

2014

Investigations on the gut microbiota of salmonids and the applications of probiotics-based feed additives

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<http://hdl.handle.net/10026.1/3089>

<http://dx.doi.org/10.24382/3406>

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**Investigations on the gut microbiota of salmonids
and the applications of probiotics-based feed
additives**

By

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A thesis submitted to the University of Plymouth in partial
fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences
Faculty of Science and Technology

This project funded by Ministry of Higher Education and Scientific Research
(MOHESR) Baghdad, Iraq

July 2014

Investigations on the gut microbiota of salmonids and the applications of probiotics-based feed additives

Ali A. Abid

A series of investigations were conducted in order to characterise the GIT microbiota of salmonids and to determine the effect of microbial modulating feed additives on the intestinal microbiota, immunity and growth of salmonids. The first experiment, Chapter three, used PCR-DGGE and 16S rRNA gene sequence analysis of cultivable bacteria were used to investigate the GIT microbiota of brown trout. 16S rRNA gene sequence analysis demonstrated that *Citrobacter freundii* and *Carnobacterium maltaromaticum* were the predominant culturable viable bacteria and lactic acid bacteria, respectively in all regions of the GIT. DGGE revealed complex communities with a diverse range of microbes from the Firmicutes and Proteobacteria phyla.

The latter chapters focused not only identifying the gut microbiota of salmonids, but also on the ability of probiotics and prebiotics to modulate these communities. In Chapter four, rainbow trout were fed a commercial diet supplemented with *P. acidilactici* for four weeks. *P. acidilactici* was detected in the GIT of the probiotic group by multiple methods and *P. acidilactici* was able to persist for at least 24h at the cessation of probiotic feeding. Histological appraisal on the intestine revealed significantly higher microvilli density in the posterior mucosa and a higher density of goblet cells in the anterior mucosa of the probiotic fed fish. RT-PCR results demonstrated that IL-1 β , IL-8 and IgT gene expression were up-regulated in the *P. acidilactici* fed fish at the end of the study. Whilst mRNA of PCNA, HSP70 and casp-3 were down-regulated in the probiotic group at both sampling points.

In Chapter five, the efficacy of dietary administration of *P. acidilactici* and short chain fructooligosaccharide (scFOS) on Atlantic salmon (*Salmo salar* L.) was evaluated at 63 and 132 days. Compared to the control group, total bacterial cell counts in all regions of the intestine with exception of the anterior digesta were significantly lower in the synbiotic group at the mid sampling point. PCR-DGGE revealed that species richness, diversity and the number of OTUs were significantly higher in the synbiotic group in the anterior digesta at the mid sampling point. Intestinal microvilli and villi length were increased in the anterior intestine of the synbiotic fed group at the end sampling point. IEL levels were increased in the synbiotic group in the posterior intestine at both sampling points. The expression of immunological genes were significantly up-regulated in the synbiotic fed salmon.

In Chapter six, rainbow trout were fed three diets fishmeal (FM), soybean meal (SBM) and PlantMix diets supplemented with or without *P. acidilactici* for 12 weeks. At both sampling points, with exception of fish fed FM, LAB levels were significantly higher in all probiotic groups compared to the control groups. Serum lysozyme activity was significantly higher in fish fed FM and SBM diets containing *P. acidilactici* than that of fish fed the control diets.

This body of research demonstrates that *P. acidilactici* can modulate immune response via up-regulation of immune genes as well as modulate IEL and goblet cell levels. Despite these benefits, *P. acidilactici* had no detrimental effects on growth performance.

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List of abbreviations

Abbreviations used	Glossary of terms used
A.	<i>Aeromonas</i>
AD	Anterior digesta
ADC	Anterior digesta control
ADS	Anterior digesta synbiotic
Al.	<i>Alcaligenes</i>
AM	Anterior mucosa
AMC	Anterior mucosa control
AMS	Anterior mucosa synbiotic
ANOVA	Analysis of variance
ACC	Aerobic colony count
B.	<i>Bacillus</i>
BLAST	Basic local alignment search tool
C.	<i>Citrobacter</i>
Ca.	<i>Carnobacterium</i>
CFU	Colony forming unit
DAPI	4, 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
E.	<i>Enterococcus</i>
EDTA	Ethylene diamine tetra acetic acid
Es.	<i>Escherichia</i>
F.	<i>Flavobacterium</i>
GIT	Gastrointestinal tract
FA	Formamide
FCR	Feed conversion ratio
FISH	Fluorescence <i>in situ</i> hybridisation
FM	Fish meal
Hb	Haemoglobin
IELs	Intra epithelial leucocytes
IL	Interleukin
La.	<i>Lactococcus</i>
LAB	Lactic acid bacteria
Lb.	<i>Lactobacillus</i>
Leu.	<i>Leuconostoc</i>
LM	Light microscopy
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCT	Microcentrifuge tubes
MCV	Mean corpuscular volume
MGW	Molecular grade water
MRS	de Man, Rogosa and Sharp
MS222	Tricaine -methyl sulphonate
NBT	Nitroblue tetrazolium
NCBI	National Centre for Biotechnology Information
NGC	Next generation sequences
OD	Optical density

OTUs	Operational taxonomical unit
P.	<i>Pediococcus</i>
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P.C.V	Percentage packed cell
PD	Posterior digesta
PDC	Posterior digesta control
PDS	Posterior digesta synbiotic
PFA	Paraformaldehyde
PMC	Posterior mucosa control
PMS	Posterior mucosa synbiotic
Ps.	<i>Pseudomonas</i>
qPCR	Quantitative polymerase chain reaction
R ²	Coefficient of correlation
RBC	Red blood cells
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SBM	Soybean meal
SCFAs	Anti-nutritional factors
scFOS	Short chain Fructooligosaccharide
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SGR	Specific growth rate
St.	<i>Streptococcus</i>
TAE	Tris-acetate- EDTA
TEM	Transmission electron microscopy
TGC	Thermal growth coefficient
Tlr	Toll-like receptors
TNF	Tumor necrosis factor
TSA	Tryptone soy agar
V.	<i>Vibrio</i>
W.	<i>Weissella</i>
WBC	White blood cells
xg	Times gravity

Dedication

*This thesis is dedicated to the memory of my parents,
may Almighty Allah bless their souls and grant them
the immortality in paradise.*

Acknowledgements

Firstly, my entire deep thanks to Almighty Allah, who blessed me with the strength, confidence and determination needed for the completion of my research. It is right to say that without doubt nothing in my life could have been accomplished without his support.

It is my pleasure to express sincere thanks to my Director of Studies, Dr. Daniel Merrifield for his guidance, help, patience, eternal optimism and many ideas. I would like to thank my supervisory committee members Dr. Graham Bradley, Prof. Simon Davies and Dr. Paul Waines for continuing to assist, support and guide me throughout my research degree.

I would like to acknowledge the Ministry of Higher Education and Scientific research in Iraq for the award of a scholarship that provided the financial support for this research project. I would like to thank the society of applied microbiology (SfAM) for awarding me the traveling student and the President's fund grants to introduce my work in Prague, Czech Republic 2012.

I would like to express my sincere gratitude to all people who have helped me during the completion of this project.

I am very thankful to all the laboratory technicians from the 3rd floor of the Davy building, the food nutrition lab and the aquarium in particular to Sarah Jamieson, Andy Atfield, Liz Preston, Ben Eynon, William Vevers and Matthew Emery for their assistance.

I would also like to extend my thanks to all my colleagues in the Aquatic Animal Nutrition and Health Research Group, who answered my request for help. I greatly appreciate the assistance and support from Prof. Waleed Al-Murrani and Dr. Wondwossen Abate Woldie. I am thankful for all Iraqi students in particular Raid Nasir, Kasim Abaas, Khwam Hussein, Husam Aljwaid and Nabil Ali.

I would like to thank Dr. Mathieu Castex (Lallemand) for his support and the provision of the Bactocell used in this series of studies.

My gratitude goes to my brothers, sisters, relatives and friends in Iraq for all their unconditional love and support that they have always given me.

Finally, I am extremely grateful to my wife for her encouragement, support and patience and my lovely son Hussain for his prayers and Godspeed to me for passing this study successfully.

My apologies if I missed anyone.

