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Effects of dietary ingredients and feed additives on the health and production of European sea bass (Dicentrarchus labrax) for applications in aquaculture

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By
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Experiment one revealed fishmeal (FM) replacement with soy protein concentrate (SPC) alone, and in combination with pea protein concentrate (PPC) and saponins (S) modulated the intestinal bacterial communities of *D. labrax*, increasing the presence of lactic acid bacteria. Intestinal histology revealed significantly reduced goblet cell's (GC's) in fish fed the SPC+S, epithelial microvilli densities (MD) in fish fed the SPC+PPC, SPC+PPC+S and SPC+S after two weeks feeding. Significant reductions in GC's and intraepithelial leukocytes (IEL's) in fish fed the SPC+S, and MD's in fish fed the SPC+S and SPC+PPC+S after four weeks feeding, relative to fish fed the FM control. Furthermore, fish fed all plant based diets appeared to exhibit a loss of membrane integrity at the microvilli tips, most pronounced in fish fed the SPC+S diet. These results suggest a sub-acute enteritis response in the posterior intestine of *D. labrax*, which was deemed to be most pronounced in fish fed the SPC+S diets.

Experiment two utilised the SPC+S diet as a sub-optimal basal diet to assess the potential of the probiotic *Bacillus subtilis* and the prebiotic Previda®, individually and in combination, in alleviating the enteritis-like effects induced by this diet, observed in the first experiment. Microbiological analyses revealed *B. subtilis* modulated the allochthonous bacterial communities. Fish fed the combination of *B. subtilis* and Previda® (synbiotic) diet exhibited a significantly increased intestinal perimeter ratio, compared to fish fed the basal. Significantly elevated GC's in fish fed the probiotic and synbiotic treatments, and significantly elevated epithelial MD's, and intestinal absorptive surface index in fish fed the probiotic diet was observed, relative to fed fish the basal. The loss of membrane integrity induced by the basal diet, was reduced in fish fed the probiotic, prebiotic and synbiotic diets. The intestinal gene expression of the pro-inflammatory cytokines IL-1β and TNFα was significantly up-regulated in fish fed all experimental diets, relative to fish fed the basal. The intestinal gene expression of HSP70, CASP3 and PCNA was significantly down-regulated in fish fed the probiotic, prebiotic and synbiotic relative to fish fed the basal. At the end of the experiment intestinal samples were exposed to one of four treatments [1. PBS (control), 2. *B. subtilis*, 3. *Vibrio anguillarum* and 4. *B. subtilis* + *V. anguillarum*], *ex vivo*, to determine if the feed additives could mitigate enteric pathogen damage. All feed additives revealed the potential to reduce the morphological damage caused by the pathogen.

Experiment three assessed *B. subtilis* and the phytobiotic Next Enhance 150® on the growth and health of *D. labrax*. *B. subtilis* modulated the allochthonous bacterial communities and reduced the presence of some potential pathogens. The intestinal gene expression of HSP70, CASP3, PCNA and CAL was significantly down-regulation in fish fed the probiotic diet relative to fish fed the control. Significantly elevated IEL's were observed in fish fed the probiotic and Next Enhance 150® diets relative to fish fed the control. Growth performance was remained unaffected.

The present research demonstrates that dietary *B. subtilis* modulates the allochthonous bacterial communities, as well as, improving the intestinal morphology and localised immunity in European sea bass. Dietary Previda® and Next Enhance 150® were also observed to confer beneficial effects on the gut health of this species. No detrimental effects were observed as a consequence of any of the feed additives used in the present research.

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List of abbreviations

ANOVA	Analyses of variance
AOAC	Association of Official Analytical Chemists
AU	Arbitrary units
BLAST	Basic local alignment search tool
CAL	Calreticulin
CASP3	Caspase-3
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
FCR	Feed conversion ratio
FISH	Fluorescent *in situ* hybridisation
FM	Fishmeal
GALT	Gut associated lymphoid tissue
HSP70	Heat shock protein 70
IL-1β	Interleukin 1β
IL-10	Interleukin 10
LAB	Lactic acid bacteria
MS222	Tricane methyl sulphonate
OTU	Operational taxonomic units
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PCV	Packed cell volume
RT-PCR	Real-time polymerase chain reaction
PPC	Pea protein concentrate
RNA	Ribonucleic acid
SEM	Scanning electron microscopy
SGR	Specific growth rate
SPC	Soy protein concentrate
S	Saponins
TAE	Tris-acetate-EDTA
TEM	Transmission electron microscopy
TE	Tris-EDTA
TLR	Toll like receptor
TNFα	Tumour necrosis factor alpha

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Dedication

I dedicate this thesis to my family and to the memory of my gran Nellie Forster for all their love and support. Also, for the financial backing from my parents, and the unexpected money left to me by my gran, which was an amazing surprise, and has been the basis with which I have been able to complete the last eight years of my university education. Many thanks for all your love and support over the years.

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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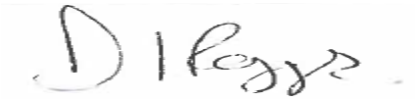
Signed..... 

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Conferences attended and work presented

Aquaculture America, The national conference & exposition of the national Aquaculture Association. Seattle, WA, 2014. Poster presentation: The effect of feed additives on the intestinal microbiome and integrity of European sea bass (*D. labrax*) fed a sub-optimal diet. Peggs *et al*.

AQUA 2012. Combination of "Aquaculture Europe" and "World Aquaculture" meetings. Prague, Czech Republic, 2012. Poster presentation: Dietary induced changes to the intestinal morphology and microbiome of European sea bass (*D. labrax*). Peggs *et al*.

Probiotic and prebiotics in veterinary sciences and aquaculture: the future. Keele University, UK, 2012. Poster presentation: Dietary induced changes to the intestinal morphology and microbiome of European sea bass (*D. labrax*). Peggs *et al*.

Biomarine convention. International conference on: Algae in aquafeeds, Marine bioresources, Aquaculture: future perspectives and Marine biotechnologies. London, UK, 2012. Participated as a note taker and report writing on various think tank sessions.

Publications

Standen, B., Rodiles, A., **Peggs, D**., Davies, S., Santos, G. & Merrifield, D. (2015) Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*, following the application of a multi-species probiotic. *Applied Microbiology and Biotechnology*, 1-15.

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Chapter 1:

General introduction

1.1 Aquaculture: feeding a growing population

The human population is estimated to reach 9.3 - 9.6 billion by 2050 (Ezeh *et al*. 2012; FAO 2014). This increase, often fastest in developing countries, inevitably leads to an increase in demand for high quality sources of protein food stuffs. For centuries, a large part of this protein has come from living resources harnessed from the oceans. Today, seafood remains an essential protein source for a large number of cultures in many countries globally. However, poorly managed wild capture fisheries over the past 40 - 50 years has brought the industry to its knees, simply unable to supply the increase in global demand. The Food and Agricultural Organization of the United Nations (FAO) reported that 61 % of wild caught fish are fully exploited and 29 % are over-exploited with only 10 % under-fished. These figures show that most wild caught species have no potential for production increases (FAO 2014). Therefore, there is a real need to alleviate the stress put on struggling species from a moral standpoint, as well as supplying adequate protein to meet consumer demand. Aquaculture is an alternative with the potential to meet this demand for protein both from marine and freshwater habitats. In fact aquaculture, defined as the culture of all aquatic animals and plants in fresh, brackish and marine environments, is the fastest growing food producing sector worldwide (Bostock *et al*. 2010; Defoirdt *et al*. 2011). Estimated by the FAO to be worth US$ 130 billion in 2012, aquaculture contributed almost half of the fish for human consumption and remains the fastest growing food production industry (FAO 2014). The development and growth of aquaculture however, like all businesses, is determined by market demand, resource availability and profitability for major investors (Bostock *et al.* 2010). It is therefore vital that aquaculture growth and expansion remains sustainable and this is

especially important with regards to aquafeeds and disease prevention. A major constituent and protein source in aquafeeds is fishmeal (FM), harvested largely from pelagic fish species, the replacement of which is of high priority in order to ensure aquaculture remains sustainable as a growing industry (Olsen & Hasan 2012). Piscivorous species such as Atlantic salmon (*Salmo salar*) and European sea bass (*Dicentrarchus labrax*), among others, require high protein diets to grow healthily and efficiently in order to meet demand and remain profitable. Replacing FM with sources such as those harnessed from plant based products e.g. soybean, rapeseed and pea products among others has been a challenge for the aquaculture industry over the last few decades. Although the protein levels and amino acid profiles in plant based products in some cases are comparable to those in FM, anti-nutritional factors (ANF's) exist within the plant protein products which are known to negatively affect the growth and/or health of some important cultured species (Van den Ingh *et al*. 1991; Knudsen *et al*. 2007; Uran *et al*. 2008). Extensive research into feed technologies has increased and improved greatly year upon year. However, replacing FM to levels considered practical and sustainable is a long way from becoming a reality. In this respect the industry faces a challenging future, if it is to supply adequate nutrients in a balanced and sustainable manner.

1.2. European sea bass (*D. labrax*): biology, production, dietary requirements and issues

The European sea bass (*D. labrax*) is silver/grey in colour with lighter sides and underbody, has a thin elongate body, and is well adapted to its environment. A coastal species common to the British Isles, most of Europe and some of north and west Africa, the European sea bass is an efficient predatory species feeding on smaller fish, prawns and crustaceans. The species is eurythermic ranging from habitat temperatures of 5 - 28 ^{o}C, and euryhaline ranging from salinities of 3 ‰ to ~ 38 ‰, which allows them to utilise coastal, estuarine and brackish

waters (FAO 2014). The European sea bass was the first marine species, other than salmonids, to be commercially cultured in Europe and is recognised, together with gilthead sea bream (*Sparus aurata*) as one of the most important commercially produced fish species in the Mediterranean. Production in 2012 reached 153,182 tonnes and was valued at just over USD 1 billion (Figure 1.1.) with Turkey, Greece, Egypt, Spain, Italy, France and Croatia the largest produces (FAO 2014). In the wild, European sea bass reproduction takes place in the winter between December and March dependent on regional distribution and temperature (Asturiano *et al*. 2000; Vázquez, & Muñoz-Cueto 2014). Females exhibit high fecundity, producing approximately 200,000 eggs/kg of body weight, reaching sexual maturity at around 2 kg in weight which occurs at around 4 years of age (Haffray *et al*. 2007). In culture, egg production/release can be manipulated through temperature and photoperiod control, as well as the administration of hormones, in order to create broodstock throughout the year (Villamizar *et al*. 2012). The majority of production is carried out in sea cages but production in ponds and lagoons are not uncommon. European sea bass generally reach a commercially marketable size of 300 - 500 g in approximately 18 - 37 months depending on the method of production with the limiting factor being their natural feeding behaviour (Haffray *et al*. 2007; FAO 2014).

A piscivorous teleost, the European sea bass requires a diet high in protein and energy. Digestible protein levels of around 50 % are required in order to generate the desired growth, however these levels can be reduced somewhat provided that adequate digestible energy levels are provided. The essential amino acid requirements of European sea bass are similar to those of other finfish and crude lipid and digestible energy requirements are approximately 15 % and 19 KJ g^{-1} respectively depending on the life stage (FAO 2014).

A major issue faced by the aquaculture industry as a whole is the loss of stock due to infectious diseases which remains a significant and difficult challenge for the industry to

overcome. European sea bass are susceptible to a variety of diseases in culture and high stocking densities and / or stress levels increase the spread of these diseases which can have a significant negative impact on commercial production. Diseases associated with European sea bass include infections from viruses (i.e. nodavirus and picornavirus), bacteria (i.e. vibriosis, photobacteriosis, myxobacteriosis, and epitheliocystis), and parasites (i.e. cryptocaryoniasis, scuticociliatosis, myxosporidiosis, microsporidiosis, gill fluke infections, anisakis infection, and isopodiasis) (FAO 2014). The often irresponsible use of antibiotics to treat these and other bacterial diseases, has led to an increase in bacterial resistance which in turn has led to concerns relating to: possible direct and indirect toxicity to non-target organisms, pathogenic resistance to the compounds, lasting residues of some compounds in the surrounding environment and potential risks to the human population on a global scale (Cabello 2006; Defoirt *et al*. 2011). As a result of this, tighter regulations on the use of antibiotics globally and the ban of antibiotics as growth promoters in Europe has been applied (Regulation 1831/2003/EC).

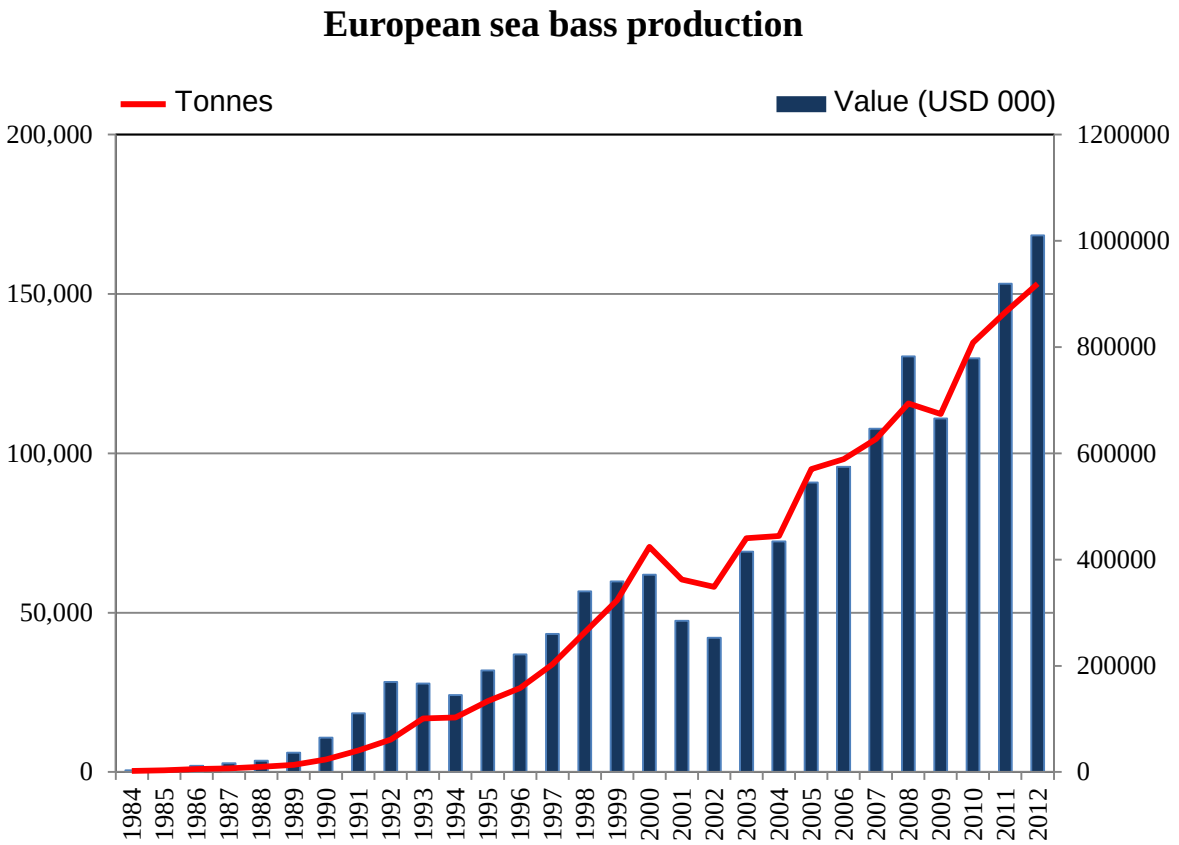


Figure 1.1. Aquaculture production and price evolution of European sea bass (1984-2012). Modified from FAO 2014.

1.3. Alternative feedstuffs for aquaculture

The aquaculture industry continues to rely heavily on FM as a primary ingredient source supplying much of the protein requirements of many high value cultured fish species. The high protein content, well balanced amino acid profile and good palatability and digestibility make FM an important and, in some cases essential, ingredient for the health and growth of the fish in aquaculture. This is particularly important with regards to piscivorous species such as salmonids, European sea bass and the gilthead sea bream. Due to their physiology and position as top predators in their respective environments, piscivorous fish require a diet high in protein and an amino acid balance representative of their diets in the wild. Therefore FM is

still a key ingredient used in the diets of piscivorous fish species in aquaculture. Issues regarding the price and sustainability of FM, have however dictated that alternative protein sources must be sought if the aquaculture industry is to continue growing at the current rate, as well as, remaining sustainable whilst doing so. In recent years alternative protein sources of interest have come from those produced by plant based products such as soybean products, rapeseed, barley, pea protein and lupin meal among others. Soybean products are perhaps the ingredient which has shown the greatest potential, and hence the greatest interest as a key source of protein and constituent in the diets of fish in aquaculture. This is largely due to the high protein content and relatively well balanced amino acid profile, as well as its competitive, stable price relative to FM (Figure 1.2.). However, like other plant based protein sources, soybean products contain ANF's which have been reported to cause various adverse effects on diet digestibility, growth and overall health of various fish species (Gatlin *et al*. 2007). These ANF's include components which interfere with digestion such as fibres, phytic acid and enzyme inhibitors, and components such as lectins and saponins, which interfere with permeability of intestinal membranes potentially affecting the influx and efflux of molecules and bacteria (Krogdahl *et al*. 2010).

Saponins are particularly important due to their presence in soybean products, typically in the range of 1-5 g kg^{-1}. Steroid or triterpenoid in nature, saponins are predominantly produced by plants, they are glycosides known for forming soap-like foams in aqueous solutions and are thought to be involved in plant defensive systems (Bouarab *et al*. 2002). One important aspect of saponins is their ability to cause membrane disruption in animals (Figure 1.3.). This action is known as haemolytic activity, due to their potential to cause lysis of erythrocytes in mammals (Augustin *et al*. 2011). In fish, saponins have been reported to cause adverse effects such as increasing gut permeability and increasing the onset of enteritis in salmonids (knudsen *et al*. 2007, 2008). Symptoms of enteritis include inflammation and subsequent

changes in gut morphology including increased vacuoles in enterocytes, shortening of mucosal folds and widening of the mucosal fold lamina propria (van den Ingh *et al*. 1991; Baeverfjord & Krogdahl 1996). However, it has been reported that intestinal inflammation as a consequence of saponin inclusion in salmonids is dependent on dose and/or the presence of other ANF's in the supplemented diets (Krogdahl *et al* 2010; Penn *et al*. 2011; Chikwati *et al*. 2012; Couto *et al*. 2014). Saponins have also been observed to significantly reduce feed intake and growth responses of Japanese flounder (*Paralichthys olivaceus*) when supplemented into diets at 3.2 and 6.4 g kg^{-1} (Chen *et al*. 2011) and play a role in influencing sex ratios to favour males in Nile tilapia (*Oreochromis niloticus*) (Francis *et al*. 2002a). Couto *et al*. (2014) reported no effects to the growth of juvenile gilthead sea bream fed diets containing purified soya-saponins at 1 g kg^{-1} and 2 g kg^{-1} after a 48 day feeding trial, but observed some structural effects to the intestinal mucosa which, the authors inferred, could compromise intestinal functionality. There is a paucity of information relating to saponin interactions in European sea bass health with only two papers, to the author's knowledge, published to date (Couto *et al*. 2015a, 2015b). The first of these studies supplemented soya-saponins into FM based diets for European sea bass (initial weights: 282 g) at a low dose (1 g kg^{-1}) and high dose (2 g kg^{-1}). Two more diets were formulated to contain phytosterols at low dose (5 g kg^{-1}) + the saponin low dose, and phytosterols at high dose (10 g kg^{-1}) + the saponin high dose. After 15 days on the experimental diets the authors reported that the high saponin dose caused a depression in maltase and alkaline phosphatase activity, as well as, some mild inflammation in the posterior intestine. Fish fed the low saponin dose did not exhibit a lower degree of inflammation after 15 days but increased severity was observed after 59 days of feeding on the experimental diets. Fish fed the high phytosterol + high saponin diets were also observed to exhibit a decrease in maltase activity and evidence of posterior intestinal inflammation. The authors also reported five - fold increases in the expression of the pro-

inflammatory gene interleukin-1 β (IL-1β) in fish fed the low and high dose saponin diets, after 59 days, compared to fish fed the control diet. Couto *et al*. (2015b) assessed the same diets in European sea bass juveniles (initial weights: 27 g) for 59 days analysing growth performance, intestinal histology and immunology, and digestive enzyme activity. The authors reported no changes in growth performance, digestive enzymes or gene expression. Mild intestinal morphological changes were observed only in fish fed the high saponin based diets. The results of these two studies reveal that European sea bass appear to exhibit a high tolerance to dietary saponins when supplemented into a FM based diet. There is limited information however, on the effects saponins, or indeed other ANF's, have on the intestinal microbiota of fish (Krodahl *et al*. 2010; Knudson *et al*. 2007, 2008), and to the author's knowledge no studies have assessed saponin effects on the intestinal microbiota of European sea bass. More research is required on this and other factors such as inclusion levels, species variation and life stage effects to validate the results of the aforementioned studies. Although these and many other questions are yet to be answered, it is clear that saponins and other ANF's have an influence on the nutritional health of fish species and are a significant factor in the consideration of alternative protein sources for finfish aquaculture.

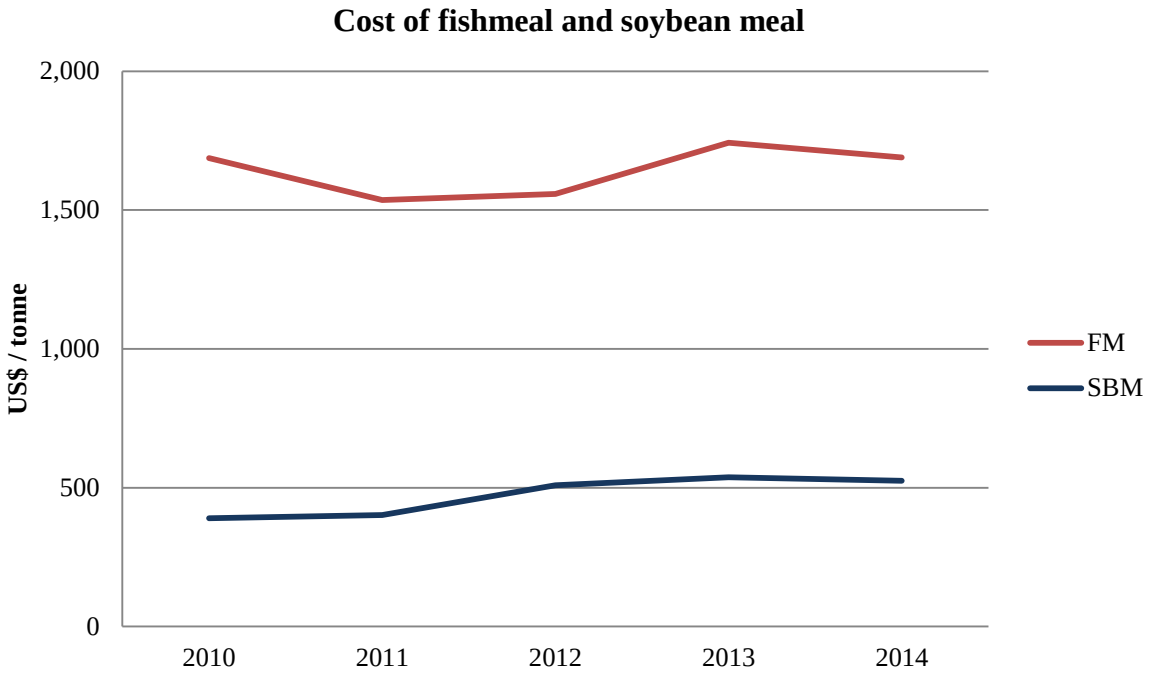


Figure 1.2. Price evolution of fishmeal and soybean meal (2010-2014). Modified from FAO 2014.

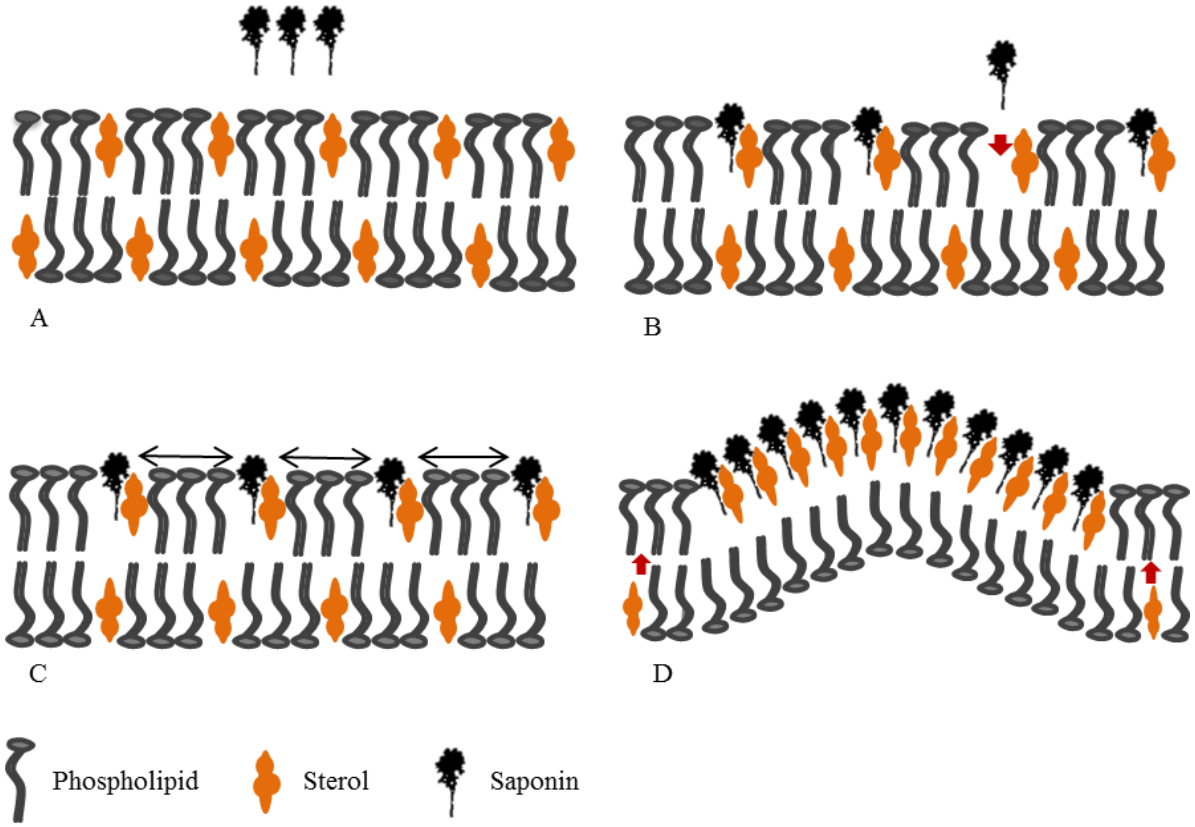


Figure 1.3. Schematic diagram showing the interactions between saponin molecules and biological membranes. Molecules of saponins penetrate into the lipid membrane (A) binding to sterols (i.e. cholesterol) in the membrane (B). The saponin molecules interact with the sterols and other saponin molecules (C). As more saponin molecules penetrate to form insoluble complexes with sterols an asymmetrical and curvature of the membrane is formed (D). Modified from Augustin *et al*. (2011).

1.4. Antibiotics in aquaculture

Prophylactic antibiotic applications are still used today in some parts of the world as growth promoters in aquaculture. Examples of antibiotics used in aquaculture practices today are displayed in Table 1.1. Antibiotics have two main mechanisms of action, either bacteriostatic (inhibition of bacterial growth) (e.g. penicillin, metronidazole and fluoroquinolones) or

bactericidal (killing of bacteria; e.g. tetracyclines, sulfonamides, chloramphenicol, erythromycin macrolides and sarafloxacin). Many antibiotics exhibit both of these mechanisms and bacteriostatic or bactericidal action is often determined by the dose administered and the bacteria targeted. The dose and treatment time required depends on the causative agent and severity of infection. Four main modes of action are employed by antimicrobials against bacteria which include: inhibition of bacterial cell wall synthesis, DNA synthesis, protein synthesis and folate synthesis (Kaufman 2011). Antibiotics have an essential role to play in aquaculture with regards to the control and treatment of diseases in fish, however problems arise when these chemicals are used consistently as precautionary measures even when diseases are not present. Regulations for antimicrobial treatment in aquaculture vary geographically; in Europe and North America for instance these regulations are relatively stringent but this is not the case for a significant proportion of finfish producing countries globally (Defoirdt *et al*. 2011). Effects of chemotherapeutic agents as disease treatments in aquaculture such as bacterial resistance and detrimental effects to surrounding environments have received increasing attention over recent years (Cabello 2006; Kim & Cerniglia 2009). There are two forms of bacterial resistance to antibiotics: inherent/intrinsic resistance, where a species is not susceptible to a specific antibiotic due either to a lack of affinity between the antimicrobial and the target bacteria so the antimicrobial cannot enter the cell, or an absence of the target in the cell itself. The second form is acquired resistance, where a bacterial strain is normally susceptible to an antimicrobial but some strains of the same bacteria are resistant. Resistance genes can be transferred to initially susceptible populations through lateral DNA transfer (transformation, transduction and conjugation), allowing the bacterial populations to adapt to the changes in conditions and potentially proliferate under the environmental conditions created by the antibiotic (Romero *et al*. 2012). However, understanding these mechanisms is only the first part in tackling the problem.

Genes and their hosts travel through their environments in complex patterns, and resistant genes can be captured in different bacteria and bacterial strains, and therefore tracking them becomes problematic (Marshall & Levy 2011).

The effect that antibiotics have on the gastrointestinal (GI) bacterial populations of fish is a relatively new area of research and our knowledge is therefore limited. Some studies have reported a shift and reduction in overall GI microbial diversity in fish exposed to antibiotic treatments (Bakke-McKellop *et al*. 2007; Sekirov *et al*. 2008; Naverrete *et al*. 2008). Bakke-McKellep *et al*. (2007) included experimental diets with oxytetracycline (OTC) at 3 g kg^{-1} conducted on Atlantic salmon reporting a significant decrease in bacterial populations in all intestinal regions. A greater reduction was observed in the allochthonous than autochthonous microbiota (500 - fold compared with 10 - fold log reductions respectively). The posterior intestine was affected to the largest extent in both digesta and mucosa samples followed by the mid intestine, with the anterior region least affected. Naverrete *et al*. (2008) also conducted a study on juvenile Atlantic salmon and using molecular microbiological techniques observed a reduction in GI bacterial diversity when treated with oxytetracyline at 0.75 g kg^{-1} per day. He *et al*. (2011) also reported a reduction in microbiota with the administration of an antibiotic this time using tilapia as the model. Juvenile hybrid tilapias were fed the growth promoting antibiotic florfenicol (0.02 g kg^{-1}) for a period of 16 weeks and although the treatment increased the growth of the fish, a significant reduction in GI microbiota was observed. Sequential decreases in intestinal microbial populations through antibiotic treatment might not come as a huge surprise as it is, after all what they are designed to do. However, when the reduction includes mutualistic and commensal bacteria as well as pathogenic bacteria this may have significant implications for the health of the host. More studies are necessary to elucidate which bacteria are actually effected and to what extent by

various antibiotic treatments. Furthermore, there is evidence to suggest routine use of antibiotics in sea based aquaculture systems has implications for the surrounding environment. A study by Buschmann *et al*. (2012) assessed the bacteria found in the marine sediment around a salmon farm in Chile. The authors reported significantly higher bacterial numbers in the sediment and fractions resistant to oxytetracycline, oxolinic acid and florfenicol were found in the sediment around the aquaculture site compared to the control site. The authors conclude that large scale use of antibiotics in Chilean salmon aquaculture may select for antimicrobial-resistant bacteria in the surrounding sediment.

In terms of human health, it is reasonable to assume that antibiotic use in aquaculture and the subsequent bacterial resistance could have direct and indirect implications (Cabello 2006; Sapkota *et al*. 2008; Marshall & Levy 2011; Aly 2014). Bacteria in the aquatic environment share a range of mobile genetic elements and resistant genes with terrestrial bacteria, and there is evidence to suggest the existence of horizontal gene transfer between bacteria from the aquatic environment and human pathogens (Cabello *et al*. 2013). Furthermore, there are a number of studies reporting the potential occurrence of antibiotic resistance genes to various commonly used antibiotics in aquaculture environments (Costello *et al.* 2001; Luo *et al*. 2010; Seyfried *et al*. 2010; Tamminen *et al*. 2010; Su *et al.* 2011; Gao *et al*. 2012). Many authors appear to share the sentiment that there is a lack of information regarding the use of antibiotics in aquaculture practices (Defoirdt *et al*. 2011; Marshall & Levy 2011; Cabello *et al*. 2013; Aly 2014). The aquaculture industry must manage the use of antibiotics as well as research alternatives for the control and prevention of diseases. Defoirdt *et al*. (2011) suggest a holistic approach would be the most effective long term measure, whereby the measure/measures take into account the pathogen, host and environment. Other potential disease control measures include: vaccines, bacteriophage therapies, probiotics, prebiotics,

synbiotics, phytobiotics and immunostimulants (Costello *et al.* 2001; Cabello 2006; Defoirdt *et al*. 2011; Muñoz-Atienza *et al*. 2013).

Table 1.1. Examples of antibiotics used in aquaculture practices today and mechanisms of actions. Modified from Serrano (2005) and Defoirdt *et al*. (2011).

Antibiotic	Class	Mechanism of action
Amoxicillin	Beta-lactams	Interfere with cell wall synthesis
Enrofloxacin	Fluoroquinolones	Interfere with nucleic acid synthesis
Erythromycin	Macrolides	Protein synthesis inhibition
Florfenicol	Amphenicols	Protein synthesis inhibition
Furazolidone	Nitrofurans	Interfere with nucleic acid synthesis
Oxolinic acid	Quinolones	Interfere with nucleic acid synthesis
Oxytetracycline	Tetracycline	Protein synthesis inhibition
Streptomycin	Aminoglycoside	Protein synthesis inhibition
Sulphadiazine	Sulphonamides	Inhibition of folic acid synthesis

1.5. Intestinal microbiota of fish

The microbiota or microbiome of animals, including humans, is a complex and diverse community, one which in recent times has become well-researched. The study of the intestinal microbiota was initiated after the discovery of *Escherichia* in the GI tract of humans in 1885 (Rajilić-Stojanović *et al*. 2007). This then paved the way for the study of microbes in the gut of various other species for many reasons of interest. Modern molecular techniques have enhanced the study of the GI tract of animals and revealed that these communities are more diverse and complex than was initially thought (Ley *et al*. 2008; Benson *et al*. 2010; Nayak 2010a). Furthermore, scientists soon realised that these host-microbe interactions are critical and play a crucial role in the health of the host (Young 2012). The intestinal microbiome and the gastrointestinal (GI) tract is a symbiotic relationship which

has co-evolved over time to create a complex community of microorganisms which are involved in several functions in animals, including: disease resistance, immunity, metabolism and digestion (Hooper & Macpherson 2010; Kaiko & Stappenback 2014; Lee & Hase 2014). These communities can be divided into two main groups: allochthonous or transient communities which pass through the GI tract with food, and autochthonous or resident communities which reside in the GI tract and are associated with the mucosal tissue (Ringø & Birkbeck 1999). However, it should be mentioned that there is likely to be some crossover between some bacterial species which could be allochthonous/autochthonous depending on conditions. Understanding the associations and the resulting host-microbe interactions is a vital part in assuring the welfare of the host. This is also true of fish, however a more intimate relationship with their surrounding environment differentiates them from terrestrial animals. The gut microbiota of fish is heavily influenced by its surrounding aqueous environment and is thought to be present in numbers of 10^7 - 10^8 bacteria (culturable) and 10^9 - 10^{11} (total) per gram of digesta (Sugita *et al*. 2005; Shiina *et al*. 2006; Pérez *et al*. 2010). The predominant phyla of the intestinal microbiota of marine fish include Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes and Fusobacteria (Gómez & Balcázar 2008). Common bacterial species present include those from the *Bacillus*, *Vibrio*, *Micrococcus*, *Clostridium*, *Bacteroides* and *Pseudomonas* genera, as well as various species of lactic acid bacteria (LAB) (i.e. *Lactobacillus*, *Enterococcus*, *Leuconostoc* and *Pediococcus*) which are thought to constitute an important part of this microbiota (Ringø & Gatesoupe 1998; Romero *et al*. 2014). Yeast, protozoa and viruses are also common constituents of the microbial communities in fish (Merrifield & Rodiles 2015).

Current information of the intestinal microbiota of fish is available through various reviews and studies of numerous farmed species, with a large portion of these studies focused on the commercially important salmonids including those by: Nayak 2010b; Hovda *et al*. 2012;

Hartviksen *et al*. 2014; Ingerslev *et al*. 2014a, 2014b; Ringø *et al*. 2014; Zarkasi *et al*. 2014; Romero *et al*. 2014). There is however a paucity of information regarding studies assessing the intestinal microbiota of European sea bass, with only a handful of studies, to the author's knowledge, published to date on this species (Kotzamanis *et al*. 2007; Carda-Diéguez *et al*. 2014; Gatesoupe *et al*. 2014; Delcroix *et al*. 2015).

Kotzamanis *et al*. (2007) assessed the effects of commercial fish protein hydrolysates (FPH) dietary inclusion (at 10 and 19 %) on growth, digestive enzymes and the intestinal microbiota. The study also assessed host resistance to the pathogen *V. anguillarum*. The authors report fish fed the diet 10 % FPH to exhibit the best growth, survival and intestinal development. The study also revealed cultivable *Vibrio* spp. to be dominant in samples of fish fed the high dose FPH (19 %) and this dose seemed to be more effective in terms of protection against *V. anguillarum* infection. The authors suggest this protection could be due to a barrier effect with the already present *Vibrio* spp. preventing the settlement of the invading pathogen, or the presence of certain *Vibrio* spp. may act as a probiotic and stimulate the immune system. The paper concludes by advising caution with regards to the use of FPH as a means of increasing the proliferation of *Vibrio* spp. in the intestine of European sea bass larvae.

Carda-Diéguez *et al*. (2014) used pyrosequencing to assess the intestinal microbiota of European sea bass fed two functional diets containing purified 1,3 and 1,6 β-glucans as immunostimulants. The authors reported two dominant genera associated with the autochthonous intestinal communities (*Dysgonomonas* and *Ralstonia*). *Dysgonomonas* levels significantly reduced in fish fed both β-glucan diets whereas levels of *Ralstonia* were observed to be significantly elevated in fish fed the 1,6 β-glucan diet compared to fish fed the control. The authors conclude that *Dysgonomonas* could be a common symbiont in the intestine of European sea bass and is involved in digestion. Furthermore, the increase in

Ralstonia found in fish fed the 1,6 β-glucan diet could be due to the presence of some growth promoting constituents contained in this diet which *Ralstonia* was able to utilise.

Gatesoupe *et al*. (2014) assessed the effects of dietary lupin, amylomaize, and waxy maize on the metabolic response and intestinal microbiota of juvenile European sea bass compared to fish fed a FM control. The study observed dietary changes to both allochthonous and autochthonous microbial communities with the dominant *Vibrio* spp. of the allochthonous communities being different depending on the diet. The authors also reported that the dominant *Vibrio* spp. differed between allochthonous and autochthonous communities regardless of treatment and the presence of *Clostridium* sp. was increased in fish fed the lupin diet. The bacterial dissimilarity profiles of fish fed the lupin meal and amylomaize were observed to be clearly separated when compared to the fish fed the control. The dissimilarity profiles of fish fed the waxy maize were not different to the control fed fish.

Delocroix *et al*. (2015) assessed the use of hydrolysates on European sea bass development and associated abdominal microbiota using polymerase chain reaction - denaturing gradient gel electrophoresis (PCR-DGGE). The authors reported that 3 of the 5 hydrolysate diets influenced the microbial communities, indicated by the ANOSIM dissimilarity values, which were significantly dissimilar to the control and within hydrolysates treatments. Three bacterial groups were observed to be particularly abundant and the main contributors to the dissimilarities between groups: Enterobacteriaceae, Moraxellaceae and Methylobacteriaceae. Unfortunately other operational taxonomic units (OTU's) were not identified by the authors. These studies supply a small insight into the microbial communities of European sea bass, however this area of research is clearly in need of further investigations.

1.6. The fish intestine and the role microorganisms play therein

1.6.1. Physiology and metabolism

The GI tract of fish is comparable to that of other vertebrates and has evolved to encompass a large variety of morphological organisation. This anatomical organisation aims to optimise nutrient utilisation and therefore is largely dependent on the diet of the fish. Fish can be categorised into four main classes depending on their diet: carnivorous/piscivorous (fish that primarily eat other fish/vertebrates and invertebrates), herbivorous (fish that primarily eat plants and algae), omnivorous (fish that eat a mix of other vertebrates, invertebrates and plant material), and detritivorous (fish that primarily eat detritus). However, these classes may change throughout the lifestyle of the species, and most species possess the ability to shift between feeding habits which is largely depending on the availability of resources (Olsen & Ringo 1997). The GI tract is a basic tube structure that stretches through the body of the fish from the mouth to the anus. This can be divided up into regions: foregut (mouth, gill arch, oesophagus, stomach and pyloric caeca), anterior intestine, mid intestine, and posterior intestine, however there are some deviations on this classification which depends largely on the dietary habits of the species (Harder *et al*. 1975; Falk *et al*. 2013; Ray & Ringø 2014). The primary function of the stomach is the storage of digested food, where acid is secreted however in the absence of this structure many species have developed a sac-like structure called the intestinal bulb for this process (Olsen & Ringo 1997). The pyloric caeca are finger-like projections which are extensions of the intestine. They are not present in all fish but in some species can account for as much as 70 % of the total intestine (Wulff *et al*. 2012). There is also a huge variation in the size of the pyloric caeca between the species which possess these structures. It is thought that the functions of the pyloric caeca are to increase absorptive surface area of the intestine and thus aid in the digestive process (Ray & Ringø 2014). Beyond the stomach/intestinal bulb and pyloric regions, the intestine is a simple cylindrical structure which continues to the anus. This organ is the primary site of digestion and absorption of feed, and plays a crucial role in the water electrolyte balance and endocrine

regulation, as well as, supporting metabolism and immunity. In order to contribute to these processes, the intestine must possess a large absorptive surface area. This is achieved with undulating structures known as mucosal folds which are lined with a brush border of microvilli (Figure 1.4). The digestive function of the intestine is thought to decrease from the anterior to posterior whereas goblet cells and hence mucus production increases (Ringø *et al*. 2003).

The development and functionality of the fish intestine depends on a number of factors such as anatomy, pH, osmolality and diet composition which help shape the microbiota (Ray & Ringø 2014). Gnotobiotic studies in fish have revealed the importance of the GI microbiota in various processes including: nutrient metabolism, providing the host with additional nutrient uptake capabilities of nutrients otherwise unobtainable to the host (Rawls *et al*. 2004; Bates *et al*. 2006; Ray *et al*. 2012; Beck & Peatman 2015); epithelial renewal and enterocyte morphology (Rawls *et al*. 2004); and immunology (Rawls *et al*. 2006, 2007). With regards to digestion, intestinal microbes have been observed to produce digestive enzymes, not produced by the host (e.g. amylase, cellulase, lipase and protease) contributing to this process in fish (Bairagi *et al*. 2002; Ramirez & Dixon 2003; Pérez *et al*. 2010). The intestinal microbiota also contributes by serving as a source of vitamins. Vitamins such as B12, have also been reported to be produced by certain GI microbiota (e.g. *Cetobacterium somerae*) of fish (Sugita *et al*. 1991), and requirements of this vitamin vary between species (Romero *et al*. 2014). Vitamin B12 is involved in erythrocyte development and fatty acid metabolism (Lin *et al*. 2010). The GI microbiota of fish also contributes to the fermentation of dietary fibre (particularly important in herbivorous species) which convert carbohydrates to short-chain fatty acids (SCFA's) (Romero *et al*. 2014). These SCFA's can be transferred directly into energy by epithelial cells making this process an important contributor to host energy needs in herbivorous species (Mountfort *et al*. 2002). The production of SCFA's has also been

observed to decrease pH in the intestine of herbivorous fish (Clements *et al*. 1997). In European sea bass, a study by Gatesoupe *et al*. (2014) assessing the effects of amylomaize and waxy maize on the intestinal microbiota, demonstrated that the highest levels of the SCFA acetate was exhibited in samples derived from these treated diets compared to the controls. The authors suggest that the maize products were, at least in part, metabolised by the intestinal microbiota.

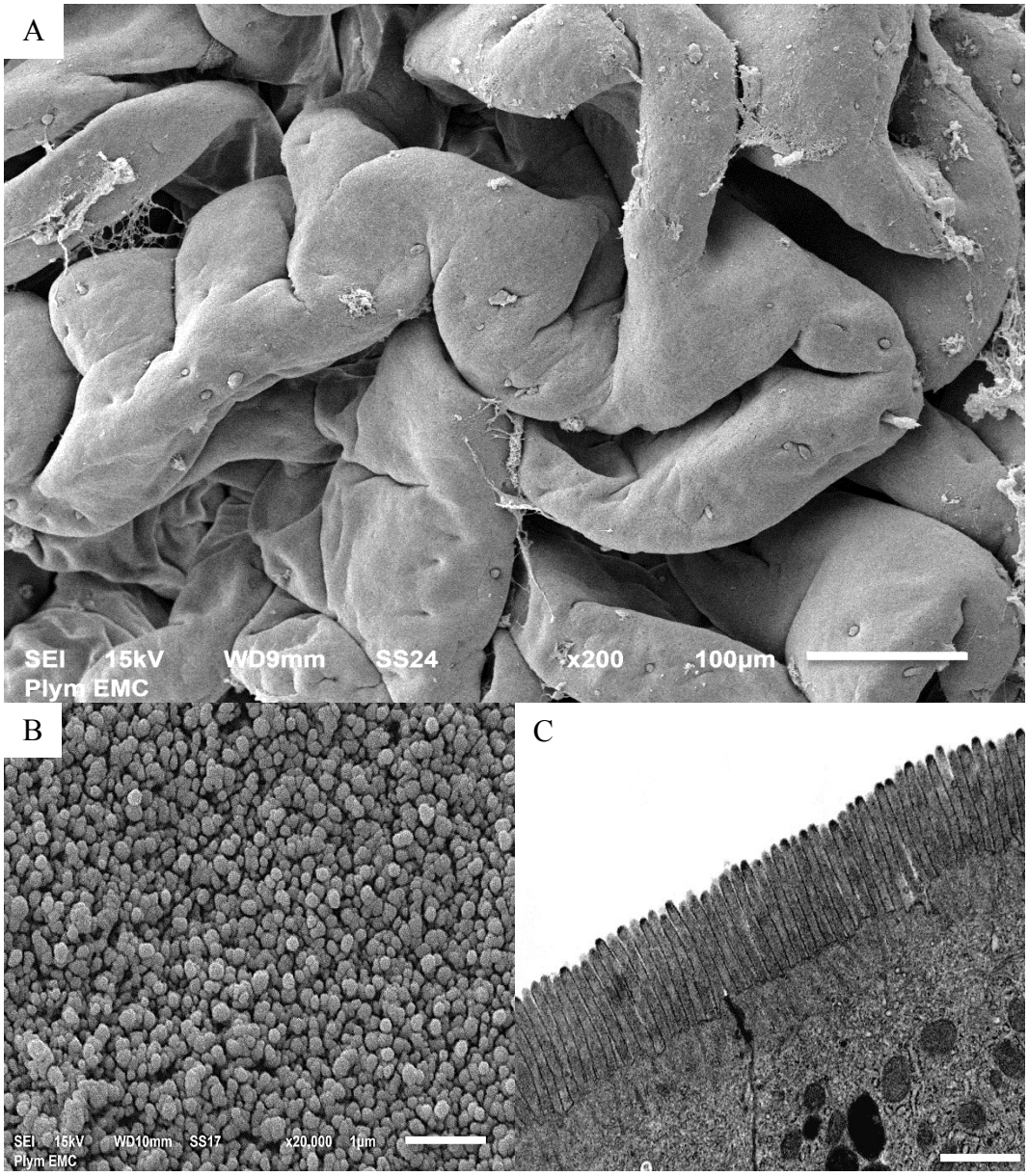


Figure 1.4. Electron micrographs of the posterior intestine of European sea bass, illustrating the mucosal folds (A) and microvilli (brush border) (B; SEM and C; TEM). Scale bars = 100 µm (A), and 1 µm (B&C).

1.6.2. Immunology

The intestinal microbiota are also recognized as key components of the fish immune system, contributing to mucosal barrier function, providing physical site-competition, as well as producing antimicrobial substances which help to protect the host against potential pathogens

(Salinas & Parra 2015). With the functionality of the digestive system (e.g. 2 - 3 days post hatch in European sea bass) (Zambonino Infante & Cahu 2001), the GI tract and associated microbiota become one of the most important interaction sites with the external microbial world. This is an antigen rich environment and consequently fish have evolved to possess an effective immune system early in their development, comparable to that of other vertebrates (Kiron 2012). The immune system of fish is therefore fundamental for defence and ultimately survival, and the intestinal microbiota play a crucial role in this system (Rombout *et al.* 2005). Intensive cross-talk interactions between the GI tract, associated microbiota and the environment depend on genetic, nutritional and environmental factors, and are integral to the development, function and maintenance of the intestinal mucosa (Montalto *et al.* 2009). The mucosal surfaces are the main sites of these interactions and are widely considered to be the first line of defence against disease. The mucus layer covers the intestinal epithelium and contains various protective substances produced by epithelial cells such as complement components, mucins, enzymes, piscidins, and defensins (Austin 2006; Kuppulakshmi *et al.* 2008). Anatomically the intestine is known collectively as the gut associated lymphoid tissue (GALT), and in fish this organ lacks specialised immune structures found in other terrestrial vertebrates such as peyer's patches. However, the gastrointestinal associated lymphoid tissue (GALT) in fish consists of lymphoid cells, macrophages, granulocytes and mucus immunoglobulin M (IgM), and various mucosal antibodies (i.e. IgM, IgT/IgZ, IgF) constituting the immune function which must distinguish between innocuous and harmful bacteria and initiate the appropriate responses (Chistiakov *et al.* 2007; Nayak 2010). This non-specific response, in ideal circumstances, would be followed by an antigen-specific adaptive response as a more comprehensive mechanism for the defence against a pathogen. The adaptive immune system of teleosts however, acts at a relatively slow rate compared to

that of higher vertebrates due, at least in part, to lower surrounding environmental temperatures, and the poikilothermic nature of fish (Whyte 2007; Foey & Picchietti 2014).

Commensal microbes, or microbiota, present in the intestinal muscosal tissue and surrounding mucus play a significant role in the factors that contribute to intestinal immune responses in fish. These complex communities are present in extremely high numbers and have even been suggested to be an extra organ in their own right (O'Hara and Shanahan 2006). The gut microbiota play an important role in the development of the host GI tract including those related to metabolism and immune functionality. Gnotobiotic work carried out by Rawls *et al*. (2004) on zebra fish (*Danio rerio*) has revealed some interesting insights into the influential roles these microbial communities play in the intestine, including those involved in the expression of certain immune related genes. For example, microbiota-associated responses were reported for the homologue of the mouse serum amyloid A1 (Saa1), C-reactive protein (Crp), complement component 3 (C3), and suppressor of cytokine signalling 3 (Socs3), and myeloperoxidase (Mpo).

The complex host-microbe interactions which occur at the intestinal barrier are only partly described in fish and the mechanisms involved therein are therefore poorly understood. However, fish are known to share certain molecules and immune processes with mammals where the depth of knowledge of this topic is far greater. The expression of pattern recognition receptors (PRR's) are responsible for the detection of microbes at the mucosal interface. Perhaps the most demonstrative of these receptors in fish are those belonging to the toll-like receptor (TLR) family. These receptors are involved in the recognition of bacterial lipopolysaccharides (LPS) and pathogen-associated molecular patterns (PAMP's) or commensal-associated molecular patterns (CAMP's). TLR recognition triggers a series of molecular pathways which include adaptor molecules such as Myd88, TRIF and SARM, transcription factor molecules such as NFκB and IRF3/7, which in turn lead to the production

of cytokines (Tlaskalova-Hogenova *et al.* 2005). Commonly studied cytokines in fish include those involved in the inflammatory response: tumor necrosis factor-α (TNF- α), IL-1β, IL-8, and IL-10. For a comprehensive review of the inflammatory pathways in fish refer to (Foey & Picchietti 2014).

With regards to probiotic (refer to section 1.7.1) applications, a number of studies have reported probiotic-associated immune responses in the GI tract of fish. Picchietti *et al*. (2009) demonstrated probiotic (*Lactobacillus delbrueckii*) -induced increases in intestinal T-cells and total body T-cell receptor-β (TcR-β) transcripts in European sea bass larvae. Transcripts of IL-1β were significantly reduced in the probiotic group intestine, and trends towards lower IL-10, Cox-2 and transforming growth factor-β (TGF-β) in the probiotic group were also reported. Multiple T-cells have been observed in fish including helper and cytotoxic T cells which are essential in cell-mediated immunity and tolerance responses (Foey and Picchietti 2014). IL-1β is one of the earliest studied pro-inflammatory cytokines which may be triggered by pathogens inducing an inflammatory response (Seppola *et al.* 2008). The anti-inflammatories TGF-β and IL-10 are important in terms of mucosal tolerance maintenance which may be expressed in the absence of pathogens. Another study focused on rainbow trout fed the probiotic *Lactobacillus plantarum*, which were subsequently challenged with the pathogen *Lactococcus garvieae* (Pérez-Sánchez *et al*. 2011). The study reported a significant up-regulation of the mRNA levels of intestinal IL-10 and IgT following *L. garvieae* infection. The authors also reported no detectable levels of either the probiotic or pathogen bacteria using PCR-DGGE and suggest direct tactile host-microbe interactions may not be necessary to induce host immune stimulation. To the author's knowledge, only three isotypes of immunoglobulins, which are important components of the humoral immunity, have been identified in fish to date: IgM, IgD and IgT/IgZ. IgT, similar to IgA in mammals, is a specialised antibody involved in mucosal function, commonly found in fish (Gomez *et al.*

2013). Zhang *et al*. (2010) demonstrated that IgT was detected in the intestinal mucus only in rainbow trout, and coated most bacteria in response to a parasite.

The intimate relationship that teleosts share with their antigen-rich aquatic environment has demanded the evolution of an effective immune system. Similar to other vertebrates fish possess innate and adaptive responses to pathogen insults through the production of a variety of immunoglobulins, antimicrobial peptides and inflammatory cytokines. The understanding of the mechanisms surrounding the innate immunity in fish is relatively well documented however, the mechanisms surrounding the adaptive immunity in fish is comparatively less well studied. The intestinal microbiota clearly play an important role in fish host immunity, however these complex host-microbe interactions are far from being completely understood, and this area warrants further investigation.

