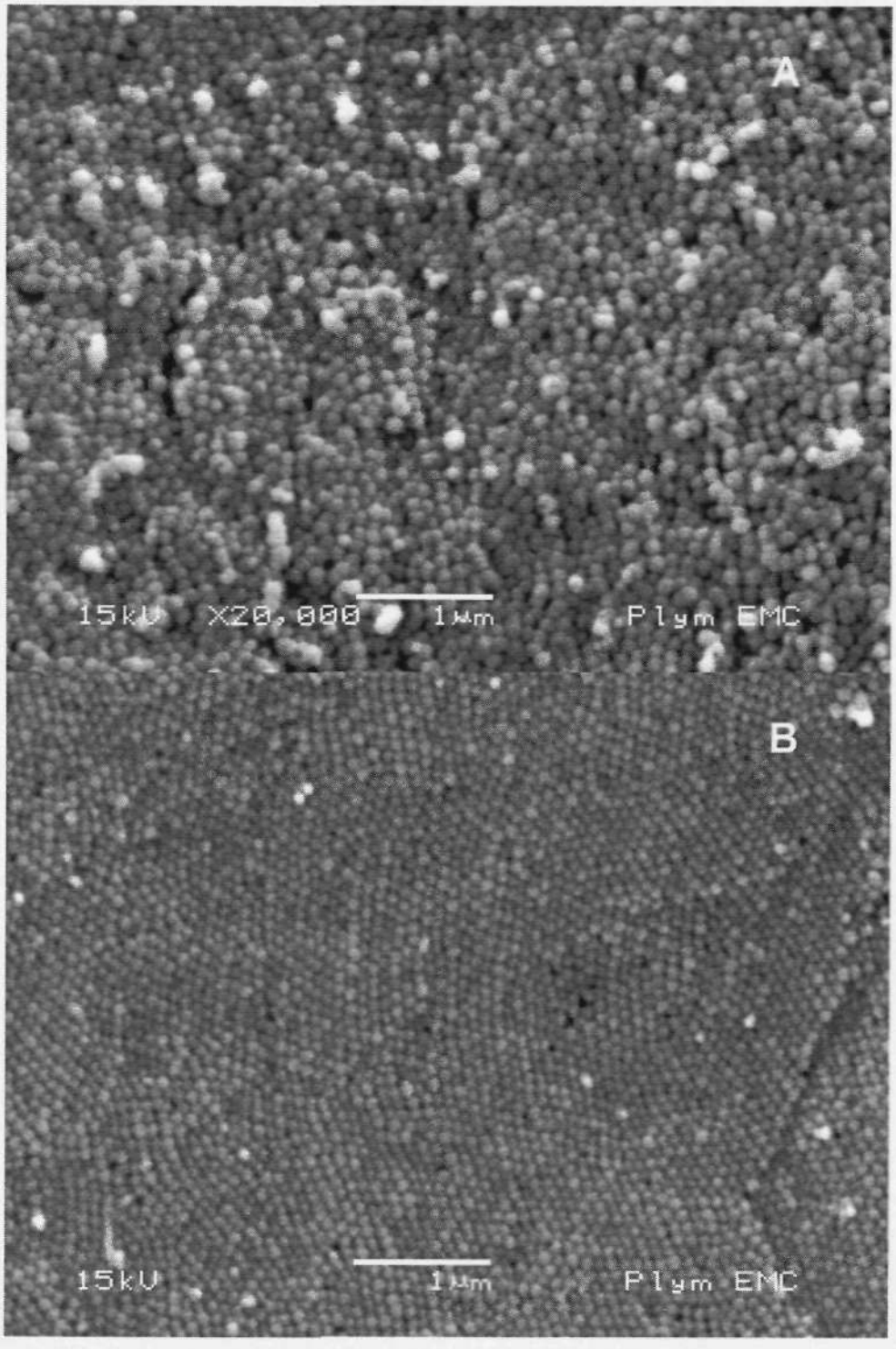

Figure 3.2 Comparative SEM micrographs of posterior region of sub-adult rainbow trout fed either the control (A) or the MOS (B) supplemented diet. Although, microvilli appear healthy in both treatments, microvilli are more regular and density is significantly higher in MOS treated fish ($P = 0.015$).

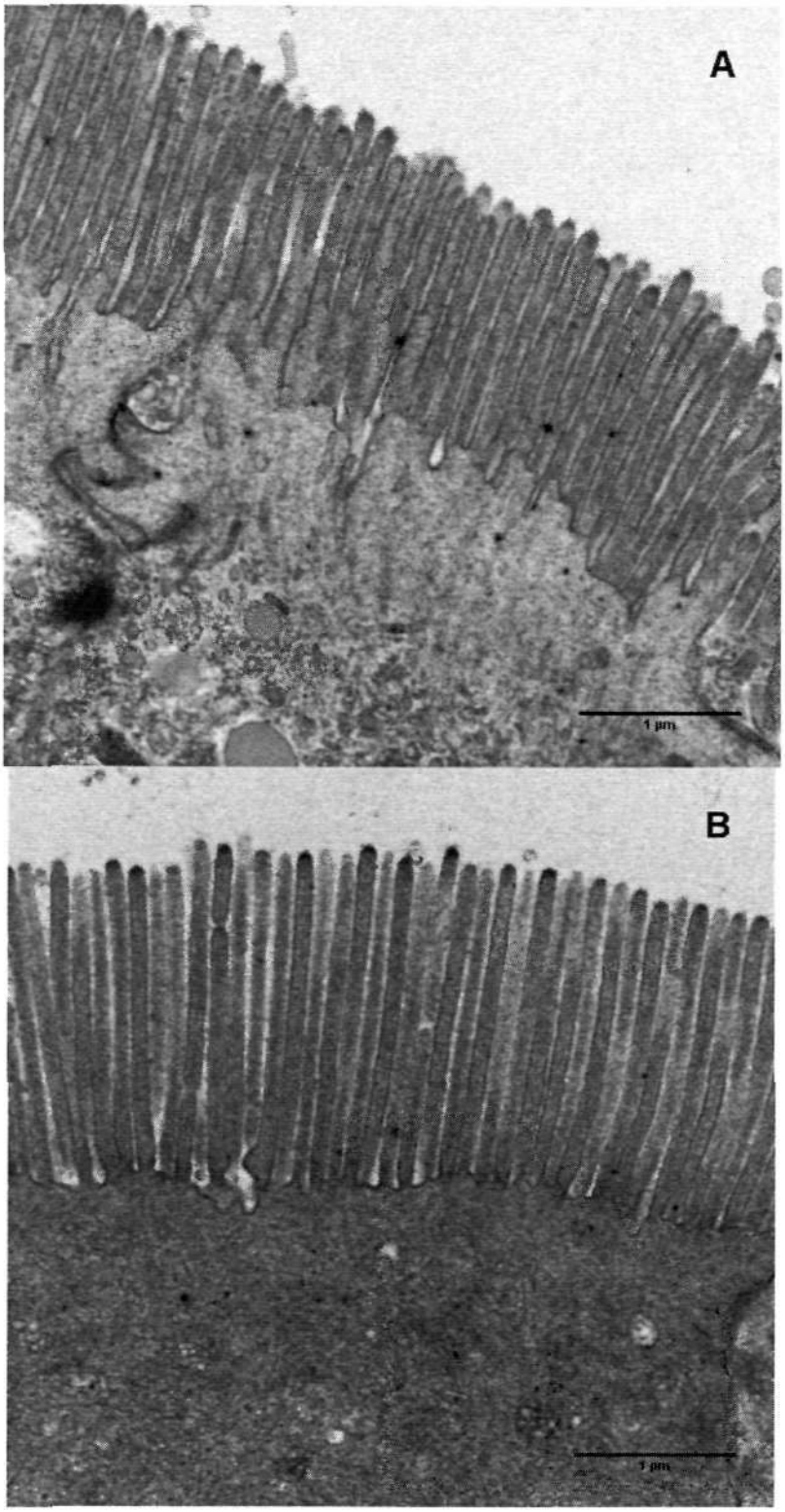

Figure 3.3 Comparative TEM micrographs of anterior region of sub-adult rainbow trout fed either the control (A) or the MOS (B) supplemented diet. Although, microvilli appear healthy in both treatments, microvilli are more regular and significantly longer in MOS treated fish ($P = 0.002$).