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 without prior agreement of the Graduate Committee. This study was fully funded by the Ministry of Higher Education and Scientific Research of Republic of Iraq (MOHESR).

Publications

- ❖ Abid, A., Davies, S., Waines, P., Emery, M., Castex, M., Gioacchini, G., Carnevali, O., Bickerdike, R., Romero, J., Merrifield, D., 2013. Dietary synbiotic application modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity. *Fish & Shellfish Immunology* **35**, 1948-1956.
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Presentations and conferences attended

Oral presentations

- Influence of dietary *Pediococcus acidilactici* on health and the microbial communities of rainbow trout (*Oncorhynchus mykiss* walbaum). NEMO conference. 10th -11th July 2012, University of Keele, UK.
- Microbial community within the intestinal mucosa of brown trout (*Salmo trutta*). CARS Postgraduate Symposium. 10th December 2012, University of Plymouth, UK.
- Microbial community within the intestinal mucosa of brown trout (*Salmo trutta*). Seminar, 10th December 2012, University of Plymouth, UK.

- Presentation in Learning and Teaching for General teaching Associates (GTA Course) 15th April 2011, Plymouth University, UK.
- Presentation of MPhil project April 9, 2011 in School of Biomedical and Biological Sciences, Plymouth University (Module Bio 5124).
- Presentation of Laboratory based teaching methods (Module ENV 5101) 18th December 2010 in School of Biomedical and Biological Sciences, Plymouth University.

Poster presentations

- ❖ Presentation for the 3rd Annual Marine Institute Conference: Spirit of Discovery, 20th December 2010, University of Plymouth, UK.
- ❖ Presentation for Rhodes conference, 18th-21st October 2011, Greece.
- ❖ Presentation for Society of Applied Microbiology conference, 1st – 5th July 2012, Edinburgh, UK.
- ❖ Presentation for AQUA, Global Aquaculture Securing Our Future conference, 1st- 5th September 2012, Prague, Czech Republic. Grant (£700) from Society of Applied Microbiology
- ❖ Presentation for CARS conference, 19th July 2013, Duchy College, UK. (Prize for best poster).
- ❖ Attendance of Next Generation Sequences conference, 4th July 2013, University of Plymouth, UK.
- ❖ Presentation for the International Symposium on Fish Nutrition and Feeding, 25-30 May 2014 Cairns, Queensland, Australia.
- ❖ Presentation for CARS conference, 6th June 2014, BBSRC North Wyke experimental station - Roth Amsted Research, UK.

The postgraduate society short conference series in University of Plymouth (poster presentations)

- Postgraduate Society Short Conference 14th March, 2012.
- Postgraduate Society Short Conference 26th June, 2012.
- Postgraduate Society Short Conference 11th March, 2013.
- Postgraduate Society Short Conference 17th June, 2014.

Word count of main body of thesis: 61.277

Chapter 1

Literature review

Chapter 1: Literature review

1.1 Introduction

Farming of aquatic animals continues to be one of the fastest growing food producing sectors world-wide. Aquaculture has grown more rapidly than other food-production industries in recent years, becoming a significant source of protein and high quality lipid biomass for human nutrition in a time when many wild fish stocks are declining.

According to the Food and Agriculture Organization (FAO) of the United Nations, world aquaculture production in 2006 was dominated by fresh water fish (54%) in comparison with low marine fish production (3%) (FAO, 2010). Aquaculture production in 2009 stood at 55.1 million tonnes contributing to 38% of the total world production of sea food (145.1 million tonnes) with a total value of US \$ 106 billion and the contribution of aquaculture will account for over 50% of the global fish supply by 2020 if this sector continues to expand at the present rate (FAO, 2013).

The global population will grow from the current 6.8 billion people to approximately 9 billion by 2050 (Garcia and Rosenberg, 2010). The growing need for nutritious and healthy food will increase the demand for fish products from marine sources. Consequently, to meet the demand for aquaculture products the evolution of fish farming has moved towards higher yields and faster production for maximum profitability. Aquatic animal production has significant economic importance and it is increasing rapidly in many countries around the world. As a result, particular species of fish such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), Nile

tilapia (*Oreochromis niloticus*) and carp (*Cyprinus carpio*) are major aquaculture species of high commercial importance (Hulata, 2001). Fish are a good source of vitamins, essential minerals (such as calcium, iron and phosphorus), polyunsaturated fatty acids, trace elements, iodine and high quality proteins with a healthy ratio of amino acids (Leroi, 2010).

The deleterious effects of inadequate nutrition and disease, which include effects of viral, protozoan and bacterial parasites are the two largest external biotic factors influencing the growth of vertebrates (Rintamaki-Kinnunen *et al.*, 2005). The profitability of aquaculture operations depends on the ability of nutritionists to formulate diets that provide all essential nutrients. At present, the greatest influence on the profitability of an aquaculture facility is the incidence and severity of diseases and deformities. Disease reduces profitability through a reduction in the numbers of fish reaching harvest size. Therapeutic agents have been used to ensure good conditions for production and further development of aquaculture by protecting fish from a variety of diseases, especially in early stages of their life (Grześkowiak *et al.*, 2011).

Traditionally, the use of disinfectants, antibiotic drugs (Hu *et al.*, 2007) and vaccinations (Vandenberg, 2004), have partially solved these problems; however, their inappropriate use has undesirable side effects. Consequently, to increase productivity of aquaculture safely more must be done to protect fish from a variety of diseases, particularly in the early stages of their lives. Therefore, it is well accepted that exploring new approaches involving biotechnology and microbiology is a high priority. One such approach that is

gaining acceptance within the aquaculture industry is the use of bio-controlling agents such as probiotics (Vázquez *et al.*, 2005).

1.2 Fish diseases

Although the aquaculture industry worldwide has been steadily growing, disease outbreaks caused by bacteria in farming are still a serious problem (Kitamura *et al.*, 2007). A generally accepted definition of disease is “any definitive morbid condition or process that has a characteristic set of symptoms or qualities” and it is well documented that stressful conditions contribute to many disease outbreaks (Groff and Lapatra, 2000). Animals including fish have complex communities of mutualistic, pathogenic and commensal bacteria in their gastrointestinal tract (GIT) and the overall health and disease outbreak can be significantly influenced by these bacteria. Thus, if the balance between the endogenous microbiota and the control mechanism of the host is disrupted, several opportunistic bacteria or transient pathogens can cause infection (Nayak, 2010b; Gómez *et al.*, 2013).

Fish diseases are caused by many pathogenic agents, e.g. bacterial, fungal, viral and parasitic pathogens (Tacchi *et al.*, 2011), since fish are in intimate contact with their environment. It is known that many of these microorganism are saprophytic i.e. fungi/ bacteria that are capable of living and feeding on non-living organic matter, whilst some are pathogenic but both have an ability to digest and degrade the tissues of fish when afforded an opportunity (Ellis, 2001).

Generally, pathogenic bacteria take one or more of the following routes to enter the host: skin, gills or GIT (Ringø *et al.*, 2007). Furthermore, the

appearance and progression of disease is influenced by host response, environment, life span and bacterial strain variations (Gómez and Balcázar, 2008). The first step of establishing diseases in fish intestines is the ability of pathogenic bacteria to colonize the intestinal mucus layer where they can damage the intestinal lining by releasing extracellular enzymes or toxins, which thereby causes a severe infection in fish (Ringø *et al.*, 2004).

However, under normal environmental conditions fish have a robust immune system and the intestinal mucosal layer covering the physical epithelial barrier which provides protection against pathogenic microorganisms, forming a first line of defence (Verschuere *et al.*, 2000; Ellis, 2001; Tacchi *et al.*, 2011); pathogens in the mucus layer are prevented from reaching the tissues by entrapment and removal by biologically active molecules such as antibacterial peptides, lysozyme, proteases and lectins (Gómez and Balcázar, 2008).