1.7. Feed additives: alternative strategies to antibiotics

1.7.1. Probiotics

Probiotic applications are an alternative approach to antibiotics for creating a healthy intestinal environment by influencing a host's microbiota. The term probiotic comes from the Greek words "pro" and "bios" simply meaning "for life", (Zivković 1998). The most widely cited definition of a probiotic was proposed by Fuller *et al*. (1989) as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". Despite continual contention Fuller's definition of the term is still widely referred to today. However, when referring to probiotic applications in aquatic systems it is important to consider certain factors which are fundamentally different from those applied to terrestrial probiotic use. The epithelium and mucosal barrier of the skin, gills (in fish) and alimentary tract are an extremely important barrier against disease in animals. In terms of fish, rearing

water generally supports a higher microbial load and the intimate relationship fish share with this potentially antigen rich environment, means a distinct definition is needed (Kesarcodi-watson *et al*. 2008; Magnadottir 2010). Merrifield *et al*. (2010) therefore suggested the following broad definition for aquatic animals: 'any microbial cell provided via the diet or rearing water which subsequently benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish'. The range of probiotics used in aquaculture is quite broad and has encompassed both Gram-positive and Gram-negative bacteria as well as yeasts and unicellular algae (Irianto & Austin 2002). The benefits of probiotics are also quite broad and incorporate potential inhibition of pathogenic microorganisms, improvement of growth performance or feed utilisation and increased immune responses (Verschuere *et al.* 2000; Spanggaard *et al.* 2001; Bairagi *et al.* 2004; Yanbo & Zirong 2006; Ringø 2008). Lactic acid bacteria (LAB) are perhaps the most widely used bacteria for probiotic applications in human and terrestrial animals. LAB are also known to be abundant in the intestine of healthy fish and therefore are commonly used probiotics in aquaculture (Ringø & Gatesoupe 1998; Kesarcodi-watson *et al*. 2008; Gatesoupe 2008). LAB commonly used in aquaculture are species from the *Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Pediococcus*, *Enterococcus* and *Streptococcus* genera. Other probiotic candidates including species from the genera: *Aeromonas*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Roseobacter*, *Vibrio*, *Clostridium*, and the yeasts *Debaryomyces* and *Saccharomyces* among others, have also been assessed in aquaculture (Nayak 2010a; Merrifield *et al*. 2010a; Lauzon *et al*. 2014). Microbial modulation in the intestine of fish induced by dietary probiotic applications has being commonly reported. Sun *et al*. (2012) demonstrated a modulation in the autochthonous intestinal microbiota of juvenile grouper (*Epinephelus coioides*) fed *Lactococcus lactis* MM1 for 60 days. The authors used PCR-DGGE to assess these effects, observing distinct clustering of replicates within the samples of

the probiotic fed fish which were different from those of the controls. The study also revealed separate clusters depending on the intestinal region and an increase in the species richness and diversity in all intestinal regions in fish fed the probiotic. Furthermore, the authors reported the growth of some potentially beneficial bacteria, as well as a decrease in the presence of the potential pathogen *Staphylococcus saprophyticus*, as a consequence of probiotic feeding. In contrast to increased intestinal microbial species richness and diversity induced by the probiotic in the aforementioned study, a number of studies have reported a decrease in these parameters. Furthermore, the number of observed species has also been reported to decrease as a consequence of dietary probiotic administration in the fish intestine. Ferguson *et al*. (2010) reported a decrease in the number of observed species as well as a decrease in species richness and diversity in the allochthonous intestinal populations of tilapia (*Oreochromis niloticus*) fed the probiotic *Pediococcus acidilactici* compared to fish fed a control after a period of 32 days. Yang *et al*. (2012) reported a marginal reduction in species richness and diversity in the autochthonous microbial populations of the mid and posterior intestinal regions of grouper (*Epinephelus coioides*) fed *Bacillus clausii* after 60 days compared to a control. Interestingly the opposite was observed in the microbial populations of the anterior intestinal region. The authors also reported the stimulation of some potentially beneficial bacteria (*Enterococcus* sp. -like and *Bacillus pumilus* -like), as well as, a reduction in potential harmful bacteria (*Staphylococcus* sp. –like and *Vibrio ponticus* –like) in samples of fish fed the probiotic diet compared to those fed the control. Cerezuela *et al*. (2013b) assessed the effects *Bacillus subtilis* and inulin on the intestinal morphology and allochthonous microbiota of gilthead sea bream after four weeks. The authors reported significant decreases in specific richness in samples of fish fed *Bacillus subtilis*, inulin and *Bacillus subtilis* + inulin compared to those fed the control. Shannon's diversity was also observed to be significantly reduced in fish fed the *Bacillus subtilis*, and *Bacillus subtilis* +

inulin diets compared to fish fed the control. Suzer *et al*. (2008) demonstrated beneficial effects, with the application of commercial *Lactobacillus* spp. (*Lactobacillus plantarium*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, and *Lactobacillus rhamnosus*) probiotic to live feeds and the rearing water of gilthead sea bream. Fish fed the probiotic treatments showed a significant increase in the specific activities of pancreatic and intestinal enzymes and an increase in both survival and specific growth rate when compared to fish fed the controls. Another study by Zhou *et al*. (2010) revealed that *Lactococcus lactis* RQ516 had the ability to inhibit the growth of the fish pathogen *Aeromonas hydrophila in vitro*. The study also observed the probiotic fed fish exhibited a significant increase in the total protein and globulin concentrations in the blood serum of tilapia (*Oreochromis niloticus*) as well as significant increases in immune parameters for respiratory burst activity, lysosome content, myeloperoxidase and superoxide dismutase activity when compared to the control fed fish. Bacterial regional specificity in the gut is also an important aspect and one which must be taken in to account when selecting a probiont. An *in vitro* study by Lazado *et al*. (2011) observed that two candidate probiotic bacteria (*Pseudomonas* sp. and *Psychrobacter* sp.) applied to intestinal epithelial cells (IEC's) of Atlantic cod adhered to different intestinal cells. The authors report that the probiotics applied exhibited the potential to interfere with the adhesion of two pathogens, *V. anguillarum* and *Aeromonas salmonicida*. The adhesion of *V. anguillarum* was affected through competition by *Psychrobacter* sp. and although affected by *Pseudomonas* sp., the mode of action was not determined. The potential pathogen *A. salmonicida* was affected through competition by *Pseudomonas* sp., while exclusion mechanisms were favoured by *Psychrobacter* sp.

Merrifield *et al*. (2010) assessed the probiotic potential of *Bacillus subtilis*, *Bacillus licheniformis* and *Enterococcus faecium* on the growth performance, feed utilisation, and intestinal microbiota of oxolinic acid treated rainbow trout for 10 weeks. The authors

reported significantly improved feed conversion ratios, specific growth rates, and protein efficiency ratios in fish fed *B. subtilis* + *B. licheniformis* compared to fish fed the control. The authors conclude, suggesting that the probiotics used here could create a stabilizing effect, and could strengthen the intestinal microbiota post antibiotic treatment. Another finding of the study was an increase in intestinal in leukocyte levels in fish fed the *Bacillus* diets as well as those fed the *Bacillus* + *E. faecium* compared to the control. Standen *et al*. (2013) also observed significantly elevated intestinal leukocyte levels, as well as increased intestinal goblet cells in tilapia fed *Pediococcus acidilactici* for six weeks when compared to the control. The authors also reported an increase in the gene expression of TNFα in fish fed the probiont at week six and suggest these collective results could be an indication of an epithelium in an increased immunological state induced by probiotic feeding.

There is in fact growing evidence that probiotics do indeed positively influence the immune response in fish, due in part at least, to the components produced by the probiont which are thought to interact with the GALT generating immune responses (Pérez *et al*. 2010; Dimitroglou *et al*. 2011). Andani *et al*. (2012) evaluated the effects *Lactobacillus casei* and *Lactobacillus plantarum* on the growth performance, immune response and antagonistic potential against *Yersinia ruckeri* in the diets of rainbow trout. The authors reported significant increases in total weight gain and SGR's of fish fed the probiotic diets compared to fish fed the control. Lysozyme activity, alternative complement activity and total immunoglobulin levels were also significantly increased in fish fed *L. casei* compared to fish fed the control. Furthermore, percentage survival rates were significantly greater in fish fed the probiotic diets compared to those fed the control after being challenged with *Y. ruckeri*. Pérez-Sánchez *et al*. (2011) investigated the effects LAB: *Lactobacillus plantarum*, *L. lactis* and *Leuc. mesenteroides* on the expression of the immune related genes: IL-1β, IL-8, IL-10 and TNFα (head kidney), and IL-8, Tlr5 and IgT (intestine) of rainbow trout fed the diets for

36 days. The authors reported a significant up-regulation of mRNA levels of IL-10, IL-8 and IgT in samples of fish fed *L. plantarum* compared to those from the control. The study also reported significantly higher mRNA levels of IL-10, IL-8 and IgT in fish fed *L. plantarum* compared to fish fed the control after fish were challenged with the pathogen *Lactococcus garvieae*. The authors concluded that *L. plantarum* has the ability to stimulate the immune response in rainbow trout; however, interestingly the authors observed undetectable levels of either probiotic or pathogen in samples of the posterior intestine using PCR-DGGE analysis. Balcázar *et al*. (2008) demonstrated a higher survival rate of brown trout (*Salmo trutta*) as a result of probiotic (*Lactococcus lactis* CLFP 100 and *Leuc. mesenteroides* CLFP 196) application in fish subjected to temperature stress. The authors also observed that fish fed the probiotics had higher phagocytic cell activation in the head kidney and decreased levels of the pathogen *Aeromonas salmonicida* compared to control fed fish. Another study on brown trout, Balcázar *et al*. (2007a) assessed the effects several LAB (*Lactococcus lactis* ssp, *Lactococcus lactis*, *Lactobacillus sakei* and *Leuc. mesenteroides*) on the humoral response, administered to the feed at 10^6 CFU g^{-1}. Fish were fed the probiotic diets for two weeks and switched back to a non-probiotic diet for another two weeks. When compared to the control group the authors observed the LAB fed fish to exhibit significantly elevated complement activity in the serum after two weeks. At the end of the third week significantly higher lysozyme activity was observed in fish fed *Lc. lactis* ssp, *Lc. lactis*, and *Leuc. mesenteroides* compared to the control fed fish. These LAB were also assessed by Balcázar *et al*. (2007b) in the diets of rainbow trout at the same levels for two weeks. Similarly, the authors reported an increase in resistance to the pathogen *A. salmonicida* in fish fed the probiotic diets. Fish fed the probiotics also exhibited significantly elevated phagocytic activity in the head kidney and alternative serum complement activity when compared to the fish fed the controls after two weeks. A number of other studies have reported immune modulation upon probiotic

application in rainbow trout (Panigrahi *et al*. 2004, 2005, 2007, 2010, 2011; Kim & Austin 2006; Newaj-Fyzul *et al*. 2007; Korkea-aho *et al*. 2011, 2012; Min *et al*. 2012). Probiotic studies in European sea bass are limited to only a few and these include studies by: Carnevali *et al*. 2006; Silvi *et al*. 2008; Abelli *et al*. 2009; Tovar-Ramírez *et al*. 2010; Touraki *et al*. 2010; Sorroza *et al*. 2012. Collectively these studies have demonstrated the potential of various candidate probiotics to modulate the sea bass intestinal microbiota (Silvi *et al*. 2008), improve growth and immunological parameters (Carnavali *et al*. 2006; Abeli *et al*. 2009; Tovar-Ramírez *et al*. 2010), and directly increase disease resistance to the pathogen *V. anguillarum* (Touraki *et al*. 2010; Sorroza *et al*. 2012). With the exception of the study by Silvi *et al*. (2008), the effects that probiotics have on the intestinal microbial communities of European sea bass, and how these effects influence the overall health of the host remains scarce and is an area in need of exploration.

1.7.2. Prebiotics

Prebiotics are also thought to offer potential beneficial health promoting effects to various fish species (Burr *et al.* 2005; Ringø *et al.* 2010). These feed additives are non-digestible feed components which may selectively stimulate potentially beneficial bacteria, thus improving host health (Gibson *et al*. 1996). Specifically, Gibson *et al*. (2004) defined prebiotics as: any foodstuff that reaches the colon, (e.g. non-digestible carbohydrates, some peptides and proteins, as well as certain lipids). Prebiotic carbohydrates can either be oligosaccharides and polysaccharides with inulin, mannanoligosaccharides (MOS), fructooligosaccharides (FOS) and galactooligosaccharides (GOS) being the most commonly used prebiotics in animal feeds, including feeds for various fish species (Ringø *et al*. 2010; Cerezuela *et al*. 2011). Prebiotics have the ability to influence the microbial community within the host, decreasing intestinal

pathogens and replacing them with beneficial bacteria such as *Lactobacillus* and *Bacillus* spp. Beneficial effects of prebiotics have been observed in mammals and are relatively well documented; however, studies relating to prebiotic applications in fish have only recently been explored (Burr *et al*. 2005; Dimitroglou *et al.* 2011).

Prebiotic studies in fresh water species have revealed some promising results in terms of growth and intestinal health (Staykov *et al*. 2007; Grisdale-Helland *et al*. 2008; Ortiz *et al*. 2013; Dimitroglou *et al*. 2011). In the study by Staykov *et al*. (2007), improved FCR's and growth, as well as increased lysozyme and complement activity were observed in fish fed a diet containing MOS at 2 g kg^{-1} compared to fish fed a control diet. MOS was also supplemented into Atlantic salmon diets in the study by Dimitroglou *et al*. (2011). The prebiotic dose was 4 g kg^{-1} in this study conducted on Atlantic salmon (smolts). The authors reported significantly increased body protein composition, as well as significantly improved absorptive surface area and microvilli density in the anterior intestine when compared to fish fed a control after 14 weeks feeding. In a 4 month study Grisdale-Helland *et al*. (2008) assessed the use of MOS, FOS in the form of inulin and GOS at 10 g kg^{-1} in the diets of Atlantic salmon. The study reported significant increases in feed efficiency (5 %) and energy retention (6 %) in fish fed the FOS relative to fish fed the control. The study also reported 11 % lower routine oxygen consumption, 5 % lower protein and 3 % higher energy concentration in whole-body and 7 % greater energy retention in fish fed the MOS supplemented diet, which were significant increases when compared to fish fed the control. Nitrogenous and energetic losses in the non-faecal nitrogen excretions were significantly higher in fish fed the GOS diet (11 and 7 % respectively) compared to fish fed the control. Additionally, the protein concentration in the wet body composition and protein retention was significantly reduced (6 and 9 % respectively) in fish fed the GOS compared to those fed the control. Neutrophil oxidative radical production and serum lysozyme activity was

significantly lower in fish fed the MOS supplemented diet compared to fish fed the control. The authors suggest the results obtained in this study to be mostly positive but indicate further research is required to assess optimal dosage and how these feed additives affect the health of fish challenged by bacterial infection or other stressors. Indeed, prebiotic dose must be a major consideration for optimum beneficial effects. Low doses may fail to confer positive effects and conversely high doses may be detrimental to the host health. High doses of inulin (150 g kg^{-1}) have been reported to be detrimental in terms of intestinal health of Arctic charr (*Salvelinus alpinus*) (Olsen *et al*. 2001). Enterocyte disruption and microvilli disorder in the posterior intestine were reported after 4 weeks feeding. Dose dependant resistance to pathogens have also been reported in fish. Ebrahimi *et al*. (2012) demonstrated this in common carp challenged with *Aeromonas hydrophila*, with lowest mortality rates observed in fish fed the 1.5 g kg^{-1} (Immunogen®) dose, compared to fish fed the 0.5, 1, and 2.5 g kg^{-1} doses. Akrami *et al*. (2012) also assessed dose of MOS supplementation in the diets of carp. The study investigated the effects 1, 2 and 3g kg^{-1} doses had on the growth, survival, body composition and some haematological parameters of juveniles fed the diets for 45 days. The authors reported significant increases in haematocrit and lymphocyte levels in fish fed the 1 g kg^{-1} dose compared to fish fed the control. Growth performance and carcass composition were also improved in fish fed the 1 g kg^{-1} MOS however, not significantly. The other parameters measured were unaffected by dietary dose. Additionally, there is further evidence that dietary prebiotics (MOS) supplementation increases the disease resistance of crucian carp (*Carassius auratus gibelio*) to the pathogen *A. hydrophila* (Zhou *et al*. 2009; Liu *et al*. 2013). MOS (1 and 2 g kg^{-1}) supplementation has also been observed to increase serum lysozyme and superoxide dismutase activities in this fish species (Zhou *et al*. 2009). The authors also report increased growth parameters in fish fed the prebiotic diets. Digestive enzyme activity has also been observed in crucian carp fed xylooligosaccharides (XOS) (Xu

et al. 2009), and increased weight gain and increased serum lysozyme and complement activities in common carp (*Cyprinus carpio*) as a consequence of MOS (2 g kg^{-1}) supplementation. There are a number of other studies assessing the effects prebiotics have on the health of various other carp species (Andrews *et al*. 2009; Lochman *et al*. 2009, 2010; He *et al*. 2011; Lin *et al*. 2012).

Prebiotic studies have also been applied to various marine fish species with beneficial effects reported. Dimitroglou *et al*. (2010) assessed the use of MOS (2 and 4 g kg^{-1}) supplemented in to the diets of sea bream of either a FM based diet or a diet with partial FM replacement with SBM for 9 weeks. The authors reported no differences in the mean final weight, SGR, FCR and protein efficiency ratio (PER) as a consequence of MOS supplementation in either FM or SBM fed fish. Other results obtained revealed a significantly lower condition factor (*K*) and hepatosomatic index (HSI) in fish fed the MOS supplemented diets of the FM based feed compared to the FM control. These parameters remained unaffected in fish fed the SBM based feed. Histological analyses revealed MOS to seemingly improve the absorptive surface area in the posterior intestine of fish fed the FM based diet. Furthermore, microvilli density and lengths were increased in the anterior and posterior regions of fish fed the MOS in both FM and SBM based diets. Microbiological analyses indicated MOS supplementation also influenced the allochthonous intestinal microbiota by increasing species richness and diversity in fish fed the FM based diets. MOS seemed to exerted little effect on the allochthonous microbial populations of fish fed the SBM based diets.

Only a small number of studies have assessed the effects of prebiotic supplementation on marine species immunity and direct protection against pathogens. In a study on Atlantic cod, Lokesh *et al*. (2012) assessed the effects of MOS (1 g kg^{-1}) on the expression of pro-inflammatory and anti-inflammatory cytokine genes in the intestine prior to, and post *V. anguillarum* infection. The study reported a significant increase in the relative mRNA gene

expression of the inflammatory cytokine IFNγ, prior to the pathogen exposure, in the anterior intestine of fish fed the prebiotic compared to fish fed the control. Post infection, prebiotic fed fish exhibited significantly increased IL8 mRNA expression in the rectum, and significantly increased IL 1β mRNA expression in the posterior intestine and rectum. In this study, MOS appeared to influence the intestinal response to *V. anguillarum* infection which may confer beneficial effects against this pathogen. A study by Cerezuela *et al*. (2008) found that dietary inulin (5 and 10 g kg^{-1}) produced significant inhibition in phagocytosis and respiratory burst in leucocytes in sea bream after one week when compared to fish fed a commercial control diet. Inulin was also reported to confer some immune-stimulating effects in another study by Cerezuela *et al*. (2012). The authors evaluated the effects that inulin (10, 15 and 30 g kg^{-1}) had on some immune parameters, immune-related gene expression and protection against *Photobacterium damselae* subsp. *piscicida* in gilthead sea bream. The authors reported 10 g inulin kg^{-1} to be the optimal dose which stimulated the serum complement activity, IgM levels and the leukocyte phagocytic activity after two weeks. Inulin also stimulated leukocyte respiratory burst activity but had no affect on immune related gene expression in the head kidney, and did not increase survival when fish were challenged with the pathogen. Another study revealed inulin supplementation effected the intestinal gene expression, with the expression of IL8, β-actin, occludin, and transferrin observed in sea bream fed the experimental diet when compared to fish fed a control (Cerezuela *et al*. 2013a). Immunity in marine fish as a consequence of prebiotic applications is an area of research warranting further investigations. Inulin however, has also been observed to negatively affect intestinal morphology in sea bream and induce changes in the intestinal microbial communities. Cerezuela *et al*. (2013b) observed signs of microvilli and enterocyte damage in fish fed inulin (10 g kg^{-1}) compared to fish fed a control diet. The authors also reported some microbiological parameters. Numbers of OTU's and the range-

weighted richness were significantly reduced in fish fed the inulin diet compared to fish fed the controls. Mahious *et al.* (2006) investigated the effect of dietary inulin, oligofructose and lactosucrose on the growth and intestinal bacteria of the marine turbot (*Psetta maxima*). The study observed the mean weight of larvae weaned on oligofructose to be significantly higher than those weaned on the other diets. A strain of *Bacillus* was observed to constitute 14 % of the total load of bacterial isolates in turbot weaned on the oligofructose. The authors suggest the presence of *Bacillus* sp. may be stimulated by oligofructose and might confer health benefits to the host. Indeed some *Bacillus* spp. are used as probiotics in the diets of fish, however the study did not identify this to species level. Although these last two studies investigated some microbiological aspects, there is a distinct lack of information relating to the effects that prebiotics have on the intestinal microbiota in marine fish species. More research into this and other areas of marine fish health are fundamentally important if the feed formulations are to be effective in culture going forward.

In European sea bass there are a very limited number of studies assessing the effects of dietary prebiotics on the health of this species (Torrecillas *et al*. 2007, 2011a, 2011b, 2012, 2013, 2015; Guerreiro *et al*. 2015). Torrecillas *et al.* (2011a) investigated the effects of dose dietary administration of MOS on growth, digestibility, liver morphology, lipid and carbohydrate metabolism enzymes, organoleptic properties, immune parameters and GI tract mucus production in European sea bass. The authors reported dietary inclusion of MOS improved feed utilization together with a reduction in feed intake. Furthermore enhanced phagocytic activity of head kidney leukocytes in fish fed 4 and 6 g kg^{-1} MOS diets at days 30, 45 and 60 was also observed. Additionally, Torrecillas *et al*. (2012) observed increased disease resistance to *V. anguillarum*, and improved innate immunological responses in sea bass fed a diet containing MOS (4 g kg^{-1}). In another study Torrecillas *et al*. (2013) reported European sea bass fed 4 g kg^{-1} MOS to exhibit significantly increased weight gain and SGR

compared to fish fed a control. Fish fed the prebiotic were also reported to exhibit significantly higher prostaglandin production, significantly reduced neutral lipid fraction, and significantly increased polar lipid fraction when compared to fish fed the control. Microscopy analyses also revealed fish fed the prebiotic to exhibit increased goblet cell's and infiltrated eosinophilic granulocytes and generally a better presented epithelium when compared to fish fed the control diet. Torrecillas *et al*. (2015) assessed the effects of concentrated MOS (cMOS) at dietary levels of 1.6 g kg^{-1} on European sea bass performance, body chemical composition, fatty acid profiles, liver and posterior gut morphology and the expression of intestinal immune and lipid metabolism genes. The authors reported increased SGR's and fish lengths in fish fed the cMOS supplemented diet compared to those fed the control. No differences were observed in tissue proximate composition analyses between treatments. In terms of gene expression a down-regulation of transforming growth factor β (TGFβ), up-regulation of immunoglobulin (Ig), histocompatibility complex II (MHCII), T cell receptor β (TCRβ) and caspase-3 (CASP3) was observed in fish fed the cMOS supplemented diets compared to fish fed the control. Guerreiro *et al*. (2015) assessed the effects of short-chain fructooligosaccharides (scFOS) and xylooligosaccharides (XOS) on the growth, feed utilisation and liver activity of the enzymes involved in the glycolytic, gluconeogenic, and lipogenic pathways of European sea bass juveniles. The study included diets based on fish meal (FM) and plant ingredients (PP) each with the inclusion of scFOS and XOS at 1 % hence dietary treatments were PP-Control, PP-FOS, PP-XOS and FM-Control, FM-FOS, FM-XOS. Fish fed the PP-XOS diet were observed to exhibit increased growth performance compared to those fed the PP-Control. Fish fed the FM-FOS and FM-XOS exhibited higher glucokinase activity compared to fish fed the FM-Control. Fish fed the XOS diets also exhibited lower lipogenic enzyme activities compared to fish fed the other treatments. FOS and XOS treatment also increased glycolytic activity in the liver of fish fed the FM based

diets. The authors concluded that XOS could be potentially used as a prebiotic in the diets of European sea bass.

The studies carried out on prebiotics in fish suggest their potential as feed additives in aquafeeds. There is however, very little information regarding their effects on the intestinal microbial communities in fish and more research is required to further increase our understanding of this important aspect of fish nutrition. Future research should also focus on optimal dosage, effects on metabolism and immunological responses to assess whether a prebiotic should be used in the diets of cultured fish species. There are also some concerns regarding the potential for several pathogens and opportunistic bacteria to utilise a wide range of carbohydrates. In this case these potential pathogens may proliferate in the gut causing detrimental effects to host health (Nayak 2010b). Therefore, research into prebiotic-potential pathogen interactions should also be conducted.

1.7.3. Synbiotics

Synbiotic applications are the use of probiotics and prebiotics in combination in order to enhance the beneficial effects either one might have individually (Cerezuela *et al*. 2011). Investigations into synbiotics have been applied to an array of fish species and have included techniques such as culture dependent/ culture independent microbial analyses, as well as growth performance, feed utilisation, body composition, and histological, immunological and disease resistance analyses as a means of assessing the potential beneficial effects. Cerezuela *et al*. (2011) provided a review on synbiotic applications in fish and readers with an interest in studies pre 2011 are referred to this review. Since 2011, there has been a number of synbiotic studies conducted on various fish species (Lin *et al*. 2012; Nekoubin *et al*. 2012a; 2012b; Abid *et al*. 2013; Cerezuela *et al*. 2012; Cerezuela *et al*. 2013a; Cerezuela *et al*. 2013b; Firouzbakhsh *et al*. 2014; Hassaan *et al*. 2014; Akrami *et al*. 2015; Beshkar *et al*. 2015;

Hassaan 2015; Hoseinifar *et al*. 2015; Nurhayati & Yuhana *et al*. 2015; Vaezi *et al*. 2015; Zhang *et al*. 2015).

Cerezuela *et al*. (2012) assessed the effects of inulin and *Bacillus subtilis* on immune parameters, immune-related gene expression and protection against the pathogen *Photobacterium damselae* subsp. *piscicida* in gilthead sea bream. The study found fish fed the synbiotic (*B. subtilis* 10^7 CFU g^{-1} + inulin 10 g kg^{-1}) to exhibit significantly higher serum complement activity after four weeks, and significantly higher IgM levels after two weeks feeding when compared to fish fed the control. Interestingly, fish fed the synbiotic were more susceptible to the pathogen with significantly higher mortality when compared to fish fed the control. In another study in gilthead sea bream Cerezuela *et al*. (2013a) demonstrated significantly increased expression of β-actin and occludin in the intestine of fish fed inulin + *B.subtilis* compared to fish fed a control diet. The authors concluded that the expression of these genes may indicate a strengthening of the tight junctions and transport of iron molecules in the intestine, but could also be indicative of a negative reaction to the initiation of the diets. Clearly, more research is required to confirm these findings and to further understand synbiotics effects on the expression of genes in the intestine and indeed other organs of fish. In a third study on gilthead sea bream Cerezuela *et al*. (2013b) assessed the effects inulin + *B. subtilis* had on intestinal morphology and microbiota. The authors observed no differences in intestinal absorptive surface area between fish fed the synbiotic and control diets. However, a reduction in goblet cells and microvilli heights was observed in fish fed the synbiotic diet compared to those fed the control. DGGE analyses revealed significant reductions in the number of OTU's, species diversity and range-weighted richness in fish fed the synbiotic diet compared to fish fed the control.

Abid *et al*. (2013) investigated the effects of a synbiotic (*Pediococcus acidilactici* 3.5 g kg^{-1} + scFOS 7 g kg^{-1}) supplemented into the diets of Atlantic salmon on the intestinal health of fish

after 63 days feeding. Results revealed fish fed the synbiotic diets exhibited significantly lower total bacterial levels in the anterior and posterior mucosal tissue and posterior digesta compared to fish fed the control diet. DGGE analyses revealed significantly increased OTU's, species diversity and richness in the anterior digesta of fish fed the synbiotic compared to those fed the control. The authors also observed a significant increase in mucosal fold length and epithelial leukocyte numbers as a consequence of synbiotic administration. In terms of immunology, a significant up-regulation of the pro-inflammatory cytokine genes, IL-1β, IL8 and TNFα, as well as the expression of the genes TLR3 and MX-1 was observed in both intestinal regions of fish fed the synbiotic treatment compared to fish fed the control. The authors concluded that the collective results obtained in this investigation suggest the synbiotic supplementation has a positive effect on the intestinal health of Atlantic salmon. In a recent study on rainbow trout fingerlings, Hoseinifar *et al*. (2015) investigated the effect that *P. acidilactici* + GOS had on the immune response, skin mucus and disease resistance in an 8 week trial. Serum lysozyme activity, alternative complement activity and leukocyte respiratory burst activity were observed to be significantly increased in fish fed the synbiotic treatment when compared to fish fed the control. Soluble protein levels were also significantly elevated in fish fed the synbiotic diet compared to those fed the control. Additionally, a bacteriocidal activity assay was carried out and revealed the skin mucus of fish fed the synbiotic diet to be significantly more effective in the inhibition of *Streptococcus faecium*, *Streptococcus iniae*, *Serratia marcescens*, *Staphylococcus aureus* and *Escherichia coli*. Furthermore, mortalities were observed to be significantly lower in fish fed the synbiotic diet compared to fish fed the control after being challenged with *Streptococcus iniae*. Firouzbakhsh *et al*. (2015) also used rainbow trout as the model for a synbiotic trial. Diets supplemented with *Enterococcus faecium* 10^{11} CFU g^{-1} + FOS (0.5, 1.0 and 1.5 g kg^{-1}) were fed to fingerlings for a period of 60 days. The study revealed significantly increased final

mean weights and SGR's of fish fed the diets containing all three synbiotic inclusion rates compared to those fed the control diet. FCR and feed conversion efficiency (FCE) was observed to be most improved in fish fed the 1.0 g kg^{-1} synbiotic treatment. Haematological parameters revealed significant increases in white and red blood cell counts as well as significantly elevated haemoglobin concentrations and haematocrit in fish fed the synbiotic treatments when compared to fish fed the control. Serum lysozyme activity was also significantly increased in fish fed the synbiotic treatments. In all haematological analyses the highest values were observed in those fish fed the 1.0 g kg^{-1} synbiotic dose. Furthermore, all synbiotic fed fish exhibited significantly higher survival compared to fish fed the control after being challenged with *Saprolegnia parasitica*. The results obtained from both of these trials suggest rainbow trout fingerling health is improved as a consequence of the respective synbiotic treatments and the optimal dose was1.0 g kg^{-1}.

Zhang *et al*. (2014) investigated the effects of *B. subtilis* + FOS on growth performance, immune responses and disease resistance in juvenile ovate pompano (*Trachinotus ovatus*). Fish were fed either a control or one of four synbiotic diets for a period of eight weeks. The synbiotics were supplemented into the diets as follows with the probiotic expressed as CFU g^{-1} + prebiotic expressed as g kg^{-1}: diet 1. 1.05 + 0.2, diet 2. 1.05 + 0.4, diet 3. 5.62 + 0.2, and diet 4. 5.62 + 0.4. Growth data results displayed fish fed all synbiotic diets to exhibit higher SGR's, significantly so in diet 3, and feed efficiency ratios (FER's), significantly so in diets 2, 3 and 4 when compared to fish fed the control. Respiratory burst activity was observed to be significantly elevated in fish fed diets 1 and 3 compared to fish fed the control. Alternative complement pathway activity was significantly elevated in fish fed diets 3 and 4 compared to fish fed the control. Serum lysozyme was significantly elevated in fish fed diets 2, 3 and 4 compared to fish fed the control. Phagocytic activity remained statistically unaffected between treatments. Furthermore, fish fed the synbiotic diets exhibited lower cumulative

mortalities after 10 days post *Vibrio vulnificus* infection compared to those fed the control diet. The results of this trial indicate that dietary *B. subtilis* and FOS may enhance the systemic immunity in juvenile ovate pompano under the conditions of the present study. The authors suggested that future work is needed to ascertain the mechanisms involved around *B. subtilis* supplementation, as well as research into the significance of this probiont and FOS in relation to the non-specific immunity and disease resistance of this fish species is also needed. Further to this, more research is required on the effects these feed additives have on the intestinal (allochthonous and autochthonous) microbial communities.

Vaezi *et al*. (2015) investigated the effects that the synbiotic: Biomin imbo application on the intestinal microbiota of Russian sturgeon (*Acipenser guldenstadti*). The study utilised five different synbiotic inclusion levels (1, 1.5, 2, 2.5 and 3 g kg^{-1}) and compared the culturable allochthonous intestinal microbiota of these fish against a control after 20, 40 and 60 days feeding. The authors assessed the total (culture dependent) bacterial levels, as well as, cultivable LAB levels. The study showed bacterial levels increased in a time dependant manor in all treatments and significant increases in LAB were observed in the synbiotic fed fish compared to control fed fish in all time points. The authors suggest the results indicate a stabilising effect towards LAB in a time dependent pattern. This study is however, only a small insight into these microbial communities given that most microbes isolated from the gut of fish cannot be cultured on traditional media, and therefore more research is needed on the culture-independent techniques in order to ascertain the true effects this synbiotic has on the intestinal microbiota of Russian sturgeon.

Even though there are a growing number of synbiotic studies in fish it's clear that most of these studies focus on various growth parameters and immunology. There seems to be a distinct lack of research focusing on how these feed additives interact with the intestinal

microbial communities. More research is required on these interactions and how they relate to various aspects of fish health.

1.7.4. Phytobiotics

Phytobiotics are plant-derived natural compounds which potentially enhance animal productivity (Antache *et al*. 2013). These feed additives are thought to exhibit antimicrobial, antioxidant, growth promoting and digestive enzyme properties (Cristea *et al*. 2012). These properties are caused by a variety of primary (e.g. protein, carbohydrates and fat) and secondary (e.g. terpenes, carvacrol, capsaicin, peperin, chicoric acid and flavonoids) ingredients and have shown some promise as alternative feed additives in the nutrition of livestock (Grashorn 2010). In fish, various phytobiotics have been observed to have immunostimulating (Dügenci *et al*. 2003; Yin *et al*. 2006; Kaleeswaran *et al*. 2012), disease resistance (Christybapita *et al*. 2007; Sahu *et al*. 2007; Abd-El-Rhman 2009; Rattanachaikunsopon *et al*. 2010; Volpatti *et al*. 2013) and growth promoting (JI *et al*. 2007; Abd-El-Rhman 2009; Thanikachalam *et al*. 2010) effects. Cristea *et al*. (2012) gives a comprehensive review of the use of phytobiotics in aquaculture. Since the publication of this review, a number of studies assessing phytobiotics in fish have been published, most notably the studies by Antache and colleagues who have published a number of studies on the effects various phytobiotics have on the health of Nile tilapia (Anatache *et al*. 2013a, 2013b, 2013c, 2014a, 2014b, 2014c, 2015). In the first of these experiments Antache *et al*. (2013a) assessed the phytobiotcs thyme (*Thymus vulgaris*), fenugreek (*Trigonela foenum graecum*), neem (*Azadirachta indica*) supplemented into diets at a concentration of 10 g kg^{-1} on the biochemical composition of Nile tilapia. The study reported differences in protein with fish fed the phytobiotics exhibiting significantly lower values when compared to fish fed the control. Moisture levels were significantly higher in fish fed the phytobiotics compared to

fish fed the control. Lipid levels were observed to be significantly higher in fish fed the thyme and fenugreek treatments but was significantly lower in fish fed the neem treatment when compared to fish fed the control. The authors concluded that these phytobiotics at a concentration of 10 g kg^{-1} significantly influence the biochemical composition of Nile tilapia. In a second study Antache *et al*. (2013b) assessed rosemary, sea buckthorn and ginger (10 g phytobiotic kg^{-1}) on growth performance of Nile tilapia. The results indicated that fish fed the sea buckthorn to exhibit the best SGR's and FCR's when compared to fish fed the control, however these were not significantly different. Antache *et al*. (2013c) also assessed the effects these phytobiotics have on oxidative stress in Nile tilapia. The study revealed significantly reduced lipid peroxidase from liver, gut and blood plasma in fish fed the sea buckthorn treatment when compared to fish fed the control. The authors suggest this to indicate a reduction in oxidative stress and the collective results obtained from the last two aforementioned studies reveal sea buckthorn at a concentration of 10 g kg^{-1} confers beneficial effects to Nile tilapia heath. More recently Antache *et al*. (2014a, 2014b) have also observed beneficial changes in haematological parameters in Nile tilapia fed various phytobiotics. Antache *et al*. (2014c) also observed some positive effects in terms of growth and reduced stress in Nile tilapia fed a diet containing sea blackthorn at 10 g kg^{-1} + vitamin E at 0.5 g kg^{-1} compared to fish fed a control. The authors suggest these results indicate a synergistic effect between these two feed additives. Antache *et al*. (2015) also observed that dietary rosemary, sea buckthorn and ginger influenced the muscle tissue biochemical composition of Nile tilapia with significantly increased percentage protein in fish fed 10 g ginger kg^{-1} feed, compared to fish fed the control. These studies report basic growth, body chemical composition, oxidative stress indicators and haematological parameters but further research is needed on various other aspects of health in order to better understand the effects these phytobiotics have on Nile tilapia. Effects on localised immunity, intestinal morphology,

metabolism, digestion and the intestinal microbiome are examples of areas in need of further research.

1.8. Conclusions and future work

It has been clear for a number of years that wild fisheries have been poorly managed and stocks have declined significantly, fuelling the growth of the aquaculture industry in the hope of meeting global demand for seafood. The aquaculture industry must continue to adapt to find new ways of becoming sustainable through feed technology and to counteract the threat of disease through methods other than those, such as antibiotics, which have serious implications to the environment and human health. The problems associated with the routine use of antibiotic compounds are well known and has pushed forward the search for alternative prophylactics. Alternatives include: phage therapy, vaccines, and various methods designed to inhibitor/disruptor pathogen virulence factors (Defoirdt *et al*. 2011; Romero *et al*. 2012). Feed additives such as probiotics, prebiotics, synbiotics and phytobiotics have shown some potential beneficial effects in many fish species, and their use is becoming common practice in many aquaculture practices. However, this is a relatively new field of research and limited knowledge is available on the mechanisms governing the dynamics between feed additives and host health. Furthermore, very few studies have focused their analyses on the intestinal microbiota and the mechanisms associated with its manipulation. More studies are necessary to validate the effects feed additives have on these microbial communities and how they affect host health. Furthermore, there are considerable gaps existing in the role that feed additives have on various aspects for metabolism, immunology and intestinal morphology, as well as specific disease resistance. It is hoped that the recent advances in molecular metagenomic and proteomic techniques will supply new information on how these feed additives impact on the microbial assemblages, and how they relate to host mucosal

responses in fish. There is very little information on the effects of feed additives on European sea bass and furthermore, with the exception of a limited number of studies, feed additive effects on the intestinal microbiota of this species is limited at best.

1.9. Thesis aims and objectives

The aim of the current research programme was to assess the effects of various plant based protein sources and feed additives on the health of European sea bass through a series of three feeding trials.

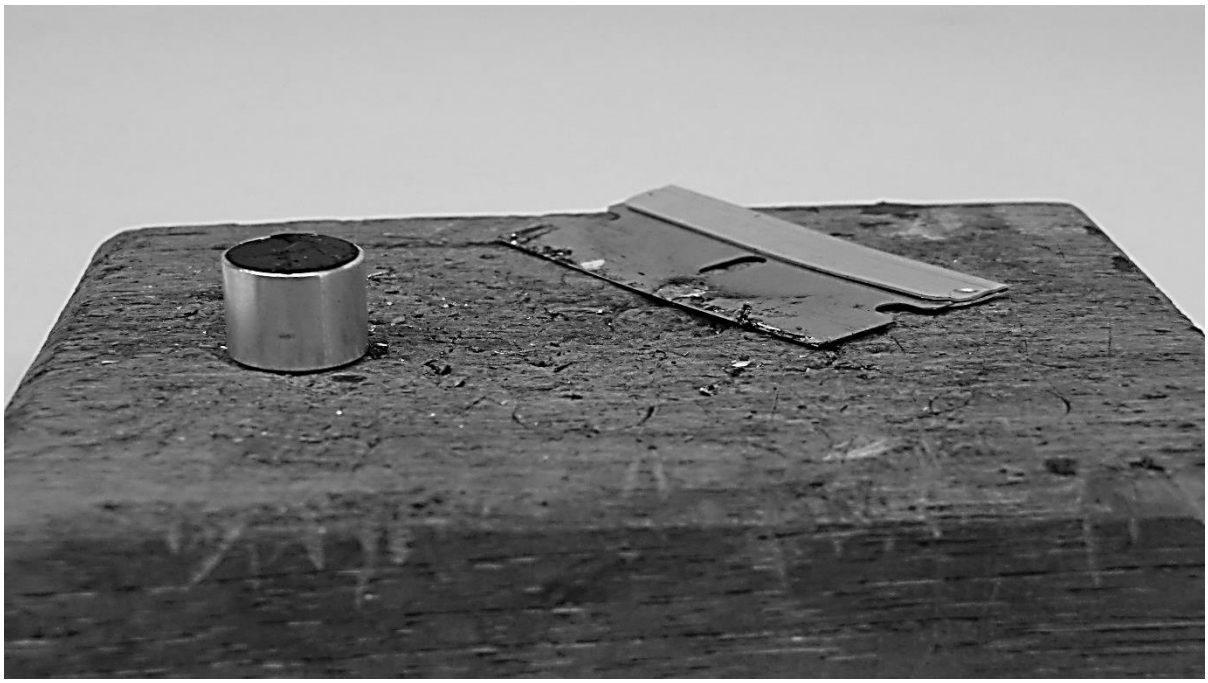
Trial 1. Assessing the potential enteritis effects as a consequence of partial fishmeal replacement with soy protein concentrate, pea protein concentrate and saponins on European sea bass.

Trial 2. Assessing the potential of probiotic, prebiotic and synbiotic supplementation on alleviating enteritis-like effects of European sea bass fed a sub-optimal diet.

Trial 3. Assessing the long-term effects of probiotic and phytobiotic supplementation on the growth, intestinal microbiota, intestinal integrity and localised intestinal and systemic immune responses of European sea bass.

Chapter 2:

General materials and methodologies



2.1. Overview

All experimental analyses were carried out with the following protocols unless otherwise stated. Chemicals, reagents and culture media were sourced from Fisher scientific Inc. (Hampton, NH, USA), Sigma Aldrich corp. (St. Louis, MO, USA) or Oxoid Ltd unless otherwise indicated. All experimental work involving fish was carried out under the Home Office project licence #30/2644 and personal licence #30/9993.

2.2. Aquarium facilities

All feeding experiments were conducted at the Aquaculture and Fish Nutrition Research Aquarium at Plymouth University. All trials were conducted in experimental system B (Figure 2.1). The system is a closed recirculation system with a total system volume of ~ 4600 L. Mechanical filtration was provided by six filter nets and a two inch gauze filter matt, cleaned every other day. Biological filtration was provided by a submerged biological filter bed in the sumps and chemical filtration was provided by UV-light. System B comprised of 18 x 110 L fibreglass tanks, each provided with recirculated aerated seawater (~ 32 ‰) at a rate of ~ 440 L hr^{-1}. A 12 hr light, 12 hr dark photoperiod was maintained throughout the trial periods. Water changes (approximately 10- 15 % of system volume) were conducted every 72 hr to minimise the accumulation of nitrogenous waste and bacterial levels. The system pH, dissolved oxygen and temperature were monitored daily using a Hach HQ 40d. The system was buffered with sodium bicarbonate ($NaHCO_3$) as required to maintain ~ pH 7. The water temperature was maintained at 25 ± 1 ^{o}C with a thermostatic controlled heater (Elecro, Titanium) and dissolved oxygen levels maintained > 80 % saturation with additional aeration provided by an air stone supplied by a low pressure side channel blower (Rietschle, UK).

Ammonia (Lange LCK 304), nitrite (Lange LCK 341) and nitrate (Lange LCK 340) were monitored on weekly basis using a Hach Lange DR 2800 with acceptable levels considered to be < 0.1 mg L^{-1}, < 1.0 mg L^{-1} and < 50 mg L^{-1} for ammonia, nitrite and nitrate, respectively.

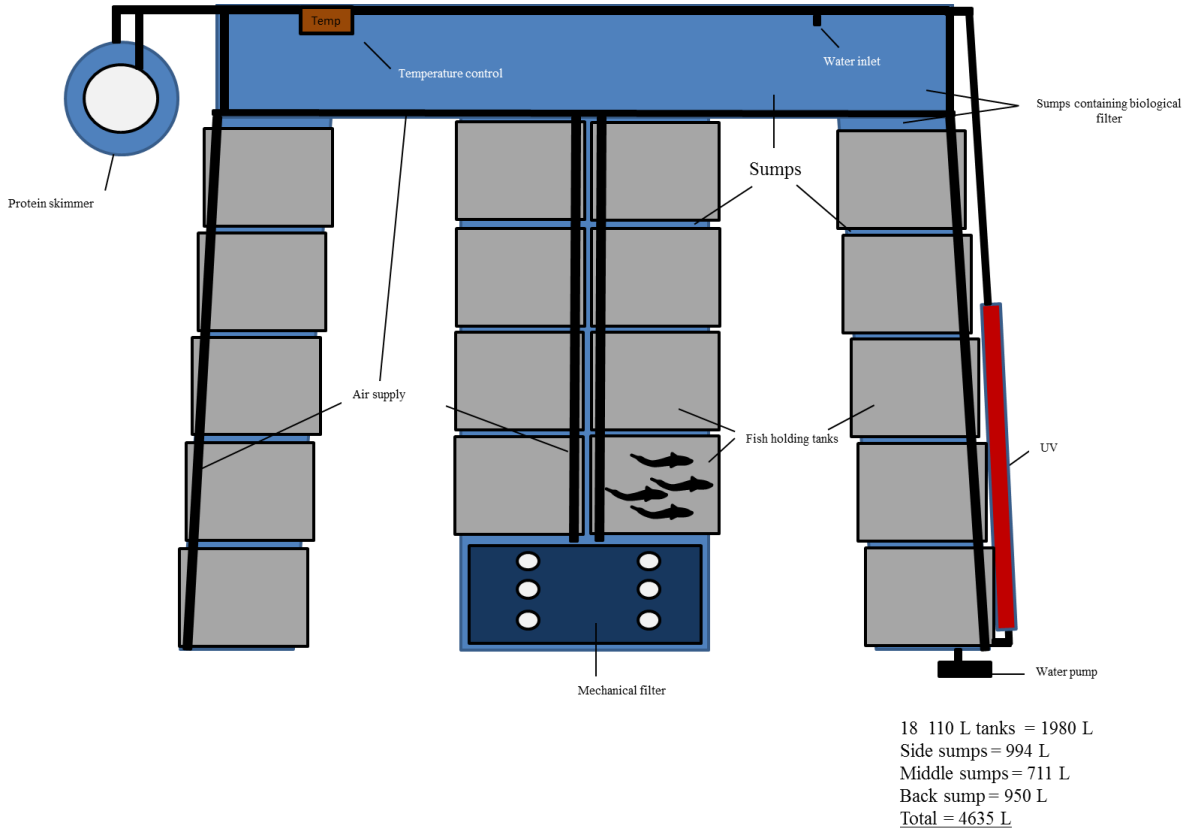


Figure 2.1. Schematic of system B with technical information and water holding capacities. A closed recirculatory system at the aquaculture facilities of Plymouth University, where all three feeding trials were carried out.

2.3. Experimental fish and feeding rates

All experimental fish were acquired from Anglesey Aquaculture Ltd, Black Point, Beaumaris, Wales, UK. An acclimation period of ~ four weeks was carried out where the fish were fed a commercial sea bass diet (Skretting) before grading and separating into tanks. A preliminary grading was carried out approximately one week prior to the final grading where batch tank weights were graded to within 1.5 % of each other. Each tank was allocated a dietary regime at random and fish were fed at a rate of 2-3 % of total tank biomass three times daily (09:00, 13:00 and 17:00). Feed was adjusted after weighing (as tank biomass) every two weeks. Feed was reduced to one feed (1.5 % biomass) the day before weighing to reduce stress.

2.4. Diet formulation and production

All experimental diets where formulated on Feedsoft professional software (version 3.1) to meet the known nutritional requirements of European sea bass (NRC 2011), and were produced at the nutritional facilities of Plymouth University. Ingredients were mixed in small amounts to ensure a homogenous mix before placing in a Hobart food mixer (Hobart Food Equipment, Sydney, Australia, model no: HL1400–10STDA). Oil and hot water were added to the mixer and cold press extrusion was conducted (PTM P6 extruder, Plymouth, UK) to produce the appropriate sized pellets. The pelleted diets were then dried for 48 hours in an air convection oven set at 45^{o}C and broken up by hand to the relevant size.

2.5. Chemical proximate analyses

Diets and carcass (where applicable) were subjected to analysis for the determination of protein, lipid, moisture, ash, and gross energy content. All samples were analysed according to AOAC (1995) protocols.

2.5.1. Crude protein

Crude protein levels were determined by the Kjeldahl method to establish the total nitrogen (N) content of samples. This amount was then multiplied by a factor 6.25 to calculate the crude protein content on the assumption that animal proteins contain 16 % nitrogen (AOAC 1995). Approximately 150 mg of sample was added to a Kjeldahl digestion tube along with catalyst tablet (3 g K_2 SO_4, 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2; BDH Ltd UK) and 10 ml of concentrated (98 %) H_2SO_4 (Sp.Gr. 1.84, BDH Ltd UK). To correct for the efficiency of nitrogen extraction two samples of acetanilide (nitrogen content 10.36 %) were used. Casein was used to validate the nitrogen content. The digestion was performed using a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 225 ^oC for 40 min and at 380 ^oC for 60 min. After digestion the samples were distilled using a Vapodest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany). Crude protein is then determined as: ((ST - BT) x 0.20 x 14 x 6.25) / SW) * 100. Where ST is sample titre (ml), BT is blank titre (ml), SW is sample weight (mg), 0.2 is the acid molarity and 1.4007 is the molecular weight of nitrogen.

2.5.2. Gross energy

Gross energy (MJ kg^{-1}) was determined using a Parr Adiabatic Bomb Calorimeter model 1356 (Parr Instrument Company, IL, USA). Approximately 1 g of sample was compressed into a cylindrical pellet which was then placed into a nickel crucible. The conductor fuse wire was then attached to electrodes and then placed on the uppermost surface of the pellet. The crucible is then placed into a decompressor vessel which is filled with 30 bar (435 PSI) of O_2. The vessel is then placed into a metal cylinder containing 2000 g of distilled water which is then placed into the calorimeter machine. The sample is electrically ignited through the fuse wire and combusted. All organic material is burnt and the subsequent heat created is transferred into the surrounding water jacket and the difference between the initial and post ignition water temperature is detected by the calorimeter. The information is then converted into the energy value of the sample.

2.5.3. Lipid

Lipid content was determined by rapid Soxhlet extraction method. Approximately 3 g of sample was placed into a cellulose thimble, lightly plugged with cotton wool and inserted into glass beakers containing bumping granules. Petroleum ether (140 ml) was then added to the beakers which were then placed onto the soxtherm unit (Gerhadt Laboratory Instruments, Bonn, Germany). The sample was then heated to 150 ^{o}C for 30 minutes and rinsed for 45 minutes and the solvent is left to evaporate for ~ 1 hr in a fume hood, after which the extracted lipid was weighed. The lipid content is then determined as: (LW / SW) x100. Where LW is the lipid weight (determined from weight increase of cup, g) and SW is the initial sample weight (g).

2.5.4. Moisture

Moisture content was determined by weighing 3-5 g of sample into a metal dish (diet) or total wet weight of fish into an aluminium foil tray and air drying in a fan assisted oven at 105 ^{o}C until a constant weight was achieved. The percentage moisture was then calculated as follows: ((WW – DW) / WW) * 100. Where WW is the wet weight (g) and DW is the dry weight (g).

2.5.5. Ash

The determination of ash (total mineral or inorganic content) was carried out by adding a known weight of sample (~500 mg) to a pre-weighed porcelain crucible. The crucibles were then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550 ^{o}C for 12 hr. The ash content was then calculated as follows: ((SR - CW) / SW)*100. Where SR is sample residue weight (g), CW is crucible weight (g) and SW is the original sample weight (g).

2.6. Fish dissection and sampling

Two fish per tank were sampled for microbiology and two per tank for histology (n = 6) at the end of each trial. Fish were euthanized by an overdose (200 mg/L water for 5 min) of MS222 (Pharmaq) and destruction of the brain. Fish were dissected under aseptic conditions and the intestine was isolated. Lipid deposits were removed and the intestine was cut just below the pyloric caeca. For molecular microbiological analyses the digesta was removed aseptically and placed into sterile microcentrifuge tubes and stored at -20 ^{o}C. For histological analyses the first 5 mm were discarded from the end of the posterior intestine, a section (5 mm) for light microscopy analysis was then excised and placed in 4 % saline formalin which was replaced with 70 % IMS after 48 h. A piece approx. 2 mm in size was then excised, cut

open and separated into two small pieces, one sample for scanning electron microscopy (SEM) and the other for transmission electron microscopy (TEM). SEM samples were washed in 1 % S-methyl-L-cysteine for approximately 20 sec to remove mucus and both sections were placed in fixative (2.5 % glutaraldehyde in pH 7.2, 0.1 M sodium cacodylate buffer). Figure 2.2. illustrates the sampling methods used.

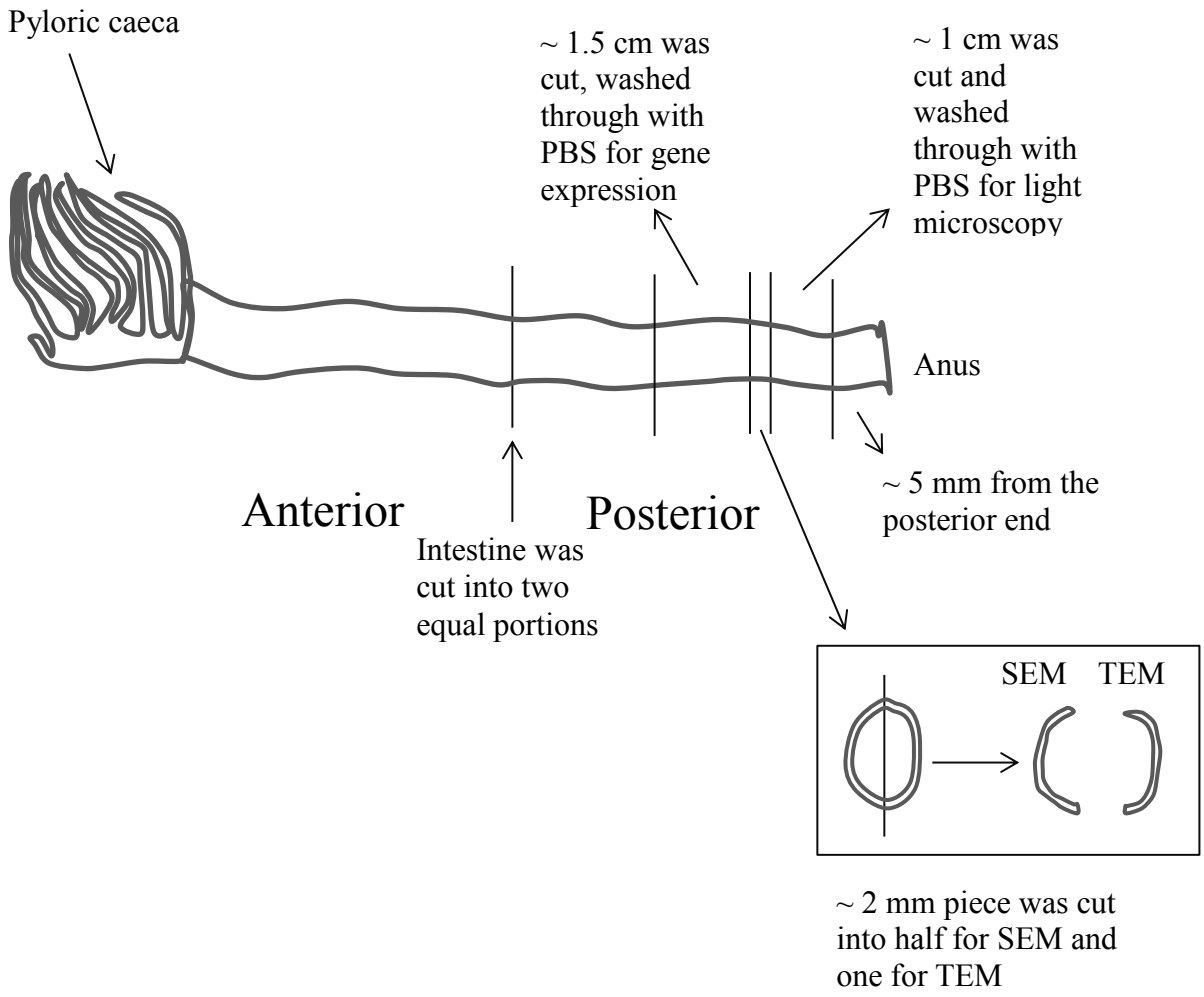


Figure 2.2. Schematic of the intestine of European sea bass and details of how samples were taken for the various analyses.