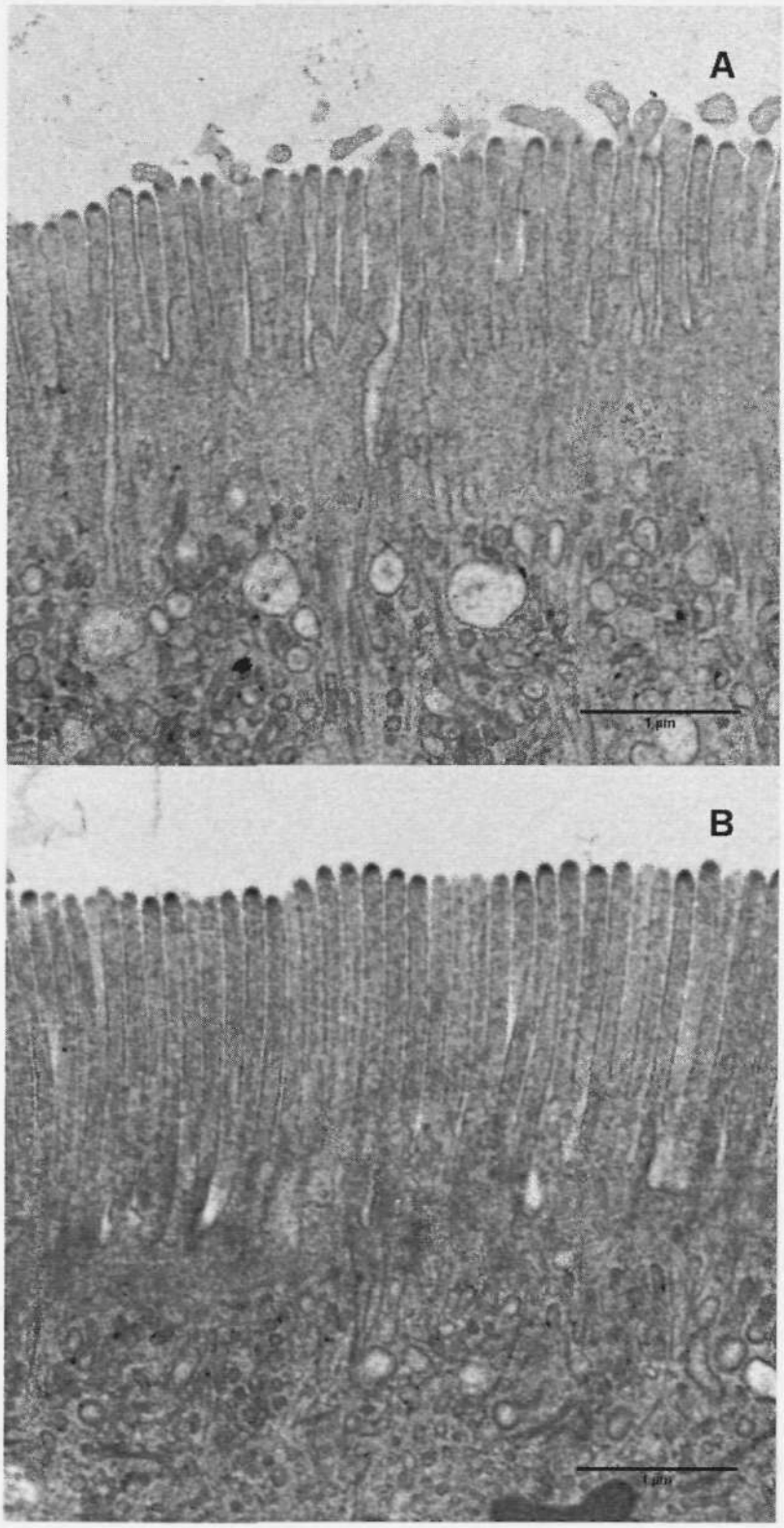

Figure 3.4 Comparative TEM micrographs of posterior region of sub-adult rainbow trout fed either the control (A) or the MOS (B) supplemented diet. Although, microvilli appear healthy in both treatments, microvilli are more regular and significantly longer in MOS treated fish ($P = 0.007$).

3.1.4.2 Culture based analysis

Table 3.2 presents the aerobic heterotrophic gut microbial composition of each group and the rearing water. Viable counts in the rearing water were determined to be 10.8 x 10^3 CFU mL^{-1}. Dominant groups in the rearing water were identified as *Aeromonas/Vibrio* spp., *Pseudomonas* spp. and members of the Enterobacteriaceae, which accounted for approximately 80% of the total culturable microbial population.

Viable intestinal populations ranged between 10^6 and 10^8 CFU g^{-1}. Viable populations in MOS supplemented groups were significantly lower than the control groups; approximately 2 log scales less (down from 10^8 to 10^6 CFU g^{-1}; $P < 0.05$). Changes in the relative abundance of the microbiota identified were also observed. Compared to the juvenile control, juvenile MOS fed fish displayed a significant reduction of the relative abundance of *Micrococcus* spp. (from 22% to 7%), *Aeromonas/Vibrio* spp. (from 37% to 9%) and unidentified Gram positives (from 25% to 6%). Coinciding with these changes a significant increase in the relative abundance of *Enterococcus* spp. (from 3% to 19%) and Enterobacteriaceae (from 5% to 39%) were found. Compared to the sub-adult control group, sub-adult MOS fed fish also displayed a significant reduction of *Micrococcus* spp. (27% to 6%). A reduction of Enterobacteriaceae (from 22% to 5%) and an increase of *Pseudomonas* spp., from 7% to 26%, were also found.

Table 3.2 Composition of sub-adult and juvenile rainbow trout culturable gut microbiota fed a control or MOS supplemented diet. Expressed as log CFU g^{-1} or mL^{-1} and percentages (calculated from percentage of total viable load).

			Juvenile groups				Sub-adult groups			
	Rearing water		Control trout		MOS trout		Control trout		MOS trout	
	CFU g^{-1}	%	CFU g^{-1}	%	CFU g^{-1}	%	CFU g^{-1}	%	CFU g^{-1}	%
Viable population	10.80×10^3		2.50×10^{8a}		2.35×10^{6b}		5.87×10^{8a}		4.83×10^{6b}	
Micrococcus spp.	-	-	5.49×10^7	22^a	1.65×10^5	7^b	1.58×10^8	27^a	2.9×10^5	6^b
Acinetobacter spp.	1.13×10^2	2	-	-	7.05×10^4	3	5.87×10^6	1	2.42×10^5	5
Enterococcus spp.	-	-	7.48×10^6	3^a	4.47×10^5	19^b	1.06×10^8	18	1.64×10^6	34
Staphylococcus spp.	-	-	-	-	-	-	2.93×10^7	5	-	-
Aeromonads/Vibrio spp.	1.13×10^2	20	9.23×10^7	37^a	2.12×10^5	9^b	9.97×10^7	17	7.73×10^5	16
Pseudomonas spp.	1.81×10^3	32	9.98×10^6	4	2.35×10^5	10	4.11×10^7	7^a	1.26×10^6	26^b
Enterobacteriaceae	1.58×10^3	28	1.25×10^7	5^a	9.17×10^5	39^b	1.29×10^8	22^a	2.42×10^5	5^b
Bacillus spp.	-	-	4.99×10^6	2	-	-	-	-	-	-
Carnobacteria/Lactobacillus spp.	1.13×10^2	2	2.49×10^6	1	-	-	-	-	1.93×10^5	4
Kurthia spp.	3.39×10^2	6	2.49×10^6	1	1.18×10^5	5	-	-	4.83×10^4	1
Unidentified Gram negative spp.	4.52×10^2	8	-	-	-	-	5.87×10^6	1	4.83×10^4	1
Unidentified Gram positive spp.	1.13×10^2	2	6.24×10^7	25^a	1.41×10^5	6^b	1.17×10^7	2	9.67×10^4	2
Yeast	-	-		-	4.7×10^4	2	-	-	-	-

[ab] Values within the same row, in the same age group, with different superscripts are significantly different ($P<0.05$).

3.1.4.3 DGGE analysis

The similarity half matrix of presence/absence banding patterns from the 12 samples run on the DGGE is shown in Table 3.3. Similarity between dietary treatments within the same age groups were high, with 77.22 ± 11.06% similarity between the sub-adult control/MOS groups and 79.22 ± 14.92% similarity between the juvenile control/MOS groups. The Primer analysis is shown in Table 3.4. Species richness was higher in the sub-adult fish than the juvenile fish. MOS supplementation resulted in the removal of certain species; species richness reduced from 11.7 in the juvenile control to 8.7 in the juvenile MOS group and from 14.0 in the sub-adult control to 12.3 in the sub-adult MOS group. The ANOSIM R statistic (values closer to 1 indicate replicates within a group are more similar than those from the groups being compared) and its significance should be used with caution due to the small number of replicates. However, the sub-adult control group appears different to both juvenile groups. The nMDS analysis (Figure 3.5) shows that the juvenile control group is clustered with SIMPER average similarity of 94.5%. The MOS treatment of juvenile group shows a distinct spatial shift (with some overlap remaining with the control) and a SIMPER of 72.2%. The sub-adult control group is again distinct from the juveniles and much less clustered (SIMPER, 72.5%). MOS treatment shows less similarity (SIMPER, 54.7%); however, spatial movement towards the juvenile groups is evident (resulting in moderate spatial overlapping). Average dissimilarity between groups also showed that the microbiota of juvenile groups were the least dissimilar (25.1%). The sub-adult control group was the most dissimilar from both juvenile groups (44.4% and 42.8%, Juvenile MOS and

control, respectively). Dissimilarity was considerably lower between the MOS groups (33.7%).

Table 3.3 Similarity half matrix between presence/absence of DGGE bands of rainbow trout intestinal microbial populations subjected to the experimental diets (values expressed as %).

Fish group			Sub-adult						Juvenile					
	Diet		Control			MOS			Control			MOS		
		Replicate	1	2	3	1	2	3	1	2	3	1	2	3
Sub-adult	Control	1	100.0	77.9	74.0	74.0	80.0	100.0	45.6	66.7	63.6	46.9	53.8	45.6
		2		100.0	80.0	68.7	73.1	71.4	47.4	61.5	57.1	54.5	60.9	66.7
		3			100.0	65.8	72.0	90.0	45.6	66.7	63.6	70.6	77.8	63.6
	MOS	1				100.0	71.4	90.0	63.6	73.3	72.7	70.6	77.8	63.6
		2					100.0	71.4	57.1	69.0	66.7	56.0	61.5	73.3
		3						100.0	63.6	73.3	72.7	82.4	88.9	72.7
Juvenile	Control	1							100.0	95.7	100.0	73.7	70.0	100.0
		2								100.0	95.7	66.7	63.2	95.7
		3									100.0	73.7	70.0	100.0
	MOS	1										100.0	93.3	73.7
		2											100.0	70.0
		3												100.0

Table 3.4 Summary of species diversity, richness and pairwise comparisons obtained from DGGE fingerprints (PRIMER analysis) of rainbow trout intestinal microbiota from each group. Feeding groups: A. juvenile control; B. juvenile MOS; C. sub-adult MOS; D. sub-adult control.