In this regard, the dermis, epidermis and scales also act as obstacles to disease-causing microorganisms (Gómez *et al.*, 2013; Lazado and Caipang, 2014). Furthermore, the GIT of fish could be considered as a hostile environment for many pathogenic microorganisms due to the existence of bile salts, acids and enzymes (Gómez and Balcázar, 2008). In addition, the microbiota of the GIT has an ability to produce a wide range of inhibitory compounds which are reported to be effective against a wide range of pathogens. For example, Robertson *et al.* (2000) reported that the growth of several fish pathogens including *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, *Photobacterium damsela*,

Vibrio anguillarum, *Vibrio ordalii* and *Streptococcus milleri* were inhibited *in vitro* by *Carnobacterium* sp. isolated from the GIT of Atlantic salmon. Pérez-Sánchez *et al.* (2011a) demonstrated that the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc* isolated from rainbow trout revealed *in vitro* antagonistic activity against pathogenic *Lactococcus garvieae*.

If pathogenic microorganisms are able to overcome these primary barriers of defence and the endogenous commensal bacteria, humoral and cellular non-specific immunity are stimulated in order to prevent and suppress a further spread of the infection. Ultimately, a microorganism must evade or cope with these defences in order to adhere to and multiply in the host tissues (Ellis, 2001). Fish in commercial farming conditions are often confronted with stress conditions such as high stocking densities and sometimes poor farming conditions that lead to diminished immune activity of fish, thereby increasing susceptibility to pathogens (Sakai, 1999; Groff and Lapatra, 2000). Indeed, pathogenic organisms have been reported to contribute large economic losses particularly during the early production stages, and are considered one of the major limiting factors in aquaculture (Kitamura *et al.*, 2007; Kiron, 2012; Heo *et al.*, 2013). The losses are summarized by Bennett (2003) as losses of production, a reduction in output quality and losses in imports, which could potentially lead to international trade restrictions due to disease and its control. Bacteria have a broad variety of virulence factors including haemolysins, cytotoxins, enterotoxins, endotoxins and extracellular enzymes (Chopra *et al.*, 2000), which give them the ability to survive inside the host and evade the immune system (Ellis, 2001). The main bacterial diseases and their causative agents are listed in Table 1.1.

Table 1.1 Important bacterial diseases and their causative agents in aquatic environments. Adapted from Austin and Austin (2007); Vendrell *et al.* (2008); Ringø *et al.* (2010a) and Tafalla *et al.* (2013).

Genus and species of bacteria	Disease (s)	Major fish species affected
<i>Aeromonas salmonicida</i>	Furunculosis	Several major families of Osteichthys, including Cyprinidae (carp and goldfish), Serranidae, Anoplopomatidae
<i>Aeromonas salmonicida</i>	Cutaneous ulcerative disease	Goldfish
<i>Vibrio (Listonella) anguillarum</i>	Vibriosis	Pacific salmon and turbot
<i>Vibrio (Aliivibrio) salmonicida</i>	Cold-water vibriosis	Atlantic salmon
<i>Streptococcus iniae</i>	Streptococcosis	Rainbow trout, tilapia and rabbitfish
<i>Lactococcus garvieae</i>	Lactococcosis	Rainbow trout
<i>Flavobacterium psychrophilum</i>	Coldwater disease (CWD) or peduncle disease	Fresh water fish, including rainbow trout, carp and goldfish
<i>Flavobacterium columnare</i>	Columnaris	Freshwater fish, including Arctic charr, bass, carp, rainbow trout, and Atlantic salmon
<i>Renibacterium salmoninarum</i>	Bacterial kidney disease	Rainbow trout, brown trout, and brook trout
<i>Yersinia ruckeri</i>	Red mouth disease or yersiniosis	Atlantic salmon, Pacific salmon, rainbow trout, brown trout, brook trout and channel catfish
<i>Mycobacterium</i> spp.	Piscine mycobacteriosis	Atlantic menhaden, rockfish, shortfin molly, striped bass, turbot and zebrafish
<i>Moritella viscosa</i>	Winter ulcer	Salmonids and cod
<i>Pasteurella piscicida</i> also named <i>Photobacterium damsela</i> subsp. <i>piscicida</i>	Pasteurellosis	Many species of wild and farmed fish in Asia, USA and Europe

1.3 The teleost immune system

Like other organisms, fish are constantly interacting with their environment, which may potentially contain pathogenic microorganisms. When microbes invade the host, cellular and humoral innate defence mechanisms including cytokines, the antioxidant defences, acute phase proteins or the cellular responses are activated (Kiron, 2012). The teleost immune system may be categorized into two types, these being 'innate' and 'acquired', as presented in Figure 1.1.

The innate immune system is further divided into two parts - cellular and humoral immunity. Antimicrobial peptides, complement components, lysozyme, pentraxins, transferrin, antiproteases, lectins and natural antibodies constitute the innate humoral effectors, whereas the innate cellular immune parameters are mediated by nonspecific cytotoxic cells and phagocytes (monocytes/ macrophages and neutrophils) (Magnadóttir, 2006; Gómez and Balcázar, 2008). Fish have a low specificity immune system with a shorter response, a limited antibody production (IgT and IgM) and a weak memory (Trichet, 2010).

The innate immune response is activated through target molecules including lipopolysaccharides (LPS), peptidoglycans, bacterial DNA and double-stranded viral RNA that are so-called pathogen-associated molecular patterns (PAMP) to microbes, which induce the immune response against infections of fish (Ringø *et al.*, 2012).

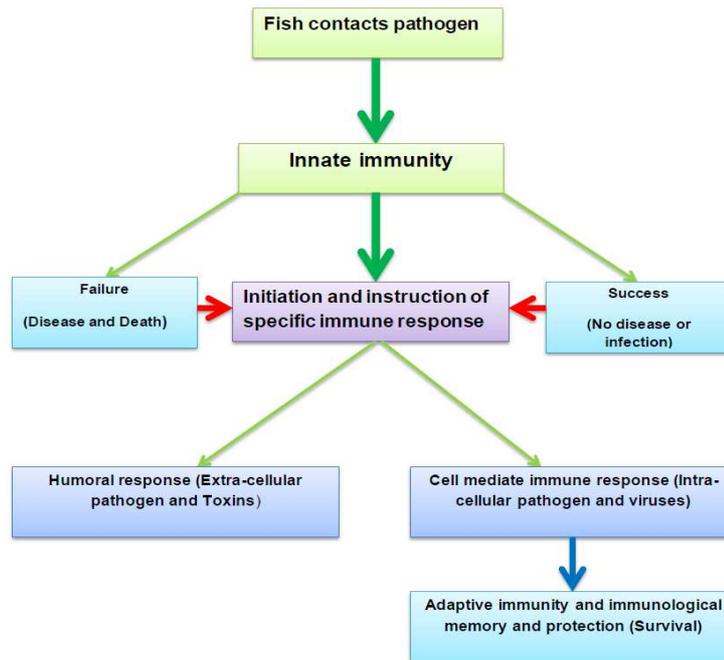


Figure 1.1 Schematic representation of immune response of fish following an encounter with a pathogen. Adapted from Shoemaker *et al.* (2001).

Mucus, which is secreted by goblet cells in the epithelial barriers including skin and the gut, plays a crucial role in preventing pathogenic microorganism from reaching the tissues due to several components that it contains: immunoglobulin, lysozyme, transferrin, complement system, antimicrobial peptides etc. (Trichet, 2010; Lazado and Caipang, 2014).

Adaptive (or acquired) immunity is comprised of two parts - humoral and cellular immunity. Immunoglobulins, which are produced by B-cells constitute the adaptive humoral effectors, whereas the cellular adaptive immune parameters are mediated by T-lymphocytes and B-cells (Denev *et al.*, 2009). Magnadóttir (2006) reported that a variety of external and internal factors, including temperature fluctuation, handling and crowding stress may negatively affect the activity of the innate immune system, whereas prebiotics, probiotics and immunostimulants can improve its activity. Additionally,

Shoemaker *et al.* (2001) reported that innate and acquired immune profiles may be influenced by factors such as the size, age and weight of the fish themselves.