2.7. Molecular microbiology

2.7.1. DNA extraction

DNA extraction was carried out using the Qiamp DNA stool mini kit (Qiagen), with some modifications. Digesta samples were weighed into microcentrifuge tubes, and with the addition of 500 µl of lysozyme (50 mg / ml in TE buffer), were incubated for 30 min at 37 ^{o}C. The samples were then homogenised in a vortex mixer and 700 µl of buffer ASL was added and samples were incubated for 60 min at 90 ^{o}C. Samples were then vortexed and centrifuged in a benchtop microcentrifuge for 1 min at 17,000 x g. The supernatant (800 µl) was added to a new microcentrifuge tube containing half of an Inhibitex tablet. The sample was immediately vortexed for 1 min and then left to stand for a further minute at room temperature. The sample was centrifuged for 3 min at 17,000 x g and the supernatant transferred to a new microcentrifuge tube. The sample was then centrifuged again for 3 min at 17,000 x g and 350 µl of the supernatant transferred to a new microcentrifuge tube. Proteinase K (15 µl) and buffer AL (350 µl) was then added to the sample which was mixed by inversion and incubated for 60 min at 56 ^{o}C. The sample was cooled to room temperature and 350 µl of molecular grade chloroform and 350 µl molecular grade phenol was added, mixed by inversion and centrifuged for 10 min at 17,000 x g. the supernatant was then removed and the chloroform step was repeated. The supernatant was then removed and 480 µl of ice cold isopropanol was added and sample was mixed by inversion. The sample was left to stand at room temperature for 10 min after which another centrifugation step was carried out for 10 min at 17,000 x g. The supernatant was then discarded and the sample washed with 500 µl ice cold 70 % molecular grade ethanol, comprising of two centrifugation steps of 10 min at 17,000 x g. The ethanol was pipetted off and the sample was left to dry at room temperature for 5 min to remove any remaining ethanol. The sample was finally re-

suspended in 30 µl of molecular grade H_2O and incubated over night at 4 ^oC. DNA recovery and quality was analysed on a Nanodrop 2000 (Thermo Scientific, Wilmington, USA).

2.7.2. PCR-Denaturing gradient gel electrophoresis (PCR-DGGE)

PCR amplification of the variable V3 region of 16S rRNA genes was carried out using the reverse primer P2 (5'- ATT ACC GCG GCT GCT GG -3') and the forward primer P3 (5'- CC TAC GGG AGG CAG CAG -3'), which had a GC clamp attached at the 5' end (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G -3'), after Muyzer *et al.* (1993). The following reagents were added to each PCR reaction: 1 µl of primer P2 and 1 µl of P3 (50 pmol ul^{-1}), 3 µl DNA template, 25 µl RedTaq™ (Bioline) and 20 µl molecular grade water. This gave a final concentration of 1.5 units Taq DNA polymerase, 10 mM TrisHCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, and 0.2 mM dNTPs. The PCR conditions employed were: 95 $^{\circ}C$ for 5 min, followed by 2 cycles at 95 $^{\circ}C$ for 1 min, 65 $^{\circ}C$ for 2 min, 72 $^{\circ}C$ for 3 min. This cycling regime was repeated with a drop in annealing temperature of 1 $^{\circ}C$ after every second cycle until a final annealing temperature of 55 $^{\circ}C$ was reached, whereupon a further 10 cycles were run. PCR products were run on 1.5 % agarose gels to assess PCR success. Denaturing gradient gel electrophoresis (DGGE) was performed using a DCode Universal Mutation Detection System (Bio-Rad laboratories, Italy). PCR products were run on an 8 % polyacrylamide gel (160 mm x 161 mm) containing 40 %–60 % denaturing gradient (where 100 % denaturant is 7 M urea and 40 % formamide). Gels were run at 65 V for 17 h at 60 ^oC in Tris-acetate-EDTA (TAE) buffer. Pooled samples were loaded in triplicate on the same gel, and DGGE gels were stained for in 100 ml TAE buffer containing 10 µl of SYBR Gold nucleic acid gel stain (Molecular Probes, UK) for 20 min. Visualization was carried out in a Bio-Rad 1387 universal hood II (BioRad laboratories,

Italy). The resulting gel was transformed into presence/absence matrices and band intensities were measured using Quantity One ™ software (BioRad laboratories).

2.7.3. Sanger sequencing

DGGE bands were excised from the gels using a pipette tip and were subjected to a re-PCR as described in section 2.7.2. using primer P1 in place of primer P3 to ensure adequate amplification. PCR success was determined by agarose gel as described in section 2.7.4. and the subsequent PCR products were cleaned using a QIAquick PCR Purification Kit (Qiagen) as per the manufacturer's protocol. Sequencing was carried out by GATC laboratories (GATC-biotech, Germany) and the resulting sequences were subjected to a BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7.4. High-throughput sequencing

PCR amplification of the variable V1-V2 region of 16S rRNA genes was carried out using the primers as follows; reverse primer 27F (5' - AGA GTT TGA TCM TGG CTC AG – 3') and the forward primer 338R (GCW GCC WCC CGT AGG WGT). The following reagents were included in each PCR tube: 1 µl of primer 338R and 1 µl of primer 27F (each 50 pmol μl^{-1}; Eurofins MWG, Ebersberg, Germany), 4 µl of DNA template (diluted 1/10 of original template), 15 µl of MyTaq^{TM} (Bioline, London, UK) and 9 µl of PCR grade water. Thermal cycling was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the following conditions: initial denaturation at 94° C for 7 min, then a touchdown of 10 cycles at 94°C for 30 s, 62 for 30 s (with the annealing temperature decreasing by 1 ^{o}C peer cycle) and 72 °C for 30 s. A further 25 cycles were performed at 94°C for 30 s, 53°C for 30 s and 72 °C for 30 s before a final extension for 7 min at 72 °C. To ensure that there was sufficient PCR amplicons for sequencing, the PCR reactions were run in duplicate and pooled into a single

sample prior to cleaning. PCR products were purified (QIAquick PCR Purification Kit; Qiagen) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Prior to sequencing the amplicons were assessed for fragment concentration using an Ion Library Quantitation Kit (Life Technologies TM, USA), then concentrations were adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles using Ion PGM Template OT2 200 kit (Life Technologies™, USA) according to the manufacturer's instructions. Multiplexed sequencing was conducted using Ion Xpress Barcode Adapters (1-16 Kit; Life Technologies™) and a 316™ chip (Life Technologies™) on an Ion Torrent Personal Genome Machine (Life Technologies™). Sequences were binned by sample and filtered within the PGM software to remove low quality reads. Data were then exported as FastQ files.

Taxonomic analyses of sequence reads were performed after the removal of reads with low quality scores (Q < 20) with FASTX-Toolkit (Hannon Laboratory, USA). Sequences were concatenated and sorted by sequence similarity into a single fasta file. Sequences were denoised and analyzed with QIIME 1.5.0 (Caporaso *et al.* 2010a). Briefly, OTU mapping was performed using the USEARH quality filter pipeline (Edgar 2010), to remove putatively erroneous reads (chimeras). Non-chimeric OTU's were identified with a minimum pairwise identity of 97% and representative sequences from the OTU's were aligned using PyNAST (Caporaso *et al.* 2010b). Taxonomic classification of each OTU was determined using the Greengenes database (DeSantis *et al.* 2006) using the RDP classifier (Wang *et al.* 2007), clustering the sequences at 97 % similarity with a 0.80 confidence threshold.

Alpha diversity metrics were calculated on rarefied OTU tables. Sampling depth and coverage were checked by constructing rarefaction curves and Good's coverage of observed species. Phylogenetic diversity (PD), Chao1 (richness) and Shannon-Wiener indices (diversity) were also calculated. The similarities between the microbiota compositions were compared using Bray-Curtis unweighted pair group method with arithmetic mean (UPGMA).

2.7.5. Agarose gel electrophoresis

PCR amplicons were loaded and ran on an agarose gel to assess product quality. All gels were 1 % agarose gel run with 1 x Trisborate EDTA (TBE) buffer in a Pharmacia electrophoresis tank. Sample's (4 - 6 µl) and loading buffer (Bioline) was loaded onto the gel as well as 5 µl of Hyper ladder IV (Bioline). Positive and negative controls were included and ran alongside the samples.

2.7.6. RNA extraction and cDNA synthesis

Total RNA was extracted from the posterior intestine using TRIzol (Invitrogen, Carlsbad, CA,USA) according to the manufacturer's instructions, with some modifications as described elsewhere (Pérez-Sánchez *et al*. 2011). RNA concentration and purity were measured spectrophotometrically (NanoDrop Technologies, Wilmigton, USA) and stored at -80 ^{o}C until use. Total RNA was treated with DNAse (10 UI at 37 ^{o}C for 10 min, MBI Fermentas), and a total amount of 1 mg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad CA, USA).

2.7.7. Quantitative Real-time PCR

PCRs were performed in an iQ5 iCycler thermal cycler (Bio-Rad) using the SYBR green method. Primer efficiencies were determined using serial dilutions (1/10) of pooled cDNA and resulting plots of Ct versus the logarithmic cDNA input, using the equation $E = 10^{(-1/slope)}$. Duplicate PCR's were carried for each sample and each PCR reaction was set on 96 or 384 well plates (7.5 µl reaction volumes) by mixing 2 µl of diluted (1/10) cDNA with 3.75 µl 2 x concentrated iQ™ SYBR Green Supermix (Bio-Rad), containing SYBR Green as a

fluorescent intercalating agent, 0.225 µl of forward and reverse primer (0.3 µM) and 1.3 µl of DEPC treated H_20 (Ambion). Quality control measures and RT-reactions were carried out according to the MIQE guidelines (Bustin *et al*. 2009). The thermal profile for all reactions was 10 min at 95 °C and then 40 cycles of 15 s at 95 °C, 60 s at 60 °C with fluorescence monitoring occurring at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak. For information relating to the housekeeping genes, genes of interest and methods for analyses refer to individual chapters.

2.8. Histology analyses

2.8.1. Light microscopy

Approximately 5 cm of the posterior intestine was sampled and fixed in 4 % saline formalin which was replaced with 70 % IMS after 48 hours. Samples were then dehydrated in a graded ethanol series and embedded in paraffin wax using a Leica EG1150H. Ultrathin sections (6 µm) were cut on a microtome (Leica) and stained with haematoxylin and eosin (H&E) and Alcian Blue-PAS. Micrographs were taken of each section and each was analysed using the software package Image J 1.45 (National Institutes of Health, USA). The perimeter ratio (PR) between the internal perimeter (IP) of the mucosal folds (absorptive surface area) and the external perimeter (EP) of the intestine was calculated (PR = IP / EP, arbitrary units) after Dimitroglou *et al*. (2010) (Figure 2.3.). Lamina propria widths were analysed by taking the average of three measurements (bottom, mid and top) along each fold, 10 folds per sample (Figure 2.4.A). Mucosal fold lengths were measured with at least 18 folds per sample (Figure 2.4.B). Intraepithelial leucocytes (IEL's) were counted per 100 µm (10 folds per sample) (Figure 2.5.A) and goblet cells were counted across a distance of 200 µm (10 folds per sample) (Figure 2.5.B).

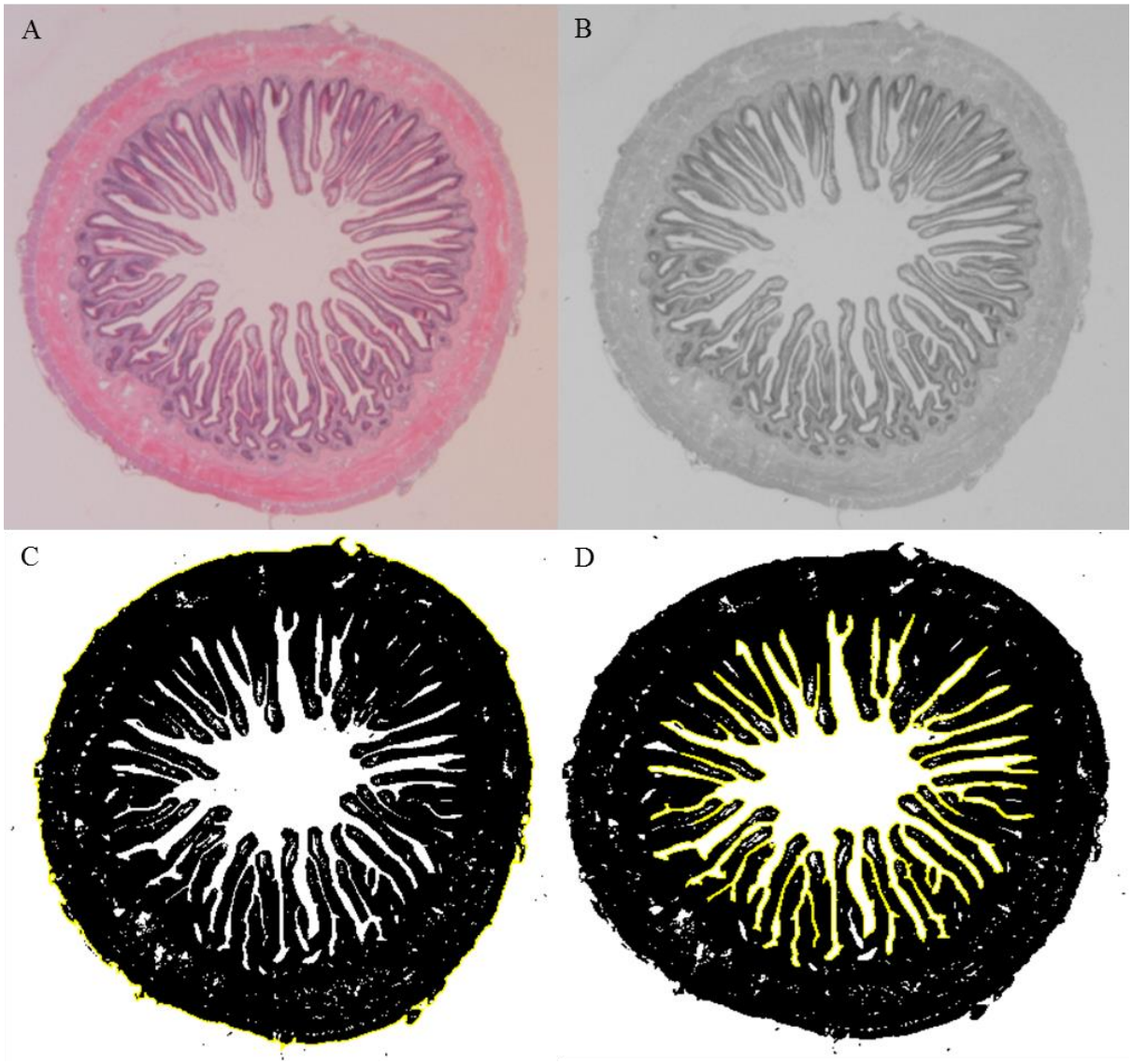


Figure 2.3. Example of the posterior intestine and how perimeter ratio measurements were carried out in image J. Transverse light microscopy images are loaded into image J (A), transformed to 8-bit (grayscale) (B) and a threshold of the image is then applied (C/D) where measurements of the outside and the inside of the intestine can be made.

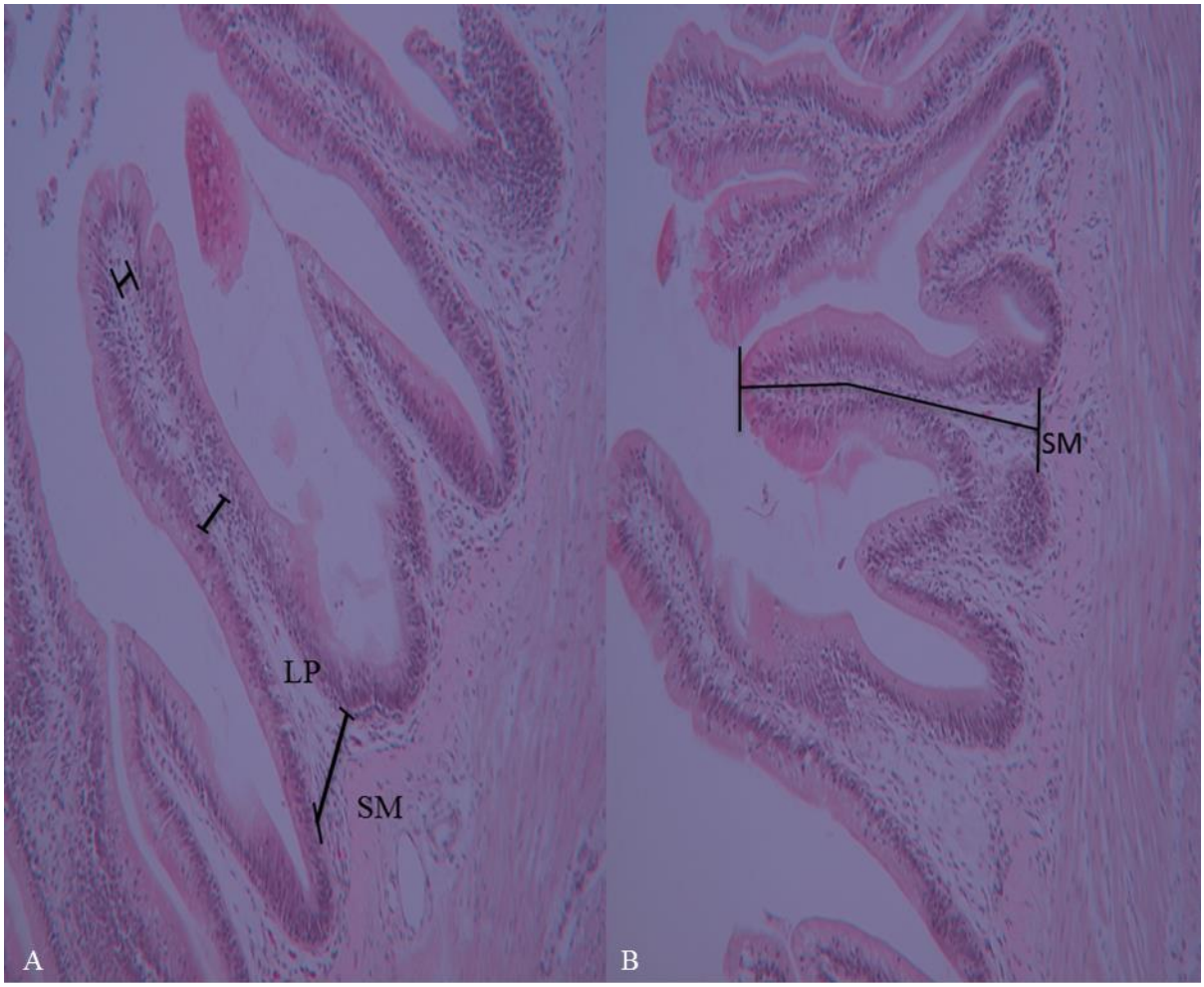


Figure 2.4. Lamina propria widths were measures in three places along complete folds (bottom, middle and top of the fold) (A). Complete mucosal folds were measured by drawing a line at the top of the submucosa (SM) (base of the fold) and measuring to the tip of the fold with care taken to keep the line within the lamina propria (B).

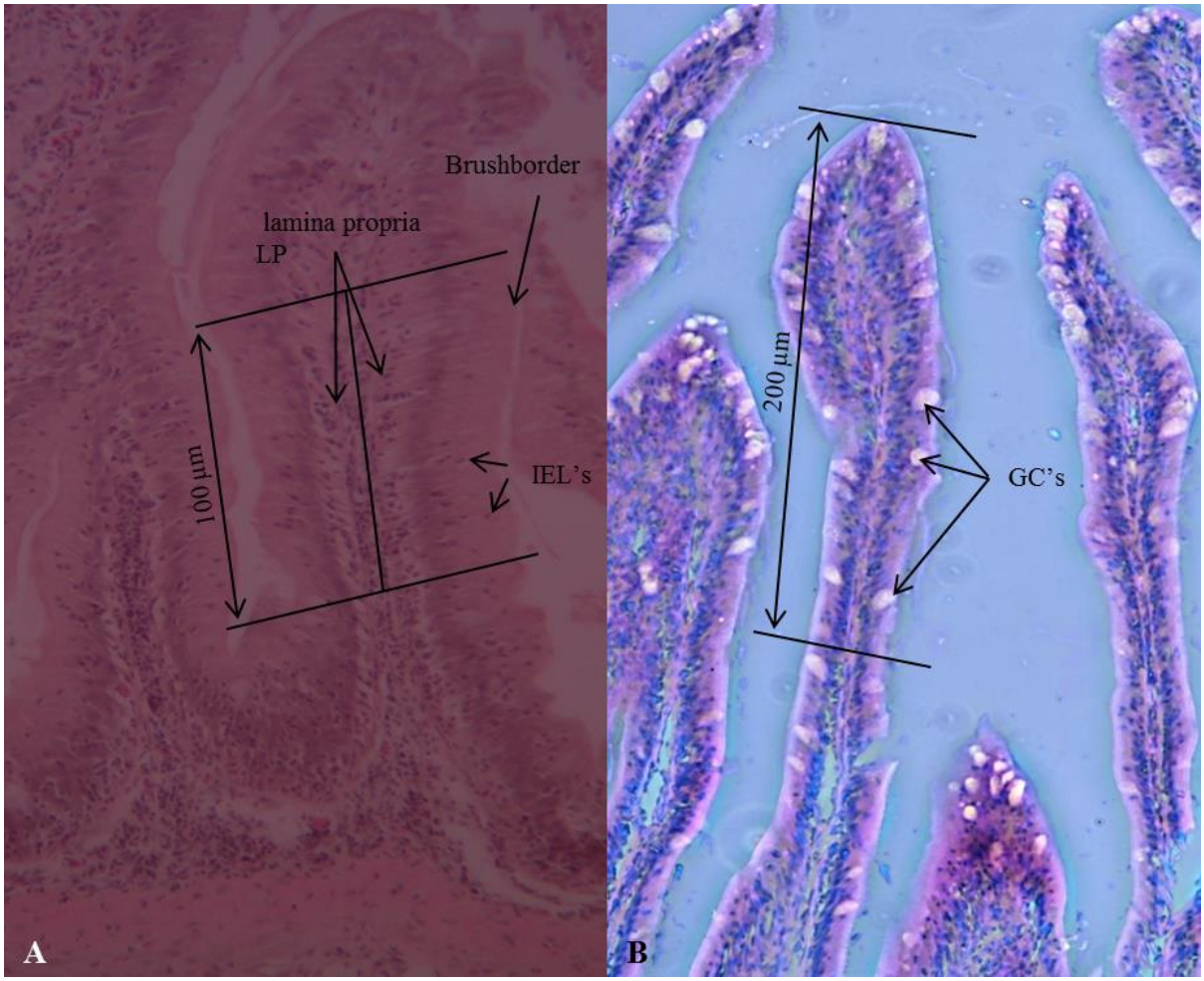


Figure 2.5. Intraepithelial leucocytes (IEL's) were counted by measuring 100 µm of the fold and counting cells in the epithelial layer between the lamina propria and brush border. An average of two counts per fold (either side of the fold) was taken (Figure A). For goblet cell (GC) counts a light microscope in phase contrast mode was used to show the GC's more clearly. A measurement 200 µm from the tip of the fold was measured out and an average GC count on either side of the fold was taken per fold measured (Figure B).

2.8.2. Scanning electron microscopy (SEM)

Samples were fixed in 2.5 % glutaraldehyde with 0.1 M sodium cacodylate buffer (1:1 vol., pH 7.2, 3% NaCl). Samples were then dehydrated in a graded ethanol series each step for 15

min (30 % alcohol, 50 %, 70 %, 90 % and 100 % x2) and critical point dried (K850 Emithech) with ethanol as the intermediate fluid and CO_2 as the transition fluid. The samples were then sputter coated (K550 Emitech) with gold and viewed with JSM 6610 LV and JSM 7001 F electron microscopes (JEOL, Tokyo, Japan). Multiple images were captured per sample at magnifications ranging from x 500 – x 20,000. Microvilli density measurements were carried out in image J 1.45 using images taken at x 20,000 magnification. A ratio of foreground (microvilli) / background (gaps between microvilli) was calculated to give a density value (arbitrary units; AU) (Figure 2.6.).

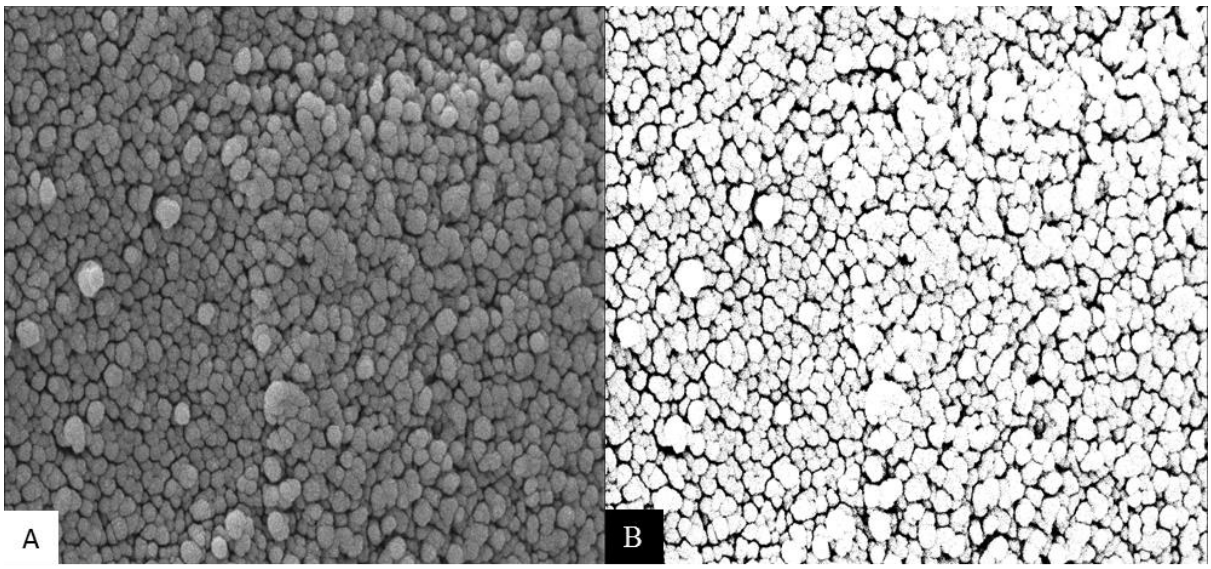


Figure 2.6. High magnification (x 20,000) electron micrographs showing the posterior intestine of European sea bass and how the microvilli measurements were carried out in image J. The image is loaded into image J (A), a threshold of the image is then applied to the image (B) and a ratio of the white and black is calculated to give a microvilli density ratio expressed as arbitrary units (AU).

2.8.3. Transmission electron microscopy (TEM)

Samples were fixed in 2.5 % glutaraldehyde with 0.1 M sodium cacodylate buffer (1:1 vol., pH 7.2, 3% NaCl). Samples were then post-fixed in OsO_4 (1 %, cacodylate buffer pH 7.2, 0.1M) for 1 hr The tissue was dehydrated through a graded alcohol series which was then replaced with agar low viscosity resin in increasing concentrations (30 Resin: 70 Alcohol, 50:50, 70:30) with 12 hours between each step until samples were in 100 % resin. Samples were then placed in beem capsules and embedded over night at 60 ^{o}C. Ultrathin sections (6 µm) were cut on a Leica Ultra-microtome with a diatome diamond knife. The sections were then stained using a saturated solution of uranyl acetate (15 min) and Reynolds lead citrate (15 min). Samples were screened using a JEOL 1200 EX II TEM (Tokyo, Japan). TEM sampling and processing was conducted after Dimitroglou *et al*. (2009). Ten well orientated microvilli were measured (Merrifield *et al*. 2009) within three different images of each sample with a total of 4 samples per treatment. Measurements were carried out using Image J 1.45 (National Institutes of Health, USA), as illustrated in Figure 2.7.

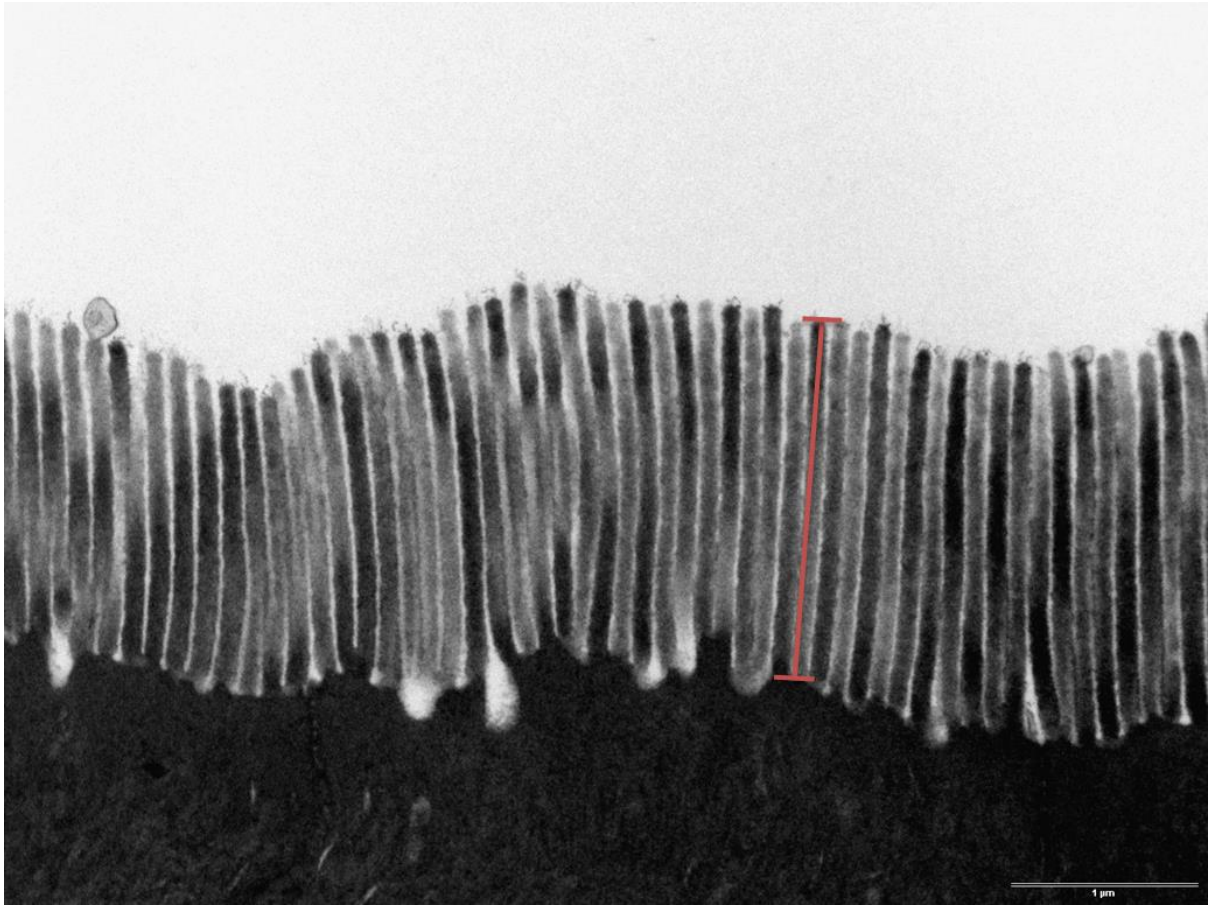


Figure 2.7. Electron micrograph of the posterior intestine illustrating for microvilli heights were measured in image J. Microvilli heights were measured by drawing a line from the base to the tip of complete well defined microvilli.

2.9. Statistical analyses

In order to evaluate similarities between treatment groups within the PCR-DGGE banding patterns were transformed into intensity matrices using the software Quantity One, version 4.6.3 (Bio-Rad Laboratories), after Schauer *et al*. (2000). Primer V6 software (Clarke & Gorley 2006) was used to analyse and measure band intensities, and to determine similarity percentages (SIMPER). Pairwise comparisons to determine differences between PCR-DGGE banding profiles were analysed by a one-way analysis of similarity (ANOSIM) (Abell and

Bowman 2005). Ecological calculations were also carried out in Primer V6: total number of operational taxonomical units OTU's (*S*).Margalef species richness ($d = (S - 1)/\ln (N)$); where *S* is the number of species and *N* is the total number of individuals (total intensity units). Shannon diversity index ($H' = 9 \Sigma (p_i (\ln p_i))$; where p_i represents the proportion of the total number of individuals in the *i*th species. These parameters were subjected to a one-way ANOVA.

For high-throughput sequencing data Good's estimator of coverage was calculated using the formula: $(1-(\text{singletons/individuals})) \times 100$. Chao 1 index was calculated using the formula: $S_{chao1} = S + (n_1\text{-}1)/ (n_2+1)$; where S_{chao1} is the estimated richness, *S* is the number of observed species, n_1 is the number of OTU's with one sequence (singletons) and n_2 is the number of OTU's with two sequences (doubletons). The Bray-Curtis was calculated using the formula; $C_n = 2jn/ (na + nb)$; where Na is the total number of individuals in site (treatment) A, *n*b is the total number of individuals in site B and 2j*n* in the sum of the lower of the two abundances for species found in both sites. The phylogenetic metric (PD) represents the minimum total branch length which covers all taxa within the sample present on the phylogenetic tree. A Kruskal-Wallis test was performed followed by pairwise comparison to compare OTU abundance and alpha diversity metrics, Vegan and ape packages of R were used to analyse the beta diversity of the groups. STAMP v2.0.8 was used to perform an ANOVA and Tukey's *post hoc* test to distinguish differences at the phylum and genus levels.

For all other data, means ± standard deviation (SD) are presented. Statistical analyses were carried out using SPSS version 18 (SPSS Inc., Chicago, IL, USA). Data were tested for normality and a one-way ANOVA was carried out thereafter. Significant differences between the control and treatment groups were determined by Tukey's *post hoc* test. In all cases, significance was accepted at $P < 0.05$.

Chapter 3:

Dietary induced changes to the intestinal morphology and microbiome of European sea bass *D. labrax*

3.1. Abstract

The present study was designed to develop an enteritis model to be used in Chapter 4A to test the efficacy of feed additives on any potential intestinal inflammation which manifest as a consequence of fishmeal (FM) replacement. Chapter 3 therefore assessed the effects of the partial replacement of dietary FM with soy protein concentrate (SPC), and pea protein concentrate (PPC), with and without the inclusion of saponins (S), on the intestinal microbiota and morphology of European sea bass. Fish (24.40 ± 0.25 g) were fed for four weeks with one of five dietary regimes: 1] FM (control), 2] SPC, 3] SPC+PPC, 4] SPC+PPC+S, and 5] SPC+S. PCR-DGGE and high-throughput sequencing analyses revealed distinct clusters between treatments suggesting differences in the intestinal microbiomes. PCR-DGGE also revealed significantly higher numbers of operational taxonomic units (OTU's) in the plant based treatments compared to the control group after two and four weeks. Histological analyses revealed a significant ($P < 0.05$) reduction in goblet cells in fish fed the SPC+S supplemented diets when compared to FM fed fish at two and four weeks, as well as significant ($P < 0.05$) reduction in intraepithelial leukocytes (IEL's) in fish fed the SPC+S supplemented diets when compared to control fed fish at week four ($P < 0.05$). Microvilli density was also significantly reduced in fish fed SPC+PPC, SPC+PPC+S and SPC+S treatments at weeks two and four ($P < 0.05$). This present study revealed that partial replacement of fishmeal with SPC and PPC, with and without saponin supplementation, cause changes to the intestinal microbiota and morphology of European sea bass.

3.2. Introduction

Replacing fishmeal (FM) in aquafeeds, especially for carnivorous species, has been challenging for the aquaculture industry. Alternative protein sources derived from various plant based products have been increasingly studied, and utilised, in commercial aquafeeds. Soy proteins such as soybean meal (SPM) and soy protein concentrate (SPC) have the potential to replace, at least in part, a considerable percentage of FM in aquafeeds. Soy protein products contain a well-balanced amino acid profile and a lower market price compared to FM. However, certain anti-nutritional factors (ANF's) contained in soy protein products limits their use in animal feeds and various negative health implications for fish have been associated with these protein source (Gatlin *et al.* 2007). Some ANF's of SPC include fibres, phytic acid, enzyme inhibitors, lectins and saponins, which can reduce nutrient digestibility and growth performance. Some of these compounds, such as saponins, are known to interfere with the permeability of intestinal membranes, affecting the influx and efflux of molecules and bacteria (Knudsen *et al.* 2008; Øverland *et al.* 2009; Krogdahl *et al.* 2010). High dietary SPC inclusion levels have been reported to induce histopathological disruption in the gastrointestinal (GI) tract of fish which include: shortening of the mucosal folds, reducing the absorptive surface area of the intestine, widening of the lamina propria, loss of supranuclear vacuolisation of the enterocytes, and up-regulation of pro-inflammatory genes such as IL-1β and TNF-α (Krogdahl *et al.* 2000; Knudson *et al.* 2007; Uran *et al.* 2008, 2009). These deleterious changes, collectively known as enteritis, observed in the GI tract have predominantly been reported in salmonid species (Baeverfjord & Krogdahl 1996; Burrells *et al.* 1999; Nordrum *et al.* 2000; Krogdahl *et al.* 2003), but more recently similar effects have been detected in common carp (Uran *et al.* 2008; Marel *et al.* 2014). Pea protein meals and concentrates (PPM's and PPC's) have also been used as a partial replacement of FM in aquafeeds. Studies in Atlantic salmon (Øverland *et al.* 2009) and rainbow trout

(Thiessen *et al.* 2003) have indicated that PPC inclusion levels of up to 20 % did not cause inflammation in the posterior intestine and supported feed conversion ratios and growth performance equal to that of the control fed fish. Higher dietary levels of PPC (35 %) have been reported to lower growth performance and induce enteritis in the posterior intestine of Atlantic salmon. Saponins are a particularly important ANF present in SPC, PPC and other plant based protein sources and the levels of which require careful consideration when designing animal feeds (Gatlin *et al.* 2007; Krogdahl *et al.* 2010). Saponins are glycosides, steroidal or triterpenoid in nature, which form soap-like foams in aqueous solutions whiah are known to cause membrane disruption in mammals due to their haemolytic nature (Francis *et al.* 2002a; Bouarab *et al.* 2002; Augustin *et al.* 2011). Salmonid studies have revealed that saponins cause an increase in gut permeability and are a causative agent in the development of enteritis (Bureau *et al.* 1998; Knudsen *et al.* 2007, 2008; Iwashita *et al.* 2008). A review by Krogdahl *et al.* (2010) concluded that saponins play an important role in the onset of enteritis in salmonids but only when other plant based compounds are present in the diet. More recently however, there is evidence to suggest that saponins induce enteritis in Atlantic salmon independent of other plant-based compounds (Krogdahl *et al*. 2015). Saponins have also been reported to affect fish growth, metabolism, cholesterol levels in males and sex ratios favouring males in Nile tilapia (Francis *et al.* 2001; Francis *et al.* 2002b).

The role of microbial communities in fish has recently received much attention and the importance of the many complex interactions which occur in the gut are a key factor affecting the health of the host (Nayak 2010; Dimitroglou *et al.* 2011). As a consequence there are a growing number of studies describing the effects of dietary plant proteins (SPC in particular) on the intestinal microbiota of fish (Heikkinen *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008; Merrifield *et al.* 2009; Cai *et al.* 2012; Desai *et al.* 2012; Silvia *et al.* 2012; Reveco *et al.* 2014). The impact of plant based protein sources on

intestinal microbial communities of fish is only partly described but readers with an interest in this topic are referred to the review by Merrifield *et al.* (2011).

Most studies regarding the effects of plant proteins on fish gut histology and microbiology have focused on long term effects at the end of growth trials (i.e. > 10 weeks) and so short-term, temporal and transitional response effects may not have been identified. Therefore, the aim of the present study was to assess the short-term effects caused by partial FM replacement with plant proteins (with and without additional saponin supplementation) on European sea bass intestinal morphology and microbiota.

3.3. Materials and Methodologies

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

3.3.1. Experimental design

European sea bass were obtained from Anglesey Aquaculture Ltd, Black Point, Beaumaris UK and transported to the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK with an acclimation period of six weeks. The fish were then graded and separated into 15 x 110 L fibreglass tanks in a closed recirculatory system at a stocking density of 40 fish per tank with an average weight of 24.40 ± 0.25 g. Each dietary treatment was randomly attributed to the tanks in triplicate and the fish were fed each experimental diet at a rate of 2 – 3 % of biomass per day in equal rations at 9:00 and 17:00. Daily feed was adjusted on a weekly basis by batch weighing following a 24-h starvation period. Fish were

held at 24 ± 1 °C and 30 ± 2 ppt salinity with a photoperiod of 12: 12 h light: dark. Water quality parameters were maintained at 7.0 ± 0.5 pH and dissolved oxygen > 85 % saturation, and were monitored daily. Ammonium, nitrite and nitrate levels were monitored weekly with weekly water changes of approx. 25 % system volume were conducted to minimise the build-up of these compounds.

3.3.2. Diet preparation

Five iso-nitrogenous and iso-lipidic dietary regimes were formulated (Table 3.1.) to meet the known requirements of European sea bass (NRC 2011). The dry ingredients were mixed in a Hobart food mixer (Hobart Food Equipment, Sydney, Australia, model no: HL1400–10STDA). The oil and hot water were gradually added to the mixer and cold press extrusion was conducted (PTM P6 extruder, Plymouth, UK) to produce 2 mm pellets. The pelleted diets were then dried to achieve ca. 5 % moisture content in an air convection oven set at 50 °C for 48 hours. The diets were then broken up to the appropriate size and the composition analysed using AOAC (1995) protocols (Table 3.1.). Experimental diets were subsequently stored in airtight containers prior to use.

Table 3.1. Dietary formulation (%) and chemical composition

	FM	SPC	SPC + PPC	SPC + S	SPC + PPC +S
Fishmeal[a]	62.93	20.00	20.00	20.00	20.00
Soy protein concentrate[b]	-	52.72	18.51	52.72	18.51
Lysamine pea protein[c]	-	-	25.00	-	25.00
Glutalys[c]	10.00	10.00	10.00	10.00	10.00
Fish oil[d]	7.25	11.08	9.81	11.08	9.81
Corn starch[e]	17.22	3.60	14.68	3.30	14.38
Mineral/vitamin premix[f]	2.60	2.60	2.00	2.60	2.00
Saponin[g]	-	-	-	0.30	0.30
Proximate analysis (%)					
Dry matter	91.2	91.3	94.1	94.6	93.5
Crude protein*	50.3	48.9	51.1	50.5	50.9
Crude lipid*	13.1	14.7	13.7	14.5	13.8
Ash*	9.1	7.1	5.1	7.4	4.8
Gross energy (MJ kg^{-1})*	20.3	21.0	21.0	20.9	21.3

[a] Herring meal LT94: CC MOORE & Co. Ltd., Dorset, UK.
[b] Hamlet HP100 (56 % crude protein), Hamlet Protein, Denmark.
[c] Roquette Company, Frêres, France.
[d] Seven seas Ltd. Hull, UK
[e] Sigma-Aldrich Company, UK.
[f] Premier Nutrition Products (PNP Ltd.) Rugeley, Staffordshire, UK.
[g] Sigma–Aldrich Company, UK. (20-30% sapogenic content)
* % wet weight basis

3.3.3. Dietary proximate analyses

Proximate analysis of diets was determined as described in section 2.5.

3.3.4. Sampling

Two fish per tank were sampled for microbiology and an additional two per tank for histology (n = 6) at weeks two and four. Fish were euthanized by an overdose (200 mg/l water for 5 min) of MS-222 (Pharmaq) and destruction of the brain. Fish were dissected under aseptic conditions and the intestine was isolated. Lipid deposits were removed and the intestine was cut just below the pyloric caeca. To assess the allochthonous bacterial

populations, digesta was removed from the posterior region of the intestine using sterile forceps and collected into sterile microcentrifuge tubes and stored at -20 ^{o}C until use.

3.3.5. Microbiological analysis

3.3.5.1. PCR-DGGE and sequencing

For PCR-DGGE analysis, digesta samples from two fish per tank were pooled and homogenised, thus providing n = 3 per treatment. DNA from 200 mg samples was extracted as described in section *2.7.1.* PCR-DGGE analysis and sequencing was carried out as described in sections *2.7.2.* and *2.7.3.*

3.3.5.2. High-throughput sequencing

For high-throughput sequence analysis, digesta samples were sampled from one fish per tank for SPC, SPC+PPC, SPC+PPC+S and SPC+S treatments (n = 3), and one fish from two tanks from the FM treatment (n = 2). DNA from 200 mg samples was extracted as described in section *2.7.1.* High-throughput sequence analysis was carried out as described in section *2.7.4.*

3.3.6. Intestinal histology

3.3.6.1. Light microscopy

Light microscopy analysis was carried out on two fish per tank (n = 6) as described in section *2.8.1.*

3.3.6.2. SEM

SEM analysis was carried out on two fish per tank (n = 6) as described in section *2.8.2.*

3.3.6.3. TEM

TEM analysis was carried out on four fish per treatment (n = 4) as described in section *2.8.3.*

3.3.7. Statistical analysis

Statistical analysis was carried out as described in section 2.9.

3.4. Results

3.4.1. Gross observations

Fish accepted the diets well and there was 100 % survival throughout the trial. No significant differences in growth were observed among the treatments at week two (33.22 ± 5.55 g) or at week four (41.82 ± 7.54 g).

3.4.2. Microbiological analyses

3.4.2.1. PCR-DGGE

The V3 16S rRNA PCR-DGGE fingerprints and respective dengrograms for weeks two and four are presented in Figure 3.1 A & B. The microbial ecological parameters for week two and four are presented in tables 3.2. & 3.3.

3.4.2.1.1. Week two

A total of 52 distinct OTU's were observed on the denaturing gradient gel. A high level of dissimilarity (46.72 – 53.91 %) was observed when the control was systematically compared to the plant based treatments (Figure 3.1.A; Table 3.2.). The number of OTU's increased incrementally with increasing FM substitution and was significantly increased from 23.67 ± 7.09 in the control to 34.67 ± 0.58 in the SPC+PPC treatment group ($P = 0.05$). The mean SIMPER value observed for the replicates within the control revealed a low degree of inter-replicate similarity at 52.95 ± 14.82 %. In contrast, SIMPER values observed for the replicates within the plant based groups were markedly higher, significantly so in the SPC (80.87 ± 6.91; $P < 0.05$), SPC+PPC (93.49 ± 2.33; $P < 0.05$), and SPC+S (88.64 ± 2.98; $P < 0.01$). Species richness and diversity remained unaffected at week two.

3.4.2.1.2. Week four

A total of 54 distinct OTU's were observed on the denaturing gradient gel at week four (Figure 3.1.B). The dissimilarity was high (38.80 – 41.38 %) when the control was compared to the plant based treatments (Table 3.3.). The number of OTU's was highest in the SPC+PPC+S treatment (49.67 ± 1.53) and was significantly higher when compared to the control (41.67 ± 2.31; $P < 0.001$), SPC (45.33 ± 0.58; $P < 0.05$), and SPC+PPC (45.33 ± 1.58; $P < 0.05$) treatment groups. Species richness increased from 3.77 ± 0.20 in the control to 4.21 ± 0.11 in the SPC+PPC+S treatment ($P < 0.01$). Shannon's diversity index also increased from 3.62 ± 0.08 in the control to 3.77 ± 0.03 in the SPC+S ($P < 0.05$), 3.77 ± 0.04 in the SPC+PPC ($P < 0.05$), and 3.86 ± 0.03 in the SPC+PPC+S ($P < 0.001$).

3.4.2.2. DGGE Sequence analysis

A number of OTU's were excised for sequence analysis from the PCR-DGGE (Tables 3.4. and 3.5.). The presence of OTU's DP3 (*Lactobacillus salivarious*), DP4 (unidentified bacteria with 93 % similarity to *L. fermentum*), DP14 (*L. buchneri*), DP16 (*Collimonas fungivorans*), DP17 (*Enterobacteriaceae* FG157) and DP18 (*Enterococcus faecalis*) were present in at least two of the three replicates of each treatment. OTU's DP1 (*B. subtilis*), DP12 (*B. subtilis*), DP2 (*Paenibacillus mucilaginous*), DP13 (*P. mucilaginous*) and DP6 (*L. johnsonii*) appeared to be affected by PPC inclusion with their presence in at least two replicates of fish fed the PPC diets and their absence from fish fed the other treatments, with the exception of DP6 which was detected in one replicate of the FM fed fish. The presence of OTU's DP5 (*L. buchneri*), DP19 (*B. subtilis*), DP20 (*B. subtilis*), and DP21 (*Desulfosporosinus* sp.) appeared to be influenced by all plant ingredients with their presence observed in all three replicates of fish fed the plant based diets compared to either absence or presence in only one replicate of fish fed the control diet.

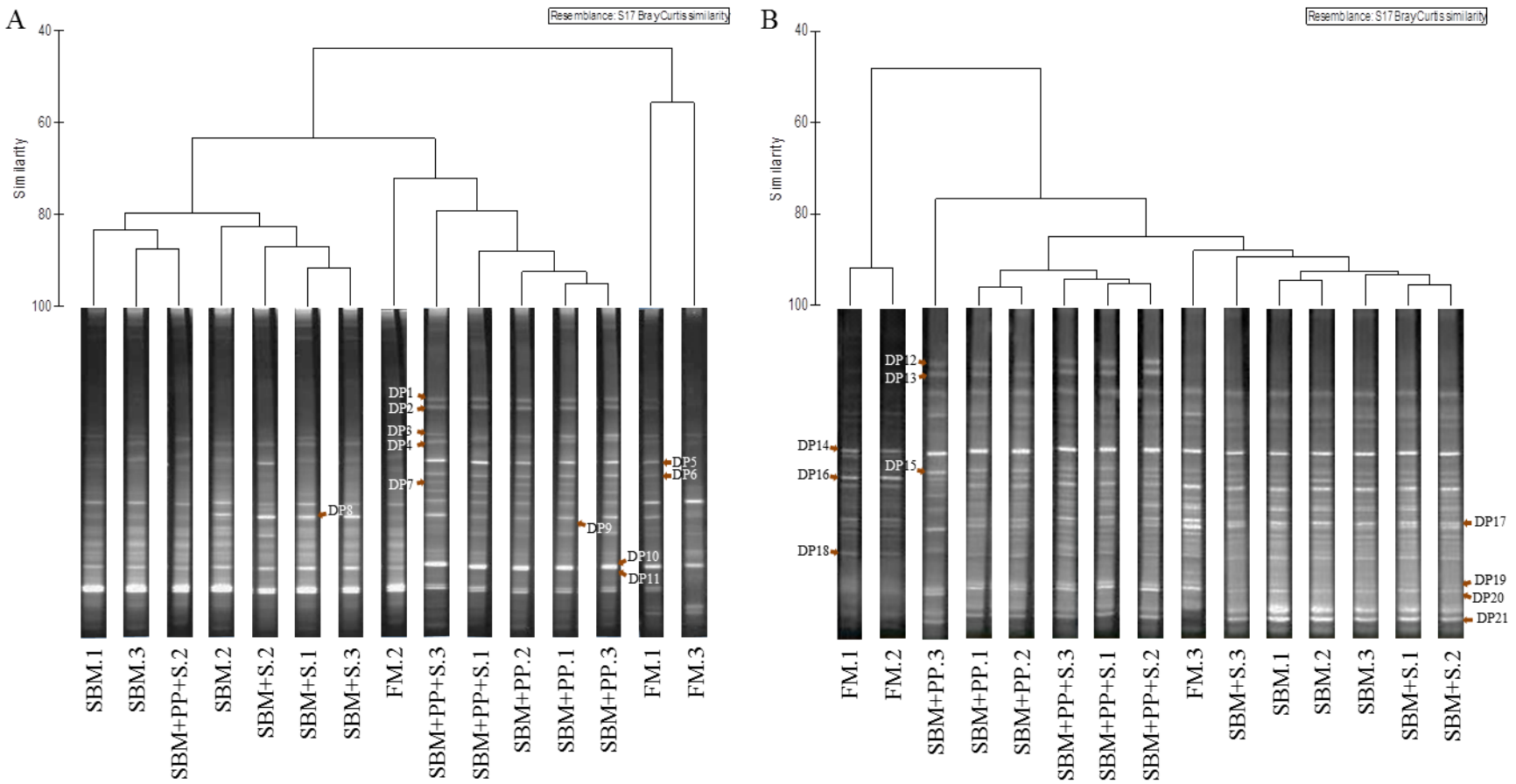


Figure 3.1. Cluster analysis dendrograms of the PCR-DGGE fingerprint profiles of the bacterial community in the posterior intestine of European sea bass after being fed the experimental diets for two (A) and four (B) weeks.

Table 3.2. Microbial community analysis from the PCR-DGGE of the allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for two weeks. (ANOVA + *post hoc* Tukey's) accepted at $P < 0.05$. Values expressed as means ± standard deviation.