	SIMPER		1 -way ANOSIM		
Group	Species richness	Similarity %	R - value	*p* Value	Dissimilarity (%)
Juvenile control (A)	11.7	94.5			
Juvenile MOS (B)	8.7	72.2			
Sub-adult MOS (C)	12.3	54.7			
Sub-adult control (D)	14	72.5			
Pairwise comparison					
A, B			0.26	0.30	25.1
A, C			0.22	0.20	38.2
A, D			1	0.10	42.8
B, C			-0.19	0.90	33.7
B, D			0.89	0.10	44.4
C, D			0.26	0.10	38.1

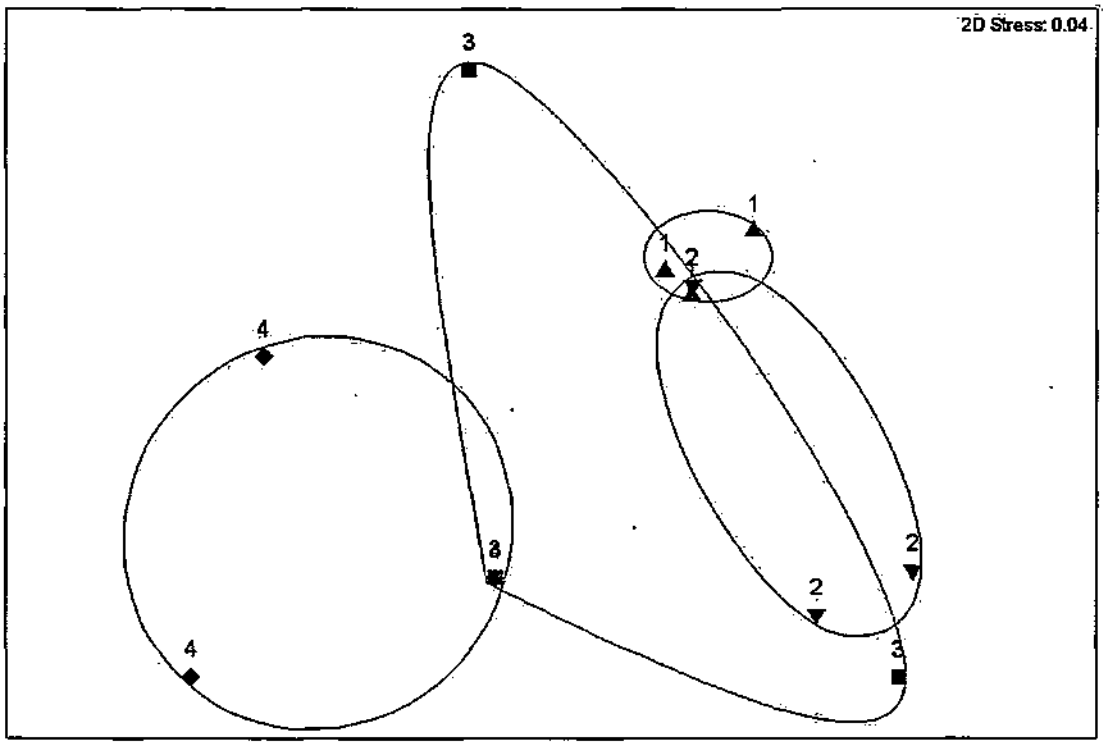

Figure 3.5 Non-metric multidimensional scaling analysis (nMDS) plot of DGGE fingerprints showing similarities between treatments. 1. juvenile control; 2. juvenile MOS; 3. sub-adult MOS; 4. sub-adult control. Clustering indicates that MOS supplementation of juvenile groups causes a shift in the bacterial community structure. Sub-adult plots are not closely clustered, but MOS supplementation appears to cause a shift towards the juvenile groups.

3.1.5 Discussion

Both light and electron microscopy showed that MOS caused significant differences of the intestinal morphology in the sub-adult trout. MOS produced a systemic improvement of gut absorptive surface area in both anterior and posterior intestinal regions. MOS increased absorptive surface by producing longer and more complex villi structures. This was confirmed at the EM level,

where micrographs showed that MOS was able to increase both microvilli density and length. Histological examination of juvenile trout showed that MOS was unable to confer significant improvements despite the fact that juveniles were receiving MOS supplementation for a longer period of time. The reason for this is not understood. Literature regarding the effect of MOS on rainbow trout gut histology is scarce; however previous experiments in gilthead sea bream (Chapter 2.1) and white sea bream larvae (Dimitroglou, 2004) suggest that 0.2% MOS supplementation alters the intestinal morphology of some fish. However, Torrecillas et al. (2007) assessed the effect of MOS on the gut villi of European sea bass and found that dietary administration of MOS at 0.2% or 0.4% for 67 days did not seem to affect villi length. Nevertheless, similar to the findings in the present study, MOS supplementation significantly increased microvilli length in cobia larvae (Salze et al., 2008). Similarly, improvements of gut morphology with MOS have been reported in poultry and swine (Iji et al., 2001; Hooge, 2004; Peet-Schwering et al., 2007; Castillo et al., 2008). An improvement in inestinal morphology is not only likely to benefit feed utilization but the maintenance of an intact, healthy mucosal epithelium reduces the chances of opportunistic indigenous bacterial infections. Indeed, this may well have been a contributory factor to the improved growth performance, feed utilization and survival of rainbow trout observed by Staykov et al. (2007).

In the current study, viable populations from the intestinal tract ranged between 10^6 and 10^8 CFU g^{-1}, which is within the range of values reported in other rainbow trout investigations (Spanggaard et al., 2000; Heikkinen et al., 2006; Kim et al., 2006). The intestinal microbial communities of rainbow trout are often highly culturable; with culturability estimates of 50 – 90 % (Spanggaard et

al., 2000; Huber et al., 2004). Dominant groups in the present study were identified as belonging to the γ subclass of Proteobacteria, in particular *Aeromonas/Vibrio* spp. and Enterobacteriaceae, which have been commonly isolated from trout (Spanggaard et al., 2000; Spanggaard et al., 2001; Huber et al. 2004; Heikkinen et al. 2006; Kim et al. 2006; Pond et al., 2006). Viable populations in MOS supplemented groups were significantly lower than the control groups (10^6 and 10^8, respectively) which agree with the findings from the previous experiments with sea bream (Chapter 2.2). As previously discussed, MOS is able to bind to certain Gram negative bacteria preventing intestinal colonization; thus, resulting in a removal mechanism of bacteria from the gut (Spring et al., 2000). Binding and removal of selected Gram negative may explain the changes of viable populations observed in the present study. Differences in the relative abundance of the microbiota identified were also observed. Compared to the juvenile control, juvenile MOS supplemented fish displayed a significant reduction of *Aeromonas/Vibrio* spp. This may be of particular interest for rainbow trout aquaculture where some of the most common diseases, such as Vibriosis and furunculosis, are caused by *Vibrio* and *Aeromonas* spp.. Such species include *V. anguillarum*, *V. ordalii*, *V. alginolyticus* and *A. salmonicida,* which are often indigenous to fish and are thought to infect via the gastrointestinal tract (Austin and Austin, 1999; Ringø et al., 2007). A recent study showed that dietary MOS can provide protection against *V. alginolyticus* infection in European sea bass, (Torrecillas et al., 2007). The exact mechanism of protection was not clear, but enhanced immune responses were observed. The findings from the present study suggest that a potential direct role of MOS against *V. alginolyticus* could have been a factor for the improved protection in European sea bass. The elevation in

the relative population of *Enterococcus* spp. in the juvenile MOS group is also interesting, as lactic acid bacteria (LAB) such as *Enterococcus* spp. may be considered as beneficial bacteria. For example probiotic administration of *Enterococcus faecium* has demonstrated an alteration of the gut microbiota of sheet fish (*Silurus glanis*) by reducing numbers of Enterobacteriaceae (including *Escherichia coli), Staphylococcus aureus* and *Clostridium* spp. (Bogut et al., 2000). Subsequently growth performance was increased. The benefits of probiotic administration of *E. faecium* has also been demonstrated in European eel, *Anguilla anguilla* L. (Chang and Liu, 2002) and rainbow trout (Panigrahi et al., 2007). The reason for this relative increase is not clear; it may be a direct result of the MOS supplementation or a secondary effect produced by altering other microbial populations, thus, reducing competition and antagonism creating a more favorable environment for *Enterococcus* spp.. If such microbial modulation occurs in other fish species, it may well have been a factor which contributed towards the enhanced intestinal epithelial morphology of cobia observed by Salze et al. (2007) and the improved soybean-meal digestibility by red drum (*Sciaenops ocellatus*) observed by Burr et al. (2008).

Diversity indices calculated from gradient gel electrophoresis analysis are an effective quantitative means of comparing microbial community profiles from different environmental samples (Eichner et al., 1999). DGGE statistical analysis shows the sub-adult and juvenile fish to be significantly different to each other. Presence/absence half matrix of the DGGE banding patterns showed similarity between the different feeding regimes within the same age group were relatively high (~80%). This indicates that the species present in the overall microbial communities, including those that were not culturable, were relatively similar in

all groups. SIMPER analysis showed that the sub-adult control group has the greatest species richness. MOS feeding appears to decrease the species richness in both the sub-adult and juvenile fish. Results from nMDS confirm that dietary MOS has a considerable effect on the gut microbiota; however, this is perhaps not always seen in all fish. Plots indicate that MOS supplementation of juvenile groups causes a shift in the bacterial community structure. Sub-adult plots are not as closely clustered, but MOS supplementation appears to cause a shift towards the juvenile groups. Thus, MOS treatment of both sub-adult and juvenile fish appears to have similar effects; it would be interesting to prolong a study of sub-adult fish to see if the microbial profile converges with that of the juvenile group as the treatment and maturation increases. Sub-adult fish were fed MOS supplemented diets for 58 days and juvenile fish were fed for 111 days, however, the accumulated effect of continual supplementation of a sub-adult group may reduce the variability of the microbiota, as was evident in the juveniles. The DGGE analysis confirms the culture based findings; MOS appears to reduce certain species as well as removing certain species entirely (as is evident from the reduction of species diversity). Despite the evident changes in populations, the spatial overlapping of each treatment demonstrates that the result of MOS addition is a subtle alteration of the microbiota as opposed to a dynamic shift.

General observations from nMDS plots, ANOSIM and SIMPER comparing the sub-adult trout with the juvenile trout indicate that microbial communities in sub-adult trout are more diverse than in the juvenile trout with reduced similarity within replicates. This would suggest that the complexity of the intestinal microbial community structure is closely related to duration of exposure to environmental microbiota.

In conclusion, dietary MOS alters the characterization of rainbow trout intestinal microbiota and enhances intestinal morphology by increasing absorptive surface area and improving microvilli structure in the sub-adult trout. Continuing from Torrecillas et al. (2007), further work should be undertaken to assess the possible potential of MOS against other *Aeromonas* and *Vibrio* infections in challenge studies. It would also be interesting to see if a synergistic effect could be achieved between applying a synbiotic of MOS and *E. faecium*. A longer duration trial would be interesting to see if the shift in microbial populations would continue indefinitely, or to some specific end point.