Lysozyme is a component of the humoral innate immune system. It is a cationic enzyme with the ability to destroy the cell walls of many Gram-positive bacteria by breaking the glycosidic bonds between N-acetylmuramic acid and N-acetylglucose-amine in the peptidoglycan cell wall. It is also active against fungal cell walls and some strains of Gram-negative bacteria (Ellis, 2001; Villa and Crespo, 2010). Although enzymatic damage of peptidoglycan represents the main mode of antibacterial activity of lysozyme, it is well accepted that bacteria could be killed by non-enzymatic techniques (Villa and Crespo, 2010). This non-enzymatic activity can be categorized into two types. Firstly, in the lytic mode, as lysozyme is cationic, it can activate bacterial autolytic enzymes (autolysins). Secondly, in the non-lytic mode, the cell is killed via disruption of the peptidoglycan portion of the membrane, ultimately leading to cell lysis (Fischetti, 2008). In fact, it is possible that non-enzymatic activity destroys more bacteria compared with the enzymatic split of peptidoglycan (Masschalck and Michiels, 2003).

In general, only Gram-positive bacteria are killed by lysozyme, whereas Gram-negative bacteria are generally protected against lysozyme's enzymatic activity by their external membranes (Villa and Crespo, 2010). However, lysozyme has some developmental modifications, which increase its ability to kill Gram-negative bacteria (Ibrahim *et al.*, 2002), by facilitating the movement of molecules through the outer membrane (Masschalck and

Michiels, 2003). It is considered that the physiology of fish, environmental condition, infections and levels of stressors all affect the activity of lysozyme (Saurabh and Sahoo, 2008).

Cellular components of the innate immune system such as neutrophils, monocytes and macrophages play an important role in the defence against bacteria, viruses, and parasites in fish and shellfish (Harikrishnan *et al.*, 2011). Phagocytosis activity has been reported to be the second line of immunity and that phagocytic cells will proliferate quickly to deactivate pathogenic microorganisms (Trichet, 2010).

Granulocytes, monocytes/macrophages and natural cytotoxic cells constitute the cellular part of the innate immune system in fish. Natural cytotoxic cells are responsible for fighting viral infections; their receptors distinguish proteins presented at the surface of viral particles (Trichet, 2010). Granulocytes are classified into three distinctive types, depending on their morphology: neutrophils, eosinophils and basophils. Neutrophils exist in the circulation in low numbers, but under activation conditions as a result of presence of cytokines, specific activation molecules or other bacterial components, neutrophils migrate toward the source of inflammation where the infection occurred whether in the circulation system or in the tissues (Suzuki and Iida, 1992). Eosinophils are ordinarily less numerous than neutrophils, but the former can be appear in greater levels as a result of presence of parasitic infections (Beutler, 2004; Alvarez-Pellitero, 2008). It has been reported that phagocytotic cells, including macrophages and neutrophils are able to engulf microbes and kill them using enzymes and reactive oxygen species (ROS),

especially, hydroxyl radicals, singlet oxygen, oxygen halides and hydrogen peroxide during the respiratory burst activity (Ellis, 2001). On the other hand, two types of cells are responsible for the function of the adaptive immune system: the B-lymphocytes and the T-lymphocytes, these are considered the third line of immune system (Trichet, 2010). B-lymphocytes are designed to produce a specific antibody on their cell surface membrane while antigens detected by macrophages stimulate T-lymphocytes, thus they provoke B-lymphocytes to produce specific antibodies against the determined antigen.

Cytokines are proteins, which are related to both the specific and non-specific immune response. Immune cells in particular produce numerous cytokines, including groups of interleukin, interferon, tumor necrosis factor, transforming growth factor- β and various chemokines (Gómez and Balcázar, 2008). Cytokines and chemokines are secreted by leucocytes (in particular neutrophils) in response to microbial antigens or substances released from injured cells, and act as intracellular signals to regulate the components of the innate and adaptive immune response (Magnadóttir, 2006). Recently, many investigators have been able to use the mRNA expression of cytokine genes in response to probiotic bacteria as a technique for measuring immune responses (Kim and Austin, 2006a; Panigrahi *et al.*, 2007; Mansfield *et al.*, 2010; Pérez-Sánchez *et al.*, 2011b). The gut associated lymphoid tissue (GALT), acts as a barrier to the entry of pathogens and contains leucocyte populations which are exposed to the external environment. GALT in teleost fish lacks specialized structures such as the Peyer's patches of mammals but the gut contains populations of leucocytes, including macrophages,

lymphocytes, mast cells, granulocytes and plasma cells (Pérez *et al.*, 2010). The immune cells are intensively present in the posterior intestine of fish, which act to be an important antigen presenting cells (APCs); IgT, a specialised mucosal antibody which is an isotype of mammalian IgA, is also present in the gut mucus of fish (Rauta *et al.*, 2012). Commensal bacteria are also present in the GALT, which are implicated in the induction of local immune responses (Pérez *et al.*, 2010). Figure 1.2 represents the structure of the gut mucosa surface with different profiles of both innate and adaptive immune systems.

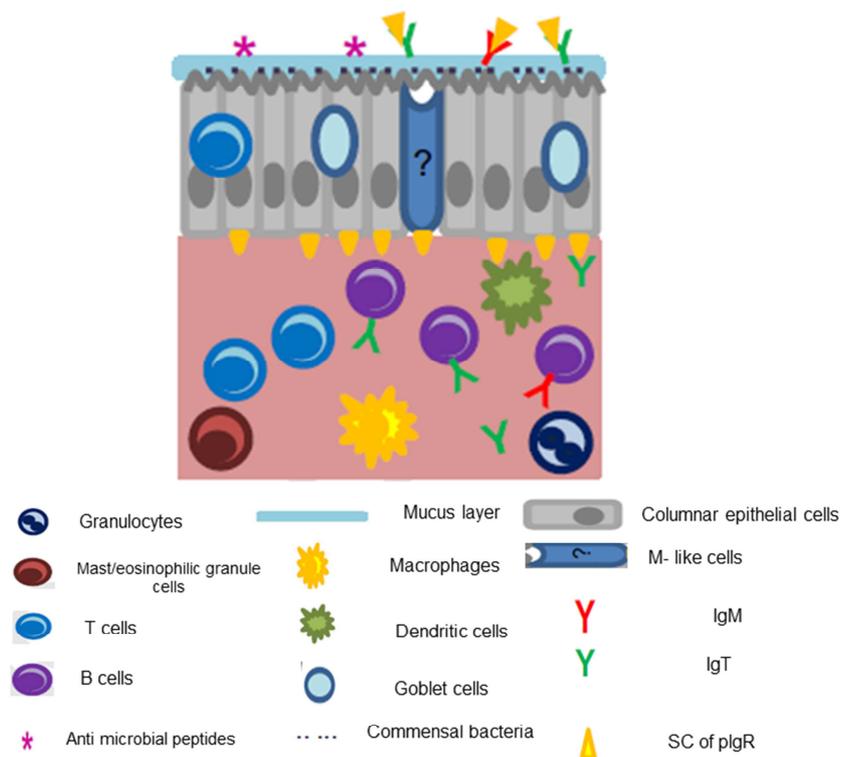


Figure 1.2 Representation of intestinal mucosal surfaces in the teleost fish. The cellular components of the innate immune system (dendritic cells, macrophages, granulocytes and mast cells, the location of B and T cells and the secretory components (SC) of the polymeric immunoglobulin receptor (pIgR) are displayed. Commensal bacteria and antimicrobial peptides (AMPs) are shown in the outer mucosal surface. Adapted from Gómez *et al.* (2013).

1.4 Fish diseases treatment

Disease outbreaks in worldwide aquaculture have increased as a result of increased fish production. Disease arising from these sources can be dealt with effectively through simple husbandry techniques, through meeting the nutritional requirements and by good management of the water and the environment (Maqsood *et al.*, 2011; Kiron, 2012). Imbalances in nutrition during the early stages of a fish's life are likely to influence growth, survival and host protection against disease. Nutritional balance plays a crucial role in maintaining fish resistance against disease and reducing disease outbreaks (Kiron, 2012). Infectious diseases often require treatment with some form of a therapeutic agent. Many viral and bacterial infections are controlled by the use of disinfectants such as malachite green and hydrogen peroxide or vaccination, antibiotics or other chemotherapeutics (Vandenberg, 2004; Ridha and Azad, 2012).