	Ecological parameters				**ANOSIM**		
Treatment	OTU's	Richness†	Diversity‡	SIMPER (similarity %)	*R*-value	*P*-value	Dissimilarity (%)
FM	24.00±7.09[a]	2.17±0.56	3.07±0.34	52.95±14.82[a]			
SPC	31.00±4.00 [ab]	2.72±0.31	3.32±0.14	80.87±6.91[b]			
SPC+S	30.00±1.53[ab]	2.66±0.14	3.32±0.05	88.64±2.98[b]			
SPC+PPC	35.00±0.58[b]	3.04±0.04	3.50±0.02	93.49±2.33[b]			
SPC+PPC+S	31.00±2.31[ab]	2.79±0.19	3.36±0.09	70.73±11.75[ab]			
Pairwise comparisons							
FM vs SPC					0.67	0.10	52.30
FM vs SPC.S					0.70	0.10	53.91
FM vs SPC.PPC					0.52	0.10	46.72
FM vs SPC.PPC.S					0.30	0.10	47.45
SPC vs SPC.S					0.33	0.10	18.71
SPC vs SPC.PPC					1.00	0.10	36.25
SPC vs SPC.PPC.S					0.41	0.30	31.26
SPC.S vs SPC.PPC					1.00	0.10	31.34
SPC.S vs SPC.PPC.S					0.52	0.10	30.50
SPC.PPC vs SPC.PPC.S					0.41	0.10	24.33

SIMPER, similarity percentage within replicates of each treatment; ANOSIM, analysis of similarities between treatments.
† Margalef species richness: $d = (S - 1)/\log(n)$.
‡Shannon's diversity index: $H' = -\text{SUM}(pi*\log(pi))$.
Values expressed as means ± standard deviation.
[ab] Different superscript letters in the same row indicate significant differences accepted at $P < 0.05$

Table 3.3. Microbial community analysis from the PCR-DGGE of the allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks.

	Ecological parameters				**ANOSIM**		
Treatment	OTU's	Richness†	Diversity‡	SIMPER (similarity %)	*R*-value	*P*-value	Dissimilarity (%)
FM	41.67±2.31^{a}	3.77±0.20^{a}	3.62±0.08^{a}	63.91±24.27			
SPC	45.33±0.58^{a}	3.84±0.05^{a}	3.74±0.01^{ab}	93.30±1.84			
SPC+S	45.67±1.53^{ab}	3.87±0.11^{a}	3.77±0.03^{bc}	92.16±3.67			
SPC+PPC	45.33±1.53^{a}	3.88±0.10^{a}	3.77±0.04^{bc}	88.40±6.67			
SPC+PPC+S	49.67±1.53^{b}	4.21±0.11^{b}	3.86±0.03^{c}	94.59±0.65			
Pairwise comparisons							
FM vs SPC					0.67	0.1	38.80
FM vs SPC.S					0.63	0.1	39.03
FM vs SPC.PPC					0.54	0.2	41.48
FM vs SPC.PPC.S					0.70	0.1	40.35
SPC vs SPC.S					0.26	0.2	08.63
SPC vs SPC.PPC					0.85	0.1	21.84
SPC vs SPC.PPC.S					1.00	0.1	14.95
SPC.S vs SPC.PPC					0.70	0.1	18.90
SPC.S vs SPC.PPC.S					0.82	0.1	11.87
SPC.PPC vs SPC.PPC.S					0.56	0.1	10.91

SIMPER, similarity percentage within replicates of each treatment; ANOSIM, analysis of similarities between treatments.
† Margalef species richness: $d = (S - 1)/\log(n)$.
‡Shannon's diversity index: $H' = -\text{SUM}(pi * \log(pi))$.
Values expressed as means ± standard deviation.
^{ab} Different superscript letters in the same row indicate significant differences accepted at $P < 0.05$

Table 3.4. Closest bacterial relatives (% similarity) of excised bands from the PCR-DGGE of the allochthonous bacterial communities from the European sea bass posterior intestine after being fed the experimental diets for two weeks. The presence of OTU's in each replicate of each dietary regime is indicated; 0 = not present in any replicate, 1 = present in one replicate, 2 present in two replicates and 3 present in all three replicates.

Band ID	**Band presence**					**Phyla**	**Nearest neighbour**	**Alignment similarity (%)**	**Accession number**
	Replicates								
	FM	SPC	SPC+PPC	SPC+PPC+S	SPC+S				
DP1	0	0	3	2	0	Firmicutes	*B. subtilis*	100	NC_019896.1
DP2	0	0	3	2	0	Firmicutes	*Paenibacillus mucilaginosus*	100	NC_017672.1
DP3	2	3	3	3	3	Firmicutes	*Lactobacillus salivarius*	98	NC_017481.1
DP4	3	3	3	3	3	Firmicutes	*Lactobacillus fermentum*	93	NC_017465.1
DP5	1	3	3	3	3	Firmicutes	*Lactobacillus buchneri*	100	NC_018610.1
DP6	1	0	3	2	0	Firmicutes	*Lactobacillus johnsonii*	100	NC_017477.1
DP7	0	0	3	2	2	Firmicutes	*Lactobacillus johnsonii*	100	NC_017477.1
DP8	1	0	3	1	3	Firmicutes	*Staphylococcus aureus*	100	NC_018608.1
DP9	3	0	3	1	0	Firmicutes	*Thermoanaerobacterium saccharolyticum*	92	NC_017992.1

DP10	3	3	3	1	0	Firmicutes	*B. subtilis*	100	NC_019896.1
DP11	0	0	3	2	3	Firmicutes	*B. subtilis*	100	NC_019896.1
DP12	0	0	3	3	0	Firmicutes	*B. subtilis*	100	NC_019896.1
DP13	0	0	3	3	0	Firmicutes	*Paenibacillus mucilaginosus*	100	NC_017672.1
DP14	3	3	3	3	3	Firmicutes	*Lactobacillus buchneri*	100	NC_018610.1
DP19	1	3	3	3	3	Firmicutes	*B. subtilis*	100	NC_019896.1
DP20	1	3	3	3	3	Firmicutes	*B. subtilis*	100	NC_019896.1

Table 3.5. Closest bacterial relative (% similarity) for sequences excised from the PCR-DGGE of the allochthonous bacterial communities from the European sea bass posterior intestine after being fed the experimental diets for four weeks. The presence of OTU's in each replicate of each dietary regime is indicated; 0 = not present in any replicate, 1 = present in one replicate, 2 present in two replicates and 3 present in all three replicates.

Band ID	**Band presence**					**Phyla**	**Nearest neighbour**	**Alignment similarity (%)**	**Accession number**
	Replicates								
	FM	SPC	SPC+PPC	SPC+PPC+S	SPC+S				
DP1	0	0	3	2	0	Firmicutes	*B. subtilis*	100	NC_019896.1
DP2	0	0	3	2	0	Firmicutes	*Paenibacillus mucilaginosus*	100	NC_017672.1
DP10	3	3	3	1	0	Firmicutes	*B. subtilis*	100	NC_019896.1
DP11	0	0	3	2	3	Firmicutes	*B. subtilis*	100	NC_019896.1
DP12	0	0	3	3	0	Firmicutes	*B. subtilis*	100	NC_019896.1
DP13	0	0	3	3	0	Firmicutes	*Paenibacillus mucilaginosus*	100	NC_017672.1
DP15	2	0	3	3	0	Firmicutes	*Lactobacillus buchneri*	100	NC_018610.1
DP16	3	1	3	2	3	Proteobacteria	*Collimonas fungivorans*	100	NC_015856.1
DP17	3	3	2	3	3	Proteobacteria	*Enterobacteriaceae* bacterium strain FGI57	100	NC_020063.1

DP18	3	3	2	3	3	Firmicutes	*Enterococcus faecalis*	100	NC_018221.1
DP19	1	3	3	3	3	Firmicutes	*B. subtilis*	100	NC_019896.1
DP20	1	3	3	3	3	Firmicutes	*B. subtilis*	100	NC_019896.1
DP21	0	3	3	3	3	Firmicutes	*Desulfosporosinus* sp.	100	NC_018515.1

3.4.2.3. High-throughput sequencing

After trimming and QC, a total of 491,354 reads were retained and subsequently used for downstream analyses. Alpha rarefaction curves for all samples reached, or were approaching, a plateau after approx. 7,000 sequences (Figure 3.2.). Further, Good's coverage estimators for all samples were > 0.99 (Table 3.6.), indicating that the bacterial community was fully sampled and that the OTU's detected in the samples were representative of the population (Sims *et al*. 2014). The Bray-Curtis UPGMA showed a clear differentiation between replicates within treatment groups indicated by the grouping of samples into three clusters: one cluster comprising samples from the FM treatment, which were distinctly different from the other treatments, the second cluster containing most of the SPC samples, and the third cluster containing most the SPC+PPC samples (Figure 3.3.). No statistical differences between species richness (Chao1) or diversity (Shannon-Wiener) were observed between treatment groups.

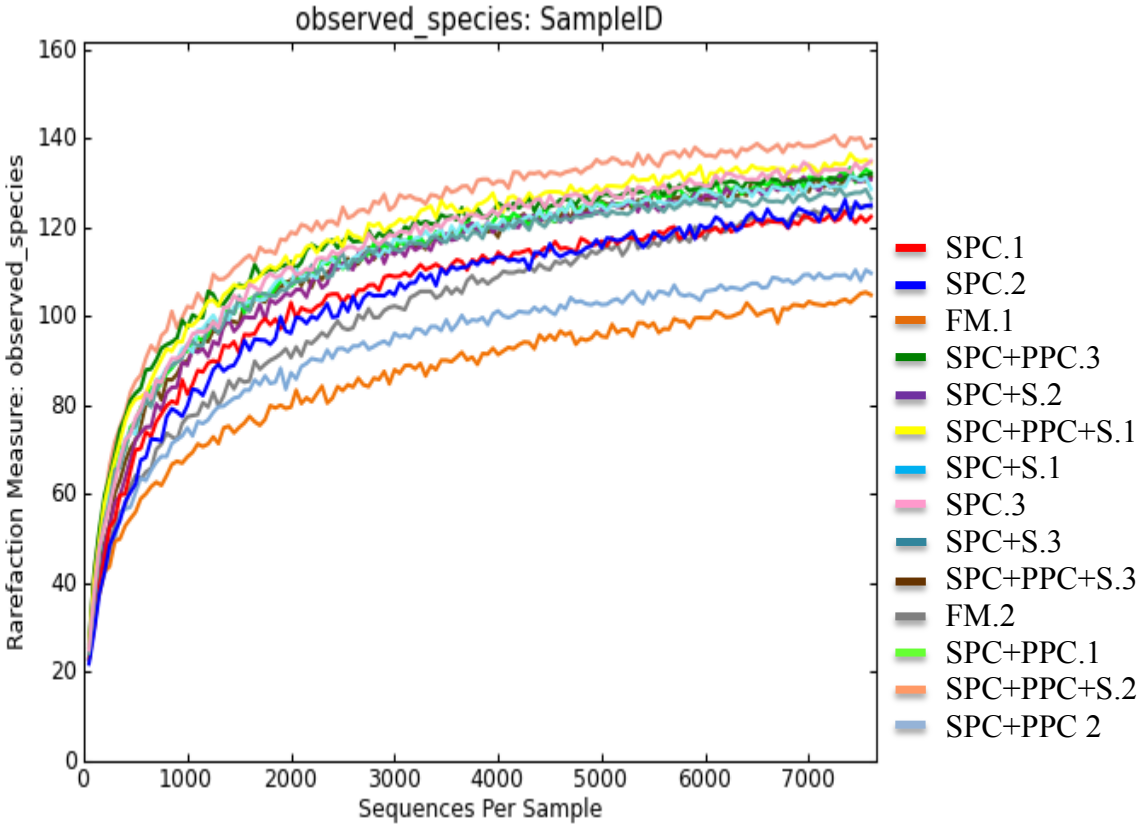


Figure 3.2. Alpha rarefaction curves representing the number of OTU's per sample, which is used as an inference of the number of species, as a function of the sequencing effort.

Table 3.6. High-throughput sequencing good's coverage estimations by treatment and alpha diversity parameters of the allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks. Data represent mean ± SD.

Treatment	Good's coverage	Observed species	Chao 1 index	Shannon index
FM	0.9971±0.0002	114.78±10.22	134.70±11.76	4.82±0.02
SPC	0.9982±0.0002	126.54±5.14	135.56±5.33	4.70±0.24
SPC+S	0.9984±0.0001	129.56±0.85	138.01±1.15	5.05±0.17
SPC+PPC	0.9982±0.0004	124.48±10.25	137.96±5.55	5.15±0.27
SPC+PPC+S	0.9983±0.0001	135.14±3.59	144.35±3.13	5.18±0.15

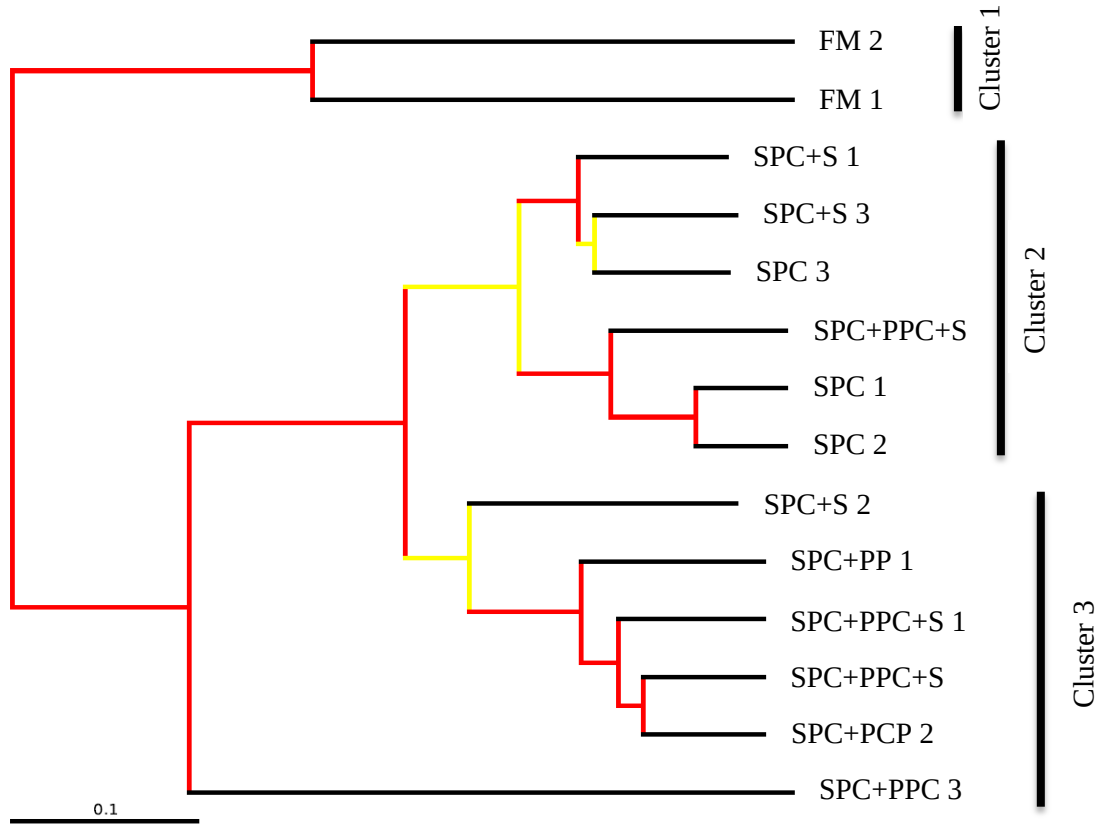


Figure 3.3. Bray-Curtis UPGMA UniFrac clustering of reads of the replicates of the allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks. Jackknife support is: Red (75-100 %) and yellow (50-75 %). Bar indicates 10 % divergence. Cluster 1 contains samples of the FM fed fish. Cluster 2 contains all samples of the SPC fed fish and cluster 3 contains all but one of the PPC fed fish.

Figure 3.4. illustrates the sequence distribution at the phylum level. A large proportion (30.60 %) of the reads, predominantly present in the samples from fish fed SPC containing diets, belonged to the order Streptophyta. BLAST results identified these reads as chloroplast nucleotides and it was therefore concluded that these reads were artefacts as a consequence of the high plant meal inclusion levels; these reads were subsequently removed from the sequence libraries, as has been described elsewhere in fish gut microbiota studies (Wong *et al.* 2013). The majority of the remaining sequences belonged to the Proteobacteria (62.40 %), followed by Firmicutes (35.30 %), Actinobacteria (1.80 %) and Bacteriodetes (0.40 %). The abundance of the reads assigned to each phylum was not significantly affected by dietary treatment.

Figure 3.5. illustrates the sequence distribution at the genus level (displaying genera that contain > 0.2 % of the total sequences). The most abundant 16S rRNA reads belonged to an unidentified genus from the *Enterobacteriaceae* family (21.10 %), followed by *Leuconostoc* (16.20 %), *Acinetobacter* (12.90 %), *Weissella* (7.70 %) and *Bradyrhizobium* (7.10 %). The relative abundance of reads assigned to *Pseudomonas*, *Clostridium*, *Acinebactacter*, *Enterococcus* and *Acidovorax* were significantly ($P < 0.001$) higher in the FM fed fish when compared to all other treatments. Reads assigned to *Janthinobacterium* were significantly ($P < 0.05$) higher in the FM fed fish when compared to SPC, SPC+PPC+S and SPC+S fed fish. Reads assigned to the genus *Diaphorobacter* were significantly higher in SPC+PPC+S and SPC+S ($P < 0.05$) when compared to FM fed fish. Reads assigned to the genera *Leuconostoc* and *Serratia* were significantly higher in SPC+PPC+S and SPC ($P < 0.05$) when compared to FM fed fish. Reads assigned to the genus *Mesorhizobium* were significantly elevated in fish fed SPC+PPC ($P < 0.05$), as well as, reads assigned to *Weissella*, which were significantly elevated in fish fed SPC+PPC+S compared to FM-fed fish ($P < 0.05$).

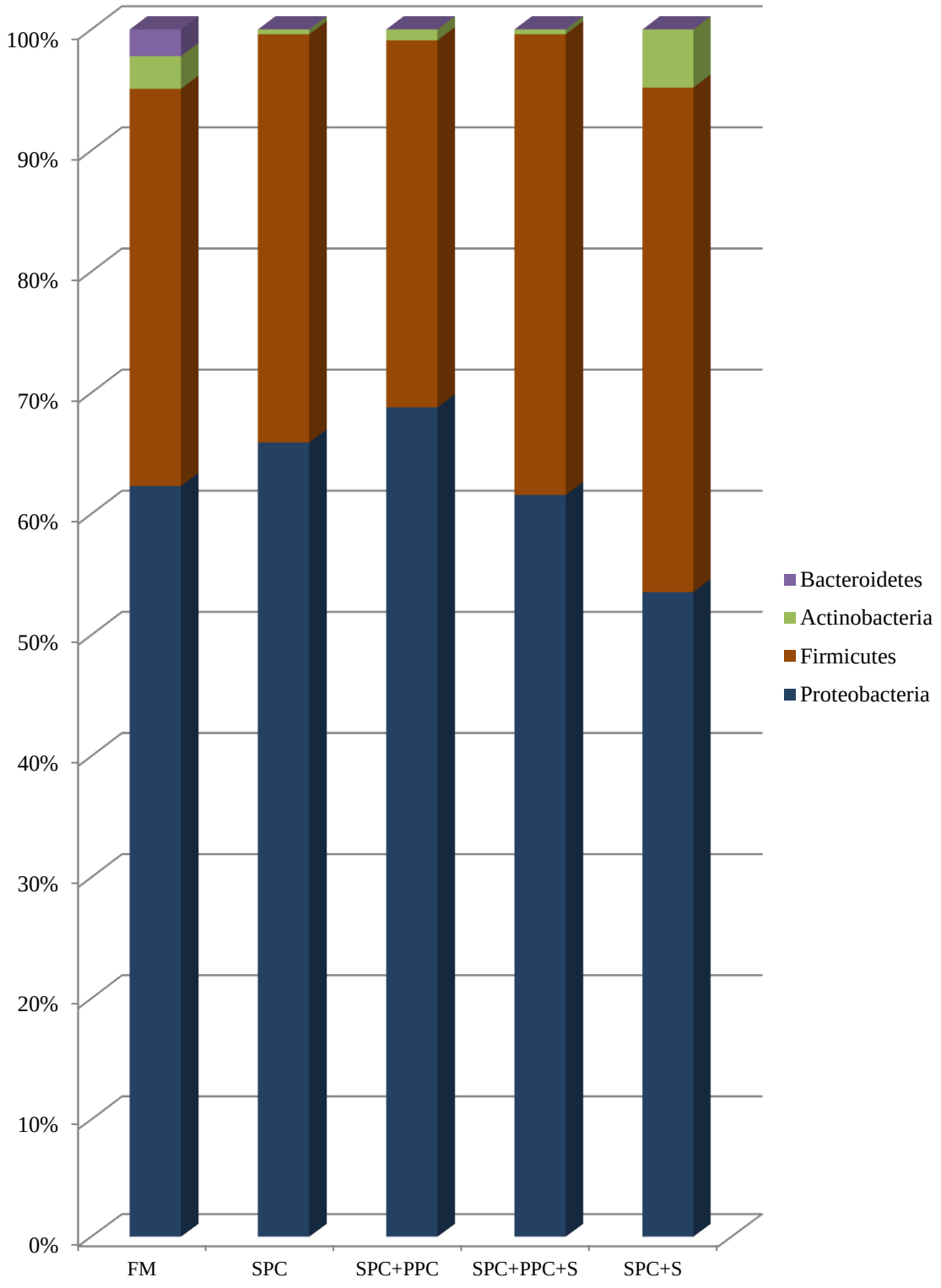


Figure 3.4. The allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks. Data represent bacterial phyla percentage.

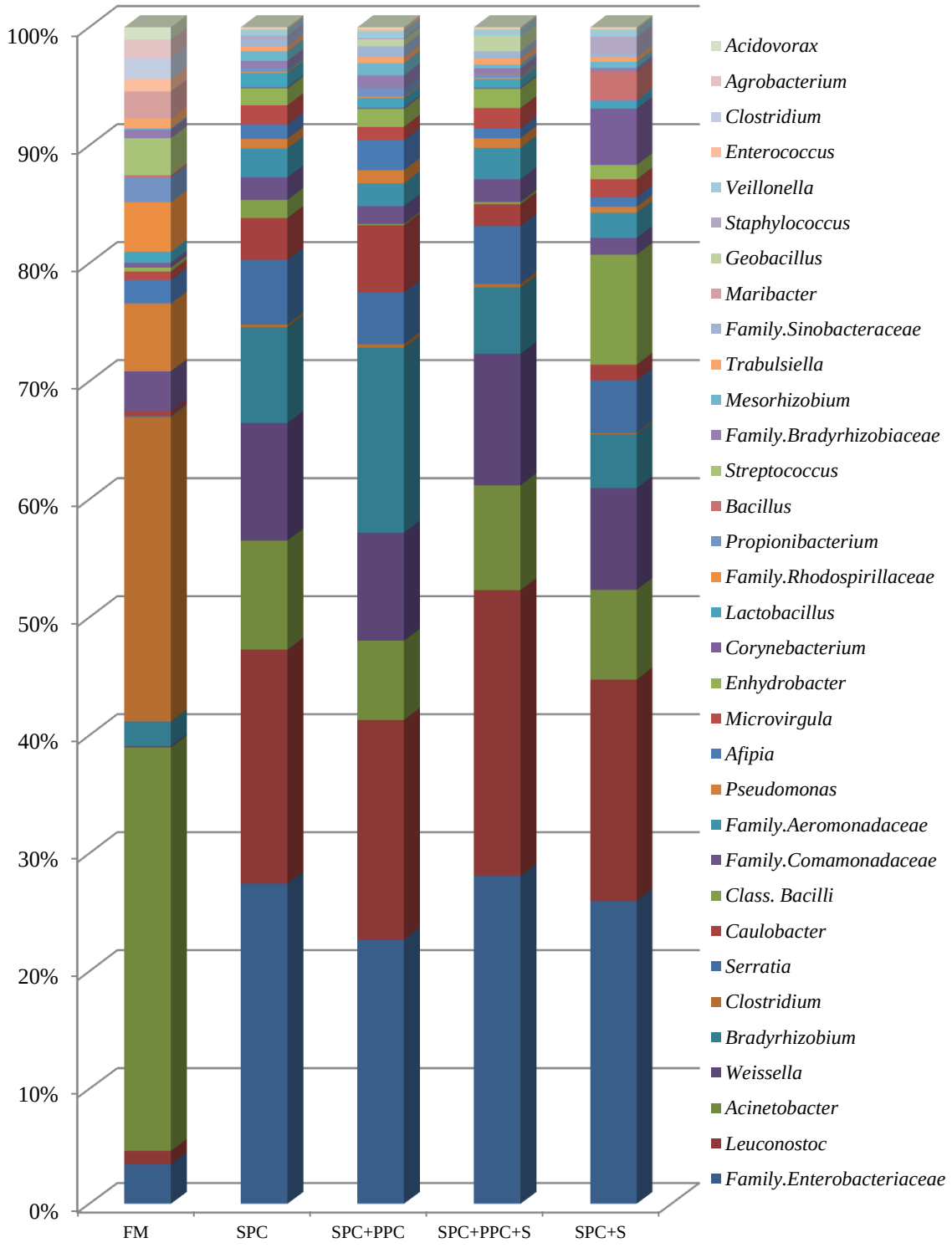


Figure 3.5. The allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks. Data represent bacterial genera with reads accounting for > 0.2 % of total reads.

3.4.3. Intestinal histology

3.4.3.1. Light microscopy

Gross morphological analysis using light microscopy revealed some qualitative differences between dietary treatments. Examples of H & E stained intestinal sections from each dietary treatment at week four are displayed in Figure 3.6. At both weeks two and four, the posterior intestine of fish fed the FM diet displayed a well-preserved epithelial mucosal barrier, with well-differentiated, long mucosal folds extending in to the lumen with a thin intact lamina propria. The intestine of the FM fed fish also exhibited an abundance of goblet cells and intra-epithelial leukocytes throughout the mucosal surfaces. Although not statistically significant, trends were observed with regards to a reduction in the mucosal fold lengths and absorptive surface area of the digestive epithelium, as well as an increase in the lamina propria widths observed in fish fed the plant based diets at both weeks two and four (Table 3.7.). The number of intraepithelial leukocytes (IEL's) in the intestine of the SPC+S fed fish was significantly reduced ($P < 0.001$) at week four (68.35 ± 15.41) compared to the FM fed fish (80.87 ± 14.52). IEL levels in the SPC, SPC+PPC and SPC+PPC+S treatments did not differ from those observed in the FM fed fish at week four and the IEL levels remained unchanged throughout treatment groups at week two (Table 3.7.). Goblet cell numbers in the SPC+S treatment were significantly reduced ($P < 0.001$) at week two (7.95 ± 2.58 compared to 6.38 ± 1.79 in the FM fed fish) (Table 3.7.). After four weeks the abundance of intestinal goblet cells were significantly reduced in the SPC+PPC ($P < 0.001$), SPC+PPC+S ($P < 0.001$) and SPC+S ($P < 0.001$) fed fish compared to the FM fed fish (Table 3.7.).

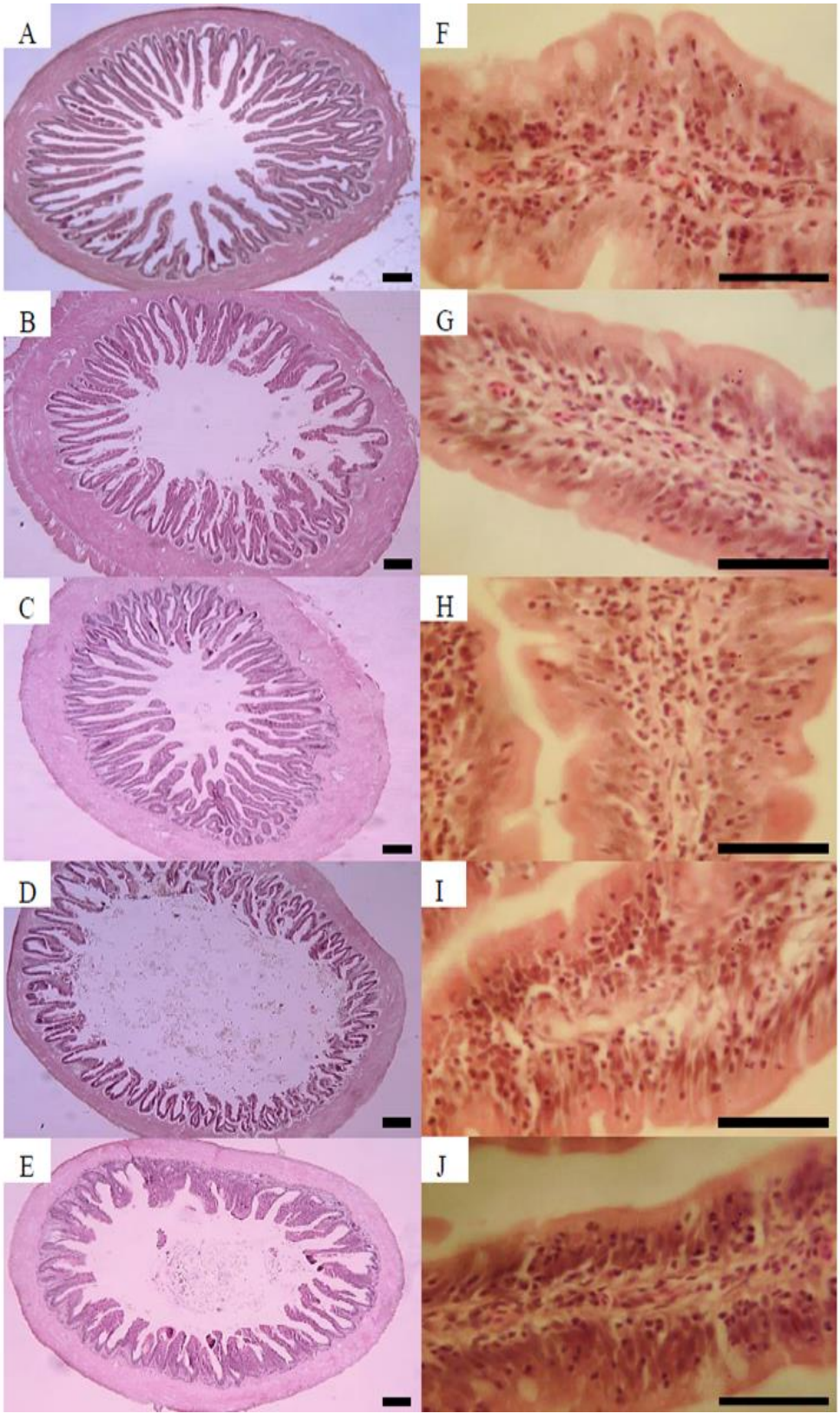


Figure 3.6. Light micrographs of the posterior intestine of European sea bass fed the control (A & F), SPC (B & G), SPC+PPC (C & H), SPC+PPC+S (D & I), and SPC+S (E & J) treatments for four weeks. Scale bars = 100 µm in A-E and 50 µm in F-J.

158 **Table 3.7.** Histological parameters of the posterior intestine of European sea bass fed the
159 experimental diets for two and four weeks. Different superscript letters within the same row
160 indicate significant differences.

Histological parameters					
	Dietary regime				
	Week 2				
Variable	**FM**	**SPC**	**SPC+PPC**	**SPC+PPC+S**	**SPC+S**
Perimeter ratio (AU)	4.06±0.86	3.97±0.81	4.11±0.69	3.94±0.84	4.21±0.67
Mucosal fold lengths (μm)	394.82 ±101.86	384.62±105.54	385.19±93.61	381.80±105.15	387.53±113.56
Lamina propria width (μm)	17.82 ±5.60	18.70 ±4.69	18.37 ±4.50	17.40 ±4.94	19.11±5.12
Goblet cells (per 100 μm)	7.95±2.58^{a}	7.17±2.40^{ab}	7.03±2.03^{ab}	6.73±2.14^{ab}	6.38±1.79^{b}
IEL's (per 100 μm)	82.4±18.51	81.30±15.33	82.07±20.46	81.36±17.87	79.19±16.88
Microvilli density (AU)	12.16±4.34^{a}	9.59±3.82^{ab}	7.97±2.71^{b}	7.14±1.88^{b}	7.76±1.03^{b}
	Week 4				
Perimeter ratio (AU)	4.46±0.89	3.97±0.38	4.16±1.20	3.77±0.92	3.75±1.06
Mucosal fold height (μm)	426.25±95.63	410.83±103.43	416.20±121.56	406.16±121.38	405.32±118.61
Lamina propria width (μm)	17.95±5.48	19.61±5.24	21.45±4.37	19.71±4.33	21.87±5.38
Goblet cells (per 100 μm)	8.12±2.32^{a}	7.34±2.51^{ab}	6.63±1.95^{ab}	6.56±2.22^{ab}	6.44±2.05^{b}
IEL's (per 100 μm)	80.87±14.52^{a}	78.78±19.03^{ab}	79.43±17.33^{ab}	76.63±16.32^{ab}	68.35±15.41^{b}
Microvilli density (AU)	13.65±3.11^{a}	10.20±2.74^{ab}	12.00±3.79^{ab}	8.08±1.96^{b}	7.92±3.23^{b}
Microvilli length (μm)	1.70±0.15	1.58±0.19	1.84±0.28	1.72±0.27	1.50±0.26

161

3.4.3.2. SEM

Analyses at the ultra-structural level using SEM revealed the posterior intestine of the FM fed fish to be healthy with uniform enterocyte formations and densely packed microvilli; no signs of enteritis were observed (Figure 3.7. A & F). In contrast, observations of the posterior intestines from fish fed the plant based diets revealed malformed microvilli, gaps between enterocytes and areas of patchy microvilli with reduced microvilli densities (Figure 3.7. B & G, C & H, D & I, E & J). These observational results were confirmed when applying quantitative analysis. The intestines from the SPC+PPC, SPC+PPC+S and SPC+S fed fish exhibited a significant reduction in microvilli density at week two when compared to the FM fed fish ($P < 0.001$). At week four the treatments SPC+PPC+S and SPC+S both continued to exhibit significantly reduced microvilli density compared to fish fed the FM diet ($P < 0.001$) (Table 3.7.).

3.4.3.3. TEM

TEM analysis of the posterior intestinal brush border morphology revealed differences between the SPC, SPC+PPC, SPC+PPC+S and SPC+S fed groups when compared to the FM group. Fish fed the control diet showed densely packed, uniform, microvillar formations with no obvious signs of damage (Figure 3.7. A). In contrast, fish fed all plant based diets showed less densely packed microvilli and signs of irregular microvilli structure (Figure 3.7. B-E). The SPC+S fed fish exhibited the shortest microvilli in comparison to all other treatments, however there were no significant differences between treatments at week 4 (Table 3.7.).

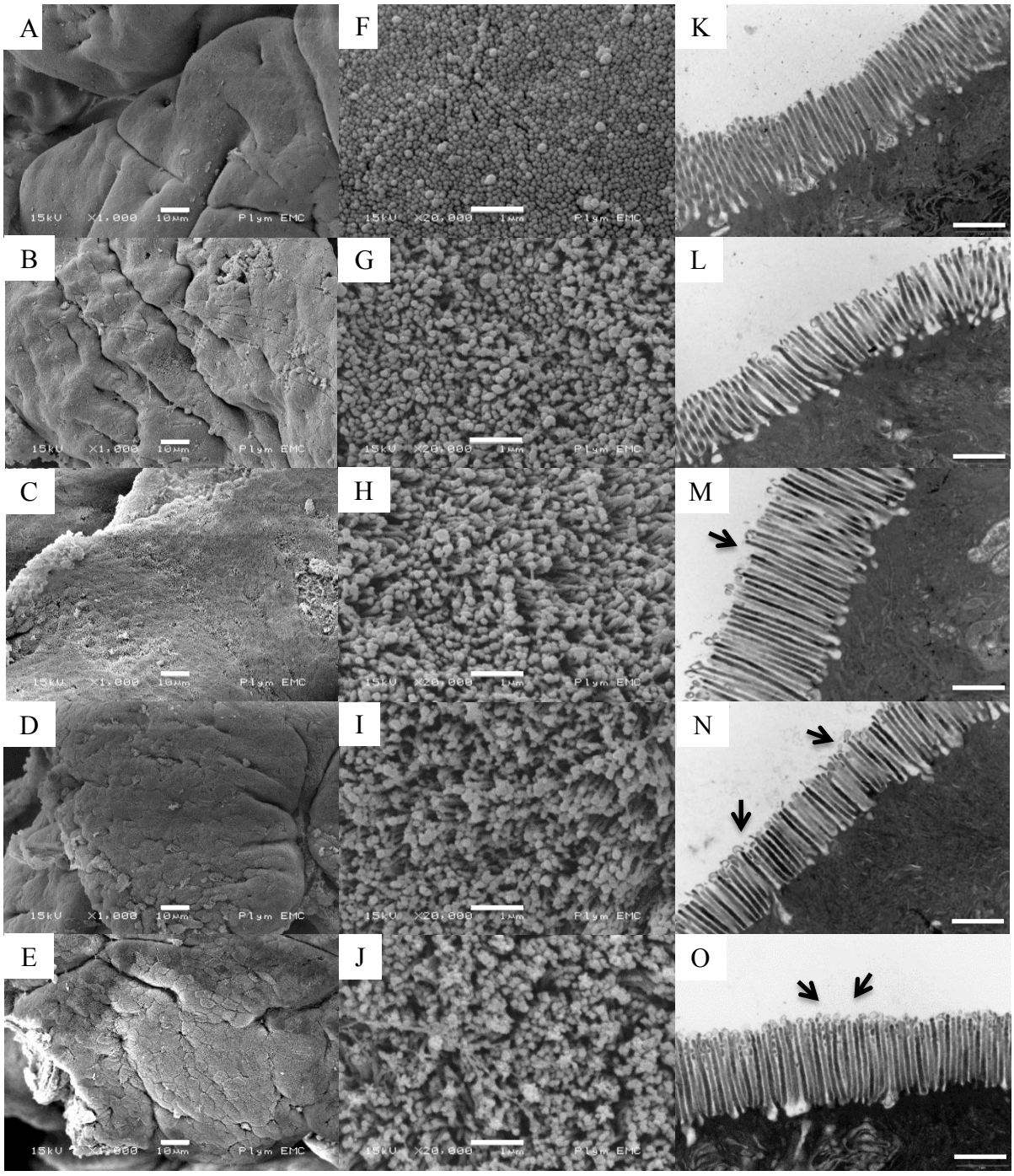


Figure 3.7. Electron micrographs (SEM; A-E and TEM; F-J) of the posterior intestine of European sea bass fed the experimental diets; FM (A, F & K), SPC (B, G & L), SPC+PPC (C, H & M), SPC+PPC+S (D, I & N) and SPC+S (E, J & O) for four weeks. Note the TEM images derived from fish fed the SPC+PPC, SPC+PPC+S and SPC+S based dietary regimes (M, N & O) which seem to reveal a loss of membrane integrity at the apical tips of the microvilli. Scale bars = 10µm in images A-E and 1 µm in images F-O.

3.5. Discussion

A number of studies have previously revealed that dietary soy protein products can alter the gut microbiota in fish (Heikkinen *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008; Merrifield *et al.* 2009; Dimitroglou *et al.* 2010; Reveco *et al.* 2014). A number of studies have previously revealed that dietary soy protein products can alter the gut microbiota in fish (Heikkinen *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008; Merrifield *et al.* 2009; Dimitroglou *et al.* 2010; Reveco *et al.* 2014).

The majority of these studies have generally reported an increase in intestinal microbial populations. Heikkinen *et al.* (2006) and Refstie *et al.* (2006) reporting an increase in the culturable populations of Atlantic salmon and Atlantic cod as a consequence of SPC based diets. However, contrary to the study by Heikkinen *et al.* (2006), the study by Refstie *et al.* (2006) reported no change in the posterior intestine. Bakke-Mckellep *et al.* (2007) reported significant increases in autochthonous and allochthonous microbial populations in posterior intestinal region of Atlantic salmon fed a SPC based diet when compared to the control after three weeks. Dimitroglou *et al.* (2010) reported an increase in species richness/presumed species in fish fed a SPC based diet compared to fish fed a FM control after nine weeks. Contrary to the above findings, Reveco *et al.* (2014) reported a reduction in species richness in the posterior intestine of Atlantic salmon fed a SPC based diet compared to fish fed a FM based control diet for 80 days. Making comparisons between studies is problematic however, and differences in, for example, fish species, SPC ingredients used, trial conditions, trial length and methodology used for the analyses, may affect the results obtained, complicating comparisons. It would appear that the effects of soy protein products on fish may be dependent on fish species and the intestinal region, as well as, the differences in soy protein, making comparisons difficult.

The majority of studies assessing soy protein products in fish have focused on long term feeding trials (i.e. > 10 weeks) and short term temporal effects are often missed. In the present study the inclusion of SPC, alone and in combination with PPC and saponins, influenced the gut microbial composition of European sea bass, when compared to a FM based diet after two and four weeks.

High-throughput sequence libraries displayed Good's coverage estimations of > 99 %, suggesting the bacterial microbiome had been fully sampled. Cluster analyses revealed distinct clusters of replicates within treatment groups, an observation which was supported by the PCR-DGGE analyses. The numbers of OTU's, species richness (Chao 1) and diversity (Shannon-Wiener) were all elevated in fish fed the plant based diets compared to fish fed the control at both weeks two and four. Fish fed the saponin supplemented diets appeared to exhibit the highest number of OTU's at week four, which PCR-DGGE analyses revealed to be significantly increased in fish fed the SPC+PPC+S treatment when compared to fish fed the FM control. The bacterial diversity was also increased in fish fed the plant based diets compared to those fed the FM diet, with PCR-DGGE analyses revealing that the SPC+PPC, SPC+PPC+S and SPC+S treatment groups exhibited significantly elevated diversity at week four. Fish fed the FM diet showed the greatest degree of inter-replicate variation at both weeks two and four. The pairwise comparisons of the PCR-DGGE fingerprints comparing the profiles of fish fed FM and fish fed the plant based diets was greater at week two than at week four.

In terms of relative sequence abundance, high-throughput sequencing revealed the phyla Proteobacteria accounted for 62.40 % of the 16S rRNA reads, followed by Firmicutes (35.30 %), Actinobacteria (1.80 %) and Bacteriodetes (0.40 %). Bands excised from the PCR-DGGE also revealed Proteobacteria and Firmicutes to be the most abundant phyla. All phyla detected in the current study have been reported as constituents of the gut microbiota of

various marine fish species, including European sea bass (Navarrete *et al.* 2008; Mansfield *et al.* 2010; Silva *et al.* 2011; Zhou *et al.* 2012; Green *et al.* 2013; Lamari *et al.* 2013; Carda-Diéguez *et al.* 2014; Gatesoupe *et al.* 2014; Kormas *et al.* 2014).

At the genera level, high-throughput sequence analyses revealed that the highest proportions of 16S rRNA reads were assigned to *Leuconostoc*, *Acinetobacter*, and *Weissella* in the treatment groups. *Lactobacillus*, *Bacillus* and *Enterococcus* spp. were also present at a lower level, which were also detected in the PCR-DGGE analyses. Reads assigned to *Enterococcus* spp. were significantly elevated in fish fed the FM diet compared to fish fed all plant based diets. Species of this genus (e.g. *E. faecium* and *E. faecalis*) have been reported to exhibit potentially beneficial effects when administrated as probiotics in various fish and shellfish species, as well as demonstrating inhibitory effects against some potential fish pathogens (Swain *et al.* 2009, Gopalakannan & Arul 2011; Avella *et al.* 2011; Sun *et al.* 2012; Sorroza *et al.* 2013; Allameh *et al.* 2014; Araújo *et al.* 2015). *Enterococcus* spp. are known to exhibit greater proteolytic and lipolyitc activity when compared to other lactic acid bacteria (LAB) (Ramakrishnan *et al.* 2012), and their increased presence in the allochthonous communities of fish fed the FM diet compared to the plant based diets in the present study may have implications relating to the hosts ability digest proteins and lipids in the intestine.

Reads assigned to the genera *Leuconostoc*, *Weissella* and *Bacillus* were observed to be elevated in fish fed the plant based treatments when compared to fish fed the FM diet, with *Leuconostoc* significantly elevated in fish fed SPC+PPC+S and SPC, and *Weissella* significantly elevated in fish fed the SPC+PPC+S when compared to fish fed the FM diet. Various species of these genera have been reported to have intestinal modulatory effects in fish when added to aquafeeds. For example, elevated immunological responses such as increasing the levels of intestinal leukocyte infiltration, increased lysozyme activities and positively effecting the expression of immune related genes, improved growth and digestive

enzyme activities have also been demonstrated in fish as a consequence of *Leuconostoc mesenteroides* supplemented feeding (Balcázar *et al.* 2007a, 2007b; Askarian *et al.* 2011). To the author's knowledge there is little information regarding the use of *Weissella* spp. as probiotics in fish, however, Mouriño *et al.* (2012) demonstrated a reduction in *Vibrio* spp. in the intestine of hybrid sorobims (*Pseudoplatystoma* sp.), as well as increased immunoglobulin levels, in fish fed dietary inclusion of *W. cibaria* for 15 days. There is an abundance of literature demonstrating the potential of *Bacillus* spp., including *B. subtilis*, *B. licheniformis*, *B. pumilis*, and *B. clausii*, as probiotics in fish (Newaj-Fyzul *et al.* 2007; Bagheri *et al.* 2008; Merrifield *et al.* 2010b, 2010c; Sun *et al.* 2011; He *et al.* 2013; Yang *et al.* 2012). However, with the exception of Touraki *et al.* (2012), who demonstrated that European sea bass larvae exhibited an increased resistance to the pathogen *V. anguillarum* as a result of *B. subtilis* feeding, there is a scarcity of information relating to the efficacy of *Bacillus* spp. as probiotics in European sea bass.

The OTU's sequenced from the DGGE generally support the high-throughput sequence data with the abundance of *B. subtilis* and *L. buchneri* observed to be elevated in fish fed the SPC+PPC, and SPC+PPC+S treatments compared to fish fed the FM control after two and four weeks. Increased LAB populations in the posteriors intestine due to dietary SPC inclusion has previously been reported in Atlantic salmon (Reveco *et al.* 2014) and may be considered to be beneficial to the host as many LAB have been used as potential probiotics in fish (Newaj-Fyzul *et al.* 2014). The reasons for this increase in LAB populations are not clear but may be due, at least in part, to the availability of oligosaccharides and polysaccharides (e.g. glucose, arabinose, galactose and cellulose) present in the plant ingredients. This has previously been suggested in a study assessing SPC in the diets of broiler chickens (Lan *et al.* 2004).

289 Histological analysis revealed distinct qualitative differences between treatment groups with
290 the control fed fish appearing healthy with no obvious signs of epithelial disruption or
291 damage. In contrast, the epithelium of fish fed the plant based diets showed signs of damage
292 and enteritis-like effects similar to those previously described in salmonids (Heikkinen *et al.*
293 2006; Krogdahl *et al.* 2003) and common carp (Uran *et al.* 2008). The epithelial damage
294 appeared to be exacerbated in fish fed the saponin supplemented diets which is in agreement
295 with previous studies conducted on salmonids (Knudson *et al.* 2007, 2008). Morphometric
296 analyses revealed a reduction in epithelial GC's in fish fed SPC based diets when compared
297 to the control at both weeks two and four in the SPC+S. Similarly, IEL numbers were
298 observed to be reduced in fish fed the SPC based diets and significantly reduced after four
299 weeks when the SPC+S treatment was compared to the control. A reduction in GC's and
300 IEL's may suggest an epithelium more susceptible to bacterial infection, with lowered mucus
301 production and fewer leukocytes present to protect against pathogenic insults. However, other
302 studies have observed an increase in IEL's as an inflammatory response to SPC induced
303 enteritis in salmonids after longer periods of feeding (Romarheim *et al.* 2008; Marjara *et al.*
304 2012). Future research is required to ascertain the reasons for the discrepancies in these
305 observations which may be a result of the temporal differences of the studies (i.e. differences
306 in the exposure duration to the diets) or differences between fish species.

307 SEM analysis also revealed distinct differences between treatments at the epithelial
308 ultrastructural scale. The epithelium of fish fed the SPC based diets all showed signs of
309 epithelial damage greater in extent when compared to the epithelium of the control fed fish.
310 This was further supported when assessing microvilli density which revealed a significant
311 reduction in the density of the SPC+PPC, SPC+PPC+S and SPC+S fed fish after two weeks,
312 and SPC+PPC+S and SPC+S fed fish after four weeks, when compared to the control fed fish.
313 This is in accordance with studies which have observed a decrease in epithelial microvilli

density as a result of SPC inclusion in salmonids (Bakke-McKellep *et al.* 2007; Merrifield *et al.* 2009). A reduction in microvilli length has also been reported to be a sign of SPC induced enteritis in salmonids (van den Ingh *et al.* 1991; Merrifield *et al.* 2009). In the present study no statistical differences in microvilli lengths were observed between treatments, however, potential differences in the membrane integrity of fish fed the SPC based diets was observed. The highest degree of disruption was observed in the fish fed the saponin diets suggesting that the saponin inclusion may, at least in part, contribute to this microvilli degradation. Saponins have the ability to integrate into intestinal membranes and form complexes with strerols such as cholesterol, creating plagues in phospholipid formation which in turn changes membrane structure and permeability and thus affecting the influx and efflux of molecules, as well as, potentially aiding translocation of bacteria from the host lumen (Krogdahl *et al.* 2010; Augustin *et al.* 2011).

3.6. Conclusion

In the present study, the gut microbiota identified by both PCR-DGGE and high-throughput sequence analyses revealed that SPC and PPC alone, and in combination with saponins, can modulate the allochthonous intestinal bacterial communities of European sea bass which is consistent with other studies on piscivorous fish species (Heikkinen *et al.* 2006; Refstie *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Merrifield *et al.* 2009; Navarrete *et al.* 2013). Signs of sub-acute enteritis were also observed in the present study but the results indicate a more moderate response than those observed in the previous studies published on salmonids (Krogdahl *et al.* 2003; Bakke-McKellep *et al.* 2007; Romarheim *et al.* 2008; Uran *et al.* 2008; Merrifield *et al.* 2009; Marjara *et al.* 2012). Future studies are required to assess whether these changes affect the microbiome functionality, and whether this change in

338 microbiota coupled with reduced epithelial morphological integrity may increase the
339 susceptibility to enteric pathogens. Further research should also focus on the long term effects
340 SPC has on the intestinal microbiota and integrity of European sea bass.

341 The present investigation revealed that the allochthonous bacterial communities were
342 influenced by dietary SPC alone and in combination with PPC and saponins. Furthermore,
343 the collective histological analyses revealed that European sea bass are susceptible to dietary
344 induced enteritis-like effects of the posterior intestine. This Chapter provides a novel enteritis
345 model for this fish species which could be used to assess the effectiveness of feed additives in
346 reducing inflammation in the intestine, and would be the focus of Chapter 4A.

Chapter 4A:

The effect of feed additives on the intestinal microbiome and intestinal integrity of European sea bass fed a sub-optimal SPC based diet

4A.1. Abstract

The aim of the present study was to assess modulatory effects of dietary *B. subtilis* and/or Previda®, and the antibiotic oxytetracycline, on the intestinal bacterial populations in European sea bass, and to assess the efficacy of these products to reduce intestinal damage caused by a high soy protein concentrate + saponin basal diet. The basal diet in this chapter (SPC+S) was used as a negative control diet referred to as basal throughout. The four experimental diets consisted of the basal dietary formulation with the addition of probiotics, prebiotics or probiotics & prebiotics (i.e. synbiotic treatment) as follows: 1] the probiotic diet contained 10^7 CFU g^{-1} *B. subtilis*, 2] the prebiotic diet contained Previda® at 0.6 g kg^{-1}, and 3] the synbiotic diet contained 10^7 CFU g^{-1} *B. subtilis* + Previda® at 0.6 g kg^{-1}. Fish (88.8 ± 1.2 g) were fed one of the experimental diets for four weeks. PCR-DGGE and high-throughput sequencing analysis revealed that the inclusion of the feed additives modulated the allochthonous gut microbiota of *D. labrax*. *B. subtilis* was abundant in all samples from both the probiotic and synbiotic treatment groups. Distinct clusters within treatment replicates confirmed differences in the bacterial communities. Gene expression analyses revealed a significant up-regulation in the expression of IL-1β and TNFα in fish fed all experimental treatments relative to fish fed the basal. HSP70, CASP3 and PCNA expression was significantly down-regulated in fish fed the probiotic, prebiotic and synbiotic, whereas fish fed the antibiotic treatment exhibited significant up-regulation of these genes relative to fish fed the basal diet. The synbiotic fed fish exhibited a significantly ($P < 0.05$) higher intestinal perimeter ratio compared to the basal fed fish. In addition, significantly ($P < 0.05$) elevated goblet cell levels were observed in the probiotic and synbiotic treatments compared to the basal fed fish. TEM revealed the loss of membrane integrity induced by the basal diet, was reduced in the probiotic, prebiotic and synbiotic treatments. Moreover, the probiotic and

prebiotic applications significantly ($P < 0.05$) increased the microvilli density when compared to fish fed the basal control.

4A.2. Introduction

Chapter 3 revealed that European sea bass juveniles are susceptible to dietary induced enteritis similar to those observed in salmonids and carp (Baeverfjord & Krogdahl 1996; Burrells *et al.* 1999; Nordrum *et al.* 2000; Krogdahl *et al.* 2003; Uran *et al.* 2008; Marel *et al.* 2014). The ANF's associated with soy protein products, including saponins, have been observed to reduce nutrient digestibility and thus growth performance, to interfere with the permeability of intestinal membranes, affecting the influx and efflux of molecules and bacteria, and disrupt intestinal morphology, of fish (Krogdahl *et al*. 2010). Furthermore, soybean based diets are known to effect the intestinal microbiota of fish which as a consequence may directly or indirectly effect the host (Merrifield *et al.* 2011). Soybean products are routinely used in aquafeeds and are therefore an important aspect associated with the successful formulation of these diets for various fish species. The intestinal disruption and microbial modulation observed in European sea bass fed the SPC+S diet in chapter 3 presented an interesting model with which to assess probiotics and prebiotics and their potential protective potential.

In recent years, the manipulation of the intestinal microbiota through probiotic and prebiotic applications has become commonplace in the nutrition of various cultured animal species including finfish in aquaculture practices (for a review on probiotics in fish refer to Merrifield & Carnevali 2014). Improved growth, disease resistance, immunological responses as well as improving the gastrointestinal morphology and modulation of the intestinal microbiota have been attributed to probiotic applications in fish (Abelli *et al.* 2009; Merrifield *et al* 2010a;

Ferguson *et al.* 2010; Dimitroglou *et al.* 2011; Perez-Sanchez *et al.* 2011; abid *et al.* 2013; Standen *et al.* 2013; Gioacchini *et al.* 2014).

Prebiotic studies have reported enhanced growth and disease resistance in striped sea bass fed a commercial prebiotic supplemented diet (Gatlin & Li 2004), significantly improved antibody production and lysozyme activity (Staykov *et al.* 2007), as well as modulation of intestinal microbiota and improvement in intestinal morphology (Dimitroglou *et al.* 2009) in rainbow trout (*Oncorhynchus mykiss*) fed a Mannan-oligosaccharides (MOS) supplemented diet. Prebiotics are also thought to play a role in innate immunity either through direct stimulation or by enhancing the growth and proliferation of commensal microbes. For a review of this topic readers are referred to Song *et al*. 2014.