3.2 The effect of a specific MOS supplementation on Atlantic salmon smolts (*Salmon salar* L.) fed diets with high levels of plant proteins

3.2.1 Abstract

An experiment was conducted in order to investigate the effect of dietary MOS supplementation on Atlantic salmon smolts (*Salmon salar* L.). The first treatment (control) consisted of fish fed a basal diet and the second treatment (MOS) consisted of fish fed the basal diet supplemented with 0.4% MOS. In the basal diet, half of the protein content was derived from fish meal and the rest from plant protein sources (mainly soy and wheat). After 14 weeks feeding on the experimental diets the results demonstrated that MOS supplementation did not affect growth performance. However, body protein composition, apparent fibre digestibility and blood haematocrit levels were significantly increased in fish fed the MOS supplemented diet. Additionally, liver histochemistry revealed that glycogen deposition in the liver was increased by ~43% in the MOS fed fish. Similarly, intestinal histology demonstrated that MOS supplementation significantly increased the mucosal folding and the microvilli density in both the anterior and posterior intestinal regions. Microvilli length was increased at the posterior intestine from 1.10 ± 0.18 μm in the control group to 1.41 ± 0.19 μm in the MOS fed fish. Furthermore, counts of sea lice attached to the fish epidermis and total number of fish infected by sea lice were significantly lower in MOS fed salmon. The present study shows that 0.4% of MOS supplementation improves intestinal morphology and increase crude fibre digestibility, carcass protein

content, glycogen deposition in the liver and improves resistance to epidermal sea lice infection.

3.2.2 Introduction

Fishmeal (FM) is the main source of protein in the aquaculture feed production industry. As the aquaculture industry continues to expand, the demand for fishmeal continues to increase. This has lead to concerns about production costs and the effects of increasing demands on the sustainability of wild fish stocks. Many plant proteins have been used as alternatives to FM and soybean meal (SBM) is the major representative because of its competitive price and relative availability compared to FM. However, plant protein sources and especially SBM have been demonstrated to induce histological and functional changes of the fish gastrointestinal tract which include enteritis, increased susceptibility to bacterial infection, changes in absorptive cells, increased presence of inflammatory cells, shortening of villi and microvilli (Baeverfjord & Krogdahl, 1996; Krogdahl et al. 2000; Bakke-McKellep et al. 2000; Krogdahl et al. 2003; Sitjà-Bobadilla et al. 2005; Heikkinen et al. 2006, Merrifield et al., 2009a). Atlantic salmon production has continually increased over the last decades and its production plays a vital role for the economy of countries like Norway, Scotland and the U.K.. Atlantic salmon has the largest production among the salmonids species (FAO, 2008).

The purpose of this trial was to investigate the effect of MOS supplementation on growth parameters, nutrient digestibility, carcass proximate analysis and flesh pigmentation using diets where FM was replaced from plant protein sources. In addition, liver and intestinal samples were histologicaly

examined using both LM and EM. Blood haematocrit and serum lysozyme activity were also evaluated. Finally, sea lice infection was also evaluated twice during the experimental period.

3.2.3 Methodology

The trial was conducted in the facilities of GIFAS (Gildeskål Research Station) in Gildeskål, Norway. The initial stocking density was ~200 fish (mean weight 46 g) per cage. Two dietary groups were randomly distributed in 8 replicate 125 m^3 cuboid net cages (4 net cages per group). The first feeding group was the control, where Atlantic salmon (*Salmon salar*) were fed a basal diet and the second feeding group was the MOS group, where salmon were fed the basal diet with the addition of 0.4% MOS. The composition and proximate analyses of the diets are shown in Table 3.5. The feed contained a high level of plant protein and was produced by Fiskeriforskning in Bergen using a Wenger type twin screw extruder supplied with standard equipment for grinding, mixing, drying, coating and cooling. Fish were fed to satiation twice a day with a minimum time interval of 4 h between the 2 feedings. In order to facilitate accurate measures of feed intake and FCR, uneaten feed was collected and registered with the feed intake calculated on a daily basis. Mortalities were removed daily and the weight was measured and registered. The duration of the trial was 14 weeks from mid July to mid October. The water temperature (measured at 3 m depth) varied with season during the trial between 8.8 °C and 15.2 °C. Fish sampling was conducted on the last day of the experiment (day 98).

Table 3.5 Diet formulation and proximate composition analysis of the experimental diets

Ingredients (%)*	**Control diet**	**MOS**
Fish meal LT	32.1	32.0
Wheat gluten	9.0	9.0
Soy protein concentrate	10.0	10.0
Soy extracted protein	6.0	6.0
Sunflower meal	8.0	8.0
Wheat	10.0	10.0
Fish oil	11.3	11.3
Canola oil	11.3	11.3
MAP[1]	0.6	0.6
Premixes vitamins & minerals	2.4	2.4
Carophyll pink	0.055	0.055
Lysin – HCL	0.2	0.2
Methionine	0.1	0.1
Ytrium Oxide	0.01	0.01
Moisture change	-1.1	-1.4
MOS		0.4
Proximate analysis		
Moisture (%)	5.7	6.3
Protein (%)	43.9	43.8
Fat (%)	26.3	26
NFE[2] (%)	14.7	14.2
Fibre (%)	1.9	2.2
Ash (%)	7.5	7.5
Astaxanthin in DM (ppm)	48.8	50.2
DP (MJ)	37.74	37.51
DE (MJ/kg)	18.22	18.10
DP/DE (g/MJ)	20.72	20.72

[1]MAP: mono-ammonium phosphate

[2]NFE: N-free extract, DM: dry matter, DP: digestible protein, DE: digestible energy.

*All ingredients were provided by the feed manufacturer suppliers

3.2.3.1 Digestibility and body composition analysis

All fish were stripped of faeces by applying gently pressure in the distal-ventral region and faeces collected. Pooled faecal samples were immediately frozen at – 20 °C until analysis. Faeces samples were used to calculate the apparent digestibility coefficients (ADCs). The ADCs for the nutrients were calculated using the formula:

$ADC_{nutrient}$ = 100 – 100 x [Ytrium Oxide in faeces (ppm) / Ytrium Oxide in faeces (ppm)] x [nutrient in faeces (%) / nutrient in feed (%)].

Additionally, at the end of the trial the carcasses of 8 fish per cage were pooled (n = 32) and analysed for energy, moisture, protein and fat composition according to standard AOAC (1995) methods as previously explained in Chapter 2.1.3.3. Briefly sample dry matter (DM) was calculated from the weight of sample before and after drying in a fan assisted oven (Genlab ltd, UK) at 105 °C until a constant weight was achieved. Crude protein analysis was performed using the Kjeldahl method with the constant relationship between N and animal protein being 6.25. Lipid content was determined using the Soxhlet extraction method and gross energy was measured using a adiabatic bomb calorimeter.

3.2.3.2 Flesh pigmentation

Astaxanthin concentration was measured on the Norwegian Quality Cut (NQC, NS9401 1994) filet from 8 fish per cage (n = 32). The NQC fillet is located between the end of the first dorsal fin and the beginning of the anal fin. Astaxanthin analysis was carried out by Nofima in Norway using an HPLC

method developed from DSM (Roche) and modified by FHL 2004 (Norwegian Seafood Federation).

3.2.3.3 Haematological parameters

Five fish from each cage (n = 20) were anaesthetized with MS-222 (Ethyl 3-aminibenzoate methanesulfonate Tricaine; Pharmaq, Fordingbridge, UK; 200 mg L^{-1}) and blood was collected from the caudal vein. Immediately, heparinised haematocrit capillary tubes, which were then sealed with Crystalseal, where filled and centrifuged for 5 min at 1,500 g. Haematocrit values were measured using a Hawksley reader as % packed cell volume (%PCV). The remaining blood was left to clot for at least 3h at room temperature prior to centrifuging for 10 min at 3600 *g* for the collection of serum. Serum lysozyme activity was measured using a modified lysoplate assay (Lie et al., 1986). Punch wells of 2 mm diameter were made in petri dishes with 1% agarose in 50 mM phosphate buffer (PB) and 500 µg mL^{-1} *Micrococcus lysodeikticus* were. Wells were filled with 8 µL of sample serum and then incubated for 16 h at room temperature (~25°C) before measuring the diameter/raius of cleared area surrounding each well.

3.2.3.4 Histology

Liver and intestinal samples from 4 fish per cage (n = 16) were retained for subsequent histological examination by LM and EM. Intestinal sections from the intestine distal to the pyloric cecae (anterior region) and the intestinal section prior to the anus (posterior region) were taken. Samples were analyzed according to the previously described methodology in Chapter 2 (§ 2.1.3.4). Briefly, samples for light microscopy were fixed in 4% saline formalin. The tissue samples were

dehydrated in graded ethanol before equilibration in xylene and embedding in paraffin wax. 8 µm transverse sections were cut and stained using alcian blue periodic acid-Schiff staining technique (AB-PAS; Kiernan, 1981). Samples for scanning and transmission electron microscopy (SEM & TEM) were fixed in 2.5% glutaraldehyde with 0.1 M cacodylate acid sodium salt solution (1:1 vol.), pH 7.2 with additional 2.5% NaCl. Prior to fixation, SEM samples were rinsed in 1% S-carboxymethyl-L-cysteine (Sigma) for 30 sec in order to remove epithelial mucus. SEM samples were dried using a K850 critical point drier (Emithech; Kent, UK) with ethanol as the intermediate fluid and CO_2 as the transition fluid. All samples were coated with gold using K550 sputter coater (Emithech; Kent, UK) and screened with a Jeol JSM 5600 LV electron microscope at 15 kV (Jeol; Tokyo, Japan). Samples for TEM were post-fixed in OsO_4 for 1 h and embedded with the standard resin procedure. Resin blocks were sectioned using a diamond knife (~90 nm). Ultrathin sections from each sample were placed in copper grids and stained with uranyl acetate, post stained with lead citrate and screened with a Jeol JSM 1200 transmission electron microscope at 120kV (Jeol; Tokyo, Japan). Liver samples were analysed with light microscopy only.