1.4.1 Antibiotics

The antibiotics amoxicillin, erythromycin, terramycin (oxytetracycline) or sulfadimethoxine have been intensively used in fish and shellfish farming (Maqsood *et al.*, 2011). In addition to deleterious effects that these agents may have on the user, many common treatments have considerable deleterious side effects on fish and the environment. Not only pathogenic bacteria are targeted by antibiotics, commensal bacteria are also affected. Furthermore, the misuse of antibiotics carries serious risks such as the increase of antibiotic resistance in marine and aquatic microbes, which may include pathogens (Yang *et al.*, 2012). Infected fish are predominantly treated with antibiotics via their food and approximately 70 - 80% of these antibiotics

will be released into the aquatic environment by urinary food waste expulsion (Hu *et al.*, 2007; Panigrahi and Azad, 2007).

Additionally, the presence of residual antibiotics in aquaculture products could result in the consumption of antibiotics by humans (Panigrahi and Azad, 2007; Yousefian and Amiri, 2009; Romero *et al.*, 2012). Low level accumulation of antibiotics and chemicals in fish tissues (Maqsood *et al.*, 2011) may lead to health problems in consumers including allergies and toxic effects (Smith *et al.*, 1994). Disruption of immune function in carp (*Cyprinus carpio*), rainbow trout, Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) were reported after treatment of these fish by oxytetracycline (Romero *et al.*, 2012). Therefore, farmed fish and shellfish that are treated with antibiotics and chemicals have been refused import permits by numerous countries in order to decrease these effects (Panigrahi and Azad, 2007; Harikrishnan *et al.*, 2011).

1.4.2 Vaccines

Another method of disease control is vaccination, which has been proven to be effective at controlling infectious diseases of farmed fish such as vibriosis, enteric red mouth disease and furunculosis (Maqsood *et al.*, 2011), but is considered as a prophylactic agent for endemic disease (Romero *et al.*, 2012), and may be a key part of future sustainable aquaculture schemes. Vaccines, which may be defined as “preparation of antigens derived from pathogenic organisms, rendered non-pathogenic by various means, which will stimulate the immune system in such a way as to increase the resistance to disease from subsequent infection by a pathogen” (Ellis, 1988), are

generally given as a preventative measure or in the early stages of many diseases (Home *et al.*, 2009; Yousefian and Amiri, 2009); for which they are usually delivered via three different ways including injection, immersion or oral vaccination and each of these approaches has advantages and disadvantages (Gudmundsdóttir and Björnsdóttir, 2007; Rombout *et al.*, 2011; Gómez *et al.*, 2013). For example, vaccination by injection generates stress whereas immersion vaccination is less stressful but it is less effective and results in a shorter period of immunity (Gudmundsdóttir and Björnsdóttir, 2007; Heo *et al.*, 2013). Additionally, vaccination by injection may cause growth reduction, changes in fish flesh quality or possible contamination of vaccine solutions and instruments (Press and Lillehaug, 1995; Ellis, 1997). During hatching and in the larval stage, the small size of the fish and the slow development of the immune system make it difficult to use vaccines.

Although oral vaccination is the most acceptable method of immunisation (it is often cheaper and less stressful than injection and immersion vaccination), vaccines are often damaged in the GIT of fish before reaching the immune sensitive areas (Gudmundsdóttir and Björnsdóttir, 2007). In addition, many vaccines induce various side-effects in different fish species and may be very costly (Yousefian and Amiri, 2009).

It can be useful to incorporate oil-based adjuvants or bioencapsulation through live food organisms, with vaccines, which may result in improving their efficiency (Panigrahi and Azad, 2007; Maqsood *et al.*, 2011). Adjuvants are defined as “helper substances that increase the magnitude of an adaptive response to a vaccine (potency), or ability to prevent infection and death (efficacy)”(Tafalla *et al.*, 2013).

1.4.3 Immunostimulants

Immunostimulants are substances have the ability to increase the nonspecific immuno profiles, which contribute to protection against disease outbreaks in fish and shellfish (Sakai, 1999; Harikrishnan *et al.*, 2011; Tacchi *et al.*, 2011). In addition, growth performance and enhanced quality of fish meat have been reported to be advantages of immunostimulation in aquaculture (Merrifield *et al.*, 2010c). Administration of immunostimulants such as intact microbes, lactoferrin, glucan, chitin, levamisole, numerous compounds from plant and animals, synthetic chemical agents, microbial cell components and nutritional factors such as vitamins B and C, and growth hormone have been used in aquaculture to control infectious diseases (Sakai, 1999; Harikrishnan *et al.*, 2011; Maqsood *et al.*, 2011; Kiron, 2012). Yeast and fungal cells are a rich source of immunostimulant compounds. Chitin and chitosan extracted from various fungal sources are reported to be immunostimulants (Sakai, 1999). The same author demonstrated that bacteria are found to be a rich source of immunostimulators or immunostimulation compounds, including lipopolysaccharide (LPS) and peptidoglycans.

The efficacy of immunostimulants is affected by many factors, including dosage, duration, the physiological status of fish and delivery method (Harikrishnan *et al.*, 2011), therefore, their degree of success may differ (Tacchi *et al.*, 2011; De *et al.*, 2014). However, administration of immunostimulants into the host through the diet typically can generate a strong response if the material remains unaltered by stomach acid or digestive enzymes, and immunostimulants which are given in this route directly increase protection against disease by promoting the innate immune

system (Sakai, 1999; Tacchi *et al.*, 2011). Injection, as a route of immunostimulant delivery, is time-consuming, labour intensive and not appropriate for juvenile and larval fish (Harikrishnan *et al.*, 2011), while the immersion method generally has no stress, requiring minimal handling. Immune profiles stimulated by immunostimulants via the immersion route may be affected by stocking density and duration of exposure (Anderson *et al.*, 1995).

In addition, it has been quoted that vitamins are micronutrients that are referred to as a kind of immunostimulants having a fundamental function in all microorganisms metabolism (Irianto and Austin, 2002a; Tacchi *et al.*, 2011).

1.5 Microbial populations in the gastrointestinal tract (GIT) of fish

The GIT microbiota is a complex and dynamic microbial ecosystem in the GIT of an animal (Nayak, 2010b). By virtue of the fact that rearing water contains abundant microbial communities, aquatic animals, including fish, are in more intimate contact with external microbes than terrestrial animals (Denev *et al.*, 2009). Therefore, the GIT microbiota of fish has received much attention in recent years, as the importance of their interactions with the host at the intestinal mucosa has become increasingly more apparent (Merrifield *et al.*, 2010c).

It is well documented that the composition and quantity of the fish gut microbiota differs between fish species and during progression through life stages (Spanggaard *et al.*, 2000; Ringø *et al.*, 2003). In particular, the

structure of the GIT influences the composition of the GIT microbiota since the gut differs in morphology between fish species and between different life stages in the same species (Ringø and Birkbeck, 1999). Due to the fact that the GIT contains different physico-chemical conditions (e.g. concentrations of acids, bile salts and enzymes) in the various gastric regions (i.e. oesophagus stomach, pyloric caeca, intestine) (Ringø *et al.*, 2003; Gómez and Balcázar, 2008), there are also likely to be variations in the composition and density of the microbial communities among different regions (Nayak, 2010b). In addition, a number of environmental factors, including the levels of dissolved oxygen, temperature, salinity, feed and stress (Ringø and Birkbeck, 1999; Leroi, 2010) may affect the gut microbiota (refer to Table 1.2).

The microbiota of fish consists of aerobic, facultative anaerobic and potentially obligate anaerobic bacteria (Nayak, 2010b), and Gram-negative bacteria have been observed to be numerically dominate over Gram-positive bacteria in the intestine of numerous fish groups (Ringø *et al.*, 1995), with *Aeromonas*, *Pseudomonas*, *Clostridium* and *Bacteroides* are predominating in the microbiota of fish (Ganguly and Prasad, 2012). Additionally, the intestinal microbiota is classified according to whether they are autochthonous or allochthonous. The former group has the ability to colonize the mucus layer and/or the epithelial surface of the gut of the host, whilst the latter group tend to remain within the GIT lumen temporarily without colonizing it (Ringø *et al.*, 2003).