The concept of synbiotics (a mixture of prebiotics and probiotics), was introduced as a means to increase the effectiveness either prebiotic or probiotic would have individually on the health of the host. A number of synbiotic studies have been conducted in various fish species over the last few years. Rodriguez-Estrada *et al.* (2009) observed an improvement in growth performance and immune response when investigating the of effect *Enterococcus faecalis* + mannan oligosaccharide in rainbow trout. Abid *et al.* investigating the application of *Pediococcus acidilactici* and short chain fructooligosaccharides on the intestinal health of Atlantic salmon (*Salmo salar*) concluded that modulation of the gut microbiota had a protective action on the intestinal mucosal cells, improving morphology and stimulating the innate immune response without negatively affecting growth performance or feed utilization. Another study found the administration of *B. subtilis* + fructooligosaccharide in the diets of juvenile ovate pompano (*Trachinotus ovatus*) to enhance immune responses and increase disease resistance against *V. vulnificus* (Zhang *et al.* 2014). Most of the aforementioned studies however, have focused their analyses on long-term feeding trials (ca. 2-3months) and

as was observed in Chapter 3 effects on the intestine brought about by high soybean based diets can be seen as early as two weeks.

Chapter 3 assessed the plant proteins SPC and PPC together with saponins based on the hypothesis that these plant based products may induce enteritis-like effects in the European sea bass intestine. The results indicated a level of intestinal inflammation as a consequence of all dietary combinations of the plant diets; however it was observed that the diet deemed most challenging was the SPC+S. This diet would therefore be used in the present chapter.

The aims of the present study was to assess the effects of the feed additives: *B. subtilis* and Previda® individually and in combination, as well as the antibiotic oxytetracycline on the gastrointestinal health of European sea bass fed a suboptimal soybean meal based diet. Oxytetracycline is a broad-spectrum antibiotic commonly used for the treatment against pathogens in aquaculture, and concerns of antimicrobial resistance are well documented (Rigos et al. 2013; Shah et al. 2014). The present study also assessed how these feed additives and antibiotics impacted the localised immune condition in the intestine, by the expression of the pro-inflammatory genes tumour necrosis factor-α (TNFα) and interleukin-1β (IL-1β), and the genes associated with: cellular level stress; heat shock protein 70 (HSP70), programmed cell death; caspase 3 (CASP3), and cell proliferation; proliferating cell nuclear antigen (PCNA). These genes were selected due to their relevance in the inflammatory response process and roles in intestinal integrity. It was hypothesised that the sub-optimal basal diet would cause an up-regulation in these cytokines and the feed additives might mitigate these responses.

4A.3. Materials and Methodologies

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

4A.3.1. Experimental design

European sea bass were obtained from Anglesey Aquaculture Ltd, Black Point, Beaumaris UK and transported to the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK with an acclimation period of six weeks. The fish were then graded and separated into 15 x 110 L fibreglass tanks in a closed recirculatory system at a stocking density of 15 fish per tank with an average weight of 88.8 ± 1.2 g. Each dietary treatment was randomly attributed to the tanks in triplicate and the fish were fed each experimental diet at a rate of 2 - 3 % of biomass per day in equal rations at 9:00, 13.00 and 17:00. Daily feed was adjusted on a weekly basis by batch weighing following a 24 - h starvation period. Fish were held at 24 ± 1 °C and 31 ± 0.5 ppt salinity with a photoperiod of 12: 12 h light: dark. Water quality parameters were maintained at 6.6 ± 0.3 pH and dissolved oxygen > 85 % saturation, monitored daily. Ammonium, nitrite and nitrate levels were monitored weekly with weekly water changes of approx. 25 % system volume to reduce build-up of these compounds.

4A.3.2. Diet preparation

Five iso-nitrogenous and iso-lipidic dietary regimes were formulated (Table 4A.1.) to meet the known nutritional requirements for European sea bass (NRC 2011). The dry ingredients were well mixed in a Hobart food mixer (Hobart Food Equipment, Sydney, Australia, model no: HL1400–10STDA). The oil and hot water were gradually added to the mixer and cold press extrusion was conducted (PTM P6 extruder, Plymouth, UK) to produce 2 mm pellets.

The pelleted diets were then dried to ca. 5 % moisture in an air convection oven set at 50 °C for 48 hours. The diets were then broken up to the appropriate size and the composition analysed using AOAC (1995) protocols (Table 4A.1.). Experimental diets were subsequently stored at 4 ^{o}C in airtight containers prior the start of the trial. Probiotic inclusion levels (probiotic treatment: 1.98 x 10^7 CFU g^{-1} and synbiotic treatment: 2.03 x 10^7 CFU g^{-1}) were confirmed by spread plating onto specific *B. subtilis* media (Starch nutrient agar with the addition of polymixin B) for 24 h at 30 ^{o}C.

Table 4A.1. Dietary formulation (%) and chemical composition

	Basal	Probiotic	Prebiotic	Synbiotic	Antibiotic
Fishmeal[a]	20.00	20.00	20.00	20.00	20.00
Soya protein concentrate[b]	52.72	52.72	52.72	52.72	52.72
Glutalys[c]	10.00	10.00	10.00	10.00	10.00
Fish oil[d]	11.08	11.08	11.08	11.08	11.08
Corn starch[e]	3.30	3.30	2.70	2.70	1.30
Mineral/vitamin premix[f]	2.60	2.60	2.60	2.60	2.60
Saponin[g]	0.30	0.30	0.30	0.30	0.30
Probiotic[h] (log CFU g^{-1})	-	7.27	-	7.62	-
Prebiotic[i] (g kg^{-1})	-	-	0.60	0.60	-
Antibiotic (oxytetracycline[j]) (g kg^{-1})	-	-	-	-	2.00
Proximate analysis (%)					
Dry matter	93.0	92.6	92.6	92.9	92.9
Crude protein*	51.5	53.5	52.2	53.1	53.0
Crude lipid*	14.0	14.2	14.0	14.0	14.2
Ash*	7.42	7.78	7.59	7.92	7.99
Gross energy (MJ kg^{-1})*	20.80	20.90	21.00	21.10	20.80

[a] Herring meal LT94: CC MOORE & Co. Ltd., Dorset, UK.
[b] Hamlet HP100 (56 % crude protein), Hamlet Protein, Denmark.
[c] Roquette Company, Frêres, France.
[d] Seven seas Ltd. Hull, UK.
[e] Sigma-Aldrich Company, UK.
[f] Premier Nutrition Products (PNP Ltd.) Rugeley, Staffordshire, UK.
[g] Sigma-Aldrich Company, UK. (20-30% sapogenic content).
[h] Probiotic (*B. subtilis*): Novus Int, St. Charles, USA. (lyophilised cells at 10^{10} CFU g^{-1})
[i] Previda® Novus Int, St. Charles, USA.
[j]Pharmaq ltd, Fordingbridge, Hampshire, UK.
* % wet weight basis

4A.3.3. Dietary proximate analyses

Proximate analysis of diets was determined as described in section 2.5.

4A.3.4. Sampling

Two fish per tank were sampled for microbiology and two per tank for histology (n = 6) at the end of the trial. Refer to section 2.6. for sampling methodology.

4A.3.5. Microbiological analyses

4A.3.5.1. PCR-DGGE and sequencing

For PCR-DGGE analysis, two digesta samples per tank were pooled and homogenised, thus providing n = 3 per treatment. DNA from 200 mg samples was extracted as described in section *2.7.1.* PCR-DGGE analysis and sequencing was carried out as described in sections *2.7.2.* and *2.7.3.*

4A.3.5.2. High-throughput sequencing

For high-throughput sequence analysis triplicate digesta samples were subjected to DNA extractions as described in section *2.7.1.* High-throughput sequence analysis was carried out as described in section *2.7.4.*

4A.3.6. Intestinal histology

4A.3.6.1. Light microscopy

Light microscopy analysis was carried out on two fish per tank (*n* = 6) as described in section *2.8.1.* An absorptive surface area index (ASI) was calculated according to the following: ASI = microvilli length (μm) × microvilli density (AU) × intestinal perimeter ratio.

4A.3.5.2. SEM

SEM analysis was carried out on two fish per tank (*n* = 6) as described in section *2.8.2.*

4A.3.5.3. TEM

TEM analysis was carried out on four fish per treatment (*n* = 4) as described in section *2.8.3.*

4A.3.7. Gene expression

4A.3.7.1. RNA extraction, cDNA synthesis and real-time PCR

RNA extraction, cDNA synthesis and real-time PCR was carried out as described in sections 2.7.6. and 2.7.7.

4A.3.7.2. Reference gene, genes of interest and analyses

β-Actin was used as the reference gene in each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality (Bustin *et al.* 2009). No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. The data obtained was analysed using the iQ5 optical system software version 2.0 (Bio- Rad) including Genex Macro iQ5 Conversion and genex

Macro iQ5 files. The calculations in this spreadsheet are derived from the algorithms outlined by Vandesompele *et al.* (2002) and from the GeNorm manual and associated calculations (http://medgen.ugent.be/ ~jvdesomp/genorm/). GeNorm calculates the stability value "M" of the reference genes by comparing the variation in expression for all other target genes. Modification of gene expression is represented with respect to the controls being sampled at the same time as the treatment. Genes of interest were the pro-inflammatory cytokines: interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNFα), and the immune-regulatory cytokines: heat-shock protein 70 (HSP70), caspase-3 (CASP3) and proliferating cell nuclear antigen (PCNA). Primers used and their sequences are presented in Table 4A.2.

Table 4A.2. Information regarding primers used for real-time PCR analysis

Gene	Forward primer	Reverse primer	Amplicon size	Genbank No	E-value	Annealling temp
β-actin	AACACTGTGCTGTCTGGAGG	CTGCTGGAAGGTGGACAGAG	177	AJ493428.1	1.9	60
IL-1β	CAACAGCGCAGTACAGCAAG	AGATGCAAGGTTGGCTCCTC	265	AJ269472.1	1.9	60
TNFα	AGGCCAAACCGAAGCACTAA	ACTCCAGCTTGGCAGTCAAA	146	DQ070246.1	1.9	60
HSP 70	GCACTCAACTACGAGCGTCT	AGTGTTGCTGGGGTTCAGAG	233	AY423555.2	2.0	58
CASP 3	CTTCGACAGGAGAACAGGCA	GCGTTGCAGCTGTGATCTTC	174	DQ345773.1	1.9	60
PCNA	GCTGGGTACAGGAAACGTCA	GCGTGGCTTTGGTGAAGAAG	137	JQ755266.1	1.9	60

4A.3.8. Statistical analysis

Statistical analysis was carried out as described in section 2.9.

4A.4. Results

4A.4.1. Gross observations

Fish accepted all dietary regimes well and fish appeared healthy with 100 % survival recorded throughout the trial. The average fish weights after four weeks were, basal: 120.30 ± 6.78 g, probiotic: 125.20 ± 6.60g, prebiotic: 121.10 ± 10.79g, synbiotic: 125.10 ± 9.77g, and antibiotic: 122.90 ± 3.77g. Growth parameters remain statistically unaffected.

4A.4.2. Microbiological analyses

4A.4.2.1. PCR-DGGE

The 16S rRNA PCR-DGGE is presented in Figure 4A.1. with arrows depicting the excised bands for sequencing. Cluster analysis and multi-dimensional scaling of the replicates in each dietary treatment group is presented in Figure 4A.1. Replicates of fish fed the probiotic and synbiotic treatments exhibited clusters with a clear separation from fish fed all other treatments. The probiotic and synbiotic treatments exhibited > 80 % similarity between all replicates. Three of the four replicates within both treatments distinctly clustered with some crossover between these two treatments apparent between the remaining replicates. The replicates of fish fed the prebiotic treatment were distinct form all the other groups and three of the four replicates within the basal were also distinct from the other treatment groups. Two replicates of fish fed the antibiotic treatment exhibited some distinction from fish fed the

other treatments with the remaining two replicates exhibiting crossover/similarity with the replicates of the basal and prebiotic fed fish.

The highest within treatment variation was observed in replicates of fish fed the antibiotic treatment with a percentage similarity (SIMPER) of 61.45 ± 9.48 which was significantly reduced compared to the other treatment groups. Replicates of fish fed the probiotic diet exhibited the highest percentage similarity (88.00 ± 3.82), followed by fish fed the prebiotic (87.94 ± 4.48), synbiotic (87.64 ± 4.78), and basal (82.56 ± 11.09) treatments (Table 4A.3). Percentage dissimilarity values were observed to be lowest when the replicates of the probiotic and synbiotic fed fish were directly compared (18.04 %), and the greatest level of dissimilarity was observed when the replicates of the probiotic fed fish were compared to replicates of basal fed fish (65.61 %). The ecological parameters remained unaffected by dietary treatment Table. 4A.3.

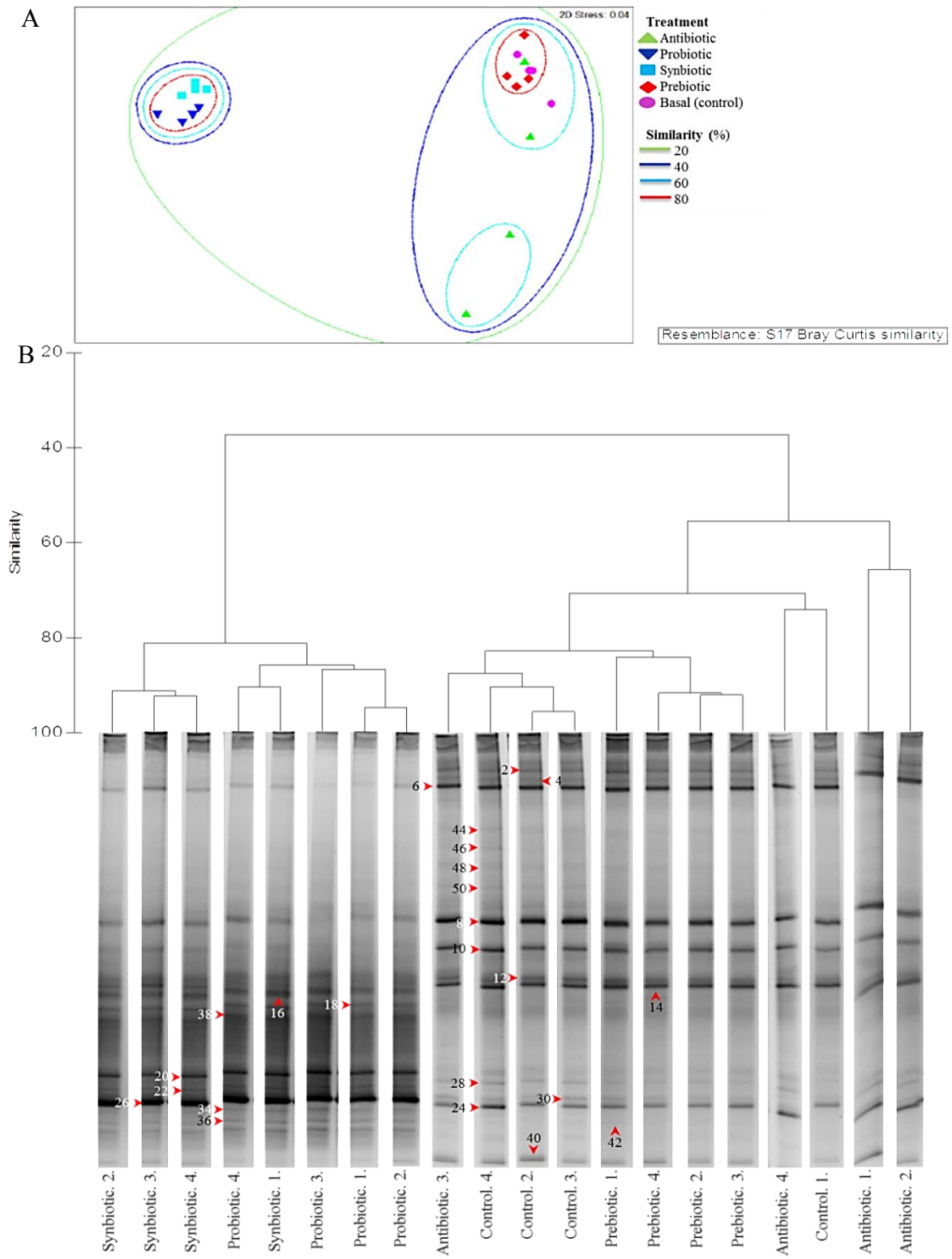


Figure 4A.1. Nonmetric multidimensional scaling analysis plots (A) showing clusters at different similarity levels and dendrogram (B) of the PCR-DGGE fingerprint profiles of the allochthonous bacterial communities associated with the posterior intestine of European sea bass fed the experimental diets for four weeks.

Table 4A.3 Microbial community analysis from the PCR-DGGE of the allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks (ANOVA + *post hoc* Tukey's) accepted at $P < 0.05$. Values expressed as means ± standard deviation.

	Ecological parameters				**ANOSM**		
Treatment	OTU's	Richness[†]	Diversity[‡]	SIMPER (similarity %)	*R*-value	*P*-value	Dissimilarity (%)
Basal	29.00 ± 4.83	2.72 ± 0.40	3.24 ± 0.18	82.56 ± 11.09[a]			
Probiotic	27.00 ± 2.16	2.46 ± 0.20	3.15 ± 0.08	88.00 ± 3.82[a]			
Prebiotic	29.00 ± 1.83	2.71 ± 0.14	3.27 ± 0.06	87.94 ± 4.48[a]			
Synbiotic	29.00 ± 1.41	2.61 ± 0.11	3.24 ± 0.04	87.64 ± 4.78[a]			
Antibiotic	22.50 ± 7.68	2.16 ± 0.69	2.98 ± 0.34	61.45 ± 9.48[b]			
Pairwise comparisons							
Basal vs. Probiotic					1.00	0.029	65.61
Basal vs. Prebiotic					0.40	0.057	18.52
Basal vs. Synbiotic					1.00	0.029	60.51
Basal vs. Antibiotic					0.27	0.057	34.30
Probiotic vs. Prebiotic					1.00	0.029	62.61
Probiotic vs. Synbiotic					0.66	0.029	18.04
Probiotic vs. Antibiotic					1.00	0.029	65.30
Prebiotic vs. Synbiotic					1.00	0.029	58.18
Prebiotic vs. Antibiotic					0.41	0.029	35.91
Synbiotic vs. Antibiotic					1.00	0.029	64.31

SIMPER, similarity percentage within replicates of each treatment; ANOSIM, analysis of similarities between treatments.

[†] Margalef species richness: $d = (S - 1)/\log(N)$.

[‡] Shannon's diversity index: $H' = -\text{SUM}(pi * \log(pi))$.

Values expressed as means ± standard deviation.

[ab] Different superscript letters in the same row indicate significant differences accepted at $P < 0.05$

4A.4.2.2. DGGE sequence analysis

A number of OTU's were excised for sequence analysis from the DGGE (Table 4A.4). The sequence data acquired from BLAST searches revealed that all of the bands sequenced belonged to the phylum Firmicutes. A number of these bands were observed to be related to *B. subtilis* (bands: 18, 20, 22, 34, 36, 38) which were present in both the probiotic and synbiotic treatment groups and absent from the other treatment groups confirming the presence of the probiotic.

Table 4A.4. Closest bacterial relative (% similarity) for sequences excised from the PCR-DGGE of the allochthonous bacterial communities from European sea bass posterior intestine after being fed the experimental diets for four weeks. The presence of bands in the replicates of each dietary regime is indicated in columns 2-6. Numbers indicate; 0 = band not present in any replicate through to 4 = band present in all replicates.

Band ID	**Band presence**					**Phyla**	**Nearest neighbour**	**Alignment similarity (%)**	**Accession number**
	Replicates								
	Basal	Probiotic	Prebiotic	Synbiotic	Antibiotic				
2	4	0	4	0	4	Firmicutes	*Lactobacillus* sp.	81	AF_159000.1
4	4	3	4	2	4	Firmicutes	Uncultured *Lactobacillus*	95	KC_354241.1
6	4	4	4	4	4	Firmicutes	Uncultured *Lactobacillus*	98	KC_354241.1
8	4	3	4	4	4	Firmicutes	Uncultured *Weissella* sp.	93/93	HM_820310.1
10	4	3	4	4	4	Firmicutes	*Lactobacillus satsumensis*	96	AB_362684.1
12	4	4	4	4	4	Firmicutes	Uncultured *Lactobacillus*	96	KC_354254.1
14	4	4	4	4	4	Firmicutes	*Lactobacillus satsumensis*	92	AB_362684.1
16	0	4	3	4	0	Firmicutes	*Bacillus* sp.	96	JQ_068110.1
18	0	4	0	4	0	Firmicutes	*B. subtilis*	96	HQ_703594.1
20	0	4	0	4	0	Firmicutes	*B. subtilis*	99	HQ_703594.1

22	0	4	0	4	0	Firmicutes	*B. subtilis*	100	HQ_703594.1
24	4	0	4	0	4	Firmicutes	*Lactobacillus frumenti*	98	JX_272061.1
26	0	4	0	4	0	Firmicutes	*B. subtilis*	100	HQ_703594.1
28	4	2	4	4	4	Firmicutes	Uncultured *Lactobacillus*	87	JN_883491.1
30	4	0	4	0	4	Firmicutes	Uncultured *Lactobacillus*	87	JN_883491.1
34	0	4	0	4	0	Firmicutes	*B. subtilis*	95	HQ_703594.1
36	0	4	0	4	0	Firmicutes	*B. subtilis*	99	HQ_703594.1
38	0	4	0	4	0	Firmicutes	*B. subtilis*	94	HQ_703594.1
40	4	3	4	4	4	Unidentified	Uncultured soil bacterium	93	GU_375004.1
42	4	4	4	4	3	Unidentified	Uncultured bacterium	87	HM_269688.1
44	4	0	4	0	4	Firmicutes	*Lactobacillus rossiae*	96	JN_680708.1
46	4	0	4	0	4	Firmicutes	Uncultured *Lactobacillus*	93	KC_354151.1
48	4	0	4	0	4	Firmicutes	*Lactobacillus rossiae*	98	JN_680708.1
50	4	0	4	0	4	Firmicutes	*Salinococcus* sp.	92	NR_044030.1

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4A.4.2.3. High-throughput sequencing

A total of 1,404,925 reads were retained after trimming and QC, which were used for downstream analyses. Alpha rarefaction analyses revealed all samples to be reaching a plateau after approx. 25,000-30,000 sequences (Figure 4A.2.), and good's coverage estimators were > 0.99 (Table 4A.5.), signifying that the bacterial communities were fully sampled and the subsequent observed OTU's were representative of the population. Alpha diversity parameters are also presented in Table 4A.5. and revealed that fish fed the prebiotic treatment exhibited the highest numbers of observed species (325.51 ± 13.18) which was significantly higher than in samples of fish fed the probiotic and synbiotic treatments (181.61 ± 35.22 and 230.93 ± 6.91, respectively). Confirming the results obtained from the PCR-DGGE, the lowest value was observed to be in fish fed the probiotic treatment. Shannon-wiener diversity index revealed fish fed the probiotic and synbiotic treatments exhibited lower bacterial diversity (3.59 ± 0.12 and 3.78 ± 0.08, respectively) compared to fish fed the other treatments, which was observed to be significantly ($P < 0.05$) lower when compared to fish fed the antibiotic treatment (5.07 ± 0.07). The phylogenetic distance (PD) was observed to be lowest in fish fed the probiotic treatment (3.15 ± 0.69) which was significantly ($P < 0.05$) lower than in fish fed the prebiotic treatment (5.51 ± 0.37). Species richness (Chao 1) remained statistically unaffected by treatment groups. Unweighted UniFrac UPGMA clustering of reads from the replicates of each treatment revealed two distinct clusters, with all replicates from the basal, prebiotic and antibiotic treatment groups clustering together in the first cluster, and all replicates of the probiotic and synbiotic treatment groups clustering together in the second cluster (Figure 4A.3.).

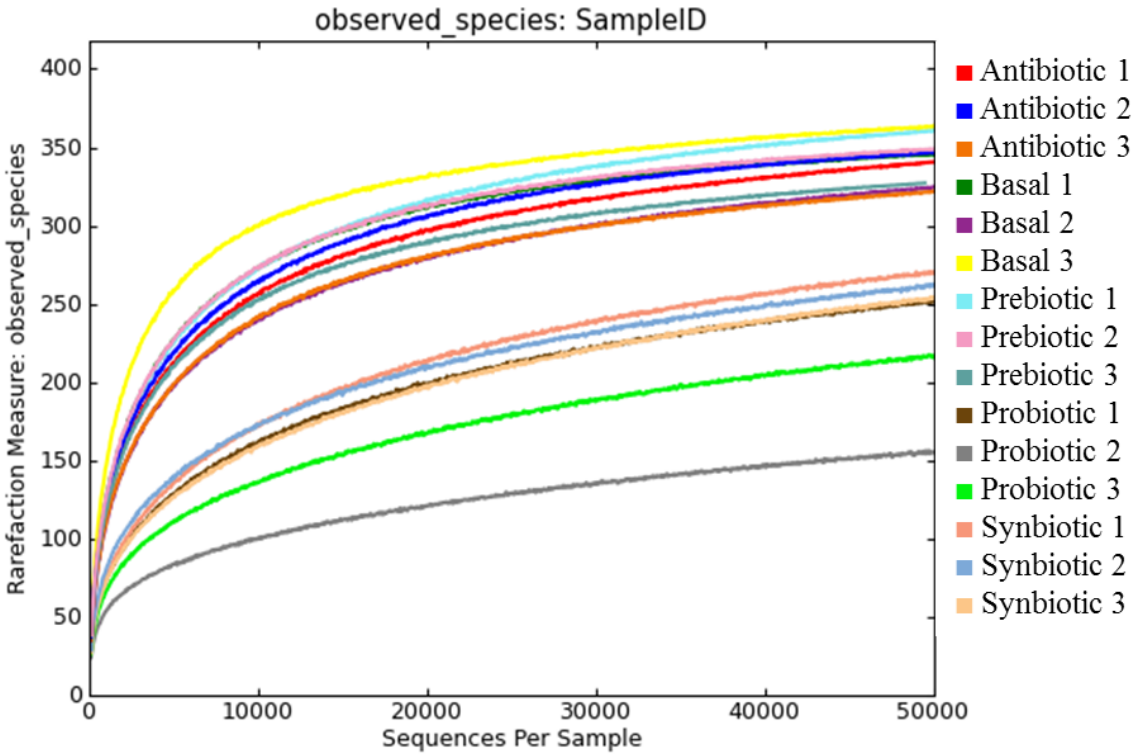

Figure 4A.2. Alpha rarefaction curves representing the number of observed species (OTU's) per sample, which is used as an inference of the number of species, as a function of sequencing effort.

Table 4A.5. High-throughput sequencing goods coverage estimations by treatment and alpha diversity parameters of the allochthonous bacteria associated with the posterior intestine of European sea bass fed each dietary regime for four weeks. Data represent mean ± SD.

Treatment	Good's coverage	Observed species	Chao1 index	Shannon index	Phylogenetic Distance
Basal	0.9972±0.0001	261.89±16.17	296.00±15.97	5.03±0.15	5.07±0.38
Probiotic	0.9978±0.0006	111.89±23.65	160.80±33.32	3.18±0.06	2.11±0.52
Prebiotic	0.9971±0.0002	254.91±4.12	286.79±8.51	4.86±0.01	4.88±0.29
Synbiotic	0.9972±0.0001	139.83±4.84	193.55±3.18	3.30±0.06	2.74±0.16
Antibiotic	0.9969±0.0002	247.58±7.02	286.21±4.85	4.76±0.17	4.73±0.12

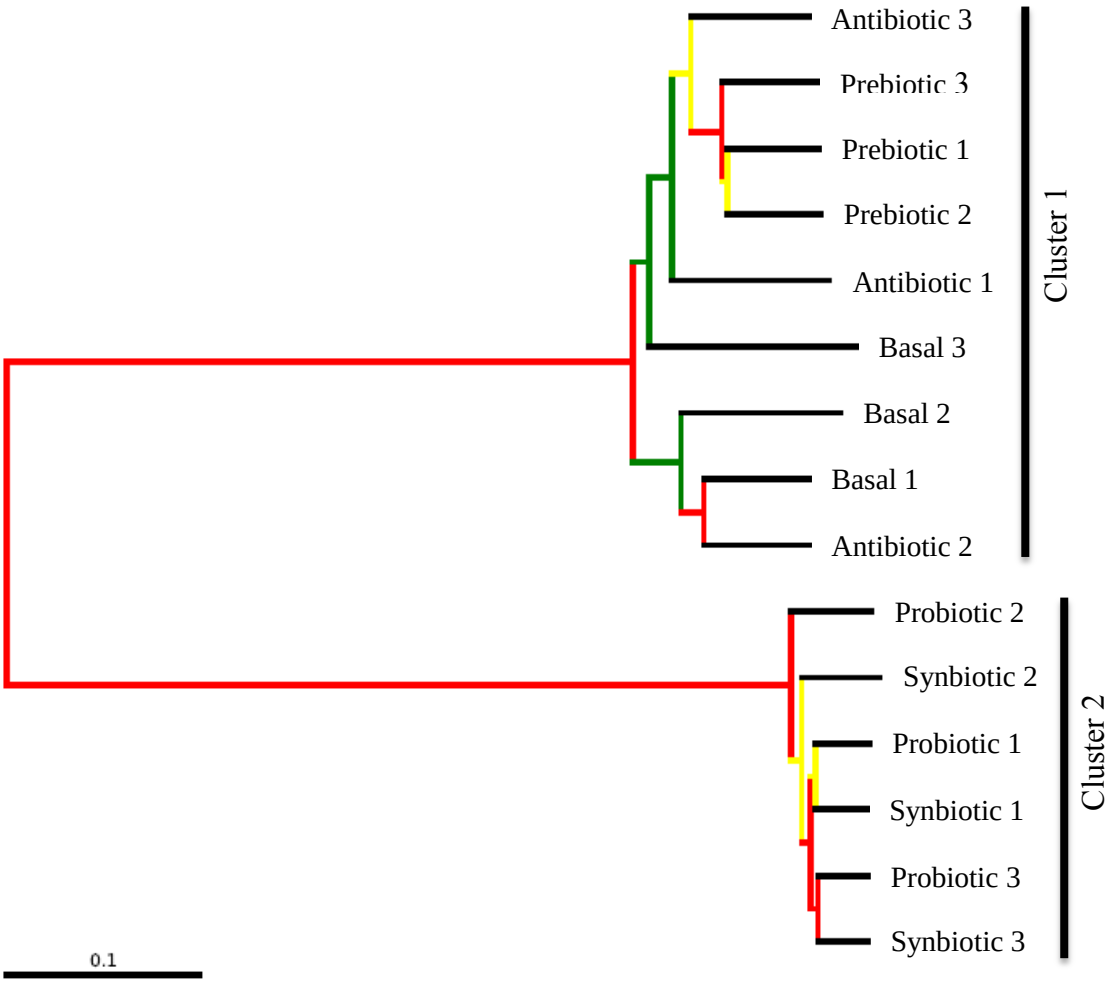

Figure 4A.3. Bray-Curtis UPGMA UniFrac clustering of reads of the replicates of the allochthonous bacterial communities in the posterior intestine of European sea bass fed each dietary regime for four weeks. Jackknife support is: Red (75-100 %), yellow (50-75 %), green (25-50 %) and blue (< 25 %). Bar indicates 10 % divergence. Cluster 1 contains all replicates within the basal, prebiotic and antibiotic treatments. Cluster 2 contains all replicates within the probiotic and synbiotic treatments.

The sequence distribution at the phylum level is displayed in Figure 4A.4. Firmicutes dominated the phyla within all treatments accounting for 97.50 % of the total read sequences, followed by Actinobacteria (1.30 %), Proteobacteria (1.10 %) and Bacteroidetes (0.10 %). Statistically, Firmicutes were significantly elevated in samples of fish fed the probiotic (99.80 %) and synbiotic treatments (99.60 %) compared to those fed the basal diet (94.90 %) ($P < 0.02$ and $P < 0.05$, respectively). The proportion of Actinobacteria reads were significantly reduced in fish fed the probiotic (0.10 %) and synbiotic (0.20 %) diets compared to fish fed the basal (2.00 %), prebiotic (2.30 %), and antibiotic (0.90 %) diets ($P < 0.001$). The phyla Proteobacteria and Bacteroidetes remained statistically unaffected by dietary treatments.

Figure 4A.5. displays the sequence distribution at the genus level. The genus *Lactobacillus* comprised the majority of the 16S RNA gene reads, comprising 42.10 % of the total reads, followed by reads belonging to the family *Bacillaceae* (identified in BLAST as *B. subtilis*) (34.90 %), and *Leuconostocaceae* (genus unidentified) (11.90 %), and the genus *Bacillus* (4.10 %) with all other genera present at 1.5 % or below. The genus *Lactobacillus* was significantly higher in fish fed the prebiotic treatment (73.20 %) compared to fish fed the basal (60.70 %; $P < 0.05$), probiotic (2.70 %; $P < 0.001$), and synbiotic (5.10 %; $P < 0.001$) diets. This genus was also observed to be significantly higher in fish fed the basal treatment compared to fish fed the probiotic and synbiotic ($P < 0.001$). The proportion of *Lactobacillus* reads was also significantly elevated in fish fed the antibiotic treatment (68.80 %) compared to fish fed the probiotic and synbiotic treatments ($P < 0.001$). The abundance of reads assigned to the genus *Bacillus* was observed to be significantly elevated in fish fed the probiotic (10.30 %) and synbiotic (9.40 %) treatments compared to fish fed the basal (0.6 %; $P < 0.001$), prebiotic (0.2 %; $P < 0.001$) and antibiotic (0.2 %; $P < 0.001$) diets. The genera *Micobacterium* and *Vagococcus* were significantly reduced ($P < 0.001$) in fish fed the

147 probiotic (undetected) and synbiotic (undetected) compared to the fish fed the basal, prebiotic
148 and antibiotic treatments which was detected at 0.30 %. Reads belonging to the genus
149 *Weissella* were significantly reduced in fish fed the probiotic (undetected) and synbiotic
150 (undetected) treatments when compared to fish fed the basal (0.80 %; $P < 0.001$), prebiotic
151 (0.60 %; $P < 0.001$) and antibiotic (0.60 %; $P < 0.01$) treatments. Reads assigned to the
152 family *Brevibacteriaceae* were also significantly higher in fish fed the basal (0.2 %; $P <$
153 0.01), prebiotic (0.1 %; $P < 0.02$) and antibiotic (0.2 % $P < 0.01$) diets when compared to fish
154 fed the probiotic treatment, and fish fed the synbiotic (undetected) treatment ($P < 0.01$, $P <$
155 0.05 and $P < 0.01$, respectively).

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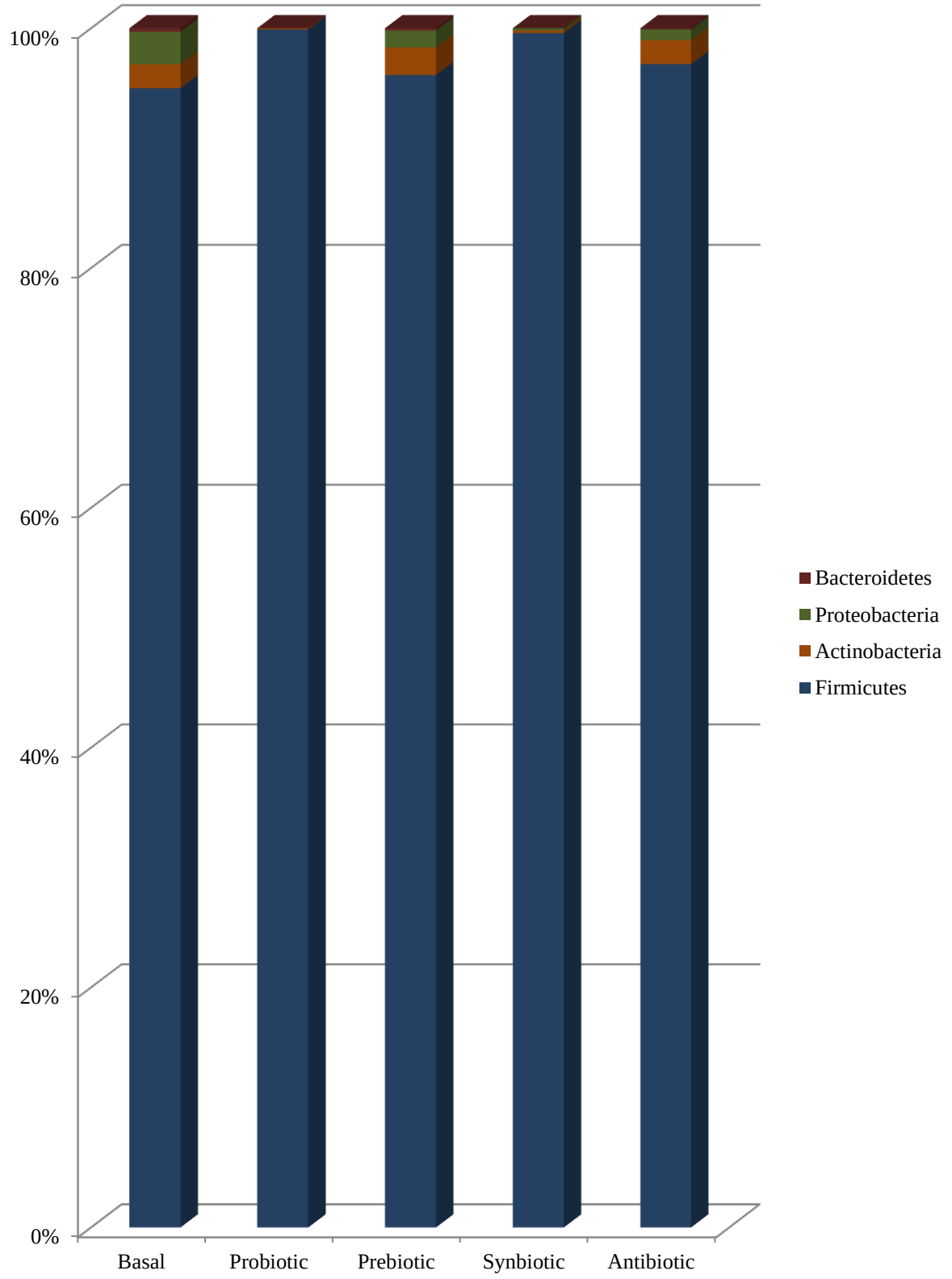


Figure 4A.4. The allochthonous bacterial communities in the posterior intestine of European sea bass fed the experimental diets for four weeks. Data represent bacterial phyla percentage.

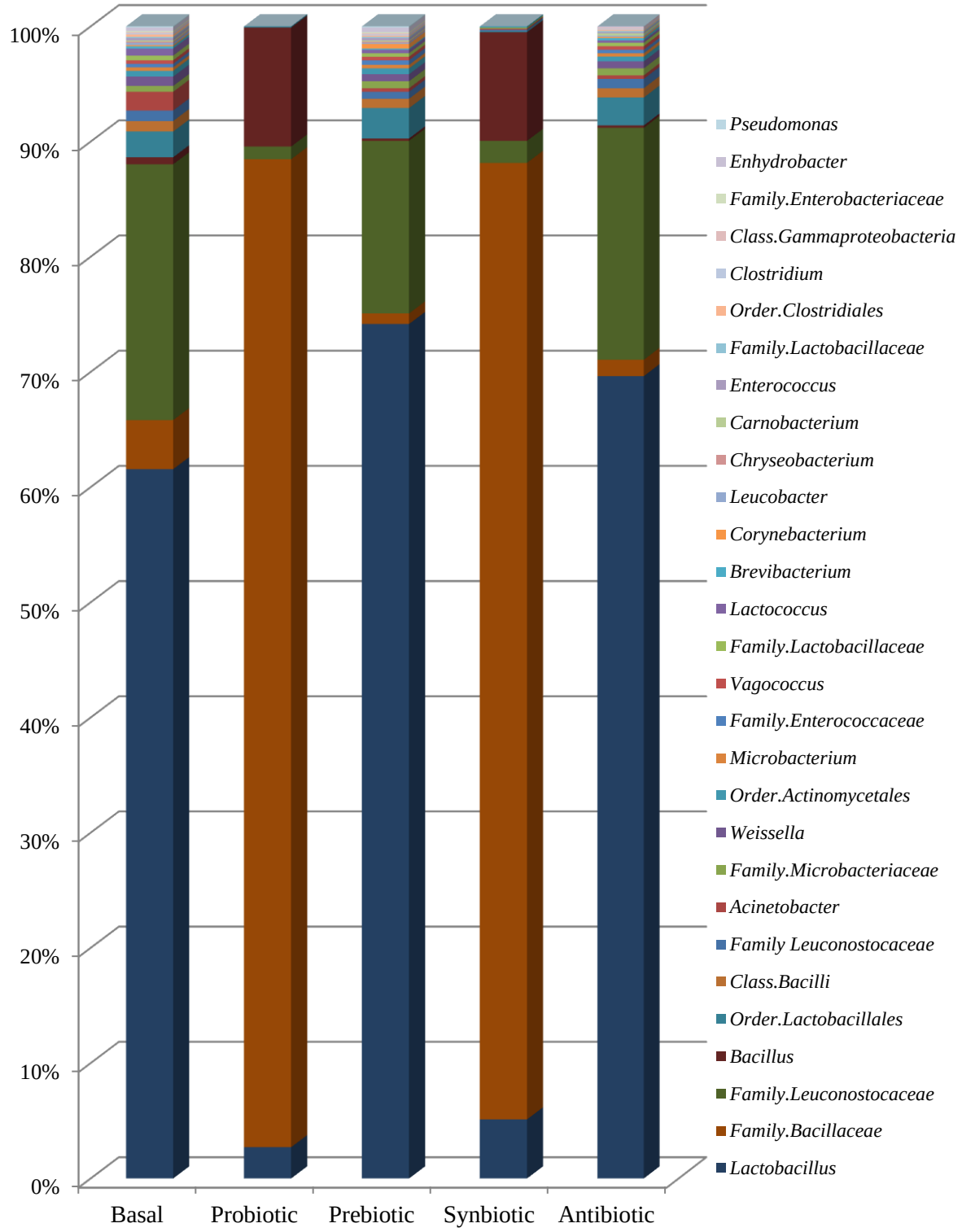


Figure 4A.5. The allochthonous bacterial communities in the posterior intestine of European sea bass fed the experimental diets for four weeks. Data represent reads assigned to bacterial genera > 0.1 %.

4A.4.2.4. Gene expression

The relative expression of the genes IL-1β, TNFα, HSP70, CASP3 and PCNA is presented in Figure 4A.6. The relative expression of the pro-inflammatory cytokine genes IL-1β and TNFα was significantly up-regulated in fish fed the probiotic, prebiotic, synbiotic and antibiotic treatments when compared to fish fed the basal diet. IL-1β was significantly up-regulated in fish fed the probiotic diet compared to fish fed the prebiotic, synbiotic and antibiotic diets. This gene was also significantly up-regulated in fish fed the prebiotic diet compared to fish fed the synbiotic and antibiotic diets. TNFα gene expression was significantly up-regulated in fish fed the probiotic, prebiotic and synbiotic diets compared to fish fed the antibiotic diet.

Changes were also observed in the expression of the genes; HSP70, CASP3 and PCNA (Figure 4A.6.). The relative expression of HSP70 was observed to be significantly down-regulated in fish fed the probiotic, prebiotic and synbiotic diets when compared to fish fed the basal. This was further significantly down-regulated in fish fed the synbiotic diet compared to fish fed the probiotic and prebiotic diets. HSP70 gene expression was also significantly up-regulated in fish fed the antibiotic diet compared to fish fed all other treatments. The relative expression of CASP3 was significantly down-regulated observed in fish fed the probiotic, prebiotic and synbiotic diets compared to the intestine of fish fed the basal. The intestine of fish fed the antibiotic diet exhibited a significant up-regulation in CASP3 compared to fish fed the all other treatments. The relative expression of PCNA followed similar trends with a significant down-regulation observed in fish fed the probiotic, prebiotic and synbiotic diets compared to fish fed the basal. The expression of PCNA was significantly down-regulated in fish fed the synbiotic diet compared to fish fed the probiotic and prebiotic diets. Fish fed the antibiotic diet exhibited a significant up-regulation in this gene when compared to fish fed all other diets.

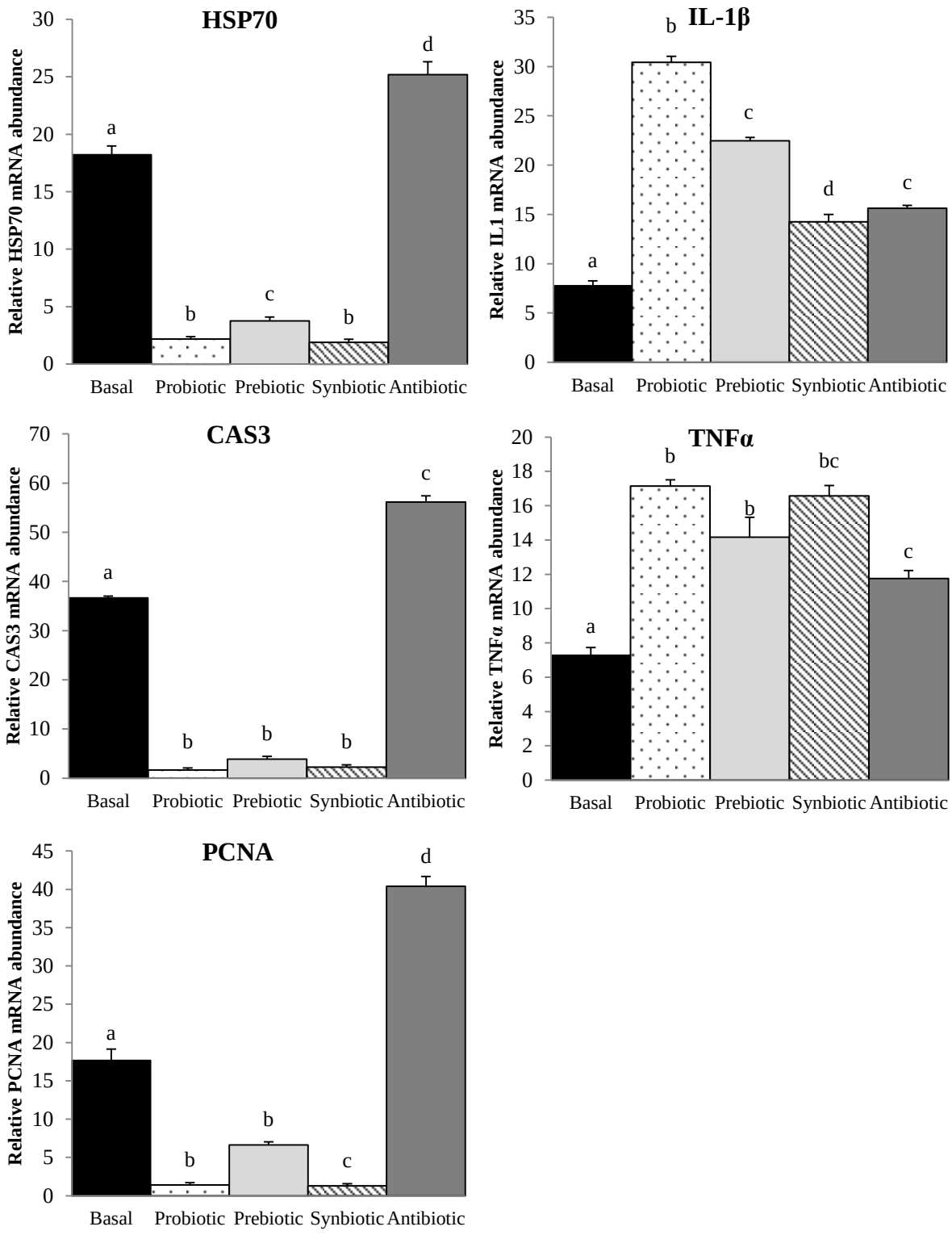


Figure 4A.6. Relative mRNA abundance of HSP70, CASP3, PCNA, TNFα and IL-1β in the intestine of European sea bass fed the experimental diets for four weeks. Different superscript letters indicate significant differences (accepted as $P < 0.05$) between treatments. $n = 6$ per treatment.

4A.4.3. Intestinal histology

4A.4.3.1. Light microscopy

Figure 4A.7. illustrates examples of H & E stained posterior intestinal sections of fish fed each dietary regime. Gross observation analyses revealed some distinct differences between treatment groups with fish fed the basal treatment appearing to exhibit reduced mucosal fold lengths. The PR values (abitrary units) of fish fed the synbiotic treatment was significantly ($P < 0.05$) higher compared to fish fed the basal (5.36 ± 0.85 vs. 4.15 ± 0.59 in conrtol fed fish). Significantly elevated GC's were obserevd in the probiotic and synbiotic fed fish (11.32 ± 1.97; $P < 0.01$ and 11.28 ± 2.89; $P < 0.05$, respectively) compared to fed fish the basal treatment (7.43 ± 0.59) (Table 4A.6.). Numbers of IEL's per 100 µm was highest in fish fed the probiotic and synbiotic treatments (70.48 ± 14.21 and 70.98 ± 10.91 respectively) however, all IEL counts per treatment remained significantly unaffected (Table 4A.6.).

223 **Table 4A.6.** Quantitative morphometric analyses of European sea bass posterior intestine
224 after four weeks on the respective dietary regimes for four weeks. Data represent mean ± SD.
225 Different superscript letters within the same rows indicate significant differences between
226 groups.

Variable	Basal	Probiotic	Prebiotic	Synbiotic	Antibiotic
Perimeter ratio (AU)	4.15±0.59^{a}	5.19±0.70^{ab}	4.80±0.85^{ab}	5.36±0.71^{b}	5.03±0.61^{ab}
IEL's (cells per 100µm)	66.02±8.54	70.48±14.21	67.03±5.87	70.98±10.91	57.63±8.19
GC's (cells per 100µm)	7.43±0.59^{a}	11.32±1.97^{b}	8.58±0.91^{ab}	11.28±2.89^{b}	9.32±3.32^{ab}
Microvilli density (AU)	6.34±0.88^{a}	8.47±1.13^{b}	8.46±1.67^{b}	7.84±1.29^{ab}	7.10±1.11^{ab}
Microvilli length (µm)	2.37±0.30	2.25±0.40	2.15±0.24	2.19±0.26	2.33±0.28
ASI	71.51±11.05^{a}	113.24±23.44^{b}	95.75±18.19^{ab}	105.65±14.84^{ab}	93.95±22.11^{ab}

227

228

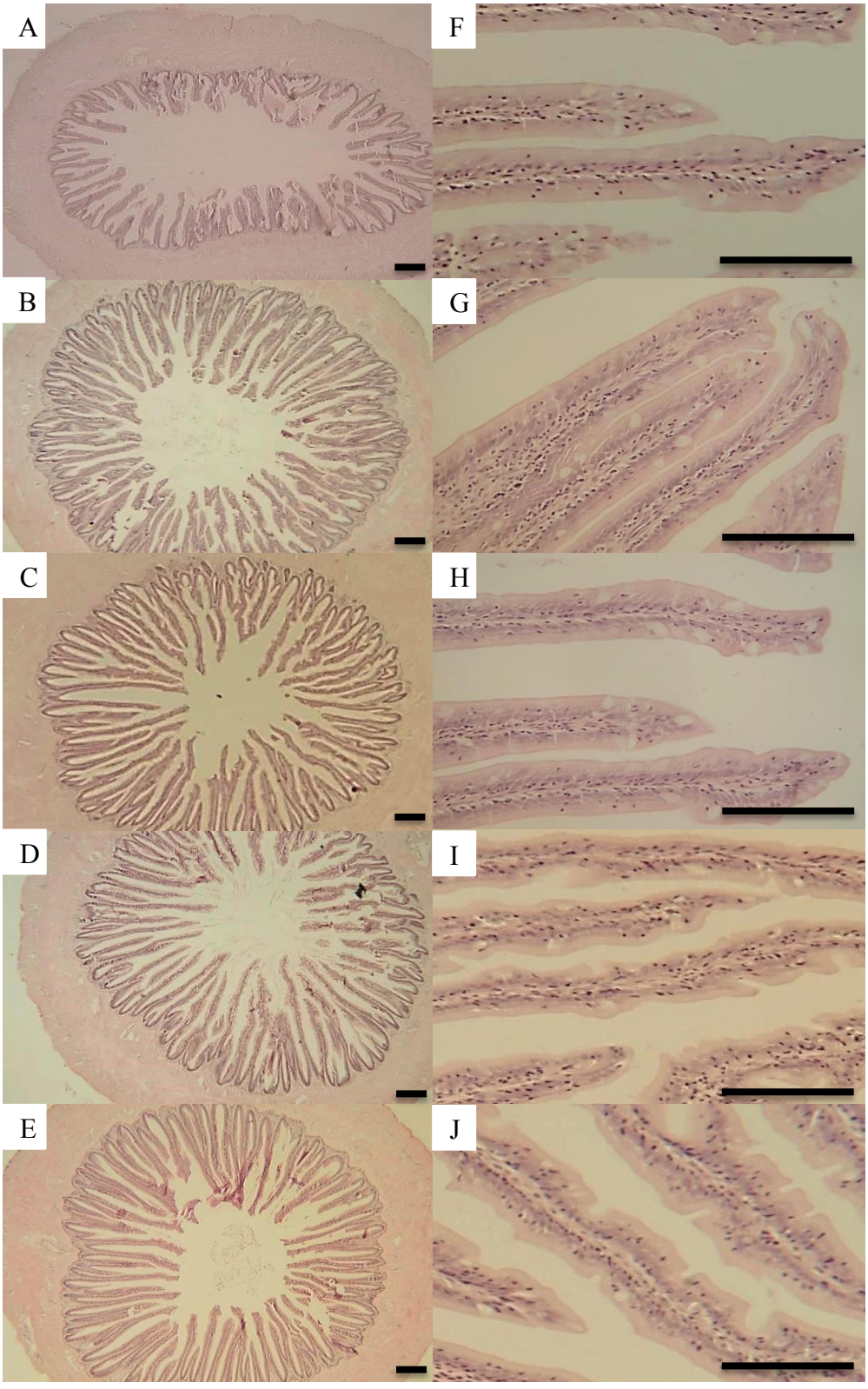


Figure 4A.7. Light micrographs of the posterior intestine of European sea bass fed the basal control (A & F), probiotic (B & G), prebiotic (C & H), synbiotic (D & I), and antibiotic (E & J) treatments for four weeks. Scale bars = 100 µm in A-E and 50 µm in F-J.

4A.4.3.2. SEM

Electron micrographs of the ultra-structural scale of the posterior intestine of fish fed each dietary treatment are presented in Figure 4A.8. Gross observations using SEM revealed distinct differences between treatments with the epithelium of fish fed the basal dietary treatment exhibiting gaps between enterocytes and areas of patchy microvilli with reduced microvilli densities and formation. In contrast the epithelium of fish fed the probiotic, prebiotic and synbiotic treatments exhibited uniform enterocyte formations and densely packed microvilli. Fish fed the antibiotic treatment exhibited similar epithelial damage to that observed in the basal fed fish. Quantitative analyses confirmed these observations with the epithelium of fish fed the basal treatment exhibiting the lowest microvilli density measurement (arbitrary units) (6.34 ± 0.88). Antibiotic fed fish displayed a value, similar to the basal fed fish of 7.10 ± 1.11. Values observed from fish fed the probiotic (8.47 ± 1.13), prebiotic (8.46 ± 1.67) and synbiotic (7.84 ± 1.29) treatments were markedly increased, significantly so with regards to the probiotic and prebiotic treatment groups with $P < 0.05$ in both cases (Table 4A.6.).