All digital images were analyzed using Image J version 1.36 (National Institutes of Health, USA). Images from light microscopy were analyzed to determine the perimeter ratio (PR) between the internal perimeter (*IP*) of the gut lumen (villi and mucosal folding length) and the external perimeter (*EP*) of the gut (PR = *IP*/*EP*). A high PR value indicates high villi length and and/or increase mucosal folding. Liver images were analysed for glycogen deposition in the hepatocytes by the ratio of a stained area by Schiff's reagent (magenta = glycogen) and the unstained area, producing arbitrary units (AU). High

magnification (x 20,000) SEM images were analyzed in order to measure the density of the microvilli (arbitrary units). TEM images (magnification x 20,000) were analyzed to measure the microvilli length.

3.2.3.5 Epidermal-sea-lice measurements

At week 5, 40 fish per cage (n = 160) were randomly selected and the epidermal sea lice were enumerated. Two copepod parasites were found and assessed. *Lepeophtheirus salmonis* (Krøyer), known as common sea lice, were assessed by counting the number of sea lice as larvae (sedentary stage), pre-adults (including males) and fertile females. Total levels of *Caligus elongates* (Nordmann), irrespective of life cycle or sex, were also enumerated. The procedure was also repeated two weeks later (week 7).

3.2.3.6 Statistical analysis

An independent sample two-tailed T-test was applied in order to evaluate the effect of MOS supplementation compared with the control treatment. Analysis were carried out on SPSS 15.0 (SPSS Inc., IL, USA) and significance was accepted at the $p < 0.05$ level. The results are presented as mean values followed by the standard deviation ($M \pm SD$).

3.2.4 Results

3.2.4.1 Growth analysis and body proximate composition

Table 3.6 presents the result from the growth study. There were no significant differences for the growth parameters at the $P \leq 0.050$ level.

Analysis of fish body composition (presented in Table 3.7) showed that the MOS fed fish contained significantly ($P = 0.050$) higher protein compared to the control fish, 16.85% ± 0.06 and 16.75% ± 0.06, respectively. Gross energy, fat and dry matter were not affected.

Table 3.6 Growth parameters of salmon fed the experimental diets for 14 weeks.

Parameter	Control	MOS
Total initial number of fish	771	771
Total final number of fish	747	751
Mortalities	24	20
Initial fish weight (g)	47.24 ± 2.31	46.24 ± 2.54
Final weight (g)	204.06 ± 9.58	204.18 ± 9.96
Initial biomass (kg / cage)	9.10 ± 0.33	8.90 ± 0.24
Final biomass (kg / cage)	34.75 ± 1.94	34.96 ± 2.62
Feed intake (kg/cage)	25.28 ± 1.57	25.39 ± 1.51
FCR	0.86 ± 0.02	0.86 ± 0.04
SGR (% day^{-1})	1.41 ± 0.09	1.44 ± 0.10

Table 3.7 Fish carcasses proximate composition analysis at the end of the trial .

Parameter	Control	MOS
Dry matter (%)	29.15 ± 0.67	29.20 ± 0.45
Protein (%)	16.75 ± 0.06	16.85 ± 0.06 *
Lipid (%)	11.35 ± 0.84	11.48 ± 0.43
Energy (%)	28.16 ± 0.36	28.32 ± 0.14
Flesh astaxanthin (ppm)	2.48 ± 0.39	2.15 ± 0.26

*Indicates statistical difference $P < 0.05$

3.2.4.2 Digestibility

There were no statistical differences ($P > 0.05$) between the control and the MOS groups in regards to digestibility of dry matter, energy, protein or lipid (Table 3.8). However, values for the crude fibre digestibility were negative. This suggests that the excretion of crude fibre was higher than the intake. The reason for the increase maybe that after digestion more indigestible NFE was measured as crude fibre. Hence, MOS supplementation produced a significantly higher crude fibre (including indigestible NFE) digestibility compared to the control (-29.03% ± 6.57 and -58.29% ± 12.10 respectively, $P = 0.010$).

Table 3.8 Apparent nutrient digestibility of Atlantic salmon fed the experimental diets.

Parameter (%)	Control	MOS
Faecal dry matter	12,24 ± 0.82	12,78 ± 0.55
Energy digestibility	78,96 ± 1.42	78,31 ± 0.95
Protein digestibility	85,97 ± 1.12	85,63 ± 0.78
Lipid digestibility	87,08 ± 1.53	86,91 ± 1.17
Crude fibre digestibility	-58,29 ± 12.10	-29,03 ± 6.57 *

*Indicates statistical difference $P < 0.05$.

3.2.4.3 Flesh pigmentation

Dietary MOS supplementation did not significantly (P = 0.220) affect flesh pigmentation (Table 3.7) of Atlantic salmon fed the experimental diets.

3.2.4.4 Heamatological parameters

MOS fed fish produced significantly (P = 0.031) higher haematocrit levels compared with the control group (Table 3.9). %PCV of the control fish = 35.91% ± 4.1 and for the MOS fed fish %PCV = 38.41% ± 1.9. Serum lysozyme levels did not differ between the treatments (P = 0.332).

Table 3.9 Haematological parameters (n = 20).

Parameter	Control	MOS
Heamatocrit (%PCV)	35.92 ± 4.07	38.41 ± 1.88 *
Lysozyme (mm^2)	139.84 ± 27.00	131.74 ± 19.12

*Indicates statistical difference $P < 0.05$

3.2.4.5 Histological examination

The results of the histological examination of liver glycogen deposition, intestinal absorptive surface area and microvilli morphology are presented in Table 3.10.

Liver sections were stained with PAS method (Periodic Schiff's reagent Staining) in order to identify the glycogen deposition in hepatocytes. The results showed that the MOS treatment significantly ($P = 0.012$) increased the glycogen deposition in liver tissue from 1.80 ± 0.73 AU in the fish fed the control diet to 2.58 ± 0.91 AU in the MOS fed fish.

Light microscopy of the anterior intestine indicated that the MOS treatment (4.63 ± 0.62 AU) produced a significantly ($P = 0.001$) higher absorptive surface (increased villi length, and more complex villi structures) compared with the control fed fish (3.35 ±0.49 AU). Similarly, in the posterior intestine the MOS treatment (3.90 ± 0.66 AU) produced a significantly ($P = 0.027$) increased absorptive surface in comparison with the control fish (3.10 ± 0.63 AU).

SEM micrographs from the anterior intestinal region showed that MOS (12.02 ± 5.95 AU) significantly ($P = 0.005$) increased the microvilli density

compared with the control (5.90 ± 1.53 AU) treatment (Figures 3.6 and 3.7). In the posterior intestinal region again the MOS treatment (10.37 ± 3.28 AU) produced significantly (P = 0.007) increased microvilli density compared to the control treatment (6.95 ± 2.12 AU).

TEM micrographs from the anterior intestinal region revealed that there was no significant effect of MOS supplementation on microvilli length compared to the control treatment (P = 0.168). However, in the posterior part of the intestine MOS (1.41 ± 0.19 μm) significantly (P = 0.008) improved the microvilli length when compared to the control (1.10 ± 0.18 μm) treatment (Figure 3.8). In the anterior intestinal region 8 out of 16 fish from the control and 0 from the MOS treatment had signs of either irregular microvilli distribution, swollen/necrotic enterocytes or enterocytes with a high density of vacuoles. In the posterior intestinal region only 4 out of 16 fish from the MOS and 10 out of 16 from the control treatment had signs of either irregular microvilli distribution or swollen/necrotic enterocytes. In the posterior intestinal region no enterocytes with high vacuole densities were observed in either feeding groups.

Table 3.10 Histological examination of liver and gut samples based on 4 fish per cage (n = 16).

Parameter	Region	Control	MOS
Liver glycogen[1]		1.80 ± 0.73	2.58 ± 0.91 *
Gut absorptive surface (PR) [1]	Anterior intestine	3.65 ± 0.49	4.63 ± 0.62 *
	Posterior intestine	3.11 ± 0.63	3.90 ± 0.66 *
Microvilli density[1]	Anterior intestine	5.90 ± 1.53	12.02 ± 5.95 *
	Posterior intestine	6.95 ± 2.12	10.37 ± 3.28 *
Microvilli length (μm)	Anterior intestine	1.94 ± 0.25	2.13 ± 0.29
	Posterior intestine	1.10 ± 0.18	1.41 ± 0.19 *