It is of further interest to state that autochthonous bacteria are able to compete with and prevent the colonization of enteric bacteria, and represent

part of the first line of defence against pathogenic bacterial invasion (Denev *et al.*, 2009). Gram-negative, facultative anaerobic bacteria including genera of *Acinetobacter*, *Alteromonas*, *Micrococcus*, *Moraxella*, *Aeromonas*, *Pseudomonas*, *Vibrio*, *Achromobacter*, *Corynebacterium*, *Flavobacterium*, *Bacteroides* and *Enterobacter* constitute the resident microbiota of numerous species of marine fish (Vázquez *et al.*, 2005). In contrast to marine fish, the endogenous microbiota of freshwater fish species are dominated by members of the genera *Aeromonas*, *Flavobacterium*, *Pseudomonas*, *Lactobacillus*, *Acinetobacter*, *Bacillus* and obligate anaerobic bacteria of the genera *Bacteroides*, *Clostridium* and *Fusobacterium* (Ringø *et al.*, 1995; Huber *et al.*, 2004; Hovda *et al.*, 2007; Kim *et al.*, 2007).

The microbiota of the GIT in fish have been widely investigated during recent years using a combination of culture-dependent and culture-independent techniques (Spanggaard *et al.*, 2000; Ringø *et al.*, 2006b; Hovda *et al.*, 2007; Kim *et al.*, 2007; Merrifield *et al.*, 2009a). Both cultivation and molecular techniques have been incorporated into several previous studies to assess the microbiota of the GIT of farmed trout (Huber *et al.*, 2004; Hovda *et al.*, 2007; Merrifield *et al.*, 2009a; Svanevik and Lunestad, 2011); these studies reported the presence of bacteria and yeasts in multiple regions of the GIT of rainbow trout. The bacterial communities were reported to be dominated by Enterobacteriaceae, Vibrionaceae, *Acinetobacter*, *Moraxella* and *Pseudomonas*, and yeasts are dominant component (Spanggaard *et al.*, 2001).

Yeasts have been isolated from the GIT of salmonids (Spanggaard *et al.*, 2000; Merrifield *et al.*, 2009a; Raggi *et al.*, 2014) and are widely distributed in different environments when appropriate sources of organic substrates are available (Raggi *et al.*, 2014). *Metschnikowia zobellii*, *Trichosporon cutaneum*, and *Candida tropicalis* have been observed to be the dominant GIT yeast species in marine fish (Gatesoupe, 2007), while *Rhodotorula* sp. was dominant in the GIT of both freshwater and marine fish (Raggi *et al.*, 2014).

Yeasts have an ability to colonize the intestine of turbot and rainbow trout (Andlid *et al.*, 1995), producing extracellular proteases and siderophores that are likely to lead to antagonism against pathogenic bacteria (Gatesoupe, 2007). Yeasts may be antagonistic to pathogenic bacteria and fungi (Schmitt and Breinig, 2002), and have been reported to stimulate of the immune response of the host, thus, they may play an important protective role against diseases (Siwicki *et al.*, 1994). In contrast, other genera, including *Trichosporon* sp., *Sporobolomyces salmonicolor* and *Cryptococcus* spp. have been reported to cause disease in fish (Gatesoupe, 2007).

Table 1.2 Overview of the main factors, and examples of relevant studies, reported to affect fish GIT microbiota.

Factors		Experimental details	Observations	References
Diet	Protein	SBM supplementation diet for Atlantic salmon	A significant decrease in bacteria numbers were found	Bakke-McKellep <i>et al.</i> (2007)
		Incorporation of dietary SBM in diets for rainbow trout	The bacterial numbers increased at the end of the trail	Heikkinen <i>et al.</i> (2006)
		Incorporation of dietary SBM in diets for rainbow trout	The total viable count was not influenced by the SBM	Merrifield <i>et al.</i> (2009b)
	Lipid	Incorporation of dietary high (27%) and low (13%) dietary lipid levels for Arctic charr	Changes in <i>Carnobacterium</i> populations	Ringø and Birkbeck (1999)
		Arctic charr fed diets containing soya bean, linseed and marine oils	Changes in <i>Carnobacterium</i> populations	Ringø <i>et al.</i> (2002)
Probiotics	<i>Pediococcus acidilactici</i>	<i>P. acidilactici</i> supplementation diet for tilapia	The ACC was not influenced, LAB levels were significantly affected	Standen <i>et al.</i> (2013)
	<i>Pediococcus acidilactici</i>	<i>P. acidilactici</i> supplementation diet for rainbow trout larvae for 56 days	Differences in microbiota compared to the control group	Ramos <i>et al.</i> (2013)
	<i>Pediococcus acidilactici</i> and <i>Lactobacillus casei</i>	Probiotics supplementation diet for sea bass larvae	The bacterial counts and LAB levels were not significantly affected	Lamari <i>et al.</i> (2013)
	<i>Bacillus</i> sp., <i>Pediococcus</i> sp., <i>Enterococcus</i> sp. and <i>Lactobacillus</i> sp.	Probiotics supplementation diet for rainbow trout larvae for 56 days	Differences in microbiota compared to the control group	Ramos <i>et al.</i> (2013)
Prebiotics	Chitin	Atlantic salmon fed diets with or without chitin	The ACC of adherent microbiota was not influenced by chitin	Askarian <i>et al.</i> (2012)
	Fructooligosaccharides	Hybrid tilapia fed diets FOS or yeast	Microbiota was affected by FOS	Zhou <i>et al.</i> (2009)
Synbiotics	<i>Bacillus</i> spp. and mannanoligosaccharide (MOS)	European lobster larvae fed diet diets supplemented with <i>Bacillus</i> spp. and (MOS)	No changes in ACC	Daniels <i>et al.</i> (2010)
	Inulin and <i>Weissella cibaria</i>	Hybrid surubins fed diets supplemented with <i>Weissella cibaria</i> and inulin	LAB were significantly higher compared to the control group	Mourino <i>et al.</i> (2012)

Stress	Handling and Starvations	Excessive handling stress and starvation on the LAB associated with the digestive tract of Atlantic salmon (<i>Salmo salar</i> L.)	No changes in ACC and <i>C. piscicola</i> populations associated with the GIT	Ringø <i>et al.</i> (2000)
	Overcrowding	Arctic charr were reared in overcrowding	Changes in adherent bacteria were found between low-ranking individuals and high-ranking individuals	Ringø <i>et al.</i> (1997)
Immuno-modulatory	Immunostimulants	Mirror carp were fed diet supplemented with β -glucan for four weeks	The ACC and LAB were not affected. LAB levels were reduced after week 4	Kühlwein <i>et al.</i> (2013)
	Vaccine /IP	Larvae of Atlantic cod were fed diet supplemented with β -glucan Atlantic salmon were intraperitoneally injected by lipopolysaccharide (LPS) from the fish pathogenic bacteria, <i>Aeromonas salmonicida</i>	The microbial community was not affected Allochthonous gut bacteria of Atlantic salmon were affected by LPS	Skjermo <i>et al.</i> (2006) Liu <i>et al.</i> (2008)
Salinity		Arctic charr fed either a capelin roe diet or a commercial feed in fresh and sea water	Differences between microbiota in fresh and sea water	Ringø & Strøm (1994)

ACC- aerobic colony count, LAB- lactic acid bacteria

1.5.1 The importance of fish GIT microbiota

The GIT microbiota of an organism usually consists of a diverse population of non-pathogenic, pathogenic and commensal bacteria which provide several important functions such as providing a defence barrier and improvement of the immune response against pathogenic microorganisms and contributing to the maintenance of the integrity of the mucosal surface (Nayak, 2010b). Moreover, the GIT microbiota of fish produce vitamins (Ramirez and Dixon, 2003) and an extensive range of enzymes that are likely to assist in digestive processes such as phosphatases, carbohydrases, lipases, cellulose, esterases, peptidases, proteases, chitinase and phytase (Bairagi *et al.*, 2002; Dimitroglou *et al.*, 2011a; Ray *et al.*, 2012). In addition, the microbiota of the GIT exhibits an ability to ferment a range of diet-derived substances, including starch, non-starch polysaccharides (dietary fibre), oligosaccharides that the host would otherwise be unable to digest (Manning and Gibson, 2004). In general, the knowledge of these functions in fish have been obtained from knowledge of germfree fish (GF) in comparison with non-germfree fish as demonstrated by the research of Rawls & workers (2004), who investigated the impact of microbiota colonization on zebrafish.