4A.4.3.3. TEM

Observations of TEM images confirmed the SEM findings with apparently uneven microvillar structures and some degradation at the apical tips in those fish fed the basal treatment. In contrast, fish fed the probiotic, prebiotic, synbiotic and antibiotic treatments all appeared to exhibit a more uniform and tightly packed microvillar morphology. Despite these morphological differences at the apical tips, the microvilli height measurements remained statistically unaffected throughout treatments. The ASI index revealed that fish fed the

synbiotic treatment exhibited a significantly ($P < 0.05$) higher value (105.65 ± 14.84) when compared to those fish fed the basal treatment (71.51 ± 11.05) (Table 4A.6.).

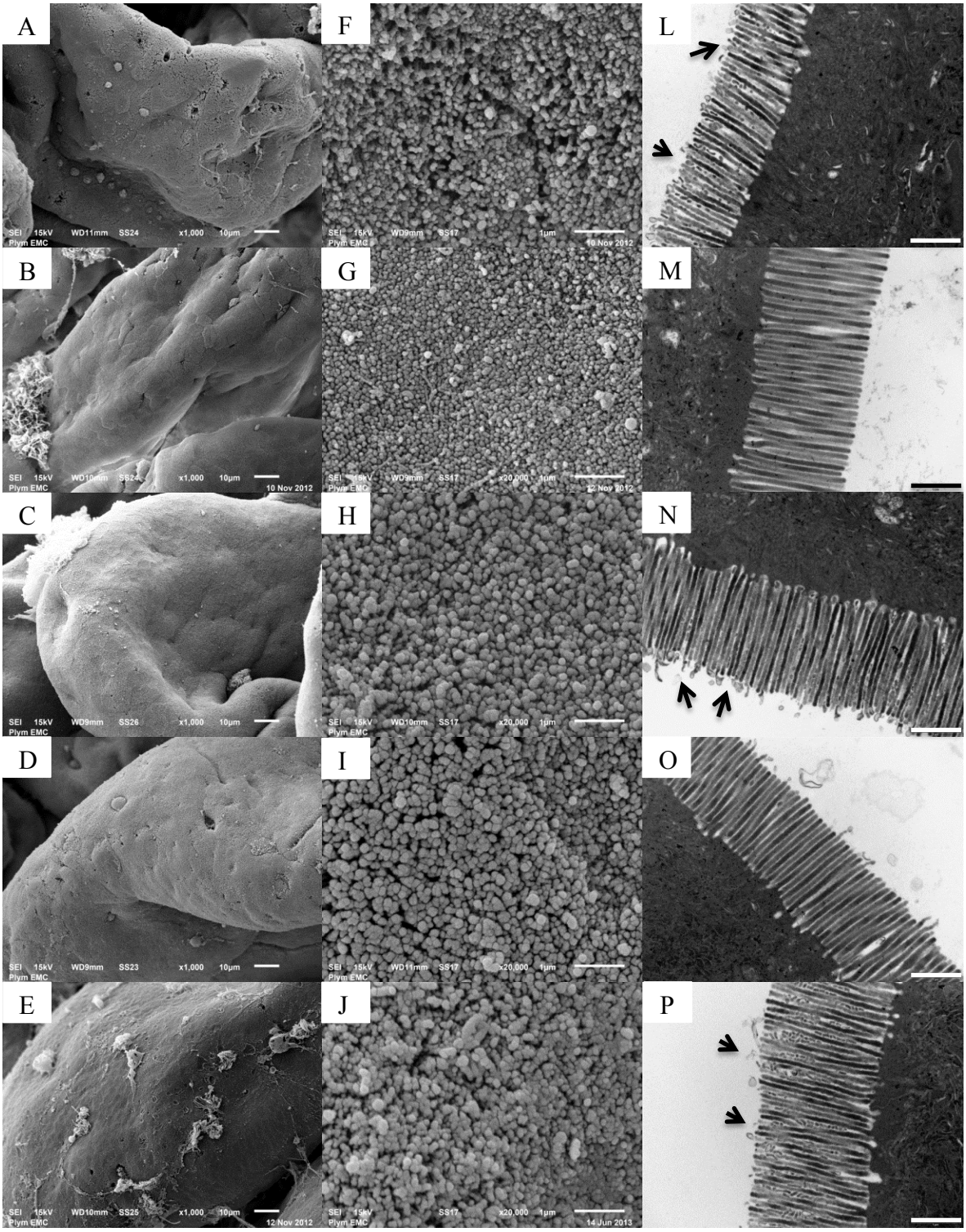


Figure 4A.8. Electron micrographs (SEM; A-J and TEM; L-P) of the posterior intestine of European sea bass fed the experimental diets; basal (A, F & L), probiotic (B, G & M), prebiotic (C, H & N), synbiotic (D, I & O), and antibiotic (E, J & P) for four weeks. Scale bars = 10µm in images A-E and 1 µm in images F-O. Note the apparent loss of membrane integrity of the microvilli apical tips in images L, N and P.

4A.5. Discussion

The present study used microbiological, gene expression and histological techniques to assess the effects the probiotic *B. subtilis*, the commercial prebiotic Previda® and the antibiotic oxytetracycline have on the intestinal microbiota and health of European sea bass fed a sub-optimal diet.

The results generated from the PCR-DGGE analyses revealed that the inclusion of *B. subtilis* modulates the allochthonous intestinal microbiota in European sea bass. Two distinct clusters with replicates from fish fed the basal, prebiotic and antibiotic treatments clustering with approx. 55 % similarity. The second cluster contained the replicates of the probiotic and synbiotic fed fish with approx. 80 % similarity. This was further confirmed by high-throughput sequence analysis, with similar cluster groupings observed in the Bray-Curtis UniFrac UPGMA. PCR-DGGE ecological parameters revealed fish fed the antibiotic treatment exhibited the lowest numbers of OTU's and significantly lower pecentage similarity between replicates (SIMPER) when compared to fish fed the other treatments. Fish fed the probiotic, prebiotic and synbiotic treatments exhibited higher SIMPER values compared to fish fed the basal, perhaps suggestive of a more stable microbiota induced by the feed additives. Species richness and diversity was lowest in fish fed the antibiotic treatment, folowed by fish fed the probiotic and synbiotic treatments though these values remained statistically unaffected. With regards to high-throughput sequence analyses, sequence libraries of all treatments exhibitied Good's coverage estimations > 0.99, indicative of fully sampled microbial populations. Similar to the PCR-DGGE analyses, high-throughput sequence ecological parameters revealed fish fed the probiotic and synbiotic treatments to exhibit lower Chao 1 (richness) and Shannon index (diversity) values when compared to fish fed the basal.

Observed species values were also observed to be lowest in fish fed the probiotc and synbiotic treatments. However, these parameters were not statistically different between treatments.

Interestingly, contrary to the PCR-DGGE analyses, fish fed the antibiotic treatment exhibited Chao1 and shannon index values similar to those observed in fish fed the basal and prebiotic treatments. Observed species values of fish fed the antibiotic treatment appeared to be lower than fish fed the basal and prebiotic treatment, but higher than in fish fed the probiotic and synbiotic treatments. The small discrepancies (although not significant) between molecular methods with regards to the Choa1 and Shannon indices of the antibiotic fed fish is perhaps not surprising and may be a result based on the higher level of sensitivity of high-throughput sequencing methods compared to the PCR-DGGE methods. Indeed, it has been reported that rare/minority bacterial groups in a given sample may not be detection by PCR-DGGE (Ercolini 2004). Leite *et al.* (2012) reported minor bacrterial constituents deteced using high-throughput sequencing, which went undetected by PCR-DGGE. Caution should also be applied to high-thrpoughput sequencing data when assessing bacterial abundance. This is particularly important in probiotic studies given the generally high numbers of probiotic 16S rRNA reads (which may occur as a consequence of the probiotic supplementation), relative to 16S rRNA reads assigned to other species. Furthermore, 16S rRNA copy numbers may differ between bacterial species potentially skewing the true bacterial abundance in a given sample (Fogel *et al.* 1999; Ercolini 2004).

Both PCR-DGGE and high-throughput sequence analysis revealed all samples to be dominated by the Firmicutes phylum. Proteobacteria, Actinobacteria and Bacteriodetes were also detected by high-throughput sequence analysis in relatively

lower abundances. These findings are in contrast to other studies on European sea bass and other marine fish species, where the intestinal microbiota appear to be dominated by Proteobacteria, with Bacteriodetes, Actinobacteria, and Firmicutes making up the smaller proportions (Izvekova *et al.* 2007; Cerezuela *et al.* 2013; Carda-Diéguez *et al.* 2014; Gatesoupe *et al.* 2014). The reasons for this warrant further investigation but could be due to the artificially high SPC content and/or saponin supplememntation of the basal formulation which had a modulatory effect to the intestinal microbiota. Indeed, dietary inclusion of soy proteins has been observed to modulate the intestinal microbiota in various fish species (Heikkinen *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008; Merrifield *et al.* 2009; Dimitroglou *et al.* 2010; Reveco *et al.* 2014; Chapter 3) Differing methodologies applied in the various studies could also be a contributing factor to the above findings.

At the genera level, LAB, and specifically *Lactobacillus* spp., appeared to dominate the bacterial community of the basal, prebiotic and antibiotic fed fish and was significantly higher than in fish fed the probiotic and synbiotic treatments. Multiple *B. subtilis* species were sequenced from PCR-DGGE of fish fed the probiotic and synbiotic treatments which were absent from fish fed the other treatments, appearing to confirm the presence of the probiotic. This was further verified through high-throughput sequence analyses, which revealed the dominance of OTU's (of those 16S rRNA reads accounting for > 0.1 %) assigned to *Bacillus* spp. in fish fed the probiotic and synbiotic treatments, which was significantly higher than in fish fed the basal, prebiotic and antibiotic treatments. Furthermore, multiple *Bacillus* sequences identified as *B. subtilis* using the NCBI database were present in the probiotic and synbiotic fed fish which were absent from the other treatments

appearing to confirm the presence of the probiotic. However, a number of sequence reads also identified as *B. subtilis* were present in all samples. This is perhaps not surprising as *B. subtilis* is commonly found in the intestine of many fish species (Mahious *et al.* 2006; Ai *et al.* 2011; Askarian *et al.* 2012; Del'Duca *et al.* 2013; 2015), and its presence in all samples in the present study may suggest this species to be part of a core bacteria found in the intestine of European sea bass. However, to the authors knowledge only a handful of studies have assessed the intestinal bacterial communities of European sea bass and hence limited data is available in this area (De Schryver *et al.* 2011; Sun *et al.* 2013; Gatesoupe *et al.* 2014; Carda-Diéguez *et al.* 2014; Delcroix *et al.* 2015; Chapter 3).

A number of genes were also evaluated in the present study to assess the affects the feed additives on the localised intestinal immunology. The cytokines IL-1β and TNFα, which are involved in the initiation of immune cells such as macrophages and neutrophils and play roles in regulating inflammation (Foey & Picchetti 2014), were assessed at the transcriptional level. It was observed that fish fed the probiotic, prebiotic, synbiotic and antibiotic treatments exhibited a significant up-regulation of the pro-inflammatory cytokine genes IL-1β and TNFα compared to fish fed the basal diet. Elevated intestinal IL-1β and/or TNFα expression has been reported in fish as a consequence of probiotic and prebiotic supplementation (Pérez-Sánchez *et al.* 2011; Pirarat *et al.* 2011; Abid *et al.* 2013; He *et al.* 2013; Liu *et al.* 2013; Román *et al.* 2013; Standen *et al.* 2013; Guzmán-Villanueva *et al.* 2014; Villamil *et al.* 2014; Yarahmadi *et al.* 2014). The elevated intestinal IL-1β and TNFα levels (relative to the basal control) in the present study may be indicative of an intestine in an elevated immunologic state. The expression of HSP70, CASP3 and PCNA was also assessed in the present study as indicators of intestinal cellular level stress.

Relative to the basal fed fish, the expression of HSP70 was significantly down-regulated in fish fed the probiotic, prebiotic and synbiotic treatments. HSP70 levels are known to increase in the presence of potential pathogens, stressors and unfavourable conditions such as inappropriate stocking densities (Gornati *et al.* 2004; Sanden *et al.* 2009; Zhang *et al.* 2014). The down-regulation in HSP70 observed in the present study in fish fed the probiotic, prebiotic and synbiotic is in agreement with a number of previous probiotic and prebiotic studies assessing this gene in various fish species (He *et al.* 2011; Lui *et al.* 2013; Yarahmadi *et al.* 2014; Chen *et al.* 2015). The relative expression of CASP3 and PCNA was also significantly reduced in fish fed the probiotic, prebiotic and synbiotic treatment compared to fish fed the basal. PCNA is a biomarker for cell proliferation and DNA repair and an increase in expression of this gene would suggest an increase in cellular division. CASP3, is part of the cysteine-dependent aspartate specific protease family, involved in programme cell death (apoptosis) (Fink and Cookson 2005). It has been observed in European sea bass that a virulence mechanism of the fish pathogen *Photobacterium damselae* ssp. *piscicida* is the ability to induce apoptosis of macrophages and neutrophils (do Vale *et al.* 2003). In the present study the relative expression of CASP3 was significantly down-regulated in fish fed the probiotic, prebiotic and synbiotic treatment compared to fish fed the basal. This is in accordance with a study where a down-regulation of CASP3 in intestinal cells of Atlantic cod was reported as a consequence of probiotic supplementation *in vitro* (Lazado *et al.* 2011). The reduced expression levels of HSP70, CASP3 and PCNA of the probiotic, prebiotic and synbiotic fed fish may be suggestive of an intestinal tissue under a state of reduced stress and a lower level of cellular turnover compared to fish fed the basal diet and the antibiotic treatment. It has been observed

previously in Atlantic salmon that the inclusion of SPC increases PCNA-positive proliferation at the base of intestinal folds, as well as a marked increase in HSP70 and CASP3 reactivity in epithelial cells at the tips of the simple folds (Bakke-McKellep *et al.* 2007). Interestingly, the opposite trend was observed with regards to fish fed the antibiotic treatment in the present study, with significant increases in the expression of HSP70, PCNA and CASP3 relative to fish fed all other treatments. This may suggest the antibiotic exerts a level of intestinal stress in the intestine of European sea bass. This could, at least in part, be due to the significant decrease in the abundance of the genus *Bacillus* in fish fed the antibiotic treatment, compared to fish fed the probiotic and synbiotic treatments, as *Bacillus* strains including *B. subtilis* have been reported to the reduce cellular level stress in gilthead sea bream by decreasing HSP70 gene expression (Avella *et al.* 2010). Future work is required on the roles antibiotics have on the expression of immune-related gene expression in the intestine of fish.

Chapter 3 revealed some histological differences between fish fed the basal diet used here and fish fed a FM control diet. This Chapter revealed reduced PR and fold lengths, as well as significantly reduced GC's and IEL's as a consequence of feeding the basal diet compared to fish fed the FM control. In the present investigation, the administration of the probiotic, prebiotic and synbiotic appeared to increased the PR relative to the PR of fish fed the basal control, significantly so in fish fed the synbiotic treatment. Furthmore, fish fed the probiotic and prebiotic treatments exhibited significantly increased microvilli density measurements when compared to fish fed the basal. These results are in agreement with previous probiotic and prebiotic studies in fish (De Rodriganez *et al.* 2009; Dimitroglou *et al.* 2009; Merrifield *et al.* 2010b). Microvilli lengths remained unaffected by dietary

433 treatments however, the combined with the PR and microvilli density indices, the
434 absorptive surface area index (ASI) was significantly increased in fish fed the
435 probiotic and generally increased in fish fed the prebiotic and synbiotic treatments
436 compared to fish fed the basal. GC numbers were significantly increased in fish fed
437 the probiotic and synbiotic treatments compared to fish fed the basal. IEL's were
438 also elevated in fish fed the probiotic and synbiotic treatments however, these
439 values remained statistically unaffected between treatments. Elevated IEL's and
440 GC's in the intestine of gilthead sea bream and European sea bass has previously
441 been reported after dietary *Lactobacillus* spp. and *B. subtilis* administration
442 (Picchietti *et al.* 2007, 2009; Salinas *et al.* 2008). Increased GC's could be an
443 indication of increased mucus production which subsequently lines the epithelial
444 surfaces creating an unreceptive environment for potential pathogens (Dharmani *et*
445 *al.* 2009). Furthmore it has been observed in rainbow trout that elevated intestinal
446 IEL's and GC's may lead to reduced epithelial damage when exposed to pathogens
447 (Harper *et al.* 2011). The colective histological results from the present study are in
448 agreement with the aformentioned experiments and the increase in IEL's and
449 significant increase in GC's exhibited by the probiotic and synbiotic fed fish may
450 suggest an intestine better protected against potential enteropathogens. Furthmore,
451 the increases in PR, microvilli density and ASI observed in fish fed the probiotic,
452 synbiotic and to a slightly lesser degree, the prebiotic, may indicate an intestine with
453 greater nutrient utilisation potential. These findings are in accordance with the
454 significantly reduced expression levels of HSP70, PCNA and CASP3 suggesting
455 that the probiotic, synbiotic and prebiotic supplementation has a positive effect on
456 the intestinal health of European sea bass fed a sub-optimal diet.
457

4A.6. Conclusion

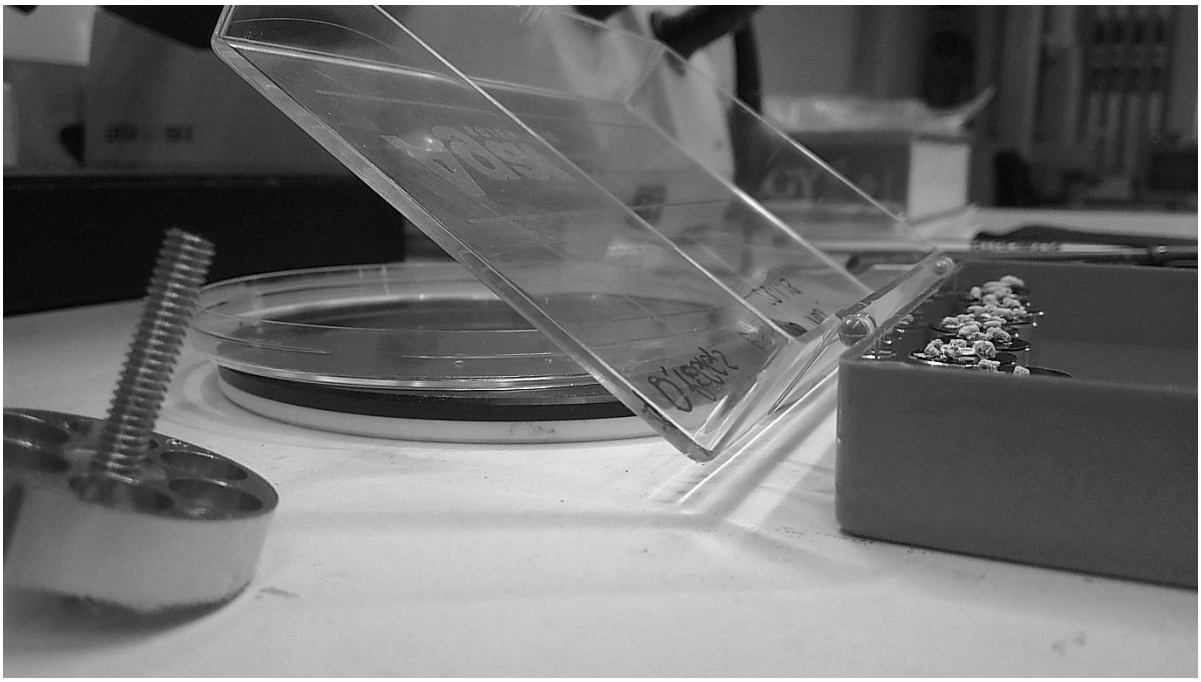
In the present investigation, the supplementation of the probiotic, prebiotic, and synbiotic was observed to modulate the allochthonous intestinal microbiota of European sea bass. Multiple *B. subtilis* strains were observed to be present in fish fed the probiotic and synbiotic treatments which were absent from fish fed the other treatments. The probiotic appeared to have a stabilising effect on the microbial communities which were observed to be less diverse and less rich compared to the other treatments. The present study also revealed the use of the probiotic and prebiotic when used individually, and in combination, significantly elevated the expression of the cytocynes IL-1β and TNFα as well as the significant down-regulation of HSP70, PCNA and CASP3 suggesting an intestine in an elevated state of immuo-readiness and under a lower level of cellular stress. These results appeared to coincide with the histological analyses where the epithelium of the intestine of fish fed the probiotic, prebiotic and synbiotic was observed to contain increased GC's and IEL's and increased PR, microvilli densities and ASI compared to fish fed the basal diet. Gene expression data and histological appraisals indicated that the antibiotic caused a level of intestinal stress in excess of those caused by the basal diet. Collectively, the results of the present investigation reveal that the probiotic and prebiotic, when incorporated individually and in combination, into a sub-optimal SPC based diet can have positive effects on the intestinal health of European sea bass juveniles.

The positive effects *B. subtilis* appeared to confer in this chapter led to the question; do these positive effects due to dietary *B. subtilis* supplementation extend to positive long-term effects on the growth and health of European sea bass juveniles. The present chapter revealed the unusually high levels of SPC and the addition of

483 saponins appeared to modulate the intestinal bacterial communities, and was not
484 representative of a diet used commercially for this species. Chapter 5 would
485 therefore use a diet more in line with one which might be used commercially in
486 European sea bass aquaculture.

Chapter 4B:

Ex vivo European sea bass Intestinal bacterial challenge trial of (*D. labrax*)



4B.1. Abstract

At the end of the trial in Chapter 4A, 20 fish per treatment (i.e. basal, probiotic, prebiotic and synbiotic) were sampled for the *ex vivo* experiment. Whole intestinal samples were removed aseptically and the posterior intestine was then isolated. Solutions containing one of four treatments [1. phosphate buffered saline (PBS) as the control, 2. probiotic at 10^7 CFU g^{-1}, 3. pathogen (*V. anguillarum*) at 10^7 CFU g^{-1} and 4. probiotic1 (10^7 CFU g^{-1}) + pathogen (10^7 CFU g^{-1}) (50:50)] was conducted with n = 5. SEM analyses revealed *V. anguillarum* caused substantial epithelial damage to the intestine of all samples. The most pronounced damage was observed in samples of fish fed the basal control and microvilli density measurements were significantly reduced in fish fed the control compared to fish fed the other treatments when exposed to the pathogen. These results appear to strengthen the findings of Chapter 4A, suggesting the improved health status of the epithelium as a consequence of *B. subtilis* and Previda® feeding may confer a level of epithelial protection against *V. anguillarum*. No significant differences in microvilli densities were observed between treatments when the intestines were exposed to the pathogen + probiotic. FISH analyses did not detect the presence of *V. anguillarum* cells on or in the epithelial tissue after the one hour exposure time. This suggests the epithelial damage caused by *V. anguillarum* may be as a result of the producing cytotoxins without directly attaching to the epithelium. The present experiment provides a novel way to assess the interactions between bacteria and host tissue

4B.2. Introduction

The GI tract of fish is an important organ involved primarily in digestion/absorption, but is also vitally important with regards to homeostasis and immunity (Ray & Ringo 2014). A common problem associated with European sea bass production is vibriosis, a bacterial disease caused by *V. anguillarum*, and it is thought the GI tract is one of the main infection site for this pathogen (Ringø *et al*. 2007; Harper *et al*. 2011). Antibiotics have traditionally been used to treat this and other bacterial diseases in fish, but more recently probiotic and prebiotic applications have come to the fore as a means of preventing bacterial infection without negatively impacting the intestinal microbiome. Probiotic and prebiotic studies in fish have presented positive effects and as a consequence are applications in the aquaculture industry are increasing year on year. For information regarding recent probiotic and prebiotic studies in fish refer to sections 1.7.1 and 1.7.2. A number of these studies provide evidence of host immunomodulation as a result of probiotic and prebiotic applications and its thought probiotics may inhibit pathogen infection through competitive exclusion (Balcázar *et al*. 2007b; Ringø *et al*. 2007a). With regards to vibriosis, most studies assessing the efficacy of probiotics to inhibit or retard this disease have been carried out on salmonids (Gram *et al*. 1999; Robertson *et al*. 2000; Spanggaard *et al*. 2001; Brunt *et al*. 2007; Ringø *et al*. 2007; Rodriguez-Estrada *et al*. 2009; Harper *et al*. 2011), with comparatively fewer studies available on European sea bass (Sorroza *et al*. 2012; Touraki *et al*. 2012). Most of these types of studies have focused on *in vivo* models, assessing survival and host immunity, and mechanisms relating to bacterial interactions and translocation are not fully understood. *Ex vivo* models have been utilised recently to assess the relationship between pathogens and the GI tract of fish. These types of studies are not only important models in terms of assessing bacterial-host interactions but are also in line with the replacement, reduction and refinement of animals in science. However, only a few studies to date are available on probiotic and

pathogen adhesion in the GI tract *ex vivo*, using variations of the intestinal sac method (Ringø *et al*. 2007b; Salinas *et al*. 2008; Kristiansen *et al*. 2011; Løvmo Martinsen *et al*. 2011; Salma *et al*. 2011; Harper *et al*. 2011; Ren *et al*. 2013). To the author's knowledge however, only one study has been carried out on European sea bass using *ex vivo* techniques (Torrecilas *et al*. 2011b), and no studies assessing probiotic efficacy using this *ex vivo* approach exist for this species. It was therefore the aim of the present investigation to utilise an *ex vivo* intestinal sac method to assess the effects the probiotic *B. subtilis* has individually, and in combination with the pathogen *V. anguillarum*, on the intestine of European sea bass fed various diets.

4B.3. Materials and Methodologies

4B.3.1. Experimental design

Prior to the *ex vivo* challenge trial, experiments were carried out in order to optimise the conditions to which the experiment would be carried out (sections *4B.2.2.* and *4B.2.3.*). For information of experimental fish and trial parameters, refer to section *4A.3.1.* At the end of the four week trial fish remained on the experimental diets for a further 3 days at which point Sampling was conducted on a total of 20 fish per feeding treatment (i.e. basal, probiotic, prebiotic and synbiotic). Each *ex vivo* treatment [phosphate buffered saline (PBS) as the control, probiotic cultured to a concentration of 10^7 CFU g^{-1}, pathogen (*V. anguillarum*) cultured to a concentration of 10^7 CFU g^{-1} and probiotic1 (10^7 CFU g^{-1}) + pathogen (10^7 CFU g^{-1}) (50:50)] was conducted with $n = 5$ (Table 4B.1.) Whole intestinal samples were removed aseptically and any contents removed with sterile PBS, and the posterior intestine was then isolated. Cotton thread was used to tie the posterior-most end of the intestine. PBS (100 µl) was then added directly to the intestine and the anterior end was subsequently tied using cotton thread. The intestine was then placed into sterile 30 ml universal tubes containing PBS

which were then incubated (Figure 4B.1.). Each sample was incubated at 20 ^{o}C for a period of 60 min, at which point samples for fluorescent *in-situ* hybridisation (FISH), and light and electron microscopy analysis were taken.

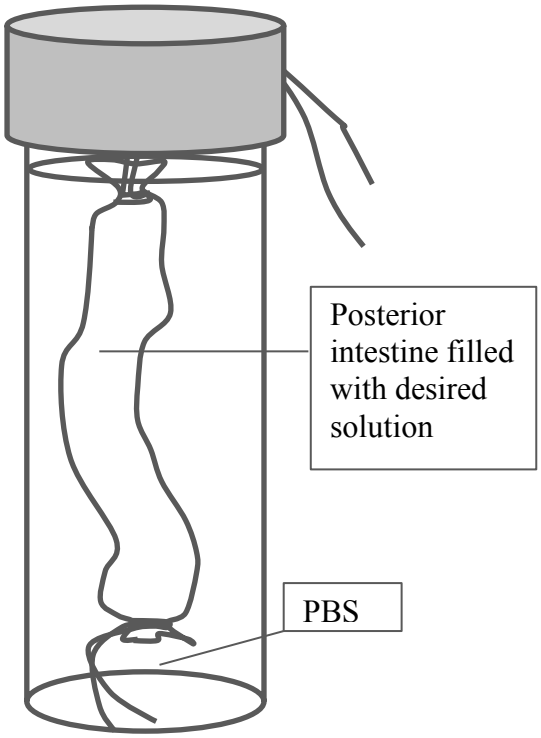


Figure 4B.1. Schematic representation of the *ex vivo* intestinal sac method used in the present experiment.

Table 4B.1. Trial information of the *ex vivo* experiment with dietary regimes and *ex vivo* treatments and numbers of fish used.

		***ex vivo* treatments**			
		PBS	PROBIOTIC	PATHOGEN	PROBKIOTIC+PATHOGEN
Dietary regimes	Basal	5	5	5	5
	Probiotic	5	5	5	5
	Prebiotic	5	5	5	5
	Synbiotic	5	5	5	5

4B.3.2. Time assessment optimisation

A preliminary experiment was conducted on the intestine of fish fed the basal diet to optimise the conditions best suited to conduct the *ex vivo* challenge trial at the end of the four week feeding trial. Intestines were incubated at 20 ^{o}C for periods of 30, 45 and 60 min to assess possible intestinal damage or artefacts caused by the *ex vivo* process (n = 4). After each time point samples were taken for SEM and TEM to determine levels of deterioration (refer to section 2.8 for methodology).

4B.3.3. Time assessment optimisation results

No obvious signs of damage were observed at the ultrastructural scale at the time points examined using both SEM and TEM analysis (Figure 4B.2.). SEM images revealed that the mucosal fold surfaces appeared to be healthy with no signs of necrosis or artefacts. Enterocytes appeared uniform as did the microvilli which were observed to be densely packed throughout all samples. TEM images also revealed a healthy brush border with well

defined, long microvilli and enterocytes bound by tight junctions with no obvious signs of ill health observed. It was concluded from the results acquired here that the *ex vivo* bacterial challenge experiment would be conducted at 20 ^{o}C and for the maximum time of 60 min. The longer time period of exposure would allow greater opportunities for the bacteria to interact with the tissue.

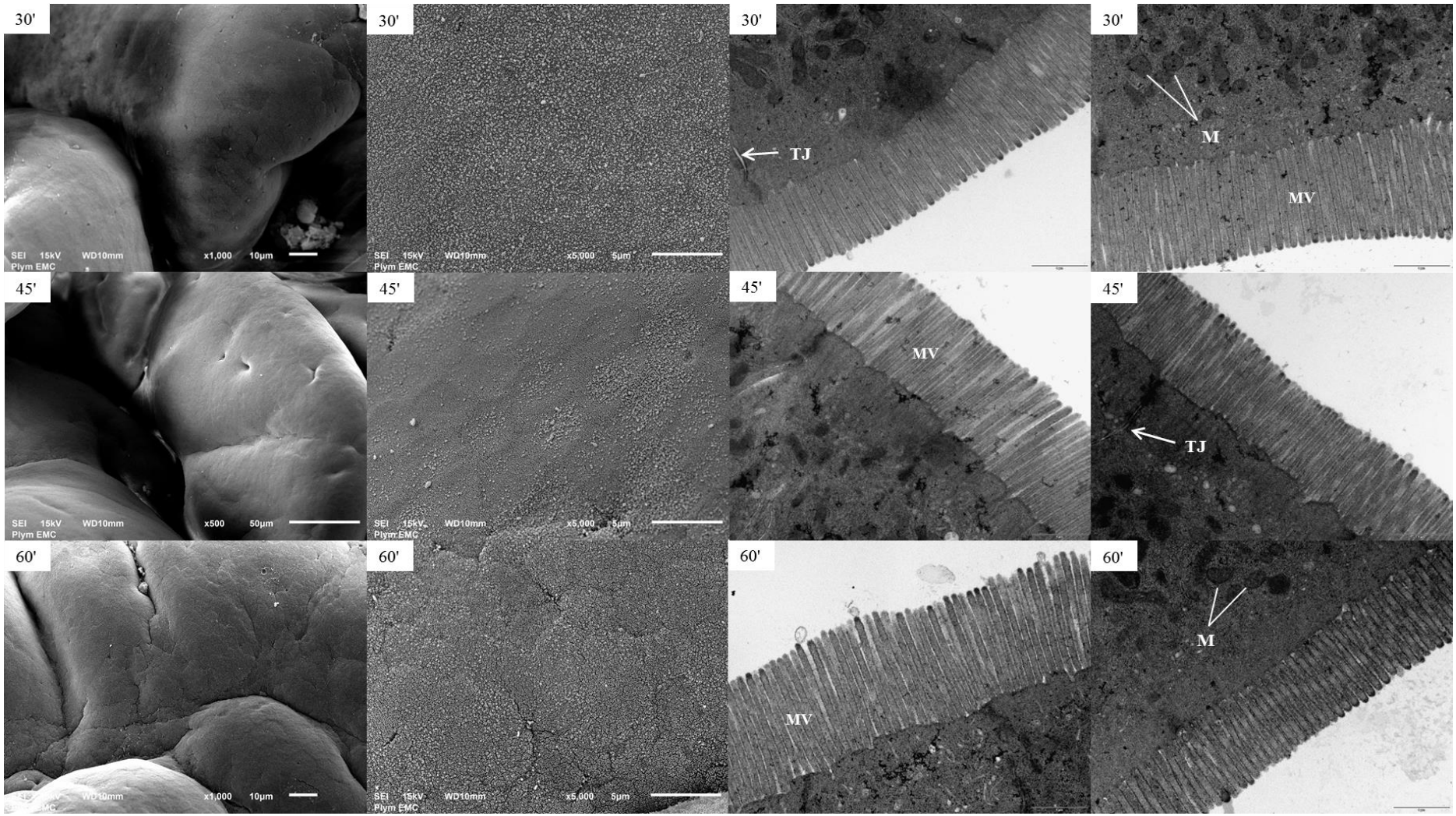


Figure 4B.2. SEM and TEM images of the posterior intestine of European sea bass in the preliminary time assessment *ex vivo* optimisation experiment, 20 ^{o}C for 30, 45 and 60 minutes. Scale bars = 1µm. Key: M = mitochondria; MV = microvilli; TJ = tight junctions.

4B.3.4. Vibrio challenge optimisation

In order to select a bacterial species for use in the challenge trial, an experiment was conducted using three known fish pathogens and intestinal tissues of fish fed the basal diet. *Vibrio harveyi*, *Vibrio alginolyticus* and *V. anguillarum* were the three *Vibrio* species assayed. The bacteria were added to the intestine in PBS at a concentration of 10^7 CFU g^{-1} and incubated at 20 ^{o}C for a period of 60 min (n = 4). Samples were taken for SEM and TEM to determine the levels of tissue deterioration (i.e. enterocyte malformation and necrosis) induced by the pathogens (refer to section 2.8 for methodology).

4B.3.5. Vibrio challenge optimisation results

Qualitative analysis acquired from the SEM micrographs of the posterior intestine showed at least some degree of damage caused by all three of the tested *Vibrio* strains. However, *V. anguillarum* appeared to show the most damaging effects including necrotic enterocytes, areas with irregular microvilli and areas lacking microvilli exposing the lamina propria and tight junctions (Figure 4B.3.). This strain was deemed to have demonstrated the highest pathogenicity and would therefore be used in the *ex vivo* experiment as the pathogen.

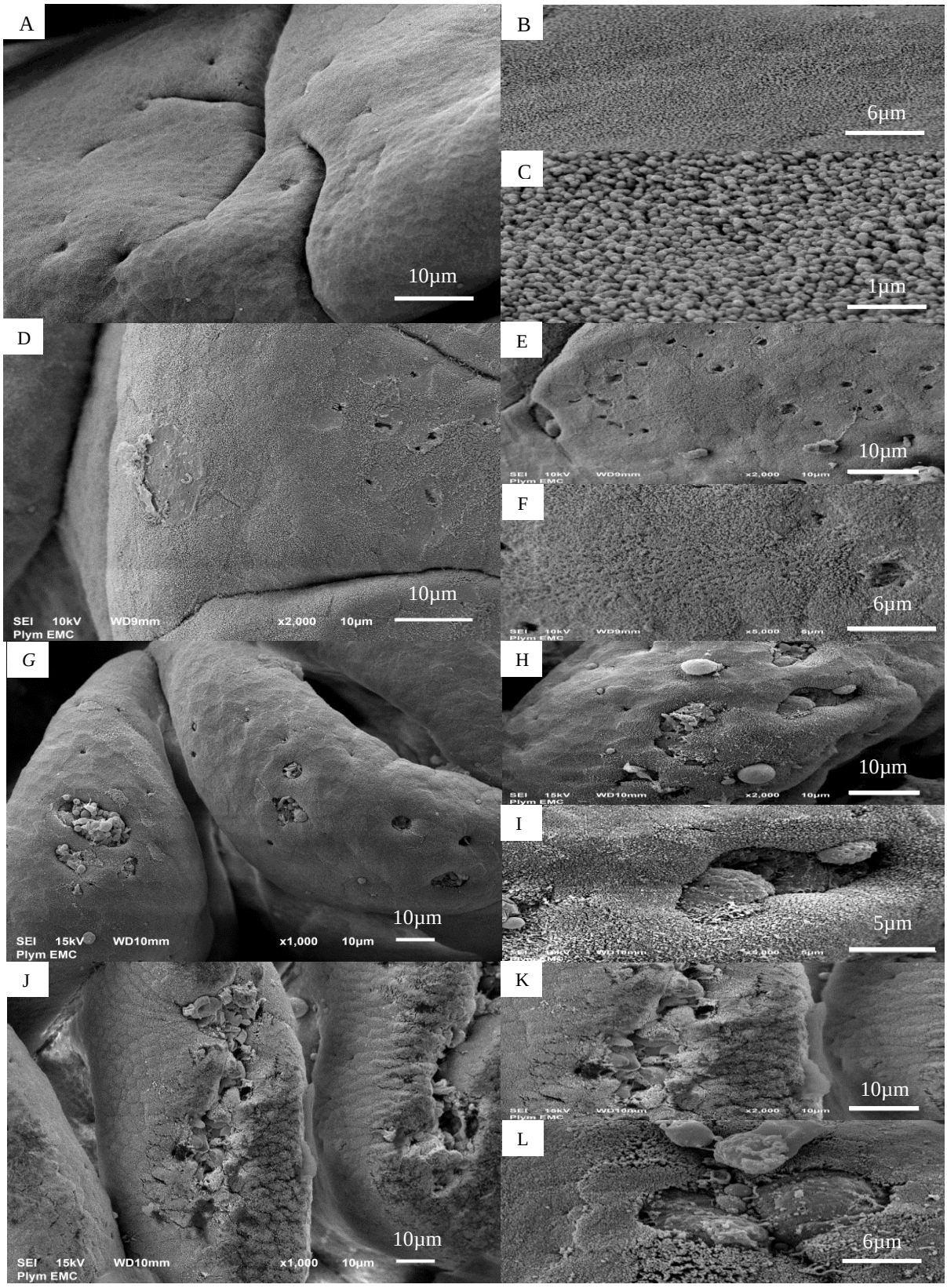


Figure 4B.3. SEM images of the posterior intestine of European sea bass exposed to either PBS (Control; no pathogen added; A-C) or *V. alginolyticus* (D-F), *V. harveyi* (G-I) and *V. anguillarum* (J-L) *ex vivo* for 60 min at 20 ^{o}C. Scale bars are presented in each image.

4B.3.6. Fluorescent in-situ hybridisation (FISH) optimisation

For optimisation of the FISH procedure, *B. subtilis* (Novus Int) and *V. anguillarum* CM31 (Plymouth University culture collection) were routinely grown at 30 ^{o}C in brain heart infusion media (Oxoid). The cells were fixed in Carnoy's solution (1 ml) for a minimum of 2 hours at 4 ^{o}C. The fixed cells were centrifuged for 5 min at 13,000 x *g* and the supernatant removed and discarded. The cells were then re-suspended in PBS (1 ml) and washed for 5 min by centrifugation (13,000 x *g*). The supernatant was discarded and cells were washed again in PBS. After re-suspension, 10 µl of the culture was placed on poly-L-lysine slides (Fisher Scientific, UK) and air dried at room temperature for 20-30 min. The slides were then dehydrated in a graded alcohol series: 50, 80, 90, 100 % (2 min in each) and washed in double distilled H_20 (DDH_20) for 2 min and left to air dry. The relevant probe solutions (1 µl) to 9 µl of hybridisation buffer ((20 mmol l^{-1} Tris–HCl pH 8.0, 0.9 mol l^{-1} NaCl, 0.02 % SDS and formamide (30 %)) was spotted onto the cultures which were circled with a wax pen. Both fluorescently labelled probes were obtained from Eurofins, UK and are presented in table 4B.2. Coverslips were added and hybridisation was carried out for 3 hours in a dark incubator at 46 ^{o}C in a humid chamber (50 ml falcon tube). Thereafter, slides were quickly added to separate falcon tubes containing preheated (48 ^{o}C) washing buffer (20 mmol l^{-1} Tris–HCl pH 8.0, 0.9 mol l^{-1} NaCl, 0.01 % SDS) and left for 15 min. Slides were then dipped in DDH_20 and then 100 % alcohol and air dried. Nucleic acid counterstain (10 µl of DAPI (1 µg 1^{-1}) (Sigma, UK)) was then spotted onto the slides which were left at room temperature for 10 min. Subsequently the slides were then dipped in DDH_20 and 100 % alcohol and then air dried. A drop of antifade (Citifluor, Ltd) was added and coverslips were applied. Slides were visualised by epi-fluorescence microscopy with a Nikon H600L microscope and Nikon Ds-Qi1Mc camera.

Table 4B.2. Fluorescent 16S probes for fluorescent *in situ* hybridization analysis

Probe	Sequence (5'-3')	Label	Hybridisation temp	Reference
Vibrio	ACAGTACTCTAGTCTGCCAG	CY5	46 ^{o}C	Moreno *et al*. (1999)
Bacillus	CGTTCAAACAACCATCCGG	FITC	46 ^{o}C	Liu *et al*. (2001)

4B.3.7. FISH optimisation results

Good fluorescence of both FITC labelled *B. subtilis* and Cy5 labelled *V. anguillarum* was observed on the slides. Non-specific binding was determined by multi-cultured slide preparations with both *B. subtilis* and *V. anguillarum* with both probes applied and visualised through epi-fluorescence multichannel image microscopy. Counter staining was carried out with DAPI. Fluorescence was observed in both species indicating good efficiencies of the respective probes, and no non-specific binding was observed (Figure 4B.4.).

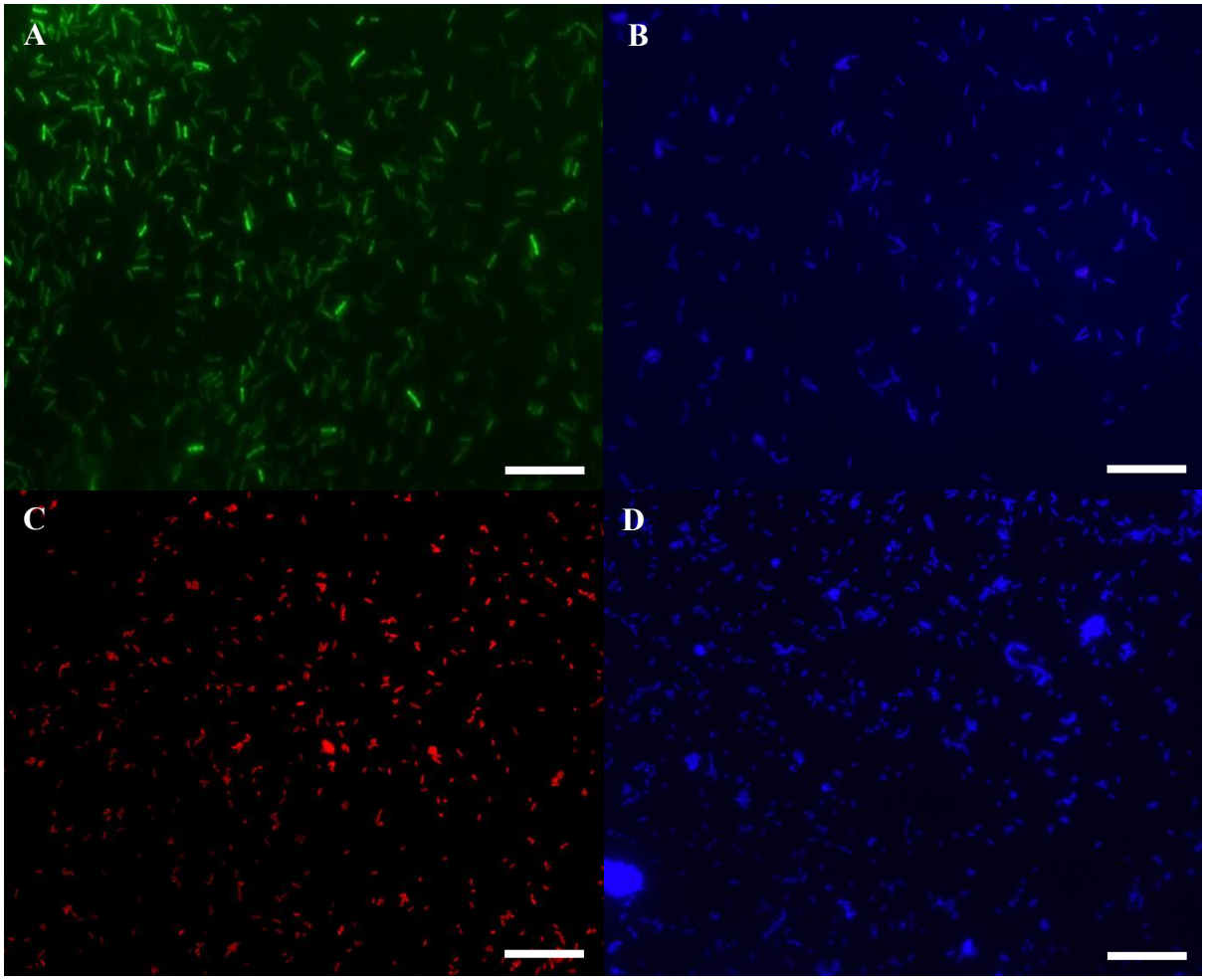


Figure 4B.4. Fluorescently labelled *B. subtilis* and *V. anguillarum* cells after hybridisation. A] FITC labelled *B. subtilis*, B] DAPI counterstained *B. subtilis*, C] Cy5 labelled *V. anguillarum* and D] DAPI counterstained *V. anguillarum*. Scale bars = 10 µm.

4B.3.8. V. anguillarum challenge experiment

At the end of the four week trial a total of 20 fish per treatment were sampled from each dietary regime to be used in the *ex vivo* experiment. Each dietary regime was exposed to the *ex vivo* treatment solutions containing: 1] phosphate buffered saline (PBS) as the Control, 2] probiotic (*B. subtilis* at 10^7), 3] pathogen (*V. anguillarum* at 10^7 CFU's g^{-1}) and 4] probiotic (*B. subtilis* at 10^7 CFU's g^{-1}) + pathogen (*V. anguillarum* at 10^7 CFU's g^{-1}) (50:50) (as

summarised in Table 4B.1). Each *ex vivo* treatment was conducted with $n = 5$. Each sample was incubated at 20 ^{o}C for a period of 60 min, at which point samples for electron (SEM and TEM), and light microscopy analyses (refer to section 2.8. for methodology)

For fluorescent in-situ hybridisation, samples of approx. 1 cm were also taken and fixed in Carnoy's solution (60 % ethanol: 30 % chloroform: 10 % glacical acetic acid) as described by Lebeer *et al*. (2010) for fluorescent *in-situ* hybridisation (FISH) analysis. Tissue samples ($n = 5$), cut to 5 µm were placed on poly-L-lysine slides (Fisher Scientific) and left overnight. Multiple (2-4) sections were cut for each intestinal sample. Deparifination was carried out using xyline and the sections were then rehydrated in a graded alcohol series: 100, 90, 80, 70, 50, 30 % (2 min in each). The samples were then post-fixed in freashly prepared Carnoy's solution for 1 hr. The relevant probe solutions (1 µl) in 9 µl of hybridisation buffer (20 mmol l^{-1} Tris–HCl pH 8.0, 0.9 mol l^{-1} NaCl, 0.02% SDS and formamide (30 %)) were spotted onto the sections which were circled with a wax pen to contain the samples. Coverslips were added immediately and hybridisation was carried out for 3 hours in a dark incubator at 46 ^{o}C in a humid chamber (50 ml falcon tubes). Thereafter, slides were immediately added to separate falcon tubes containing preheated (48 ^{o}C) washing buffer (20 mmol l^{-1} Tris–HCl pH 8.0, 0.9 mol l^{-1} NaCl, 0.01 % SDS) and left for 15 min. Slides were then dipped in DDH_20 and then 100 % alcohol and air dried. Nucleic acid counterstain (10 µl of DAPI (1µg 1^{-1}) (Sigma)) was then spotted onto the slides which were incubated at room temperature for 10 min. Subsequently the slides were then dipped in DDH_20 and 100 % alcohol and then air dried. A drop of antifade (Citifluor, Ltd) was added and coverslips were applied. Slides were visualised by epi-fluorescence microscopy with a Nikon H600L microscope and Nikon Ds-Qi1Mc camera.

4B.3.9 Statistical analysis

Statistical analysis was carried out as described in section 2.9.

4B.4.Results

4B.4.1. V. anguillarum challenge experiment

4B.4.1.1. Light microscopy

Examples of light microscopy sections stained in H&E of fish fed each dietary regime within the control *ex vivo* treatment are displayed in Figure 4B.5. The sections are representative of each dietary regime and were comparable to those samples taken at the end of the four week trial (refer to section *4A.4.3.1.*). Perimeter ratio measurements for each sample are displayed in Table 4B.3. Fish fed the probiotic and synbiotic treatments generally exhibited the highest PR values independently of *ex vivo* treatment. The highest values were exhibited in fish fed the probiotic and synbiotic dietary regimes within the probiotic *ex vivo* treatment and the lowest value was observed in fish fed the basal regime within the pathogen *ex vivo* treatment, however, these were not significant different.

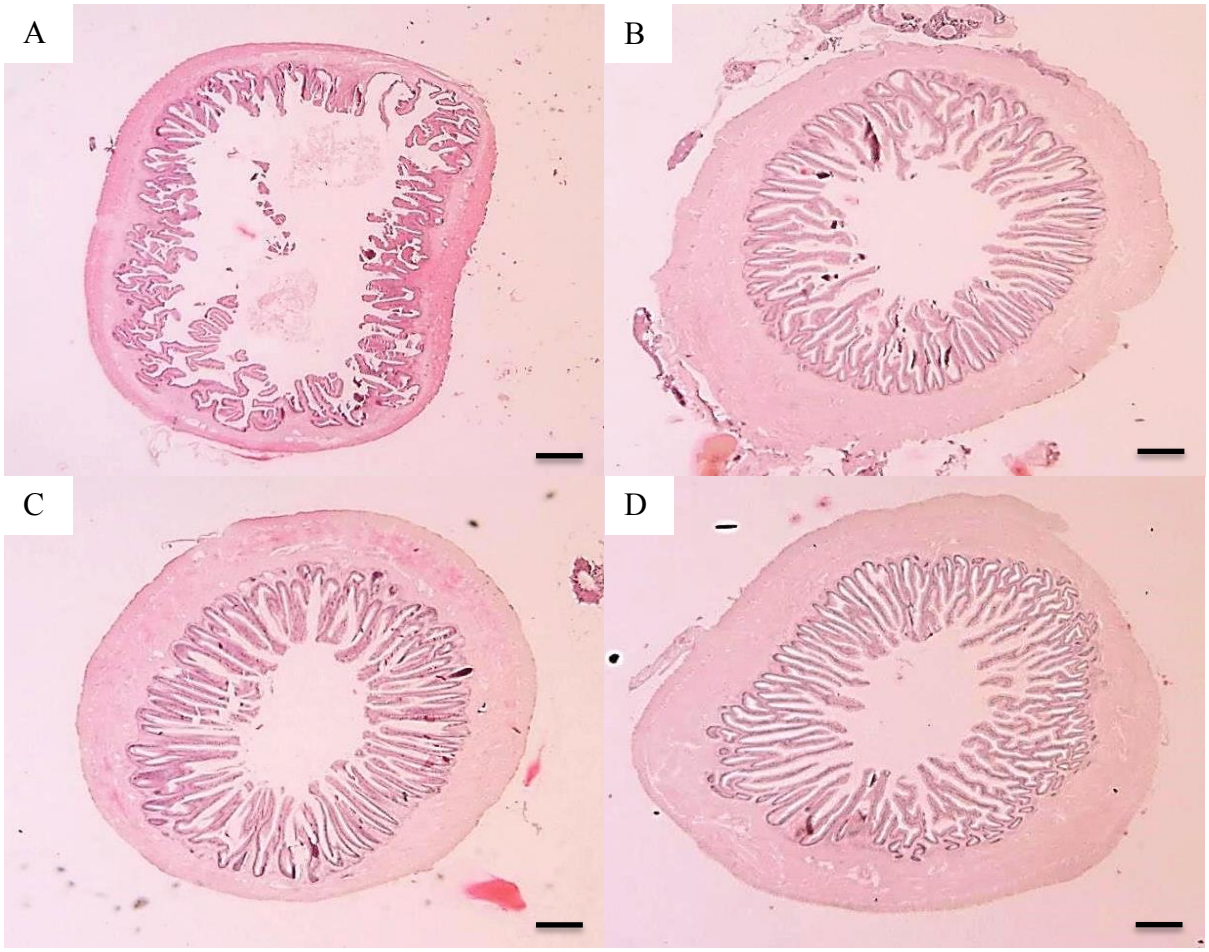


Figure 4B.5. Light micrographs of the posterior intestine of European sea bass fed the basal (A), probiotic (B), prebiotic (C) and synbiotic (D) diets for four weeks and subsequently exposed to the control *ex vivo* treatment for 60 min at 20 ^{o}C. Scale bars = 200 µm.

Table 4B.3. Perimeter ratio measurements of posterior intestine of European sea bass fed different dietary regimes, and exposed to the various *ex vivo* treatments. Data represent mean values expressed as arbitrary units (AU) ± SD.