[1]Arbitrary units

*Indicates statistical difference $P < 0.05$

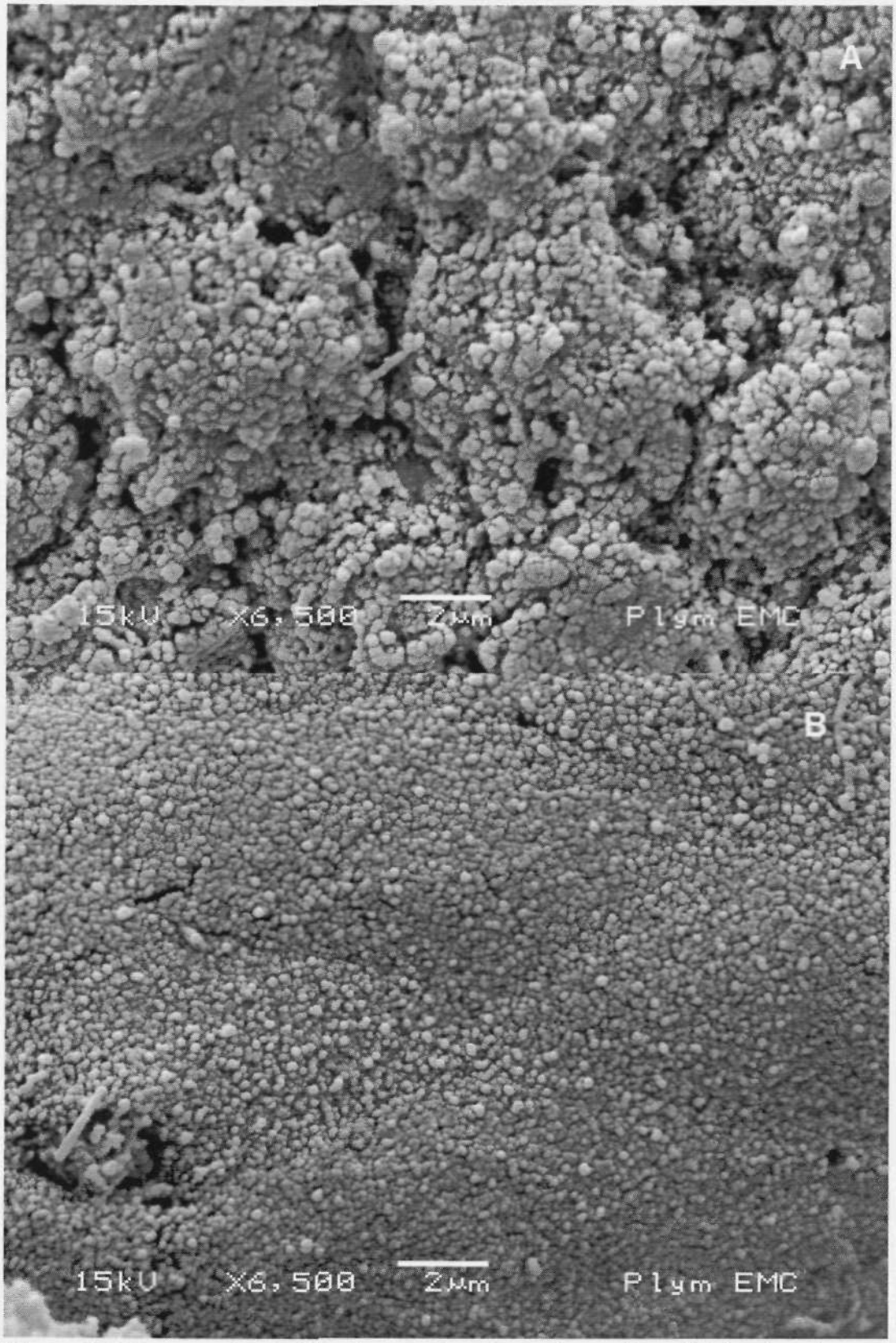

Figure 3.6 Comparative SEM micrographs of anterior region of Atlantic salmon fed either the control (A) or the MOS (B) supplemented diet. There are distinctive differences of the microvilli lay out between the treatments.

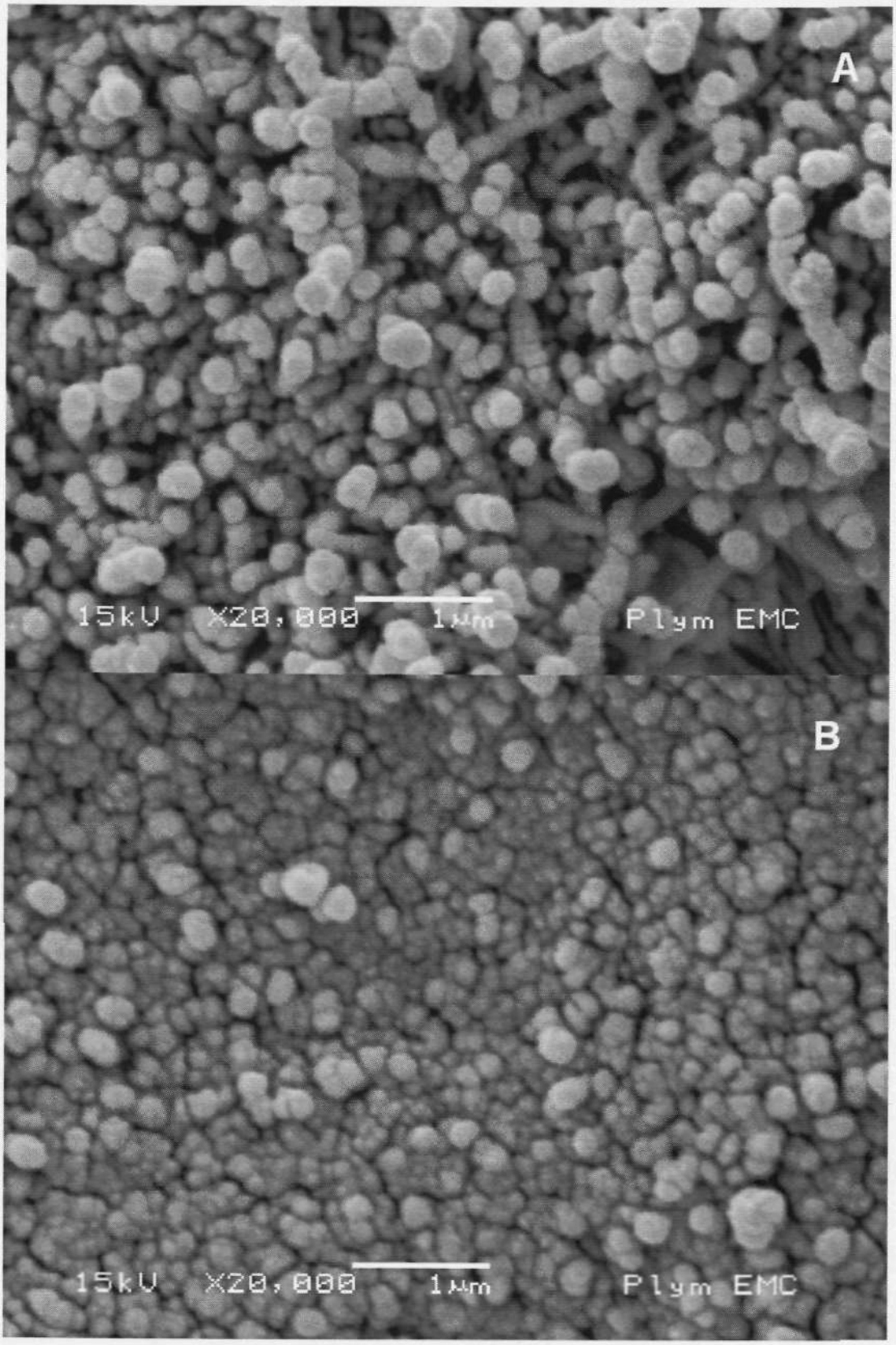

Figure 3.7 Comparative SEM micrographs of anterior region of Atlantic salmon fed either the control (A) or the MOS (B) supplemented diet. Microvilli are more regular and density is significantly higher in MOS treated fish ($P = 0.005$).

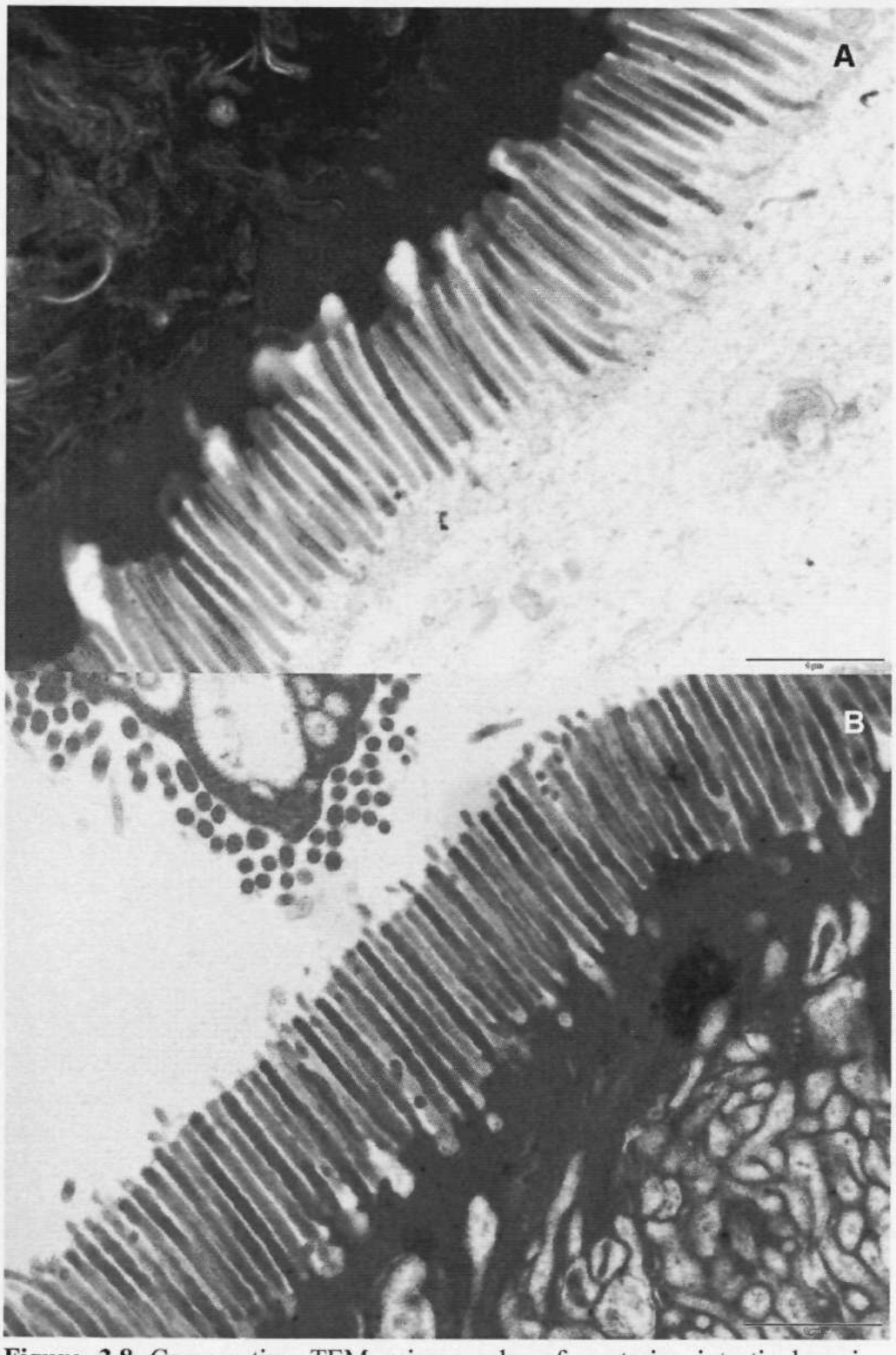

Figure 3.8 Comparative TEM micrographs of posterior intestinal region of Atlantic salmon fed either the control (A) or the MOS (B) supplemented diet. Although, microvilli appear healthy in both treatments, microvilli are more regular and significantly longer in MOS treated fish (P = 0.008).