In the last few years, considerable attention has been devoted to use of 'gnotobiotic studies' for the determination of the role of specific bacteria within the gut of fish hosts. This gnotobiotic is defined as 'to raise animals under germ-free (GF) conditions-to colonize them at varying points in their life cycle with a single microbe or more complex collections, and to then observe the effects of host habitat on microbial community structure and function and of the community on the host' (Rawls *et al.*, 2006). Zebrafish are

widely used in such studies due to the fact that the intestine larvae remain transparent until later maturational stages thus resulting in the possibility of visualizing morphological development and microbes in the GIT (Rawls *et al.*, 2006). Additionally, the growth of zebrafish is rapid and their GIT is similar in its organization to that of mammals (Rawls *et al.*, 2004). As opposed to mammals where the GIT is exposed to microbes at birth with the delivery of the neonatal fluid, the establishment of microbiota in the intestine of larval fish commences directly after opening of the mouth (Bates *et al.*, 2006).

Gnotobiotic studies in zebrafish indicate that the microbiota serves to aid development and maturity of the intestine, modulate cholesterol metabolism in the same way in both zebrafish and mice, and to up-regulate some gene expression encoding for products that have an ability to influence processes of innate immunity, nutrient metabolic pathways and digestive epithelial cell (Rawls *et al.*, 2004). Furthermore, Rawls *et al.* (2006) observed that the microbiota of zebrafish regulated several genes that contribute to the modulation of the metabolic pathways of fatty acids, amino acids, bile salts and butyrate. In contrast, when GF zebrafish were studied, the intestine failed to take in protein macro-molecules and revealed rapid mortality compared to the conventional reared zebrafish (i.e exposed to natural microbiota). In addition, the intestinal alkaline phosphatase (AP) activity, which is a marker of epithelial maturation, goblet cells, enteric endocrine cells, epithelial proliferation and the secretory cell lineages in the GIT of GF animals, was significantly lower in comparison with the intestine of conventionally reared animals (Rawls *et al.*, 2004; Bates *et al.*, 2006). Additionally, the tested bacteria in GF zebrafish exhibited a role in the

bioremediation of undesirable compounds that accumulated in the gut after feeding (Rawls *et al.*, 2006), and the GIT of GF fish showed a normal morphology and had no noticeable increase in the amount of cell death (Rawls *et al.*, 2004).

The diversity of the microbial communities in the GIT of fish could be affected by diet and feeding condition particularly during the larval stages (Reid *et al.*, 2009); however, it is important to maintain microbial balance if the growth and health of fish is to be sustained (Mansfield *et al.*, 2010; Gómez *et al.*, 2013). In this respect, maintaining the host animals in healthy status via the manipulation of GIT microbiota of the host using diet together with including desirable microorganisms (“probiotic”) and/or indigestible substance (“prebiotic”) has been widely employed (Nayak, 2010b).

1.6 Defining “Probiotics” for aquaculture

The definition of the term “probiotic” has changed over time. It is derived from the Greek words “pro”, meaning “for;” and “bios”, meaning “life” so the basic meaning is “for life” (Denev *et al.*, 2009). Parker (1974) was apparently the first to use the term probiotic in the context of animal feed supplementation and defined probiotics as “organisms and substances which contribute to intestinal microbial balance”. The definition of the term probiotic has evolved over the years. The first generally accepted definition was proposed by Fuller (1989) as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. A similar description was suggested by Holzapfel *et al.* (1998) as a “live culture of bacteria, whether single or in combination, which once used in the host will give

advantageous results by modulation of the host's microbial community composition".

A variety of definitions of the term probiotic have been suggested and the precise definition of a probiotic differs significantly according to the source. Schrezenmeir & De Vrese (2001) stated that "the probiotic concept is confined to effects exerted by viable microorganisms and is applicable independent of the site of action and route of administration". Many of these definitions are limited solely to situations involving humans or other terrestrial animals, where the probiotic is supplied as part of the feed (Verschuere *et al.*, 2000). However, these definitions are not quite so applicable in aquaculture, where the probiotic is sometimes added to the rearing water itself.

Potential pathogens are able to maintain themselves in the external environment of aquatic animals (i.e. the rearing water and sediments) and may proliferate independently of the potential host aquatic organisms (Verschuere *et al.*, 2000). Due to the complex relationship between aquatic microorganisms and their external rearing environment, the definition of the term "probiotics" has been modified for the context of aquaculture during last decades. Aquatic organisms have a much closer relationship with their external environment, it is possible to notice a big difference between aquatic and terrestrial organisms regarding the relationship between the intestinal microbiota and the surrounding environment in each of them as has been reported previously (Denev *et al.*, 2009).

Based on this observation, Verschuere *et al.* (2000) formulated another widely accepted definition of probiotics for aquaculture taking into account

the correlation between the microbe and its ambient environment i.e. “a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment”. This definition covers the supplementation of a probiotic organism to the host environment or the feed.

Live microbial cultures may be administrated in aquaculture for different purposes, e.g. disease prevention or mitigation, to improve water quality or to improve digestive performance. All of these will directly or indirectly benefit the health and/or survival of the aquatic organism. Based on this assertion, Gram & Ringø (2005) proposed that the effect of a probiotic could be identified by its capability to reduce an incidence of disease and/or increase survival from diseases. Gatesoupe (1999) proposed another definition of probiotics in aquaculture as “a microorganism which is given by different ways, to survive in the GIT and have beneficial effects on the host’s health”. The last definition emphasizes the oral administration of the probiotic and its action to enhance the well-being of the host due to its existence in the GIT of the host.

Probiotics are commercially available both in live and dead forms. However, the majority of studies show that live probiotics perform equal to, or better than dead probiotic preparations in terms of stimulation of the innate immune system. Although inactivated probiotics have an ability to assist fish in resisting disease (Irianto and Austin, 2003), live probiotics are believed to

exert greater stimulation of the non-specific immune system and potentially contribute to digestive function in fish (Panigrahi *et al.*, 2005a; Panigrahi *et al.*, 2007).

Based on this kind of observation, Merrifield *et al.* (2010c) formulated another definition that covers these forms of probiotic as “a probiotic is any microbial cell provided via the diet or rearing water that 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 applications of probiotics in aquaculture can be categorised into two groups:

- 1- digestive tract probiotics, are incorporated into the feed to enhance the beneficial microbiota of the digestive tract
- 2- water probiotics, which can proliferate in the rearing water and outcompete pathogenic bacteria to improve water quality (Denev *et al.*, 2009; Heo *et al.*, 2013). These types of applications encompass applications that may also be defined as: bioremediation and bio-control.

Additionally, probiotics can be administered continuously as a substitution approach to the aquatic animals or their ambient water in numerous ways such as: adding to culture water, bathing, using live supplementary diet or adding to synthetic diet (Verschuere *et al.*, 2000; Gómez *et al.*, 2013). In fact, the use of bio-controlling agents in fish has offered protection against different diseases. The effectiveness of probiotics depends on their persistence in the gut, which is affected by several factors relating to the

water, such as: water quality, hardness, dissolved oxygen, pH, temperature and osmotic pressure (Das *et al.*, 2008).

1.6.1 Selecting probiotic strains

It is important to get comprehensive information to understand the mechanisms by which probiotics can compete either with other autochthonous microbes and/or with pathogens in order to ascertain the potential efficacy. Although we do not have a clear comprehensive understanding about probiotics mechanisms, many mechanisms have been used to explain the modes of probiotic action. Figure 1.3 shows some desirable criteria for probiotic microorganisms.