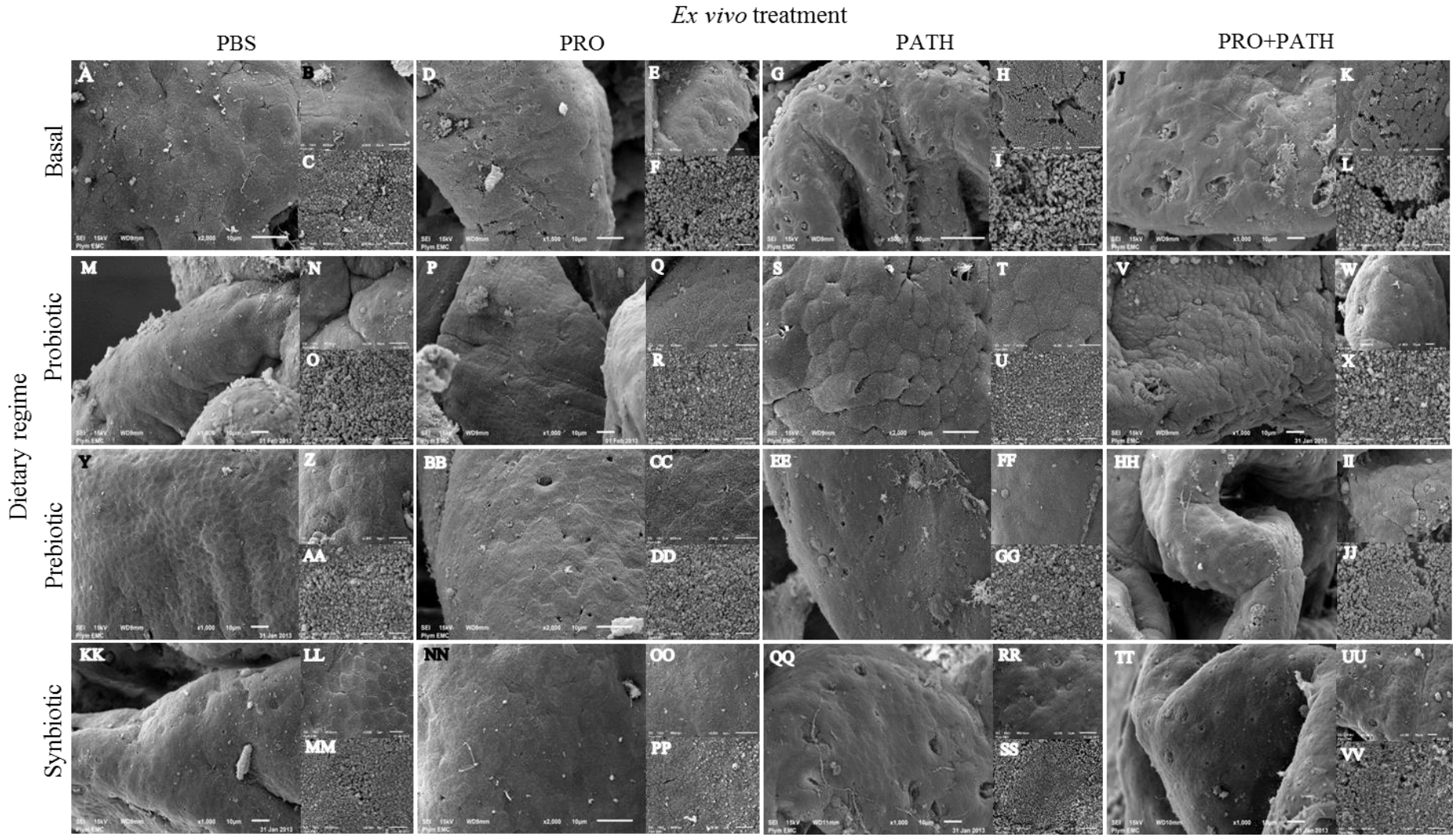
		ex vivo treatments			
		PBS	PRO	PATH	PRO+PATH
Dietary regimes	Basal	3.77±1.06	3.74±0.77	3.45±0.78	3.59±0.44
	Probiotic	4.15±0.91	4.46±0.47	3.95±0.45	4.18±0.26
	Prebiotic	4.09±0.65	4.37±0.70	3.68±0.53	3.99±0.66
	Synbiotic	4.30±0.44	4.54±0.36	3.93±077	3.94±0.58

4B.4.1.2. Electron microscopy

An example of the intestinal epithelium of each dietary regime, within each *ex vivo* exposure is presented in Figure 4B.6. Qualitative assessment revealed that within the *ex vivo* control (PBS exposure) the basal dietary regime exhibited the most epithelial damage which included malformed microvilli, patches of reduced microvilli density and gaps between enterocytes (A, B & C). There were no obvious signs of extra damage or artefacts caused by the *ex vivo* process. In contrast, the probiotic (M, N & O), prebiotic (Y, Z & AA) and synbiotic (KK, LL & MM) fed fish displayed relatively little epithelial damage within the PBS *ex vivo* exposure. The probiotic *ex vivo* exposure revealed no obvious deviation from the PBS treatment with the probiotic (P, Q & R), prebiotic (BB, CC & DD) and synbiotic (NN, OO & PP) fed fish, all exhibiting a uniform epithelium, with no signs degradation. The basal dietary regime exposed to the probiotic (D, E & F) exhibited some degree of epithelial damage similar to that observed in the basal control (i.e. PBS exposure; A, B & C) treatment. The pathogen *ex vivo* exposure caused substantial damage to all of the replicates within the basal dietary regime, which was characterised by necrotic enterocytes, areas with irregular microvilli and areas lacking microvilli which caused exposure of the lamina propria. However, the probiotic

(S, T & U), prebiotic (EE, FF & GG) and synbiotic (QQ, RR & SS) dietary regimes subsequently exposed to the pathogen exhibited less damage when compared to the basal dietary regime exposed to the pathogen (G, H & I). The probiotic + pathogen *ex vivo* exposure of the intestine of the fish fed the basal dietary regime exhibited the greatest signs of epithelium degradation (J, K & L). The probiotic (V, W & X), prebiotic (HH, II & JJ) and synbiotic (TT, UU & VV) fed groups all exhibited less damage than those observed in the basal fed fish group when exposed to both the pathogen and probiotic. It was apparent that less epithelial damage was observed in the probiotic + pathogen exposures than was observed with the pathogen exposure alone for the probiotic, prebiotic and synbiotic fed fish.

Quantitative assessment of microvilli density is presented in Table 4B.3. Within the basal dietary regime the probiotic *ex vivo* exposure group exhibited the highest microvilli density which was significantly higher than both the pathogen and the probiotic + pathogen exposures. The microvilli density of the pathogen *ex vivo* exposure was significantly reduced compared to all other treatments. Within the probiotic dietary regime the intestines of the probiotic *ex vivo* exposure treatment exhibited the highest microvilli density which was significantly higher than the microvilli density of the pathogen exposure. Within the prebiotic dietary regime the probiotic *ex vivo* exposure exhibited a significantly higher microvilli density than the pathogen and probiotic + pathogen exposures. The microvilli density within the prebiotic treatment exposed to the pathogen *ex vivo* treatment was also observed to be significantly lower than the PBS and probiotic *ex vivo* exposures. Within the synbiotic dietary regime the probiotic and PBS *ex vivo* exposures were observed to exhibit significantly higher microvilli densities when compared to the pathogen exposure. The microvilli density of the probiotic exposure was also significantly higher than the microvilli density of the probiotic + pathogen exposure.



166
167 **Figure 4B.6.** SEM images of varying magnifications of the posterior epithelium of European sea bass fed the four dietary regimes for four
168 weeks which were subsequently exposed to each *ex vivo* treatment for 60 min at 20 ^{o}C.

Table 4B.4. Microvilli density assessment of posterior intestine of European sea bass fed different dietary regimes, and exposed to the various *ex vivo* treatments. Data represent mean values expressed as arbitrary units (AU) ± SD. Different superscript letters within rows (lowercase) and within columns (uppercase) indicate significant differences between groups.

		ex vivo treatments			
		PBS	PRO	PATH	PRO+PATH
Dietary regime	Basal	5.67±1.40^{ab}	7.49±0.53^{a}	3.27±0.21$^{c(A)}$	5.29±1.42^{bc}
	Probiotic	7.61±2.45^{ab}	8.78±5.40^{a}	5.40±0.80$^{b(B)}$	6.60±1.30^{ab}
	Prebiotic	6.78±0.37^{ab}	8.49±1.85^{a}	4.52±0.60$^{c(B)}$	5.31±0.48^{bc}
	Synbiotic	7.69±2.15^{ab}	8.07±0.54^{a}	4.54±0.19$^{c(B)}$	5.43±0.82^{bc}

4B.4.1.3. FISH

Pure bacterial cultures of *B. subtilis* (Novus Int) and *V. anguillarum* (the strain used in the *ex vivo* assay) were prepared and probed on separate slides for use as positive controls (Figure 4B.7.A&B). Figure 4B.7.C presents examples of mucosal folds of the intestine of fish fed each dietary regime exposed to each *ex vivo* treatment. The eukaryotic tissue is clearly visible. Multiple areas of the mucosal folds were scanned and subjected to the different fluorescent conditions necessary for the visuallisation of both probes.

Good fluorescence was acquired for samples of tissue from all tested samples. Fluorescence of the tested strains of *B. subtilis* and *V. anguillarum* was also acquired through preparation of the pure cultures (Figure 4B.7.A&B). The tested strains were not observed to be asociated/attached with/to the mucosal folds within each dietary regime, nor were their any signs of translocation into the tissues (Figure 4B.7.C).

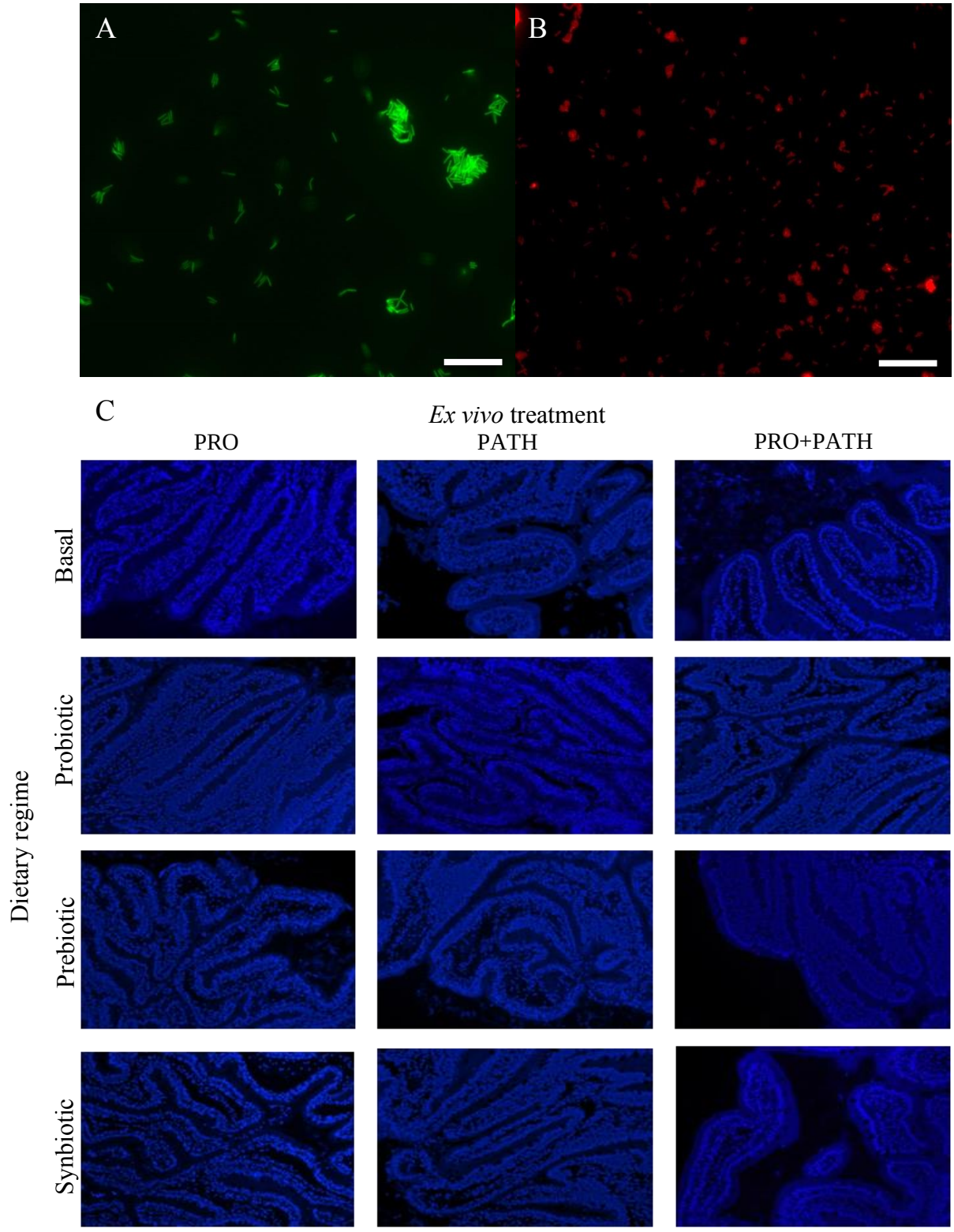


Figure 4B.7. Fluorescently labelled *B. subtilis* and *V. anguillarum* cells after hybridisation. A] FITC labelled *B. subtilis*, B] Cy5 labelled *V. anguillarum.* Scale bars = 10 µm. Image C displays examples of intestinal mucosal folds of the posterior epithelium stained with DAPI of European sea bass fed each dietary regime exposed to the *ex vivo* treatments.

4B.5. Discussion

The gastrointestinal tract of fish has been proposed as a major infection site for potential pathogens (Groff & Lapatra 2000; Ringø *et al*. 2007). Hence, *ex vivo* (and *in vitro* gut cell line culture) assays are important tools for assessing bacterial-host interactions in the intestine of fish and are crucial with regards to limiting the numbers of fish used in bacterial challenge trials. However, to the author's knowledge only a few studies have utilised this approach to assess bacterial pathogenesis in the intestine of fish species (Harper *et al*. 2011; Salma *et al*. 2011; Løvmo Martinsen *et al*. 2011; Ren *et al*. 2013). In the present study, histological and FISH techniques were employed to assess the effects of *V. anguillarum* on the intestine of European sea bass, as well as assessing any antagonistic effects between the pathogen and the *B. subtilis* probiotic. The pathogen was observed to cause extreme damage to the epithelium at the ultrastructural scale as demonstrated by the electron micrographs. The morphometric analyses of microvilli densities revealed that fish exposed to the pathogen exhibited a reduced microvilli density, which was significantly reduced when fish fed the basal, prebiotic and synbiotic treatments were exposed to the pathogen. This is in line with Harper *et al*. (2011) who observed in an *ex vivo* study the degradation of the epithelium of rainbow trout intestines when exposed to *V. anguillarum*. Conversly, Løvmo Martinsen *et al*. (2011) reported no intestinal cell damage in the mid intestine of Atlantic cod when exposed to *V. anguillarum ex vivo*. The authors also observed *Carnobacterium maltaromaticum* exerted some competitive pressure against the pathogen. However, conclusions were drawn that the mid intestine of Atlantic cod appears not to be a major infection site for this pathogen. In the present study, fish previously fed the probiotic, prebiotic and synbiotic supplemented diets, and then subsequently exposed to the pathogen *ex vivo*, exhibited significantly increased microvilli coverage when compared to fish fed the basal control diet. This may indicate a level of epithelial protection provided by the feed additives. Indeed antagonistic effects against *Vibrio*

spp. by *B. subtilis* have been reported previously *in vitro* (Vaseeharan *et al*. 2003; Nakayama *et al*. 2009; Zokaeifar *et al*. 2014) and *in vivo* (Balcázar & Rojas-Luna 2007; Touraki *et al*. 2012). With regards to prebiotic applications, there are a number of studies which indicate that prebiotic applications may improve the disease resistance of some aquatic species (Li *et al*. 2004; Costa *et al*. 2008; Rodrigues-Estrada *et al*. 2008). The beneficial effects are unlikely to be directly related to inhibition or antagonism of the pathogen, but in fact may be due to the general beneficial effects conferred by the prebiotic, particularly in terms of the intestinal health of the fish. Various studies have reported improvements in microvilli coverage and heights, as well as, increased fold heights and mucus production in fish fed diets supplemented with MOS (Dimitroglou *et al*. 2009, 2010; Torrecillas *et al*. 2011a, 2011b, 2012). In the present study, fish were fed the experimental diets for four weeks prior to the *ex vivo* challenge and it was observed that fish fed the probiotic, prebiotic and synbiotic diets exhibited increased microvilli density measurements compared to fish fed the basal control diet. Furthermore, fish fed the feed additives were observed to be under apparently less cellular stress as indicated by a significant reduction in HSP70, as well as a significant reduction in CASP3 (cell proliferation) and PCNA (apoptosis) compared to fish fed the control (Chapter 4A). The *ex vivo* experiment confirmed these results in terms of microvilli density measurements and also revealed that fish exposed to the *B. subtilis ex vivo* treatment exhibited the highest microvilli density measurements suggesting the probiotic has a positive effect on the epithelium of European sea bass.

The present *ex vivo* study also used fluorescently labelled probes for *B. subtilis* and *V. anguillarum* cells to assess any bacterial adherence to, and translocation into, the intestine. The presence of the probiotic and pathogen was not detected on or in the tissue of fish exposed to either treatment. This is perhaps not surprising given the conditions of the trial. The tissue was exposed for a period of one hour which could be a factor, as well as,

potentially loose association between baceria and tissue may have been interupted by the washing through process with PBS prior to sampling. *V. anguillarum* is reported to exhibit various virulence factors including the production of repeat-in-toxin gene cluster (Li *et al*. 2008), hemolysin gene cluster (Rock & Nelson 2006), zinc metalloprotease EmpA (Denkin & Nelson 2004) and flagellin A gene (Milton *et al*. 1996). The present study revealed through SEM that *V. anguillarum* had time to induce damage via the production of these cytotoxins, but may not have sufficient time to respond chemotactically to the mucus/epithelium and form robust attachements. Furthermore, it also appears from the electron microscopy analyses that *B. subtilis* may act antagonistically against *V. anguillarum* providing a level of epithelium protection without adhering directly to the epithelial surfaces. Multiple antibiotic biosynthesis genes, including those involved in bacilysin, plipastatin, subtilosin and surfactin production, have been reported to be produced by *B. subtilis* strains (Stein 2005; Zokaeifar *et al*. 2014; Sumi *et al*. 2015). Plipastatin has been observed to exhibit strong lipopeptide biosurfactants produced by *Bacillus amyloliquefaciens* has been reported to exhibit antibacterial action against pathogenic *Vibrio* spp., including *V. anguillarum* (Xu *et al*. 2014). As previously mentioned *B. subtilis* appears to have the potential to act antagonistically against *Vibrio* spp. however, the mechanisms involved in this process merit further investigation.

4A.6. Conclusion

Ex vivo techniques are important tools to assess bacterial interactions in the intestine of fish. Furthermore, these types of studies may minimise the numbers of animals used in bacterial challenge trials, as well as, reducing suffering and impairment of animal welfare which is in line with the 3 R's (replacement, reduction and refinement). In the present study the fish pathogen *V. anguillarum* was observed to induce intestinal damage to the epithelium of

European sea bass at the ultrastructural scale as evidenced by electron microscopy. The study also revelaed *B. subtilis* had no detrimental effects to the epithelium and in fact increased the microvilli density with higher values observed in samples of intestine exposed to the probiotic *ex vivo* treatment. It should also be mentioned that fish fed the probiotic, prebiotic and synbiotic treatments exhibited an increased intestinal health status compared to fish fed the basal, prior to the *ex vivo* study (Chapter 4A). The study demonstrates that *B. subtilis* and Previda® individually and in combination may act to provide a degree of protection to intestinal epithelium of European sea bass in the presence of *V. anguillarum*. However, further studies are required to validate these results.

Chapter 5:

The effect of *B. subtilis* and Next Enhance 150® on the growth, intestinal microbiota, intestinal integrity, and immunity of European sea bass

5.1. Abstract

The aim of the present study was to assess the effects of dietary *B. subtilis* individually, and in combination with a phytobiotic (Next Enhance 150®) on the health of European sea bass after ten weeks feeding. Five iso-nitrogenous (50% crude protein) and iso-lipidic (14% crude lipid) diets were produced, based on a commercial formulation, to meet the known nutrient requirements of European sea bass. The four experimental diets consisted of the basal dietary formulation with the addition of the feed additives as follows 1] the probiotic diet contained 10^8 CFU g^{-1} *B. subtilis*, 2] Next Enhance® low dose at 1.5 g kg^{-1} (NE 1.5), 3] Next Enhance® high dose at 3.0 g kg^{-1} (NE 3.0), and 4] Probiotic + Next Enhance 150® (10^8 CFU g^{-1} *B. subtilis* + Next Enhance 150® at 1.5 g kg^{-1}. European sea bass (40.6 ± 0.28 g) were reared in triplicate tanks (23 per tank at 25 ± 1 °C, 30 ± 2 ppt salinity and 7.0 ± 0.5 pH) and were fed the experimental diets three times daily for ten weeks. At the end of the study, growth performance was assessed, and the posterior intestine was sampled for microbiological assessment, gene expression and histological analyses.

High-throughput sequence analyses revealed that the inclusion of the probiotic modulated the allochthonous gut microbiota of *D. labrax*. Multiple *Bacillus* spp. were detected which were significantly increased in fish fed the probiotic compared to all dietary treatments and significantly increased in fish fed the probiotic + Next Enhance 150® 1.5 relative to fish fed the control, Next Enhance 150® 1.5 and Next Enhance 150® 3.0 treatments. A BLAST search revealed the presence of *B. subtilis* in fish fed the probiotic and probiotic + Next Enhance 150® 1.5 which were not present in fish fed the other treatments. High-throughput sequencing analyses revealed distinct clusters of the bacterial profiles, with fish fed the probiotic and probiotic + Next Enhance 150® 1.5 generally clustering together and fish fed the control, Next Enhance 150® 1.5 and Next Enhance 150® 3.0 treatments clustering together, confirming differences in the bacterial communities. Gene expression analyses revealed

significant down-regulation of the expression of HSP70, CASP3, PCNA and calreticulin (CAL) in fish fed the probiotic treatment relative to fish fed the control. Histological analyses revealed significantly elevated intraepithelial leukocytes in fish fed the probiotic, Next Enhance 150® 3.0 and probiotic + Next Enhance 150® 1.5 treatments compared to fish fed the control. The present observations indicate that these feed additives may have a beneficial effect on the gut health of European sea bass without having a detrimental effect, after 10 weeks feeding.

5.2. Introduction

Chapter 4A demonstrated that the *B. subtilis* modulated the intestinal microbiota of European sea bass, as well as conferring some protective effects at the intestinal epithelium against a challenge by a sub-optimal diet. However, Chapter 4A was an intestinal integrity trial designed to assess the short term effects of the feed additives on European sea bass health. The diet used in Chapter 4A was also designed to be challenging due to the high levels of soy protein and saponin products; it would be fair to assume this diet was not representative of a diet used commercially for this species. It was therefore the aim of present trial to assess the effects *B. subtilis* on European sea bass growth and health, when supplemented into a non-challenging diet which was designed to be more reflective of a diet that might be used commercially for this species. Furthermore, the present study was designed to assess these effects over a longer period (i.e. 10 weeks) and additionally, the phytobiotic Next Enhance 150® was also investigated.

Phytobiotics are plant-derived natural compounds which potentially enhance animal productivity (Antache *et al.* 2013). These feed additives are thought to exhibit an array of properties including antimicrobial, antioxidant, growth promoters and digestive enzyme

activies among others (Cristea *et al.* 2012). These properties are caused by a variety of primary (e.g. protein, carbohydrates and fat) and secondary (e.g. terpenes, carvacrol, capsaicin, peperin, chicoric acid and flavonoids) ingredients and have some demonstrable promise as alternative feed additives in the nutrition of livestock (Grashorn 2010). In fish, various phytobiotics have been observed to have immunostimulating (Dügenci *et al.* 2003; Yin *et al.* 2006; Kaleeswaran *et al.* 2012), disease resistance (Christybapita *et al.* 2007; Sahu *et al.* 2007; Abd-El-Rhman 2009; Rattanachaikunsopon *et al.* 2010; Volpatti *et al.* 2013) and growth promoting (JI *et al.* 2007; Abd-El-Rhman 2009; Thanikachalam *et al.* 2010) effects. Next Enhance 150® is an encapsulated product containing the essential oils carvacrol and thymol. These essential oils have been observed to promote beneficial effects in various species of livestock (Li *et al.* 2012; Hashemipour *et al.* 2013; Roofchaee *et al.* 2013; Arsi *et al.* 2014; Ghasemi *et al.* 2014) including fish (Ahmadifar *et al.* 2011; Giannenas *et al.* 2012; Volpatti *et al.* 2013; Pérez-Sánchez *et al.* 2015; Peterson *et al.* 2015). In fish, a recent study demonstrated that Next Enhance 150® could improve FCR's and intestinal immune function when supplemented into the diets of gilthead sea bream (Pérez-Sánchez *et al.* 2015). There are however, fewer reports on the efficacy of utilising phytobiotics in combination with probiotics. To the author's knowledge there are currently no published studies assessing the effects of probiotics in combination with phytobiotics on fish health. Therefore, the present study aimed to assess the effects the probiotic *B. subtilis* and the phytobiotic Next Enhance 150® have individually and in combination on the growth and health of juvenile European sea bass. The study utilised high-throughput sequencing for bacterial community analysis, as well as assessing systemic immunity parameters: haematocrit, haemaglobin and serum lysozyme activity, and the expression of the immune related genes: IL-1β and IL-10, and genes associated with cellular stress, apoptosis and cell turn-over related genes (HSP70, CASP3 and PCNA). In addition, the expression of the calreticulin (CAL) was also assessed. CAL is an

important binder protein involved in the regulation of Ca^{2+} homeostasis, lectin binding and molecular chaperoning in the endoplasmic reticulum in animal systems. There is evidence to suggest CAL is involved in the immune function in fish (Luana *et al.* 2007; Duan *et al.* 2014; Liu *et al.* 2011), and was therefore assessed and hypothesised that the probiotic *B. subtilis* would not induce an up-regulation of this gene in present study. Chapter 4A revealed that IL-1β was significantly up-regulated, and HSP70, CASP3 and PCNA were significantly down-regulated as a consequence of probiotic, prebiotic and synbiotic feeding. It was therefore hypothesised that the expression of IL-1β would be up-regulated and HSP70, CASP3 and PCNA would be down-regulated as a consequence of probiotic feeding.

5.3. Materials and Methodologies

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

5.3.1. Experimental design

European sea bass were obtained from Anglesey Aquaculture Ltd, Black Point, Beaumaris UK and transported to the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK with an acclimation period of four weeks. The fish were graded and separated into 15 x 110 L fibreglass tanks in a closed recirculatory system. Average fish weights were 40.6 ± 0.28 g and fish were stocked to a density of 23 fish per tank. Tanks were allotted a dietary treatment randomly and each treatment was conducted in triplicate. Fish were fed the diets at a rate of 2 – 3 % of biomass per day in equal rations at 9:00, 13:00 and 17:00. Daily

feed was adjusted on a weekly basis by batch weighing following a 24 - h starvation period. Rearing conditions were as follows: 25 ± 1 °C, 30 ± 2 ppt salinity and 7.0 ± 0.5 pH with a photoperiod of 12: 12 h light: dark and dissolved oxygen was maintained to > 85 % saturation. These parameters were monitored daily. Ammonium, nitrite and nitrate levels were monitored weekly with weekly water changes of approx. 25 % system volume to minimise the potential build-up of probiotics and phytobiotic compounds.

5.3.2. Diet preparation

Each experimental diet was formulated to be iso-nitrogenous, iso-lipidic and iso-caloloric and to meet the known nutritional requirements for European sea bass (NRC, 2011) (Table 5.1.). Briefly, dry ingredients were well mixed in a Hobart food mixer (Hobart Food Equipment, Sydney, Australia, model no: HL1400–10STDA) and the oil and hot water gradually added to the mixer. Cold press extrusion was conducted (PTM P6 extruder, Plymouth, UK) to produce ~ 2 mm pellets. The pelleted diets were then dried to ca. 5 % moisture in an air convection oven set at 50 °C for 48 hours. The diets were then broken up to the appropriate size and the composition analysed using AOAC (1995) protocols (Table 5.1.). All experimental diets were stored at 4 ^{o}C in airtight containers prior to the start of the trial. Probiotic inclusion levels (probiotic treatment: 8.36 x 10^{7} CFU g^{-1} and probiotic+ Next Enhance 150® treatment: 8.08 x 10^{7} CFU g^{-1}) were confirmed by spread plating onto nutrient agar for 24 h at 30 ^{o}C. The diets without probiotic supplementation were also plated out to confirm these diets did not contain the probiotic.

Table 5.1. Dietary formulation (%) and chemical composition

Ingredient (%)	Control	Probiotic	Next Enhance 150® 1.5	Next Enhance 150® 3.0	Probiotic + Next Enhance 150® 1.5
Fishmeal (LT 94)[a]	25.00	25.00	25.00	25.00	25.00
Concentrated soy protein 60[b]	20.00	20.00	20.00	20.00	20.00
Corn starch[c]	14.12	14.12	13.97	13.82	13.97
Glutalys[d]	12.00	12.00	12.00	12.00	12.00
Soybean meal (HP100)[e]	10.00	10.00	10.00	10.00	10.00
Hydrolysed wheat gluten[f]	5.00	5.00	5.00	5.00	5.00
Lysamine pea protein[d]	2.00	2.00	2.00	2.00	2.00
Fish oil[g]	6.88	6.88	6.88	6.88	6.88
Corn oil	4.00	4.00	4.00	4.00	4.00
Vit/Min premix[h]	1.00	1.00	1.00	1.00	1.00
Next Enhance 150®[i]	-	-	0.15	0.30	0.15
Probiotic (log CFU's g^{-1})[j]	-	8.36	-	-	8.08
Proximate composition (%)					
Crude protein*	50.11	50.42	50.15	51.08	50.70
Crude lipid*	14.44	13.89	14.50	14.12	13.87
Dry matter	96.23	96.01	96.48	97.79	96.29
Ash*	6.59	6.20	6.22	6.95	6.54
Gross energy (MJ kg^{-1})	21.10	20.98	21.20	21.09	21.21

[a] Herring meal LT94: CC MOORE & Co. Ltd., Dorset, UK.

[b] SPC60: sourced from Biomar, Edinburgh, UK

[c] Corn starch: Sigma-Aldrich Company, UK.

[d] Glutalys and lysamine pea protein: Roquette Company, Frêres, France.

[e] Soybean protein concentrate: Hamlet HP100 (56 % crude protein), Hamlet Protein, Denmark.

[f] Vital wheat gluten: Tereos syral, Marckolsheim, France.

[g] Fish oil: Biomar, Edinburgh, UK.

[h] Vitamin/mineral premix: Premier Nutrition Products (PNP Ltd.) Rugeley, Staffordshire, UK.

[i] Next Enhance 150®: Novus Int, St. Charles, USA.

[j] Probiotic (*B. subtilis*): Novus Int, St. Charles, USA. (lyophilised cells at 10^{10} CFU g^{-1})

* % wet weight basis

5.3.3. Dietary proximate analyses

Proximate analysis of diets was determined as described in section 2.5.

5.3.4. Growth and feed utilisation parameters

At the end of the trial the fish were batch weighed by tank. Growth performance and feed utilisation were monitored by assessing the final body weight (BW), weight gain (WG), specific growth rate (SGR) and feed conversion ratio (FGR). These parameters were calculated as follows:

- *BW* (g) = *TB / n*. Where *TB* = tank biomass and *n* = number of fish in tank.
- *WG* (g) = *Wf – Wi*. Where *Wf* = final weight and *Wi* initial weight.
- SGR (%) = 100 ((ln *Wf* - ln *Wi*) / *t*). Where *Wf* = final weight and *Wi* = initial weight and *t* = number of experimental days.
- FCR (g/g) = *FI* / *WG*. Where *FI* = feed intake and *WG* = weight gain

5.3.5. Sampling

Two fish per tank were sampled for microbiology and two per tank for histology (*n*=6) at the end of the trial. Refer to section 2.6. for sampling methodology. For haematology analyses blood was extracted from the caudal vein of fish using a 25 gauge needle and a heparinised 1 ml syringe. Whole blood was used for blood smears, and after additional blood was allowed to clot for 12 hr (at 4 ^{o}C), serum was isolated by centrifugation at 3600 g for 5 min and stored at -80 ^{o}C until further analyses. Haematocrit, measured as % packed cell volume (PCV), haemoglobin, erythrocyte counts (red blood cells), serum lysozyme activity, leucocyte counts (white blood cells) and differential leucocyte proportions were determined according to standard methods described by Rawling *et al.* (2009).

5.3.6. High-throughput sequencing

For high-throughput sequence analysis, digesta samples (n = 6) were subjected to DNA extractions as described in section *2.7.1.* High-throughput sequence analysis was carried out as described in section *2.7.4.*

5.3.7. Gene expression

5.3.7.1. RNA extraction, cDNA synthesis and real-time PCR

RNA extraction, cDNA synthesis and real-time PCR was carried out as described in sections 2.7.6. and 2.7.7.

5.3.7.2. Reference genes, genes of interest and analyses

GAPDH, β-actin, ELF-1α and RSPA were used as the reference genes for Q-PCR in each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality (Bustin *et al.*, 2009). No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. The software GeNorm (v 3.4, 145 Center for Medical Research, Ghent University, Belgium) was used to assess the optimal number and selection of reference genes. GeNorm calculates the stability value "M" of the reference genes by comparing the variation in expression for all other target genes. GeNorm results indicated genes GAPDH and RSPA to be the best two reference genes and were subsequently used as the reference genes in the present study. Modification of gene expression is represented with respect to the controls being sampled at the same time as the treatment. The threshold cycle (Ct), defined as the point at which the fluorescence rises appreciably above the background fluorescence, was determined manually for each run. PCR efficiencies for each set of primers were determined using serial dilutions

of cDNA (n = 3) and resulting plots of Ct versus the logarithmic cDNA input, using the equation E (PCR efficiency) = 10(-1/slope) (Rasmussen 2001), see Table 5.2. The normalised expression level of a target gene was calculated on the basis of Ct deviation (ΔCt) of the unknown sample versus a control sample, and expressed in comparison to the reference genes, according to the method outlined by Vandesompele *et al.* (2002). Data were subjected to statistical analyses carried out using the software package R as described by Hothorn & Honick (2015). Genes of interest were heat-shock protein 70 (HSP70), caspase 3 (CASP3), proliferating cell nuclear antigen (PCNA), the calcium binding protein Calreticulin (CAL), the pro-inflammatory cytokine interleukin-1β (IL-1β) and the anti-inflammatory cytokine interleukin-10 (IL-10). The Primers used were designed in Primer 3 version 4.0.0 as described by Untergasser *et al*. (2012). The sequences are presented in Table 5.2.

Table 5.2. Information regarding primers used for real-time PCR analysis.

Gene	Forward primer	Reverse primer	Amplicon size	Genbank No	E-value	Annealing temp
RSPA	ACTTGGACTTCCAGATGGATCA	AGCTTCTCCCAGGTCTTCTTC	88	HE978789.1	2.3	59.0
GAPDH	CCGCCAAATATGACGACATCAA	TGTATCCCAGAATGCCCTTCAT	75	AY863148	2.3	59.3
ELF-1α	CGCCACCGTTGCCTTTGTA	TTCAAGGGATGGAAGGTTGAGC	98	AJ866727.1	2.2	58.8
β-actin	ATCCACGAGACCACCTACAA	ACAGCACAGTGTTGGCATAC	79	AJ493428	2.3	60.1
HSP70	CCCTCTGTCCCTGGGTATTG	AAGGTCTGGGTCTGCTTTGT	93	AY423555	2.3	59.2
CASP3	GACCAGACAGTCGAGCAGAT	GCGTTGCAGCTGTGATCTT	68	DQ345773	2.3	59.2
PCNA	TGAAGTGTGCAGGAAACGAAGA	GGCGAGTGTGTCTGCATTGT	65	JQ755266	2.3	60.8
CAL	AGCAACATGCACGGAGATTC	TTGTGCTGTAGCCACAGATG	67	JX235975	2.3	57.3
IL-1β	TTACCCACCACCCACTGACA	AAGCCCTTCCAGTCTCTCCAT	70	AJ269472	2.4	58.4
IL-10	GCTGGGTCTGCTGTTCAACTA	GCTGCATGGTTTCTGTGTTGTT	66	AM268529	2.1	60.4

5.3.8. Haematological and immunological parameters

5.3.8.1. Serum lysozyme activity

Serum lysozyme activity was assessed using a turbidometric assay method (Ellis 1990). Briefly, 10 µl of serum was added to 190 µl of lyophilised *Micrococcus lysodeikticus* at a concentration of 0.2 mg ml^{-1} in 0.04 M sodium phosphate buffer (pH 6.2). After mixing in a 96 well plate, the reduction in turbidity was measured between 1 and 6 min at 540 nm at 25 ^{o}C in a microplate reader (Molecular Devices, VERSAmax). One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 units per minute.

5.3.8.2. Haematocrit

Fresh whole blood was collected into heparinised capillary tubes by capillary action and sealed. The tubes were subsequently centrifuged for five minutes at 10 000 x g in a Centurion haematocrit centrifuge. Determination of haematocrit was measured as percentage packed cell volume (PCV) with a Hawksley haematocrit reader.

5.3.8.3. Haemoglobin

Haemoglobin was determined based on Drabkin's cyanide - ferricyanide solution (Sigma-Aldrich Ltd. UK). Whole, fresh blood (5 µl) was added to 1 ml of Drabkin's solution (dilution factor: 1/ 200) and mixed and measured using a spectrophotometer at 540 nm. The sample haemoglobin levels (g dl^{-1}) were determined against a standard curve of lyophilized porcine haemoglobin powder (Sigma-Aldrich Ltd. UK) and calculated using the formula: $HC = ((OD_{540} - 0.0002) / 6.6137) \times 200$ where HC = haemoglobin concentration (g dl^{-1}), OD_{540} = absorbance at 540 nm, 0.0002 = Absorbance of standard, and 200 = dilution factor.

5.3.9. Intestinal histology

Light microscopy analysis was carried out on two fish per tank (n = 6) as described in section *2.8.1.*

5.3.10. Statistical analysis

Statistical analysis was carried out as described in section *2.9.*

5.4. Results

5.4. 1. Gross observations and growth parameters

Fish accepted all dietary regimes well and 100 % survival was recorded throughout the trial. No significant differences were observed in growth parameters by dietary regime. (Table 5.3.).

Table 5.3. Growth parameters of European sea bass fed the experimental diets for 10 weeks. n = 3 per treatment group.

	Con	Pro	NE 1.5	NE 3.0	Pro+NE 1.5
Initial weight (g)	40.65±0.45	40.67±0.33	40.46±0.18	40.64±0.27	40.58±0.31
Weight after 10 weeks (g)	109.44±1.48	110.25±3.44	107.74±4.29	108.49±0.16	108.33±2.99
Weight gain (g)	68.79±1.54	69.58±3.63	67.27±4.14	67.85±0.37	67.75±2.93
SGR	1.28±0.02	1.31±0.03	1.27±0.04	1.29±0.00	1.27±0.04
FCR	1.35±0.02	1.32±0.03	1.36±0.04	1.34±0.02	1.36±0.05

5.4.2. High-throughput sequencing

A total of 346,903 reads were retained after trimming and QC, which were used for downstream analyses. Alpha rarefaction analyses revealed all samples to be reaching a plateau after approx. 6,000 sequences (Figure 5.1.), and Good's coverage estimators were > 0.99 (Table 5.4.), signifying that the bacterial communities were fully sampled and the subsequent observed OTU's were representative of the sampled population. Alpha diversity parameters presented in Table 5.4. revealed no significant differences in observed species, Chao 1 (richness), and phylogenetic distance between treatments. Shannon-Wiener indices revealed fish fed the probiotic and probiotic + Next Enhance 150® 1.5 (4.19 ± 0.13 and 4.32 ± 0.31 respectively) to be significantly ($P < 0.05$) more diverse when compared to fish fed the NE 1.5 and Next Enhance 150® 3.0 treatments (Next Enhance 150® 1.5: 2.86 ± 0.33 and Next Enhance 150® 3.0: 3.16 ± 0.58). Bray-Curtis UniFrac UPGMA clustering of reads from the replicates of each treatment revealed three distinct clusters, with all replicates from the control and NE 1.5 treatments, and five of the six replicates of the Next Enhance 150® 3.0 treatment clustering together in the first cluster, four replicates of the probiotic + Next Enhance 150® 1.5 in the second cluster and all replicates of the probiotic treatment groups clustering together in the third cluster (Figure 4A.3.). Some crossover was observed with replicate Next Enhance 150® 3.0-3 and replicate Probiotic + Next Enhance 150® 1.5-2 which appeared in cluster 3. Replicate Probiotic + Next Enhance 150® 1.5-1 appeared to demonstrate low similarity with any of the other replicates and did not cluster with the three main clusters (Figure 5.2.).

The relative sequence distribution of the 16S rRNA reads at the phyla level is presented Figure 5.3. The 16S rRNA sequence reads assigned to the phylum Cyanobacteria accounted for 19.30 % (control), 19.40 % (probiotic), 9.80 % (Next Enhance 150® 1.5), 7.20 % (Next Enhance 150® 3.0), and 13.70 % (probiotic + Next Enhance 150® 1.5). These reads were

removed as described in Chapter 3 (section *3.4.2.3*). After the removal of these reads, the greatest number of reads in all samples were assigned to the Firmicutes phylum which accounted for 78.40 % of the total sequence reads, followed by Fusobacteria (12.80 %), Proteobacteria (7.50 %), Spirochaetes (0.50 %), Actinobacteria (0.40 %), and Bacteriodetes (0.30 %). The proportion of sequence reads assigned to the Fusobacteria was observed to be significantly elevated in fish fed the Next Enhance 150® 1.5 treatment (19.20 %) compared to fish fed the probiotic treatment (8.10 %) ($P < 0.02$). All other phyla remained statistically unaffected between treatment groups.

Figure 5.4. displays the relative sequence distribution of the 16S rRNA reads (> 0.1 %) at the genus level. The genus *Bacillus* accounted for the majority of the 16S rRNA reads (50.30 %) followed by reads assigned to the *Leuconostoc* and *Cetobacterium* genera (22.30 % and 12.70 %, respectively). The relative abundance of reads assigned to *Bacillus* were significantly elevated in fish fed the con (73.80 %; $P < 0.001$), Next Enhance 150® 1.5 (71.50 %; $P < 0.001$) and Next Enhance 150® 3.0 (59.80 %; $P < 0.01$) treatments compared to 16.10 % in fish fed the probiotic treatment. The majority of these reads were identified as *B. coagulans* with 69.28 %, 70.30 %, 60.95 % abundance observed in fish fed the control, NE 1.5 and Next Enhance 150® 3.0, respectively, which was significantly ($P < 0.001$) higher when compared to fish fed the probiotic and pro+NE 1.5 (14.45 % and 20.68 %, respectively) Next Enhance 150® 1.5. A BLAST search conducted on the remaining *Bacillus* reads revealed the presence of the probiotic (*B. subtilis*) in fish fed the probiotic (1.31 %) and probiotic + Next Enhance 150® 1.5 (10.68 %) treatments. The relative abundance of these reads were 0.27 %, 0.22 % and 0.16 % in fish fed the control, Next Enhance 150® 1.5 and Next Enhance 150® 3.0, respectively, which significantly lower when compared to fish fed the probiotic and probiotic + Next Enhance 150® 1.5 treatments ($P < 0.01$).

The relative abundance of reads assigned to *Leuconostoc* genus was significantly higher in fish fed the probiotic (59.80 %) diet compared to fish fed the control diet (3.20 %; $P < 0.001$), Next Enhance 150® 1.5 (3.40 %; $P < 0.001$), Next Enhance 150® 3.0 (17.50 %; $P < 0.001$), and probiotic + Next Enhance 150® 1.5 (27.40 %; $P < 0.01$). The relative abundance of reads assigned to *Cetobacterium* was significantly higher in fish fed the NE 1.5 diet compared to fish fed the probiotic diet (19.10 % vs 8.10 %; $P < 0.02$). The relative abundance of reads assigned to *Lactococcus* was significantly higher in fish fed the probiotic diet (2.80 %) compared to fish fed the control (0.20 %; $P < 0.001$), Next Enhance 150® 1.5 (0.90 %; $P < 0.001$), Next Enhance 150® 3.0 (0.90 %; $P < 0.01$) and pro+NE 1.5 (1.20 %; $P < 0.001$). The relative abundance of reads assigned to *Streptococcus* was significantly higher in fish fed the probiotic diet (2.50 %) compared to fish fed the control (0.10 %; $P < 0.001$), Next Enhance 150® 1.5 (0.10 %; $P < 0.001$), Next Enhance 150® 3.0 (1.00 %; $P < 0.01$) and probiotic + Next Enhance 150® 1.5 (1.20 %; $P < 0.02$). The relative abundance of reads assigned to *Actinetobacter* was significantly higher in fish fed the probiotic diet (1.30 %) compared to fish fed the control (0.20 %; $P < 0.01$), Next Enhance 150® 1.5 (0.10 %; $P < 0.001$), and probiotic + Next Enhance 150® 1.5 (0.70 %; $P < 0.02$) diets. The relative abundance of reads assigned to *Enhydrobacter* was significantly higher in fish fed the probiotic (0.60 %) compared to fish fed the control (0.10 %; $P < 0.02$), Next Enhance 150® 1.5 (0.10 %; $P < 0.01$), and probiotic + Next Enhance 150® 1.5 (0.30 %; $P < 0.05$) diets. The relative abundance of reads assigned to *Staphylococcus* was significantly higher in fish fed the control diet (1.40 %) compared to fish fed the probiotic (0.10 %), Next Enhance 150® 1.5 (0.30 %), Next Enhance 150® 3.0 (0.20 %) and probiotic + Next Enhance 150® 1.5 (0.10 %) diets ($P < 0.05$ in all cases).

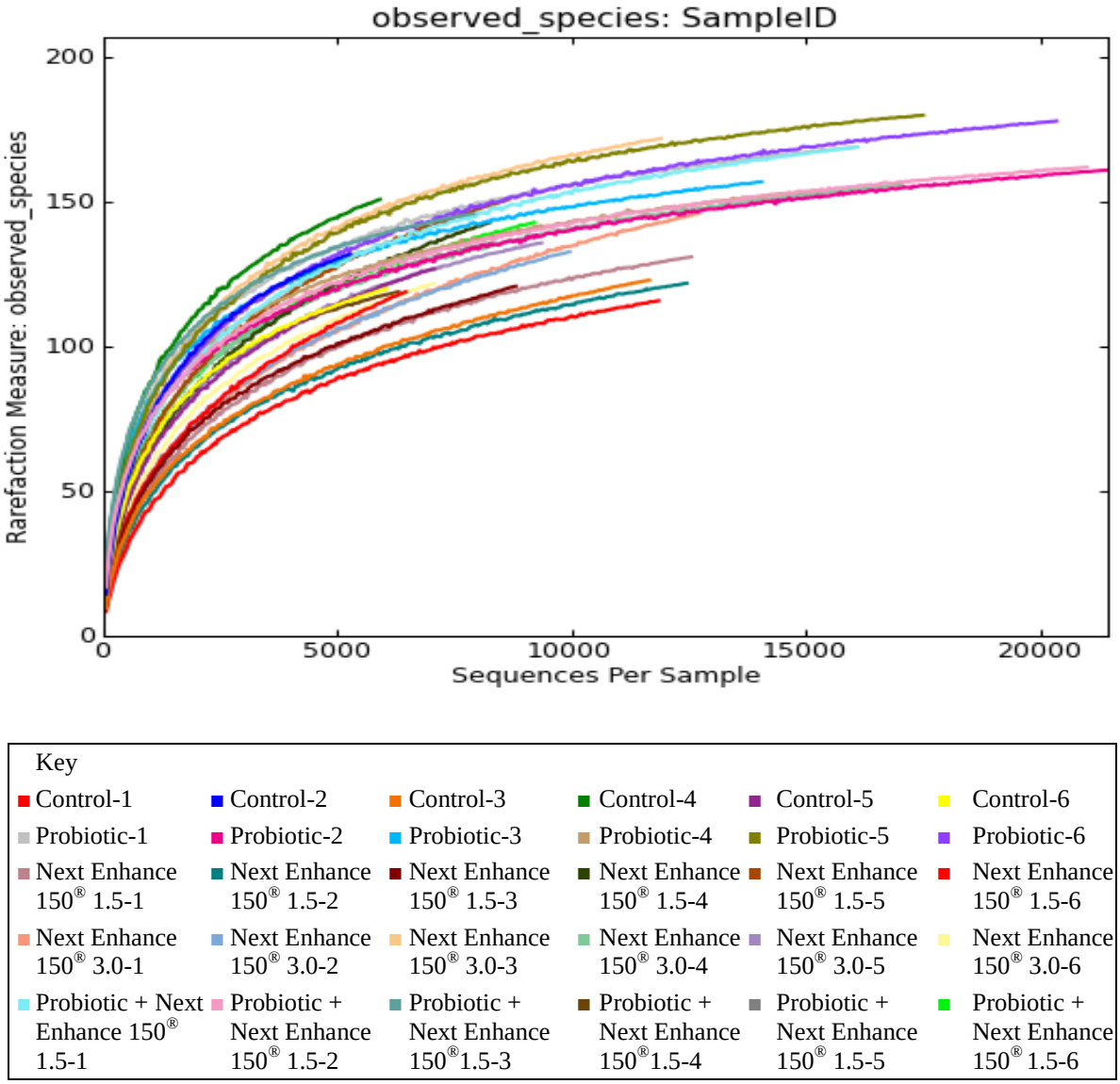


Figure 5.1. Alpha rarefaction curves representing the number of observed species (OTU's) per sample, which is used as an inference of the number of species, as a function of sequencing effort.

Table 5.4. High-throughput sequencing good's coverage estimations by treatment and alpha diversity parameters of the allochthonous bacterial associated with the posterior intestine of European sea bass fed each dietary regime for 10 weeks. Data represent mean ± SD.

	Good's coverage	Observed species	Chao1 index	Shannon index	Phylogenetic Distance
Control	0.9938±0.0005	116.06±19.39	146.87±18.74	3.08±0.71^{ab}	4.37±0.46
Probiotic	0.9938±0.0007	131.59±6.56	160.68±8.41	4.19±0.13^{b}	4.75±0.22
NE 1.5	0.9929±0.0008	110.31±12.59	152.98±16.65	2.86±0.33^{a}	4.50±0.35
NE 3.0	0.9932±0.0005	117.68±12.51	155.35±9.56	3.16±0.58^{a}	4.67±0.41
Probiotic+Next Enhance 150® 1.5	0.9941±0.0008	124.81±6.63	151.90±10.06	4.32±0.31^{b}	4.88±0.33

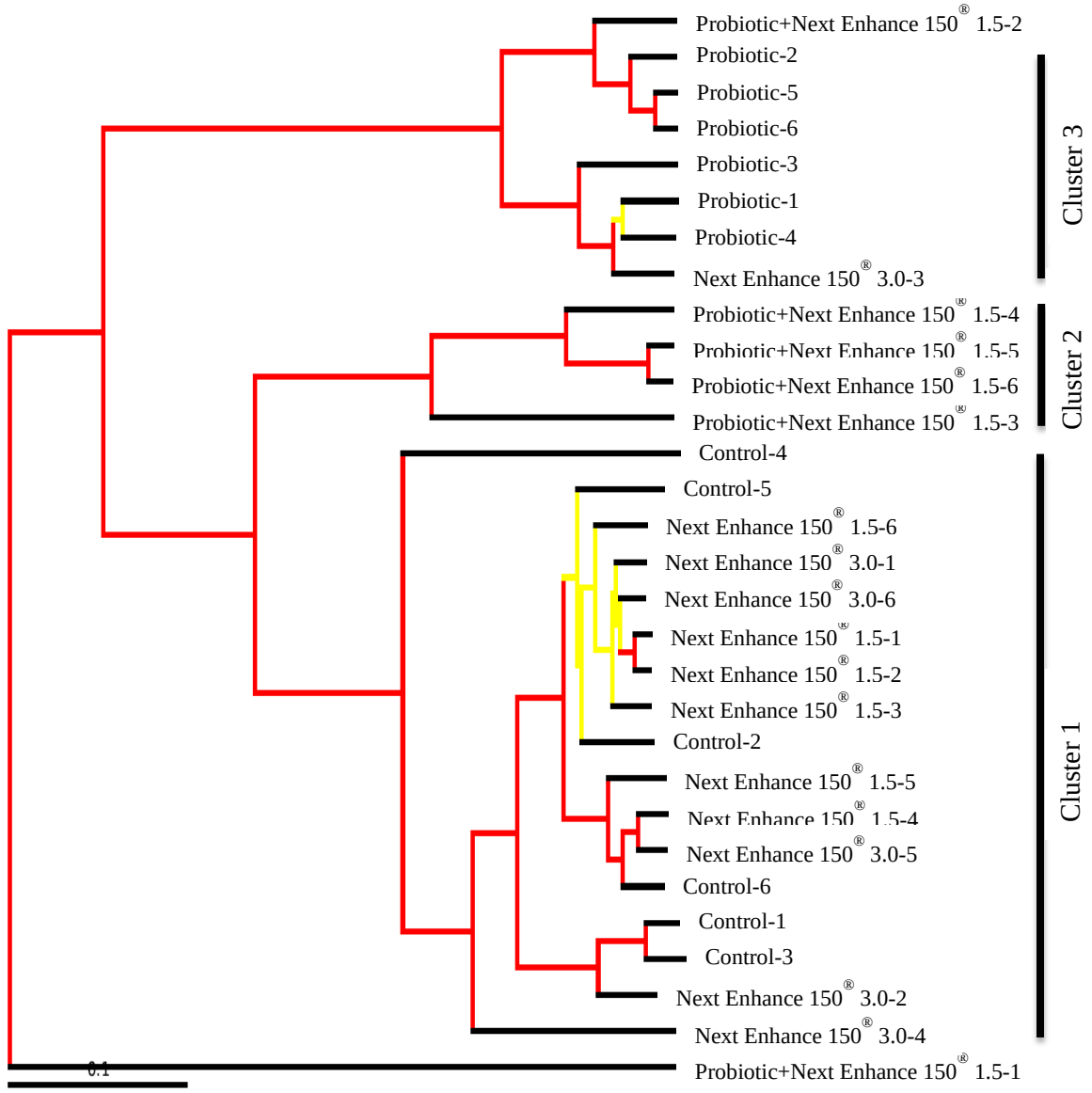


Figure 5.2. Bray-Curtis UniFrac UPGMA clustering of reads from the replicates of each treatment of the allochthonous bacterial communities of the posterior intestine of European sea bass fed each dietary regime for 10 weeks. Jackknife support is: Red (75-100 %), yellow (50-75 %). Bar indicates 10 % divergence. Cluster 1 contains all replicates of the control, Next Enhance 150® 1.5 and five of the six Next Enhance 150® 3.0 treatments. Cluster 2 contains four of the six replicates of the probiotic + Next Enhance 150® 1.5 treatment, and cluster 3 contains all replicates from the probiotic treatment and one replicate each of the probiotic + Next Enhance 150® 1.5 and Next Enhance 150® 3.0 treatments. The replicate probiotic + Next Enhance 150® 1.5-1 was not similar to any of the three clusters.