3.2.4.6 Epidermal sea lice attachment

The number of *Lepeophtheirus salmonis* and *Caligus elongates* attached to the epidermis were not significantly affected by the MOS supplementation at either of the samplings points (week 5 and 7) (Table 3.11). Additionally, *Lepeophtheirus salmonis* individual assessment depending on life stage and sex remained unaffected. Despite this, the total numbers of sea lice were lower in fish fed the MOS supplementation. At week 5, 34.75 ± 17.23 sea lice were counted on fish from the control group while 18.00 ± 2.45 ($P = 0.051$) sea lice were present on MOS fed fish. At week 7, total sea lice on the control fed fish were 59.25 ± 17.50 and which was significantly reduced to 40.50 ± 5.92 on MOS fed fish ($P = 0.044$). Additionally, the total number of fish infected by sea lice (irrespective of species, life stage or sex) was significantly reduced by the MOS supplementation. At week 5, 56.8% ± 14.8 of the control fish and 32.5% ± 2.0 of the MOS fed fish were infected by sea lice ($P = 0.009$). At week 7, 74.4% ± 10.5 of the control fish and 57.5% ± 4.6 of the MOS fed fish were infected by sea lice ($P = 0.013$).

Table 3.11 Salmon sea lice infection scoring was assessed twice within a two-weeks period. Scoring was based on 40 fish per cage (n = 160).

Week 5	**Sub-group**	**Control**	**MOS**
Lepeophtheirus Salmonis (per fish)	Fertile females	3.50 ± 3.70	0.50 ± 0.58
	Pre-adult & males	14.25 ± 17.48	1.75 ± 2.36
	Larvae	5.00 ± 6.22	0.75 ± 0.50
Caligus elongates (total per fish)		12.00 ± 3.77	15.00 ± 3.74
Sum of sea lice per fish		34.75 ± 17.23	18.00 ± 2.45
Number of infected fish		22.75 ± 5.91	13.00 ± 0.82 *
Number of infected fish (%)		56.8 ± 14.8	32.5 ± 2.0 *
Week 7	**Sub-group**	**Control**	**MOS**
Lepeophtheirus salmonis (per fish)	Fertile females	1.00 ± 0.82	0.50 ± 0.58
	Pre-adult & males	30.75 ± 16.56	22.00 ± 5.94
	Larvae	15.00 ± 10.65	9.25 ± 3.20
Caligus elongates (total per fish)		12.50 ± 5.20	8.75 ± 3.59
Sum of sea lice per fish		59.25 ± 17.50	40.50 ± 5.92 *
Number of infected fish		29.75 ± 4.19	23.00 ± 1.83 *
Number of infected fish (%)		74.4 ± 10.5	57.5 ± 4.6 *

*Indicates statistical difference $P < 0.05$

3.2.5 Discussion

Currently there is a highly focused research interest on the use of prebiotics in the aquaculture industry (Burr et al., 2008; Grisdale-Helland et al., 2008; Salze et al., 2008) as well as the induction of plant proteins in fish feeds (Hansen et al., 2007; Olsen et al., 2007; Burr et al., 2008). Both of these interests are examined in the present study, where the effect of MOS supplementation on salmon fed high plant protein diets was evaluated. Both LM and EM showed that MOS caused significant differences on the intestinal morphology of salmon. MOS supplementation increased the absorptive surface area by producing longer mucosal folding in both the anterior and posterior intestinal regions. Similarly, SEM micrographs revealed that MOS was able to increase the microviili density in the anterior and posterior intestinal regions. TEM analysis showed that MOS produced significantly increased microvilli length in the posterior intestine. Literature regarding the effect of MOS on salmon gut histology is scarce; however, Torrecillas et al. (2007) assessed the effect of MOS on the gut villi of European sea bass and found that dietary administration of MOS at 0.2% or 0.4% did not seem to affect villi length. Nevertheless, similar to the findings in the present study, MOS supplementation has been shown to significantly increase villi folding in rainbow trout, (Yilmaz et al., 2007) and hybrid tilapia, (Genc et al., 2007b) using similar dosages of MOS supplementation (0.15% and 0.3%). Additionally, microvilli length in cobia larvae was increased with 0.2% of MOS supplementation (Salze et al., 2008). In the previous experiment, 0.2% dietary MOS can increase the microvilli length, the villi and the mucosal folding in both examined intestinal regions of sub-adult rainbow trout as well as increase microvilli density in the posterior intestinal region (Chapter 3.1). Additionally, the

gilthead sea bream trial in Chapter 2.1 revealed that MOS supplementation even at the level of 0.2% can alter the intestinal morphology. Generally, an improvement in intestinal morphology is likely to benefit feed utilization and growth performance. However, as growth performance was not affected in the current study but this may have to do with the increased level of plant protein inclusion. Additionally, the maintenance of a healthy mucosal epithelium reduces the chances of opportunistic indigenous bacterial infections. This may have been a contributory factor to the improved growth performance, feed utilization and survival of rainbow trout (Staykov et al. 2007), European sea bass (Torrecillas et al., 2007) and green tiger prawn (Genc et al., 2007a) observed in previous studies.

The present results show that MOS did not affect the growth performance of salmon smolts which is similar to previous MOS studies on salmon (Grisdale-Helland et al., 2008), cobia larvae (Salze et al., 2008), hybrid tilapia (Genc et al., 2007b) and Gulf sturgeon *Acipenser oxyrinchus desotoi* (Pryor et al., 2003). However in contrast, previous studies on carp (Culjak et al., 2006), European catfish (Bogut et al., 2006), rainbow trout (Staykov et al., 2007), European sea bass (Torrecillas et al., 2007) and green tiger prawn (Genc et al., 2007a) have demonstrated that MOS can improve aquatic animal growth performance. Apparent digestibility of energy, protein and lipid were not affected by the MOS supplementation. Only apparent fibre digestibility was significantly improved by the MOS supplementation. Previous studies by Grisdale-Helland et al. (2008) on salmon and Burr et al. (2008) on red drum, *Sciaenops ocellatus,* showed that MOS supplementation can increase the apparent energy and apparent protein digestibility in FM and SBM based diets, respectively. Unfortunately, none of the previous studies gave any information on the apparent fibre digestibility. In the

present study, fish body composition analysis showed increased protein concentration of the MOS fed fish compared to the control fish. This agrees with studies on rainbow trout (Yilmaz et al., 2007) and hybrid tilapia (Genc et al., 2007b) using the same level of MOS supplementation (0.4%). In contrast, larger salmon (starting weight 200 g) (Grisdale-Helland et al., 2008) with higher level of MOS supplementation (1%) displayed significantly lower carcass protein concentration compared to the control. It appears that either fish size or the level of MOS supplementation is an important factor that may influence the outcome of MOS supplementation on carcass composition.

Furthermore, MOS supplementation did not influence the astaxanthin deposition in the NQC fillet. According to the author's knowledge there is no published literature regarding prebiotics and astaxanthin deposition in flesh.

Haematocrit levels were higher with the MOS supplementation whereas the blood serum lysozyme remained unchanged. To the author's knowledge there are no other studies of MOS in aquatic animals with regards to other species haematocrit levels. Grisdale-Helland et al. (2008) showed that serum lysozyme activity was reduced with a higher dose of MOS supplementation (1%) in larger salmon (ca 600 g). On the other hand studies with the same level of MOS supplementation (0.2% and 0.4%) in European sea bass showed that lysozyme activity was unaffected by the MOS supplementation compared with the control, but there was a positive correlation between the dosage of MOS supplementation and the blood serum lysozyme activity (Torrecillas et al., 2007). Additionally, Staykov et al. (2007) showed increased lysozyme activity of rainbow trout fed diets supplemented with 0.2% MOS. These studies indicate that there may be a relationship between the level of MOS supplementation and lysozyme activity.

Additionally, physiological characteristics due to different habitat (marine and fresh water) of each species may influence the corresponding lysozyme activity.

Histological evaluation of the glycogen deposition in the liver tissue revealed that MOS supplementation may affect the quantity of glycogen in the hepatocytes. Glycogen is stored in hepatocytes from where it can be used easily as an energy source in cases where the feeding schedule is interrupted. Prolonged interruption of feeding schedule often occurs in commercial rearing facilities especially during storm conditions. Hence, the increased glycogen deposition in the MOS fed fish might be a useful energy store during periods of feeding interruption. Future studies are required to determine this.

Sea lice scoring showed that the number of both species of sea lice attached to salmon was reduced by the dietary MOS supplementation. In addition, the total number of fish that were infected by sea lice was significant lower in the MOS supplemented diet. The reason for this is not yet understood, but the author believes that this may be related with the epidermal mucus. Mucus is a complex secretion which comprises mainly by carbohydrates and proteins. The mucus secretion can be increased by antigens thus a thick layer of mucus will be produced in order to prevent the pathogens from getting into the organism. Additionally, lysozyme is included within the mucus matrix so the mucus quantity and quality parameters should be investigated in future studies.

In conclusion, 0.4% MOS supplementation seems to have no effect on salmon growth performance. Fibre digestibility increased with MOS supplementation which suggests that MOS could be used in combination with plant protein sources to increase their digestibility. Additionally, under the conditions described MOS improves liver condition and promotes healthy livers,

which agrees with previous work by Torrecillas et al. (2007). In the present study, FM was partially replaced by plant protein sources (mainly SBM) in the experimental diets. The intestinal morphology improved significantly with the dietary administration of MOS which suggests that MOS can effectively protect the mucosal epithelium of salmon and possibly reduce the risk of morphological alterations from SBM. However further work will need to be carried out to verify this.

Chapter 4 Summary

4.1 Summary and conclusions

Summarizing the findings for the present research it would appear that dietary MOS supplementation can modulate the intestinal morphology as well as the intestinal microbiota of fish. The studies described in Chapter 2 revealed that studies in Mediterranean species with different feeding strategies or fish size can either support or restrict the effect of MOS supplementation on growth parameters of fish reared in closed circulation marine systems. When fish of average initial weight ~110 g were fed to satiation FCR, SGR and PER were significantly improved (Chapter 2.2). Using the same fish species with an average initial weight ~25 g fed based to a fixed % of biomass (with the same MOS supplementation levels) there was no significant effect on growth parameters (Chapter 2.1). This indicates that either: (i) feeding duration, or (ii) feeding strategy (feeding to satiation or to % biomass) or (iii) fish maturation influences the effects of MOS supplementation, which are mediated via complex biological mechanisms which are not always reproducible. Light microscopical evaluation of sea bream revealed that neither level of MOS supplementation could influence the villi folding of either intestinal regions investigated. However, evaluation of the intestinal ultrastructure demonstrated that 0.2% MOS supplementation produced a more constant improving effect of microvilli density as well as microvilli length in both examined regions. The higher dose of 0.4% MOS had similar effects to the 0.2% but failed to improve the microvilli length in the anterior intestinal region. This suggests, along with the findings of Torrecillas et al. (2007), that dietary

MOS does not effects villi length of marine species. Instead, the findings of the study in Chapter 2.1 demonstrate that MOS supplementation provides a localised effect on the apical brushborder which agrees with the previous observations in white sea and cobia bream larvae (Dimitroglou, 2004; Salze et al., 2008). In both sea bream trials (Chapter 2.1 and 2.2) only the species relative abundance of the intestinal microbiota was affected, thus, species richness remained unaffected. Additionally, haematological parameters showed that neither of the dietary MOS levels could affect serum lysozyme and alternative complement activity. To the author knowledge the sea bream trial (Chapter 2.1 – 2.2) is the only trial where total and differential leukocyte counts have been assessed in fish fed MOS. Total leukocytes counts and the relative proportion of lymphocytes and granulocytes were increased while the relative proportion of monocytes was reduced. This immunomodulatory affect indicates a possible role in maintaining a general level of good health which may reduce susceptibility to disease. Indeed, MOS supplementation led to significantly reduced mortalities in sole infected with *Photobacterium damselae* subsp. *piscicida*. Similarly, dietary MOS supplementation reduced mortalities of disease challenge European sea bass (Torrecillas et al., 2007) and reduced stress induced mortalities of white sea bream larvae (Dimitroglou, 2004) and cobia larvae (Salze et al., 2008). Contrary to previous findings (Chapters 2.1, 2.2; Torrecillas et al. (2007) histological examination of sole intestine demonstrated that MOS supplementation improved the morphology of the villi structures in both anterior and posterior intestine. Additionally, the microvilli density was significantly increased in the anterior intestinal region. These findings suggest that MOS supplementation may either prevent or reduce intestinal damage induced by pasteurellosis or is able to

accelerate the healing process of the gastrointestinal tract after infection. On the basis of these studies with marine species it could be hypothesized that MOS induced improvements at the villi level only occurs when villi are damaged and unhealthy.

The study described in Chapter 3 assessed the effect of MOS in rainbow trout, provided further evidence that fish size (maturation) could be related with the effect of MOS supplementation. The histological findings support this suggestion because MOS supplementation had no effect on the intestinal histology of the juvenile trout. However, in the sub-adult group the histological examination showed that MOS supplementation was able to improve the intestinal condition, i.e. villi folding, microvilli length and density, for both light and electron microscopy analysis in MOS fed trout (only the microvilli density on the anterior part of the gut was not improved). Despite this, microbiological findings suggest that MOS affects the intestinal microbiota of rainbow trout, irrelevant of fish maturation. MOS affects trout intestinal microbiota agrees with the findings from the studies in Chapters 2.1 and 2.2 where MOS altered the relative species abundance and species richness of the intestinal microbiota of gilthead sea bream after both 2 and 9 weeks feeding on supplemented diets. The sea bream experiment also indicated that 2 weeks is sufficient time for MOS supplementation to reduce the intestinal microbial load. In the trout experiment MOS supplementation had a predominant effect on the intestinal microbiota (reduce the species richness was observed as well as a reduction in the total microbial load). This difference between the two fish species may be related with the different gastrointestinal microbial physiology or with the different rearing conditions (e.g. temperature, salinity, rearing facilities). Most of these factors

have been shown to effect the composition of the intestinal microbiota of fish species (Cahill 1990; Ringø and Birkbeck, 1999; Gatesoupe 1999). Therefore, the indigenous microbial populations are likely to be distinctive with evidently different responses to dietary inclusion of MOS. In addition, it is likely to be more difficult to affect the intestinal microbiota of fish reared in close circulation systems than fish reared in outdoor flow through systems. Environmental fluctuations and microbial input into closed re-circulation systems and thus into the fish intestine are more controlled and less extreme which likely results in a more stable and established microbial community which may be more difficult to modulate. Controlling the more fluidic and fluctuating changes in open systems is likely to be more beneficial. Further research is required in order to validate this hypothesis.

The salmon trial (Chapter 3.2) supports the suggestion that the effect of MOS supplementation may relate with the size of the fish too and is able to reduce the negative effects of SBM inclusion is fish diets (i.e. poor growth rates and alterations of the gastrointestinal tract). Both salmon and sea bream first trial (Chapter 2.1) where juvenile fish (initial fish weight $\leq$ 45 g) fed supplemented diets for at least 9 weeks without significant effects on growth parameters. Despite this, carcass protein retention, fibre apparent digestibility, haematocrit and liver glycogen deposition were all significantly increased. Histological findings demonstrated that 0.4% MOS supplementation improved the intestinal health by increasing the mucosal foldings and microvilli density in both intestinal regions as well as increasing microvilli length in the posterior region. The histological findings suggest that MOS supplementation has a predominant effect in the marine species that were examined in the present research. Additionally, salmon

sea lice infection appears to be restricted in the MOS fed fish, which suggests that MOS supplementation may either affects the epidermal mucus secretion or/and production.

Hence, the effects of MOS supplementation on fish as extracted from the present research are:

1. The fish species (fish physiology), rearing conditions and feeding strategies are related with the effect of MOS supplementation on fish growth performance.
2. 0.4% MOS supplementation is more effective than 0.2% at improving intestinal morphology of fish. Every time that 0.4% MOS was used there was a clear effect on the intestinal histology almost in every parameter (PR, MD and microvilli length) on both examined intestinal regions (anterior and posterior).
3. 0.2% MOS supplementation is sufficient to alter the intestinal microbiota within 2 weeks of feeding (of gilthead sea bream at least).
4. 0.4% of MOS may improve feed utilization, as was observed in the Atlantic salmon trial.
5. MOS supplementation may influence some immune parameters (i.e. lymphocytes, monocytes and granulocutes), acting as a possible immunostimulant.
6. MOS may have a role by reducing mortalities induced by bacterial infections.
7. MOS supplementation has a predominant effect in marine species.

8. MOS supplementation reduces the negative effects of alternative plant proteins (i.e. SBM) inclusion in fish diets.

Comparing the conclusions from the present research with the little available literature it is suggested that fish feeding habits and size is not clearly related with the effect of MOS supplementation on growth performance. In fresh water, juvenile fish have shown improved growth performance when fed MOS supplemented diets (Zhou and Li, 2004; Culjak et al., 2006; Staykov et al., 2007). There is only one previous study in fresh water using hybrid tilapia with a lack of improvement in growth performance (Genc et al., 2007b). In marine water species there are only 2 studies which disagree with the effect of MOS inclusion in fish growth performance using similar levels of supplementation: Torrecillas et al. (2007) fed European sea bass and observed increased growth performance, and Pryor et al. (2003) fed Gulf sturgeon MOS without any effect on growth performance. Furthermore, there are a limited number of studies which investigate the effect of MOS supplementation with respect to the intestinal histology. There is one study with European sea bass in which 0.4% MOS supplementation did not produce any effect on the villi morphology (Torrecillas et al., 2007). There are two studies with larvae which suggest that 0.2% of MOS supplementation improves the microvilli condition (Dimitroglou, 2004; Salze et al., 2008) and villi morphology (Dimitroglou, 2004). In addition, there are no other studies with regards to the effect of MOS supplementation on the intestinal microbiology of fish. The statement that 0.4% MOS supplementation can improve feed efficiency in terms of apparent nutrient digestibility is partially supported by two studies in red drum and Atlantic salmon in which the level of MOS supplementation was

1% and apparent protein, organic matter and energy digestibility coefficient were increased (Burr et al., 2008; Grisdale-Helland et al., 2008).

4.2 Future research

Further research is required in order to fully understand how MOS supplementation mediates host benefits. More trials should be conducted in order to find the optimal dose for the fish in respects to growth performance, intestinal morphology and microbiology as well as immune parameters. This may relate with the physiology of the fish (i.e. fresh or marine water), fish feeding habits (i.e. carnivorous, omnivorous and herbivorous), as well as fish development stage (i.e. larvae, juvenile, adult); rearing conditions (i.e. indoor / outdoor, close system / flow trough). By improving intestinal morphology we set the base for potentially superior nutrient absorption. Altering the intestinal microbiota in favour of beneficial bacteria we may improve the digestive performance of the intestine, which in combination with the improved morphology may produce systemic responses leading to superior nutrient absorption and utilization with potential subsequent benefits in respect to performance. However, this is not always seen either in the present study or in the literature. Perhaps the potential benefits will be greater expressed under poor rearing conditions, with poor quality diets or situations conducive of stress. Future studies should address these possibilities. Additionally, by altering the intestinal microbial population in order to increase the beneficial bacteria against the harmful bacteria we promote animal health and reduce the danger for disease outbreaks caused by opportunistic bacteria in farming conditions. Furthermore, the role of MOS supplementation in combination with probiotics should also be investigated (synbiotics), especially

from an immunomodulatory and disease control point of view. Growth performance and fish health are both important factors in the aquaculture industry as benefit is related with the quality and the price of the final product. The present series of investigations begin to show the potential benefits of MOS applications in important aquacultured species (i.e. gilthead sea bream, sole, rainbow trout and Atlantic salmon). Furthermore, current literature provides information regarding European sea bass, rainbow trout, cobia, Gulf sturgeon, carp and European catfish, yet there is no information available for other important aquaculture species such as Atlantic cod (*Gadus morhua*), red sea bream (*Pagrus major*), Nile tilapia (*Oreochromis niloticus*), grey mullet (*Mugil cephalus*) and Japanese amberjack (*Seriola quinqueradiata*). Future work should expand research applications to these key species.

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