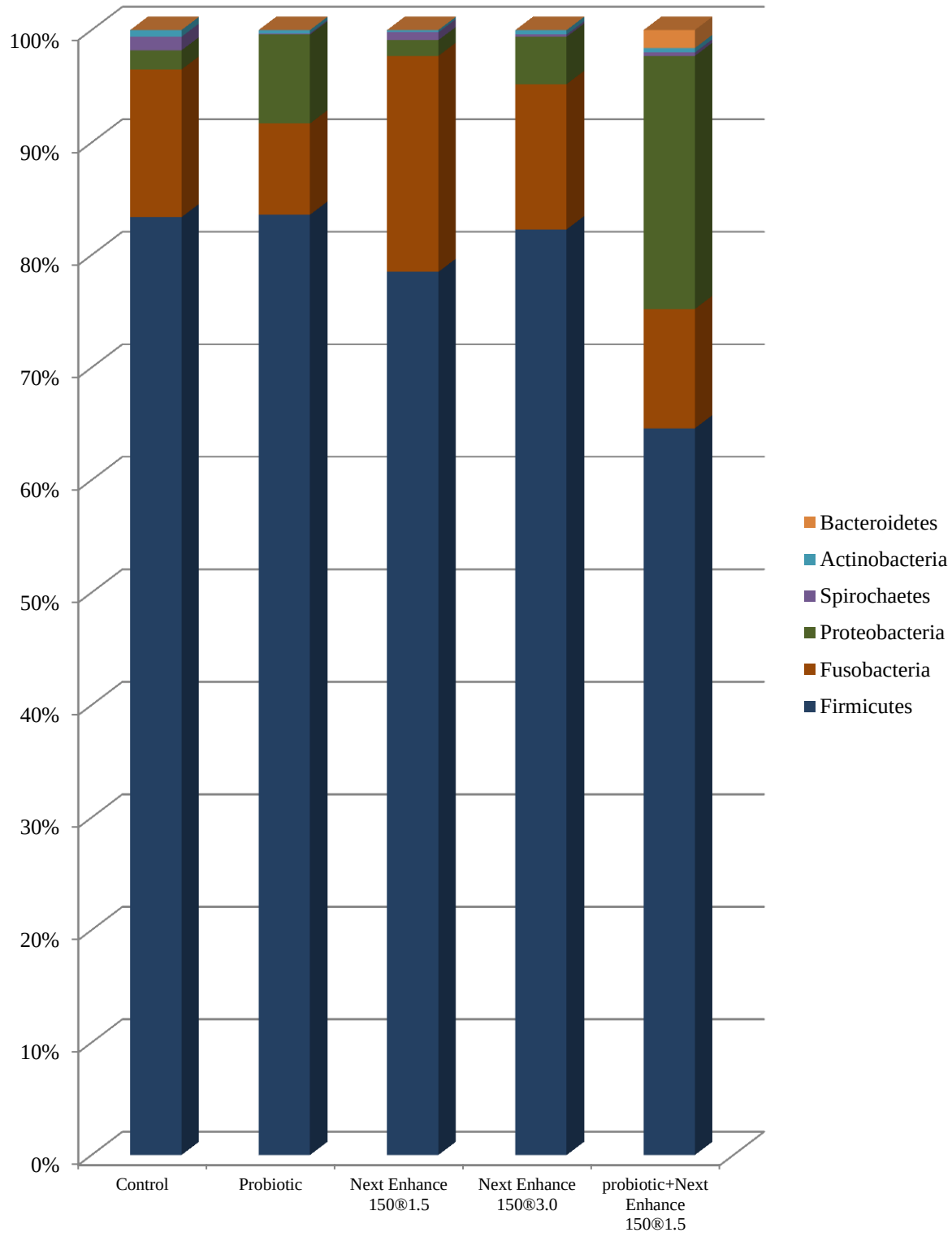


Figure 5.3. The allochthonous bacterial communities in the posterior intestine of European sea bass fed the experimental diets for 10 weeks. Data represent bacterial phyla percentage.

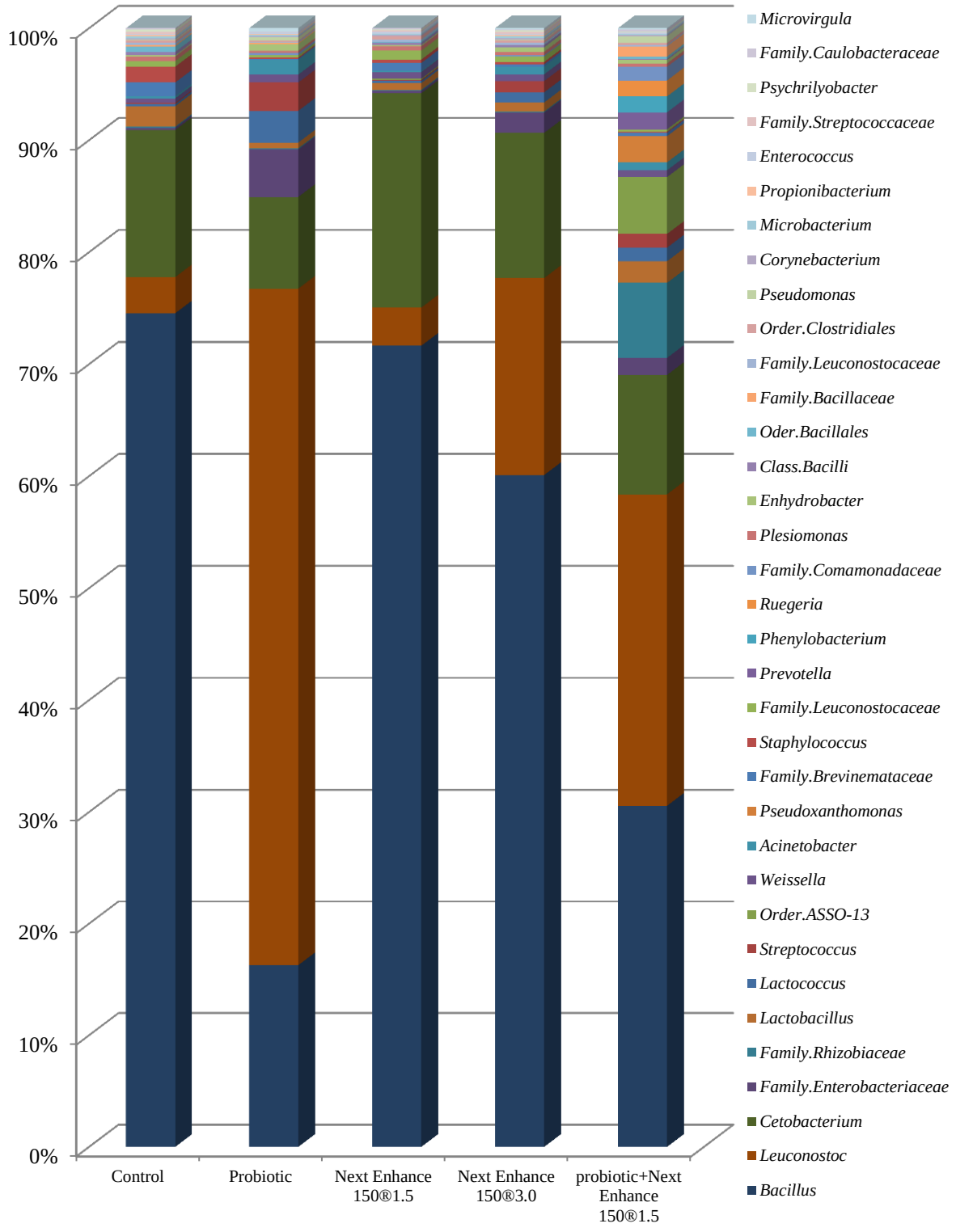


Figure 5.4. The allochthonous bacterial communities in the posterior intestine of European sea bass fed the experimental diets for 10 weeks. Data represent reads assigned to bacterial genera > 0.1 %.

5.4.3. Gene expression

The relative mRNA expression of the genes HSP70, CASP3, PCNA, IL-10, IL-1β and CAL was analyses and is presented in Figure 5.5. The relative expression of HSP70, CASP3 and PCNA was significantly down-regulated in fish fed the probiotic treatment compared to fish fed the control. The relative expression of CAL was also significantly down-regulated in fish fed the probiotic treatment relative to fish fed the control. The relative expression of the pro-inflammatory cytokine IL-1β and the anti-inflammatory cytokine IL-10 were unaffected by dietary treatment.

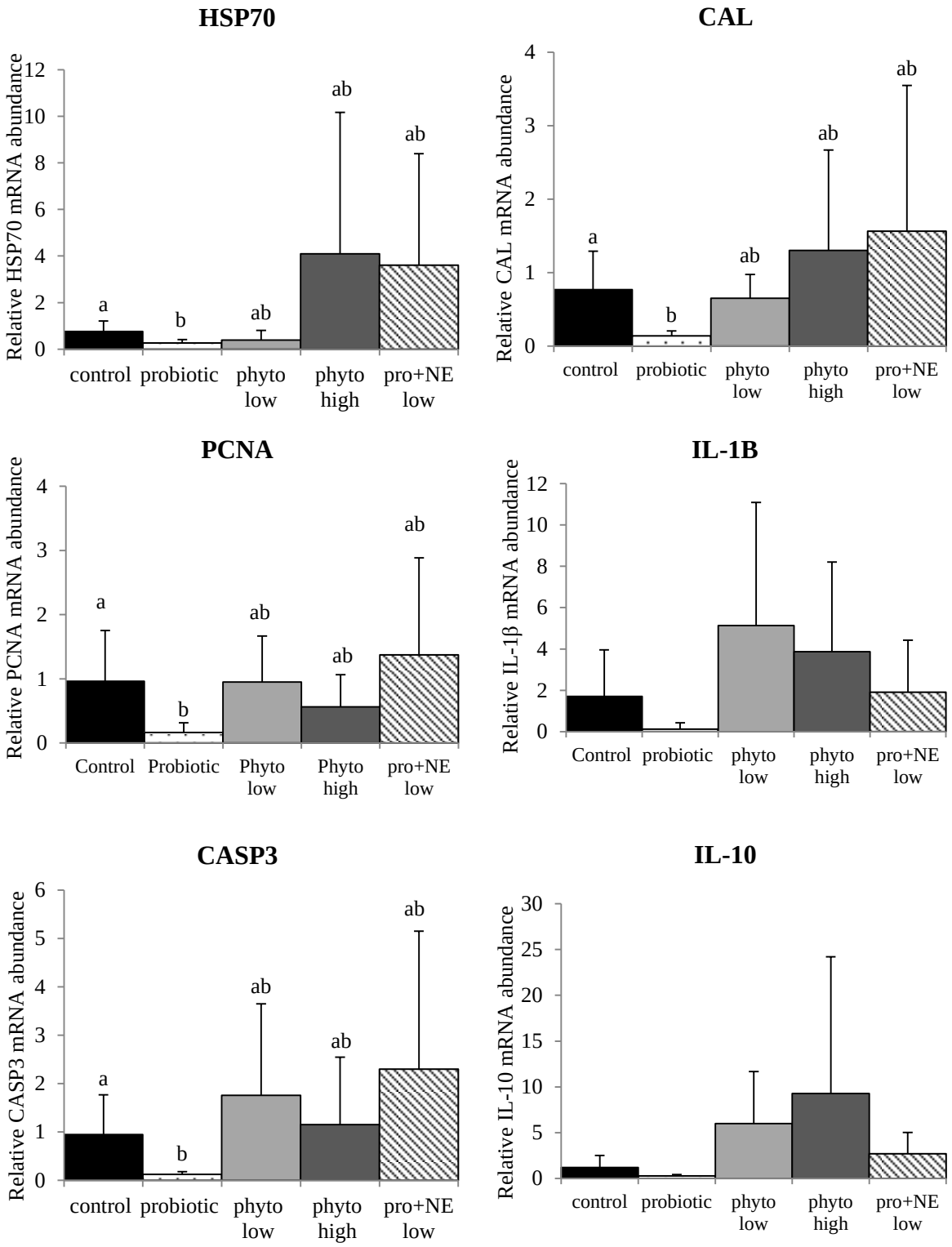


Figure 5.5. Relative mRNA abundance of HSP70, CASP3, PCNA, CAL, IL-1β and IL-10 in the posterior intestine of European sea bass fed the experimental diets for 10 weeks. Different superscript letters indicate significant differences (accepted as $P < 0.05$) between treatments. $n = 6$ per treatment.

5.4.4. Haematological parameters

Values for haematocrit, haemoglobin and lysozyme activity are presented in Table 5.6. Haematocrit, haemaglobin and serum lysozyme activity were not significantly affected by dietary treatments.

Table 5.6. Haematological parameters of European sea bass fed the experimental diets for 10 weeks. *n* = 15.

	Con	Pro	NE 1.5	NE 3.0	Pro+NE 1.5
Haematocrit (% PCV)	50.92±1.05	53.22±4.17	54.75±4.16	53.33±0.29	54.98±8.01
Haemoglobin (g dl^{-1})	9.63±0.51	10.59±0.54	9.86±0.38	10.36±1.68	10.89±1.26
Lysozyme activity (U ml^{-1})	248.99±35.54	296.40±49.10	259.21±63.61	258.26±35.71	287.17±58.95

5.4.5. Intestinal histology

Figure 5.6. illustrates examples of H & E stained posterior intestinal sections of fish fed each dietary regime. The general morphology of the intestine of all sampled fish appeared healthy with no obvious signs of intestinal inflammation or ill health. The number of IEL's per 100 µm was significantly elevated in fish fed the probiotic (76.02 ± 18.65), NE 3.0 (82.05 ± 17.11) and pro+NE (74.32 ± 16.51) dietary regime compared to fish fed the control (56.7 ± 12.88). IEL counts in fish fed the NE 1.5 remained unaffected. The number of GC's per 100 µm and PR measurements were not affected by dietary treatment (Table 5.7.).

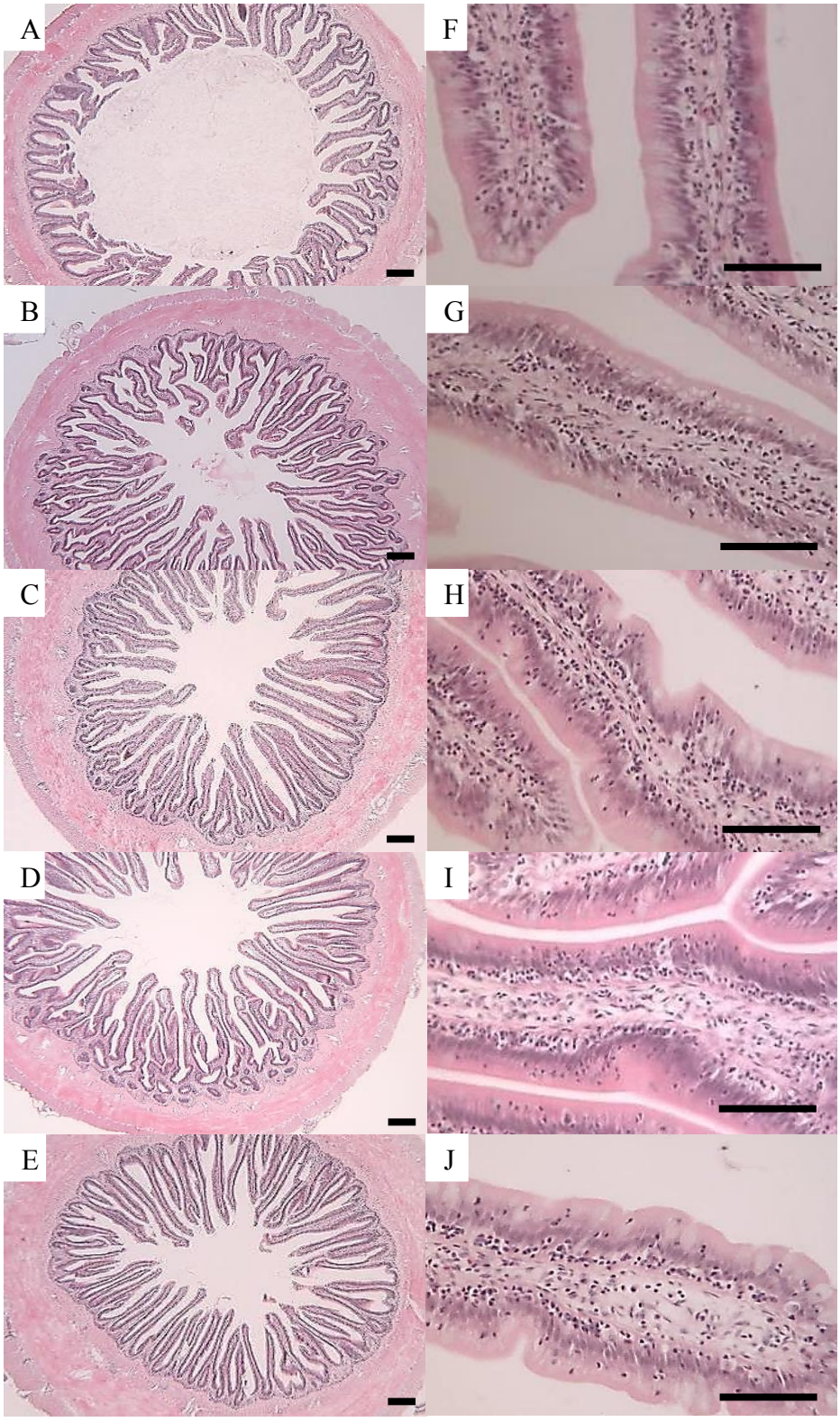


Figure 5.6. Light micrographs of the posterior intestine of European sea bass fed the control (A & F), probiotic (B & G), NE 1.5 (C & H), NE 3.0 (D & I), and pro+NE 1.5 (E & J) experimental diets for 10 weeks. Scale bars = 100 µm in A-E and 50 µm in F-J.

Table 5.7. Quantitative morphometric analyses of histological parameters of European sea bass posterior intestine after 10 weeks on respective dietary regimes. Data represent mean ± SD. Different superscript letters within the same rows indicate significant differences between groups. *n* = 6 per treatment group.

	Con	Pro	NE 1.5	NE 3.0	Pro+NE 1.5
Perimeter ratio (AU)	4.19±0.64	4.35±0.76	4.32±0.75	3.50±1.58	3.94±0.40
IEL's (cells per 100 µm)	56.7±12.88^{a}	76.02±18.65^{b}	57.78±11.10^{a}	82.05±17.11^{b}	74.32±16.51^{b}
GC's (cells per 100 µm)	7.18±3.04	8.87±3.04	9.11±3.60	8.10±3.00	9.38±3.25

5.5. Discussion

The present study assessed the effects of the probiotic *B. subtilis* and the phytobiotic Next Enhance 150®, individually and in combination, on the growth performance, haematology and intestinal health of European sea bass. The feed additives were applied to a diet formulated to reflect commercial diets for European sea bass. In terms of the growth parameters, the SGR's and FCR's observed in the present study were in line with previous studies on European sea bass juveniles (Torrecillas *et al.* 2007, 2015; Haas *et al.* 2015). The growth parameters remained statistically unaffected by dietary treatment.

With regards to microbiological analyses, high-throughput sequencing was successfully utilised to characterise the allochthonous intestinal microbiota, and along with chapters 3 and 4, is one of the first studies to apply this approach to studies of European sea bass gut microbiota. Furthermore, to the author's knowledge, this is the first study assessing the

effects Next Enhance 150®, an encapsulated combination of carvacrol and thymol on European sea bass allochthonous bacterial populations. Sequence libraries displayed Good's coverage estimations of > 99 %, which is indicative of a microbiome that has been fully sampled. Bray-Curtis cluster analyses revealed three distinct clusters separating the bacterial profiles, the first containing the control, NE 1.5 and NE 3.0, the second containing fish fed pro+NE 1.5 and the third containing fish fed the probiotic. Values for numbers of OTU's, species richness (Chao 1) and phylogenetic distance (PD) were observed to be highest in fish fed the probiotic and pro+NE 1.5 treatments. Bacterial diversity values (Shannon-Wiener) were observed to be significantly elevated in fish fed the probiotic and pro+NE 1.5 treatments when compared all other treatments

With regards to relative sequence abundance, the Firmicutes phylum accounted for a large portion of overall reads (78.40 %); this was followed by 16S rRNA reads assigned to Fusobacteria (12.80 %), Proteobacteria (7.50 %), Spirochaetes (0.50 %), Actinobacteria (0.40 %), and Bacteriodetes (0.30 %). These phyla have all been reported as constituents of the intestinal microbial communities of fish (Navarrete *et al.* 2008, Mansfield *et al.* 2010, Carda-Diéguez *et al.* 2014; Gatesoupe *et al.* 2014; Kormas *et al.* 2014; Chapter 3; Chapter 4A). The 16S rRNA sequence reads assigned to Fusobacteria were significantly elevated in fish fed the NE 1.5 treatment (19.20 %) compared to fish fed the probiotic treatment (8.10 %).

With regards to bacterial genera, the majority of the 16S rRNA reads (of the reads accounting for > 0.1 %) were assigned to *Bacillus* sp. These were observed to be significantly elevated in fish fed the control (69.28 %), NE 1.5 (70.30 %) and NE 3.0 (60.95 %), when compared to fish fed the probiotic (14.45 %) and pro+NE 1.5 (20.68 %) treatments. *B. subtilis* was identified through a BLAST search, revealing the presence of the probiotic in fish fed the probiotic (1.31 %) and pro+NE 1.5 (10.68 %) treatments. The relative presence of *B. subtilis*

was < 0.27 % in fish fed the control, NE 1.5 and NE 3.0 treatments. These reads were significantly elevated in fish fed pro+NE 1.5 compared to fish fed the other treatments.

High-throughput sequencing identified the majority of the *Bacillus* reads to be *B. coagulans*. *B. coagulans* has been reported to produce antimicrobials such as bacteriocin (Abdhul *et al.* 2015), and has been investigated as a probiotic in Koi (Lin *et al.* 2012) and common carp (Xu *et al.* 2014). Lin *et al.* (2012) reported significant increases in growth of fish fed a diet supplemented with *B. coagulans* in combination with chitosan oligosaccharides. The study also reported significantly elevated respiratory burst, phagocytic, lysozyme activities were also reported as a consequence of these feed additives. Xu *et al.* (2014) reported improved growth, lysozyme and respiratory burst activities in fish fed *B. coagulans* compared to fish fed the control. To the authors knowledge no studies have reported *B. coagulans* to be part of the allochthonous bacterial communities in European sea bass and hence its potential positive effects are not known in this species. Interestingly, *B. coagulans* was significantly reduced in fish fed the probiotic and pro+NE 1.5 compared to fish the other treatments, suggesting *B. subtilis* has the potential to reduce the dominance of *B. coagulans* observed in the control, NE 1.5 and NE 3.0. This could be due to a number of factors such as: direct antagonism (i.e. the production of substances by *B. subtilis* to inhibit the growth of *B. coagulans*), or through competitive exclusion. To the author's knowledge, antagonism between these two species in the fish intestine has not been assessed and warrants further investigation.

Reads assigned to the genus *Leuconostoc* were significantly elevated in fish fed the probiotic compared to fish fed all other treatments. The family *Leuconostocaceae* has previously been reported as a constituent of the intestinal microbiota of European sea bass (Carda-Dieguez *et al.* 2013; Chapter 4A), and *Leuconostoc* was also reported as part of the allochthonous microbiota in Chapter 3. *Leuconostoc mesenteroides* has been proposed as a potential probiotic species and its supplementation in the diets of fish has been associated with

improvements in immune responses, growth and digestive enzyme activities (Balcázar *et al.* 2007a, 2007b, 2008; Askarian *et al.* 2011). Balcázar *et al.* 2007b reported the supplementation of the probiotic strains; *Lactobacillus sakei*, *L. lactis* and *L. mesenteroides* possessed the ability to adhere and survive in the intestinal mucus of rainbow trout. The authors also reported the probiotic supplementation enhanced the humoral and cellular immune response and reduced the severity of furunculosis. *Leuc. Lactis* is comparatively less well documented, but there is evidence to suggest this species also could also have potential probiotic effects in fish (Zhang *et al.* 2013). *Leuconostoc* spp. were also observed in the bacterial communities European sea bass in Chapters 3 and 4A suggesting that this genera may be part of a core microbiota in this species.

Reads assigned to the genus *Lactococcus* were also observed to be significantly elevated in fish fed the probiotic compared to fish fed all other treatments. Species from this genus, most notably *L. lactis* have been demonstrated as a potential probiotic in fish (Sun *et al.* 2012; Touraki *et al.* 2013; Zhang *et al.* 2013). Strains of this species have been reported to produce bacteriocins (e.g. nisin production in the case of *L. lactis*), which has been observed to inhibit some Gram positive bacteria (Pasteris *et al.* 2014). *L. lactis* has also been reported to confer positive effects such as improved feed utilization and immune function when supplemented in the diets of fish (Sun *et al.* 2012; Touraki *et al.* 2013; Zhang *et al.* 2013). In the study by Touraki and co-authors (2013), European sea bass fed *L. lactis* enriched artemia nauplii exhibited increased survival rates when challenged with *V. anguillarum*.

Reads assigned to the genera *Acinetobacter* and *Enhydrobacter* accounted for a small portion of the overall reads but were observed to be significantly elevated in fish fed the probiotic when compared to the other treatments. Although some species of *Acinetobacter* (e.g. *A. johnsonii* and *A. Iwoffii*) are considered fish pathogens, there are a number of studies reporting *Acinetobacter* spp. to be part of the intestinal microbiota of healthy fish (Hovda *et*

al. 2007; Askarian *et al.* 2012). *Enhydrobacter* spp. is not as commonly reported in the fish intestine, but has been reported to be associated with fish generally, including as part of the skin microbiotia (Franchini *et al.* 2014; Falcinelli *et al.* 2014; Leonard *et al.* 2014). However, to the author's knowledge the roles *Enhydrobacter* spp. play in the fish intestine have not been investigated and hence more research is required on this topic.

Fish fed all of the experimental diets were observed to exhibit significantly reduced levels of the genus *Staphylococcus* compared to the control. The *Staphylococcus* genus contains a number of human/fish pathogens (e.g. *S. aureus*).The relative abundance of reads assigned to this genus were observed to be significantly reduced in fish fed all experimental treatments relative to fish fed the control, suggesting the feed additives may act to inhibit species of this genus. Indeed *B. subtilis* is known to produce an array of antimicrobial substances including bacilysin, subtilosin, surfactin and plipastatin (Stein 2005). The bioactivity of surfactin and plipastatin has been observed to inhibit strains of *S. aureus in vitro* (Gonzalez *et al.* 2011). There is also evidence to suggest strains of *B. subtilis* have the potential to inhibit specific fish pathogens such as *V. anguiilarum* and *Photobacterium damselae* subsp. *piscicida* (Touraki *et al.* 2012b). Interestingly, in the present investigation the relative abundance of reads assigned to *Vibrio* spp. was < 0.1 % in all treatments. Reads assigned to *Vibrio* spp. in Chapters 3 and 4A was also < 0.1 % of the total reads in all treatments suggesting this genera may be only a minor constituent of the allochthonous communities in European sea bass.

The relative abundance of reads assigned to *Cetobacterium* was significantly elevated in fish fed the NE 1.5 diet compared to fish fed the probiotic diet. Members of this genus have been commonly reported to be associated with the intestine of various fish species (Tsuchiya *et al.* 2008; Van Kessel *et al.* 2011; Larsen *et al.* 2014; Etyemez & Balcázar 2015; Li *et al.* 2015; Pedrotti *et al.* 2015; Standen *et al.* 2015). *Cetobacterium somerae* is known for its production vitamin B12 (Tsuchiya *et al.*, 2008), and has been observed to possess antibacterial properties

(Sugita *et al.* 1996). *Cetobacterium* was not detected in the 16S rRNA reads representing > 0.1 % in Chapters 3 and 4A. The reasons for this are not clear, however could be due to the differences in the basal formulations used in the respective studies. Furthermore, to the authors knowledge this is the first time this genus has been reported as part of the allochthonous bacterial communities in European sea bass, and its presence and subsequent effects should be further researched.

The gene expression of heat-shock protein 70 (HSP70), caspase 3 (CASP3), proliferating cell nuclear antigen (PCNA), and calcium binding protein calreticulin (CAL), interleukin-1β (IL-1β) and interleukin-10 were assessed in the present study. A significant down-regulation was observed in the mRNA abundance of HSP70, CASP3 and PCNA in fish fed the probiotic treatment compared to fish fed the control. These results are in agreement with Chapter 4A where a down-regulation of these was also reported in fish fed the probiotic relative to fish fed the control. As discussed in Chapter 4A, the down-regulation of HSP70, CASP3 and PCNA in fish fed a probiotic has been reported in the intestine, as a consequence of probiotic feeding of fish (Avella *et al.* 2010; Liu *et al.* 2013; Abid 2014; Chen *et al.* 2015; Chapter 4A). Collectively, the down-regulation of these genes in the present study reinforces the results obtained from Chapter 4A, suggesting *B. subtilis* has the potential confer beneficial intestinal health of European sea bass when supplemented into both challenging and non-challenging basal diets. Intestinal CAL gene expression was also significantly down-regulated in fish fed the probiotic treatment relative to fish fed the control. In mammals, calreticulin is an important binder protein involved in the regulation of Ca^{2+} homeostasis, lectin binding and molecular chaperoning in the endoplasmic reticulum. However, information on this gene and the roles it plays in fish are extremely limited (Rubinstein *et al.* 2000; Kales *et al.* 2004, 2007; Liu *et al.* 2011; Pinto *et al.* 2013; Duan *et al.* 2014). There is evidence to suggest CAL is involved in the immune function in fish and the up-regulation in expression of this gene has

been observed during bacterial infection in Chinese shrimp (*Fenneropenaeus chinensis*) (Luana *et al.* 2007), ridgetail white prawn (*Exopalaemon carinicauda*) (Duan *et al.* 2014), and channel catfish (*Ictalurus punctatus*) (Liu *et al.* 2011). In the present study, the reasons behind the significant down-regulation of CAL in fish fed the probiotic compared to fish fed the control are not fully clear, however, it further suggests that *B.subtilis* does not have a detrimental effect when applied to the feed of European sea bass.

Intestinal histological analyses revealed no detrimental effects to the posterior as a consequence of the feed additives. Significant increase in IEL's was observed in fish fed the probiotic and pro+NE 1.5 compared to fish fed the control. These results are similar to those observed in Chapter 4A when using *B. subtilis* and Previda®, and other probiotic studies on fish (Picchietti *et al.* 2007, 2009; Salinas *et al.* 2008). As discussed in Chapter 4A, elevated IEL's in the intestine may help to create an unreceptive environment for potential enteropathogens (Harper *et al.* 2011). Therefore, fish fed the probiotic and pro+NE 1.5 treatments in the present study appear to exhibit an intestinal epithelium potentailly better equiped to counteract pathogenic insults. Furthermore, the comparable results observed in the present study and those obtained from Chapter 4A appear to be present when fish were fed both a challenging diet and a non-challenging diet. In the current study the haematocrit, haemoglobin and serum lysozyme activity were also assessed. These parameters were not affected by dietary treatment.

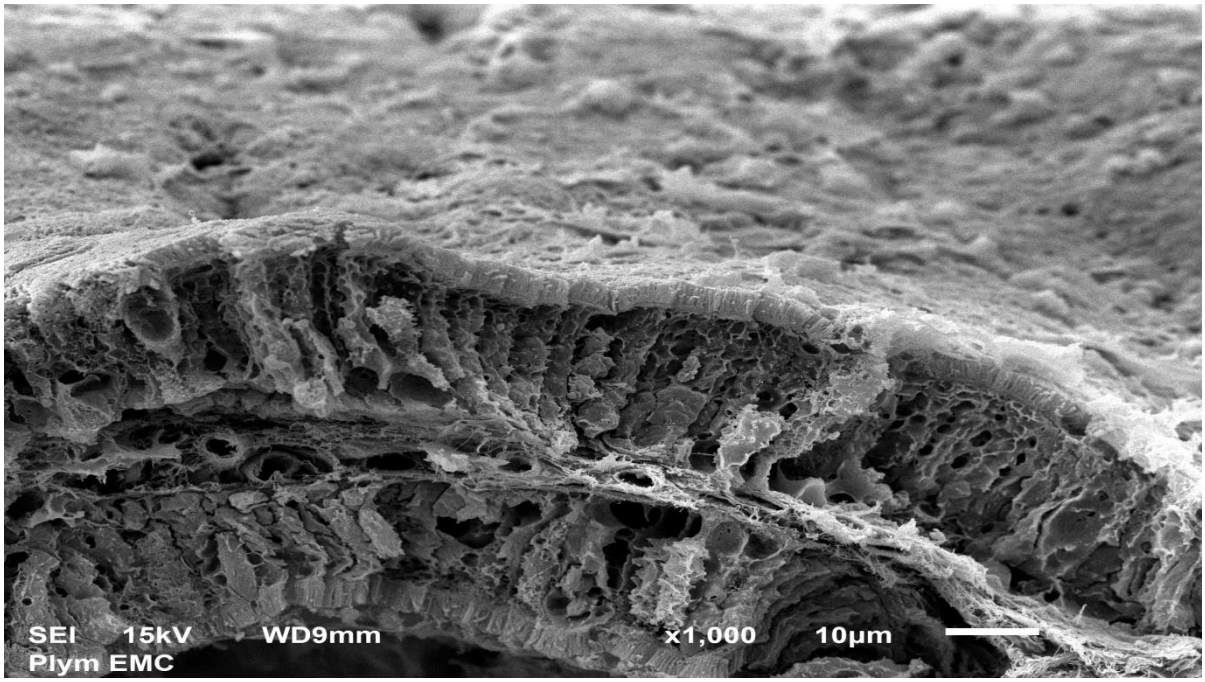
5.6. Conclusion

The results from the present investigation, similar to those in Chapter 4A, reveal that dietary *B. subtilis* supplementation modulates the allochthonous intestinal bacterial populations of European sea bass. In comparison to Chapter 4A, the level of the probiotic appeared to

present in much lower abundance. Even at the lower levels, the probiotic had modulatory effects reducing the dominance of *B. coagulans*, which dominated the bacterial populations of fish fed the control, NE 1.5 and NE 3.0 treatments, as well as, reducing the abundance of some potential pathogens. The present study also revealed the probiotic significantly reduced the intestinal gene expression levels of HSP70, CASP3, PCNA and CAL, confirmimg the results from Chapter 4A, and suggesting a postivie effect in relation to intestinal integrity. The IEL's levels were increased in fish fed the probiotic treatment which was also observed in Chapter 4A, potentially creating an intestine in an increased immunological state. This study expands on the results of Chapter 4A suggesting that dietary *B. subtilis* has the potetial to confer beneficial effects with regards to the intestinal health of European sea bass beyond four weeks and independently of dietary regime. Furthermore, these improvements in host health appeared with no detrimental effects on growth.

Chapter 6

General discussion



This research project comprised of three nutrition based experiments conducted to assess the effects the plant proteins SPC, PPC and saponins, and various feed additives, on the health of European sea bass juveniles. Additionally, an experiment was carried out *ex vivo*, to assess probiotic and pathogen interactions in the sea bass intestine. Collectively, this research provides novel information relating to dietary induced enteritis effects in European sea bass, as well as, how feed additives affect the overall health of this species, with a particular emphasis placed on the intestinal microbial populations and how their potential modulation impacts on the host. Furthermore, the *ex vivo* model provides information on bacterial host interacts and presents potential as a model to assess bacterial effects on host tissues devoid of host suffering which is in line with the 3 R's.

The first experiment (Chapter 3) investigated the effects SPC and PPC alone, and in combination with additional saponin supplementation, on the intestinal microbiota and health of European sea bass after two and four weeks feeding. The data from this chapter revealed that the allochthonous microbial populations of these fish were modulated as a consequence of dietary SPC inclusion alone and in combination with PPC and saponins. These changes were observed as early as two weeks after dietary provision, and were also observed after four weeks feeding. PCR-DGGE analyses at week two, and PCR-DGGE and high-throughput sequence analyses at week four revealed the phyla Proteobacteria and Firmicutes to be the dominant phyla, with the Actinobacteria and Bacteriodetes phyla constituting a minor proportion of the allochthonous microbial communities. Sequenced OTU's from the DGGE revealed the abundance of *B. subtilis* and *L. buchneri* was elevated as a consequence of the SPC+PPC, and SPC+PPC+S treatments compared to fish fed the FM control after two and four weeks. Similarly, 16S rRNA reads assigned to the LAB (*Leuconostoc* and *Weissella*) and *Bacillus* spp. were observed to be elevated in fish fed the plant based treatments when compared to fish fed the control, significantly with regards to *Leuconostoc* in fish fed

SPC+PPC+S and SPC treatments, and *Weissella* in fish fed the SPC+PPC+S relative to fish fed the control. LAB were also detected in fish fed the FM control with *Enterococcus* spp. significantly higher in fish fed this treatment compared to fish fed the plant based diets. However, species belonging to LAB were generally increased as a consequence of the plant based ingredients. As discussed previously, multiple LAB have been proposed as potential probiotics in fish and it may be regarded as a positive result that LAB populations were increased in fish fed the plant based diets. This is in agreement with a previous study on Atlantic salmon (Reveco *et al.* 2014), and may be due to the nutrient source availability of carbohydrates (i.e. oligosaccharides and polysaccharides) in the plant based diets. However, this remains an area in need of further research with regards to whether or not these nutrient sources are indeed being utilised by the LAB, and if so how this relates to their proliferation and ultimately host health. Intestinal histology was also assessed in Chapter 3 after two and four weeks. Deleterious intestinal changes as a consequence of the plant based diets was apparent after two weeks feeding with a significant decrease in GC's in fish fed the SPC+S, and significant decreases in microvilli density measurements in fish fed the SPC+PPC, SPC+PPC+S and SPC+S relative to fish fed the control. After four weeks feeding, GC and IEL levels were significantly decreased in fish fed the SPC+S, and microvilli measurements also decreased in fish fed the SPC+PPC+S and SPC+S relative to fish fed the control. Saponins are known to interfere with phospholipid formation in membranes affecting their permeability and structure (Augustin *et al.* 2011). The results obtained in Chapter 3 support the observations of previous studies on the effect of saponins on the intestinal morphology of fish (knudsen *et al.* 2007, 2008; Chikwati *et al.* 2012; Kortner *et al.* 2012; Couto *et al.* 2015). Additionally, Chapter 3 describes a potential loss of membrane integrity at the apical tips of the microvilli. To the author's knowledge, this is the first time this has been described in the intestine of European sea bass, as a consequence of dietary saponin supplementation. These

novel findings warrant further investigation with particular attention placed on how these interactions affect the influx and efflux of molecules into epithelial cells. Furthermore, research should focus on the interactions between bacteria and intestinal membranes compromised by saponins and whether this could lead to increased risk of bacterial infections in the intestine of European sea bass.

The enteritis-like effects as a consequence of dietary plant based protein sources were perhaps of a more moderate nature compared to previous studies on other fish species (Krogdahl *et al*. 2003; knudsen *et al.* 2007, 2008; Uran *et al*. 2008). Indeed this may be due to the relatively short trial period of the present study versus long-term (i.e. > 10 weeks) growth trials of those aforementioned studies, and increased detrimental effects may occur over time. Differences in ingredients may also be a contributing factor and hence making comparisons between studies problematic. The enteritis-like effects observed in Chapter 3 however, appeared to be at their most pronounced in fish fed the SPC+S diet, after four weeks feeding. This diet was deemed to be the most challenging with regards to intestinal health of these fish, and was therefore selected to be used as the sub-optimal basal diet in Chapter 4A.

Chapter 4A was designed to assess the potential of probiotic (*B. subtilis*) and the prebiotic Previda®, individually, and in combination, with regards to alleviating the sub-acute enteritis effects caused by the sub-optimal SPC+S basal diet used in Chapter 3. Additionally, an antibiotic (oxytetracycline) supplemented diet was also assessed in Chapter 4A. Oxytetracycline is a broad-spectrum antibiotic commonly used for the treatment against pathogens in aquaculture. With concerns of antimicrobial resistance well documented (Rigos *et al.* 2013; Shah *et al.* 2014), the inclusion of oxytetracycline in this chapter would advance our knowledge surrounding the effects of this antibiotic on European sea bass intestinal health.

In Chapter 4A the allochthonous microbial communities and intestinal histology was investigated, as well as, the expression of the genes: TNFα and IL-1β (important in the inflammatory response), and the genes: HSP70, CASP3 and PCNA (important in intestinal integrity/health). Firstly, Chapter 4A demonstrated after four weeks feeding similar deleterious changes to the intestine observed in fish fed the SPC+S in Chapter 3. As demonstrated by TEM analyses, the epithelium brush-border of fish fed the basal control (SPC+S) presented similar, apparent loss of membrane integrity at the microvilli tips, reinforcing the results observed in Chapter 3. PCR-DGGE and high-throughput sequencing analyses revealed the modulatory effects of the dietary provision of *B. subtilis*. The PCR-DGGE and high-throughput sequencing dendrograms revealed that the bacterial profiles were grouped into two distinct clusters, with one cluster containing all replicates of fish fed the control, prebiotic and antibiotic treatments, and the second cluster containing all replicates of fish fed the probiotic and synbiotic treatments. High-throughput sequencing revealed that the phylum Firmicutes dominated the bacterial communities, accounting for > 97 % of the total 16S rRNA read sequences. This is in contrast to Chapter 3 where, the phylum Proteobacteria were the dominant group. The phyla; Actinobacteria, Proteobacteria and Bacteroidetes were also detected as minor constituents. PCR-DGGE also confirmed the dominance of Firmicutes with all sequenced bands identified belonging to this phylum. Interestingly, these findings appear to contradict the literature, which generally suggests the intestinal microbiota of European sea bass to be dominated by Proteobacteria, with Bacteriodetes, Actinobacteria, and Firmicutes making up minor constituents (Carda-Diéguez *et al.* 2014; Gatesoupe *et al.* 2014). Further investigations are necessary to elecidate the overwhelming dominace of Firmicutes, in terms of 16S rRNA abundance, observed in Chapter 4A. These results may, at least in part, be due to the high levels of SPC and the inclusion of saponins in the basal control feed. Indeed, intestinal microbial modulation in fish has previously been reported as a consequnce

of soy protein products and saponin inclusion (Heikkinen *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Ringø *et al.* 2008; Merrifield *et al.* 2009; Dimitroglou *et al.* 2010; Reveco *et al.* 2014; Chapter 3). However, as mentioned previously, making comparisons between studies and different species presents difficulties, and hence future investigations on the effects soy proteins and saponins have on the intestinal bacteria of European sea bass requires more attention.

Chapter 4A reported similar observations with regards to genera present in the European sea bass posterior intestine to those observed in Chapter 3. LAB apperaed to dominate the microbial communities of fish fed the control, preboitic and antibiotic treatments. More specifically, the dominance of *Lactobacillus* spp. was reported to be significantly higher in fish fed the basal control, prebiotic and antibiotic treatments relative to fish fed the probiotic and synbiotic treatments. Multiple *B. subtilis* strains were identified from the sequenced bands extracted from the DGGE which were present in the probiotic and synbiotic fed fish and absent in fish fed the other treatments. High-throughput sequencing anayses reflected this data, revealing a significant elevation in reads assigned to *Bacillus* spp. in fish fed the probiotic and synbiotic diets, relative to fish fed the other treatments. Probiotic feeding appeared to reduce the presence of some potential pathogens, with *Salinococcus* spp. absent in the PCR-DGGE profiles of fish fed the probiotic and synbiotic treatments and present in all of the replicates from fish fed the other treatments. High-throughput sequencing data also revealed the potentially pathogenic genus *Micobacterium* to be significantly reduced as a consequence of probiotic feeding. It is clear from the microbiological data that dietary *B. subtilis* feeding influenced the allochthonous intestinal microbial communities of European bass, and demonstrated the potential to reduce some potential fish pathogens within these communities.

The probiotic modulation of the intestinal microbiota also apperaed to lead to improvements in host immunity. An up-regulation of the pro-inflammatory cytokines IL-1β and TNFα was observed in the posterior intestine of fish fed the probiotic and synbiotic treatments relative to fish fed the control. An increase in the expression of these genes was also evident in fish fed the prebiotic and antibiotic treatments relative to fish fed the control. IL-1β and TNFα play important roles in immune regulation and inflammatory responses. The up-regulation in these pro-inflammatory cytokines as a consequence of probiotic and prebiotic feeding may be representative of increased immune capacity. These results are in agreement with previous probiotic and prebiotic studies in fish (Pérez-Sánchez *et al.* 2011; Pirarat *et al.* 2011; Abid *et al.* 2013; He *et al.* 2013; Liu *et al.* 2013; Román *et al.* 2013; Standen *et al.* 2013; Guzmán-Villanueva *et al.* 2014; Villamil *et al.* 2014; Yarahmadi *et al.* 2014). The relative expression of HSP70, CASP3 and PCNA was significantly down-regulated in fish fed the probiotic, prebiotic and synbiotic treatments relative to fish fed the control diet. These genes are involved in cellular stress, apoptosis and cell proliferation/DNA repair, and the up-regulation of these genes has been associated with enetritis in fish (Bakke-McKellep *et al.* 2007). The significant down-regulation of these genes observed in fish fed the probiotic, prebiotic and synbiotic treatments suggests the potential of these feed additives in mitigating enteritis-like effects by reducing celluar level stress and improving epithelial integrity in the intetsine. Interestingly, fish fed the antibiotic treatment, as well as, exhibiting a significant up-regulation in the pro-inflammatory cytokines TNFα and IL-1β, were observed to exhibit a significant up-regulation in HSP70, CASP3 and PCNA relative to all other treatments. These findings are in line with previous studies assessing antibiotic effects in fish. Caipang *et al.* (2009) demonstrated the antibiotics florfenicol and oxolinic acid induced an up-regulation of IL-1β in Atlantic cod. Furthermore, HSP70 has been observed to increase in response to oxytetracycline in zebra fish larvae (Romero *et al.* 2012). The present study suggests the

potetntial of oxytetracycline to induce a level of intestinal stress in European sea bass, however further research is required to test this hypothesis.

Histological analyses appeared to coincide with the improved immunity described above. Epithelial goblet cell levels were significantly elevated in fish fed the probiotic and synbiotic treatments compared to fish fed the control, which has previously been reported in fish fed dietary *Lactobacillus* spp. and *B. subtilis* administration (Picchietti *et al.* 2007, 2009; Salinas *et al.* 2008). Chapter 4A also revealed, significant increases in: intestinal perimeter ratio of (probiotic and synbiortic treatments), epithelial microvilli density (probiotic and prebiotic treatments), and absorptive surface index (probiotic), relative to fish fed the control. The histological appraisals further support the potential of these feed additives to confer beneficial improvements in the intestinal health of European sea bass fed a sub-optimal basal diet.

Chapter 4B utilsed an intestinal sac method to assess the potential of *B. subtilis* to mitgate enteric pathogen (*V. anguillarum*) damage. Histological and FISH techniques were employed to assess these effects in the posterior intestine at the end of the trial conducted in Chapter 4A. SEM analyses revealed that *V. anguillarum* exposures caused severe epithelial damage characterised by necrotic enterocytes, irregular microvilli and areas where the lamina propria and tight junctions were exposed to the lumen. A significant reduction in microvilli density was observed in the intestines exposed to the pathogen in fish fed all dietary regimes. These results are in agreement with the study by Harper *et al.* (2011) where *V. anguillarum* was also reported to reduce epithelial microvilli density in the intestine of rainbow trout *ex vivo*. Microvilli density measurements of intestines exposed to the pathogen were significantly elevated in fish fed the probiotic, prebiotic and synbiotic treatments compared to fish fed the control. This result is in agreement with Chapter 4A, suggesting the feed additives may improve the epithelial health in the posterior intestine of European sea bass, and provide a level of protection against *V. anguillarum.* Interestingly, FISH analyses revealed the lack of

adhesion to the epithelial surfaces by either *B. subtilis* or *V. anguillarum*. This would suggest that the damage to the epithelium caused by the pathogen was the result of the production of extracellular toxins (Li *et al.* 2008; Rock & Nelson 2006; Denkin & Nelson 2004; Milton *et al.* 1996), and was independant of direct pathogen-enterocyte interactions. The direct interactions between *B. subtilis* and *V. anguillarum* in the intestine of European sea bass and the mechanisms involved therein requires further investigation. Chapter 4B demonstrates the potential to utilise *ex vivo* models to assess bacterial interactions in the intestine of European sea bass, and could be used prior to bacterial challenge experiments. This would provide important information on pathogen interactions in the intestine before large numbers of fish were used in challenge experiments, which is in line with the 3 R's with regards to the use of animals in science.

Collectively the results of Chapters 4A and 4B indicate that *B. subtilis*, and to a lesser extent Previda®, confer health benefits to the intestine of European sea bass juveniles fed a sub-optimal diet. Furthermore, no signs of detrimental effects were observed as a consequence of probiotic and prebiotic feeding. The challenging diet used in this chapter was revealed to cause a degree of intestinal enteritis European sea bass and provided an interesing model to assess probiotic and prebiotic applications. However, the unusually high levels of SPC and the addition of saponins used in Chapter 4A appeared to modulate the intestinal microbial communities, and was not representative of a diet used commercially for this species. Therefore the diet used in Chapter 5 was designed to be more representative of a commecial formulation for this species. Chapter 5 investigated the effects dietary probiotic (*B. subtilis*) and phytobiotic (Next Enhance 150®) supplementation individually, and in combination on the intestinal microbiota and health of European sea bass fed a commercially based basal diet for 10 weeks.

Chapter 5 revealed no differences in growth performance by dietary treatment. The allochthonous microbial communities were observed to cluster into three disticnt groups. Cluster one contained most replicates of fish fed the control, NE low and NE high treatments, cluster two contained most replicates of fish fed the pro+NE low and cluster 3 contained all replicates of fish fed the probiotic treatment. Observed species, species richness and phylogenic distance remained unaffected by dietary regime. Shannon wiener diversity index was significantly elevated in fish fed the probiotic and pro+NE low treatments relative to all other treatments. Of these bacterial communities, high-throughput sequencing revealed Firmicutes to dominate the 16S rRNA reads of all samples. This is in line with Chapter 4A where the dominatant phylum of the allochthonous microbial communities was also Firmicutes, which suggests this phyla to be a the major constituent of the allochthonous bacterial communities of European sea bass juveniles under the rearing conditions used in these studies. The species *B. coagulans* appeared to dominate the 16S rRNA reads and was significantly higher in fish fed the control, NE low and NE high treatments compared to fish fed the probiotic and pro+NE 1.5 treatments suggesting this species was reduced as a consequence of probiotic supplementation. The probiotic inclusion also appeared to promote some potentially beneficial genera such as *Leuconostoc* and *Lactococcus*, while supressing the potential pathogenic genus *Staphylococcus* spp.

With regards to the localised immune response, a significant down-regulation was observed in the mRNA abundance of HSP70, CASP3 and PCNA in fish fed the probiotic treatment compared to fish fed the control suggesting an epithelium under a lower level of cellular stress and turnover, which is in line with Chapter 4A. Chapter 5 also revealed a significant down regulation in CAL, a gene that has been observed to be up-regulated as a response to bacterial infection in fish and shellfish (Luana *et al.* 2007; Duan *et al.* 2014; Liu *et al.* 2011).

The pro-inflammatory gene IL-1β and the anti-inflammatory IL-10 remained unaffected by dietary treatment. Probiotic supplementation also increased the abundance of GC's and significantly increased the number of IEL's in the posterior intestine. The increase in mucus production and IEL's is in agreement with the results obtained in Chapter 4A, suggesting the probiotic increases the presence of leukocytes in the epithelium, and thus potentially elevating the intestinal immunological state of these fish.

This programme of research provides a wealth of information relating to the health of European sea bass juveniles in response to dietary ingredients and feed additives. Molecular techniques were adopted for the microbiological analyses in the present research, and it should be mentioned that these approaches, like other techniques, have their limitations. The caveats include sample variation in DNA extractions and PCR amplification bias which may be introduced prior to downstream analyses and sequencing. With regards to PCR-DGGE, it is possible that multiple sequences migrating to the same position in the gel may be wrongly considered as one species. There are also some potential pit-falls with respect to high-throughput sequence analyses. These include problems associated with short reads and inconsistences in the process of sequences with regards to sequence coverage. Furthermore, care must be taken when assessing relative abundances in a given sample as 16S rRNA copy numbers may vary between bacterial species, which may lead to the overestimation of bacteria with high copy numbers, and the underestimation of bacteria with low copy numbers. Readers with an interest in the strengths and weaknesses of these molecular techniques are referred to the papers by Jackson *et al.* (2000), Kuczynski *et al.* (2012), Ghanbari *et al.* (2015) and Zhou *et al.* (2014).

The present research focused on the allochthonous microbial communities and provides important information on these communities in European sea bass. Future research should focus on the interactions between feed additives, in particular probiotics, and the autochthonous microbial communities in this and other fish species. Furthermore, research should also focus on the functional roles the microbial communities play in the intestine of fish by applying metatranscriptomics, metaproteomics and metabolomics to assess their relevance and relationship with the host (Ghanbari *et al.* 2015). Metatranscriptomics provides information on the active bacteria by assessing the expression of genes in these complex communities. Metaproteomics and metabolomics addresses the expression of proteins and metabolites in a microbial ecosystem, and would advance our knowledge on the potential roles these important microbes play in the intestine, and indeed other organs of fish (Franzosa *et al.* 2014; Ghanbari *et al.* 2015). Future research is also required on the role feed additives play on the localised immunity with regards to expression pathways. Feed additive effects on systemic immunity, such as serum immunoglobulin levels, lysozyme, and complement activities among others, is relatively well documented, including a limited number of studies on sea bass (Piccolo *et al*. 2014; Ranjan *et al*. 2014; Abdelmalek *et al*. 2015). However, our understanding of the immune interactions at the mucosal surfaces with regards to pattern recognition receptors, adapter molecules and ultimately cytokine expression is extremely limited in fish, and should be addressed in future studies. Furthermore, an up-regulation in cytokine expression may not necessarily indicate the presence of actual proteins, and hence proteomic approaches would greatly improve our understanding in this area (Rodrigues *et al*. 2012; Almeida *et al*. 2015). There is also a paucity of information on SCFA levels in the intestine of fish, and should also be the focus of future studies. SCFA's are the end products of the fermentation of fibres, and the roles microbes play in this process in fish has been previously documented (Clements *et al*. 1997; Ray *et al*. 2012; Romero *et al*. 2014). However,

more research is required to further our understanding of microbial SCFA metabolism in fish, and furthermore how feed additives impact on this process. Identifying bacterial species in the fish gut through 16S rRNA metagenomics provides important information on their presence and potential, however this approach fails to provide information on the actual roles the microbes play within the community ecosystem. Information acquired using these types of techniques will greatly advance our knowledge, not only with regards to bacterial presence/abundance, but also bacterial functionality, ultimately leading to a better understanding of fish health.

Conclusion

The present research provides novel information on the European sea bass response to soy protein, pea protein and saponins, as well as, the effects various feed additive supplementation has on the growth and health of this species. Chapter 3 revealed an enteritis-like response in the posterior intestine of European sea bass as a consequence of dietary soy and pea protein inclusion, a response which appeared to be amplified with the addition of saponins. Chapters 3, 4A and 5 all revealed the allochthonous microbial communities of European sea bass to consist of a complex bacterial ecosystem. Dietary ingredients appeared to modulate these communities and *B. subtilis* was also observed to populate the intestine and influence the presence of various allochthonous bacterial species. Importantly, the feed additives utilised in the present study generally appeared to confer health benefits to the host fish, and no obvious signs of detrimental effects as a consequence of the feed additives were reported throughout the research programme. Feed additive research in aquaculture remains an interesting topic, one which has presented the potential to improve fish production and

health. However, much more research is still required particularly research relating the mechanisms underpinning feed additive effects in fish.

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