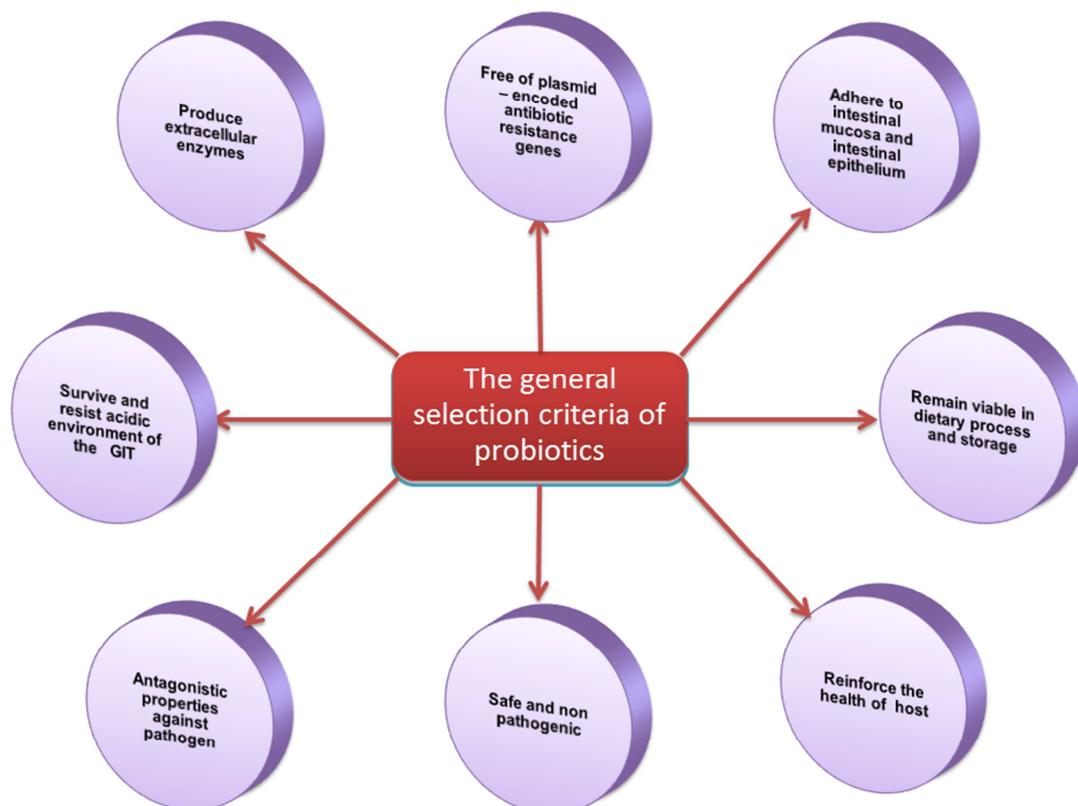


Figure 1.3 General selection criteria of probiotics.

The general characteristics for the selection of probiotic bacteria have been listed in the literature as:

- They should display adhesive properties to the GI mucosa and epithelium to decrease or prohibit colonization of pathogens.
- They should show antagonistic properties towards pathogens, either by themselves or by the production of specific extracellular substances.
- They should be able to survival processing and storage prior to administration.
- They should have an ability to colonize gut mucosa in sufficient numbers to cause these effects.

Additionally, Verschuere *et al.* (2000) added some other important criteria. For example, the probiotic should not be rejected by the host e.g. through digestion, possible colonization and replication within the host, it should reach the area where the effect is required.

Although *in vivo* testing presents a more reliable and representative means to investigate the role of probiotic bacteria (Gram 2001), it is not always available since it needs to be authorised by ethical committees and always expensive and time consuming. In addition, there is a clear recognition for scientists to minimise and reduce the number of animals used in scientific studies in order to support the 3Rs (replacement, reduction and refinement) ethos. Therefore several *in vitro* techniques have been used to study the characteristics of potential probiotic bacteria, these include, tolerance of bile salts and acidity, the ability to survive within fish mucus, hydrophobicity and antimicrobial activities. Although not in all cases (Spanggaard *et al.*, 2001),

the positive *in vitro* properties can sometimes be confirmed *in vivo* (Balcázar *et al.*, 2007c; Pérez-Sánchez *et al.*, 2011a; Burbank *et al.*, 2012). *Ex vivo* models have been used to evaluate the probiotic properties prior to use *in vivo* studies. For example, an attempt was made to investigate the competitive relationship of the probiotic *P. acidilactici* and the pathogen *V. (Listonella) anguillarum* in the intestine of rainbow trout by using the *ex vivo* intestinal sac method (Harper *et al.*, 2011). Results showed that *P. acidilactici* had an antagonistic role against *V. anguillarum* in the rainbow trout intestine and *P. acidilactici* was able to adhere the mucosa.

Importantly, probiotic bacteria have to be non-pathogenic to the host and also should not contain plasmid-encoded antimicrobial resistance genes, which could be passed to pathogenic microbes in the host or rearing environment (Dimitroglou *et al.*, 2011a). They should also be able to reinforce host health by competitive exclusion of pathogens or immune stimulation, and one of the most important criteria of probiotic organisms are that the survival rate of probiotic in the GIT of the host (Dicks and Botes, 2010; Šušković *et al.*, 2010).

Specifically, probiotics should possess the ability to resist bile salts, pancreatic secretions, proteases and low pH whilst still exerting their advantageous effects upon the host (e.g. improved digestion or immune system response) (Balcázar *et al.*, 2006). Moreover, probiotics should be able to produce one of the many antimicrobial substances which can, in sufficient concentrations, inhibit the growth of pathogenic bacteria such as acetic acid, lactic acid, propionic acid, hydrogen peroxide, bacteriocins, siderophores, ethanol, diacetyl, acetaldehyde, acetone, carbon dioxide,

ammonia and/or other antibiotic peptides/proteins (Šušković *et al.*, 2010; Dimitroglou *et al.*, 2011a).

1.6.2 Genera and species of probiotic bacteria

Fish have been found to have the following genera of probiotic microorganisms present in their GIT: *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Bacillus* and *Saccharomyces*. Additionally, genera of the family *Enterobacteriaceae*, *Aeromonas* and species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Carnobacterium*, *Shewanella*, *Bacillus*, *Clostridium*, *Alteromonas*, *Aeromonas*, *Vibrio*, *Pseudomonas*, *Flavobacterium*, *Enterobacter*, *Psychrobacter* and *Saccharomyces* spp. are reported to exert beneficial effects in different aquatic animals (Nayak, 2010a; Ringø *et al.*, 2010a; Kiron, 2012).

1.6.3 Lactic acid bacteria (LAB)

LAB are a large heterogeneous group of microorganisms sharing the property of converting fermentable carbohydrates primarily to lactic acid. In general, LAB are non-spore forming, Gram- positive and have an ability to grow both in the presence and absence of oxygen. LAB are rods or cocci, usually non-motile, and are often catalase- and oxidase-negative (Ringø and Gatesoupe, 1998). They produce lactic acid as a product of a fermentative metabolism, are able to tolerate bile / pancreatic enzymes and are fastidious microorganisms requiring many amino acids, minerals and vitamins for optimal growth (Leroi, 2010).

LAB have been reported as allochthonous and autochthonous bacteria in the GIT of healthy animals, including humans and fish, and in both cases they are used as probiotics (Ringø, 2004; Leroi, 2010). However, they are often considered as non-dominant bacteria in the normal intestine of fish in spite of some demonstrations that strains can colonize the GIT of fish (Ringø and Gatesoupe, 1998). The genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, *Weissella* and *Bifidobacterium* are members of the LAB group (Leroi, 2010). Among many groups of bacteria used as probiotics in aquaculture, LAB are the most common. Indeed, *Lactobacillus sporogenes*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Lactococcus lactis*, *Carnobacterium divergens* and *Pediococcus acidilactici* have been reported to beneficially affect aquatic species (Ringø, 2004).

1.6.4 Probiotic modes of action

Although some probiotics are currently used in aquaculture, the mechanisms by which these bacteria induce host benefits are not fully understood. No reports have comprehensively explained the modes of action in probiotics bacteria when employed in aquaculture.

However, the production of inhibitory compounds (e.g. lactic acid, hydrogen peroxide, carbon dioxide, siderophores, antibiotic peptides/proteins, organic acids, ammonia and diacetyl), competition with bacterial pathogens for nutrients and adhesion sites, stimulation of the immune response, enhancement growth parameters, improvement of water quality, production

of nutrients and enzymes which contribute to digestion, assistance in maintaining the integrity of the GIT mucosa and provision of nutritional and health benefits and have been widely accepted as the mechanisms of action of probiotics (Panigrahi and Azad, 2007; Daniels *et al.*, 2010; Nayak, 2010b; Dimitroglou *et al.*, 2011a; Ray *et al.*, 2012). Figures 1.4 and 1.5 show some mechanisms by which probiotic strains might exert their effects.

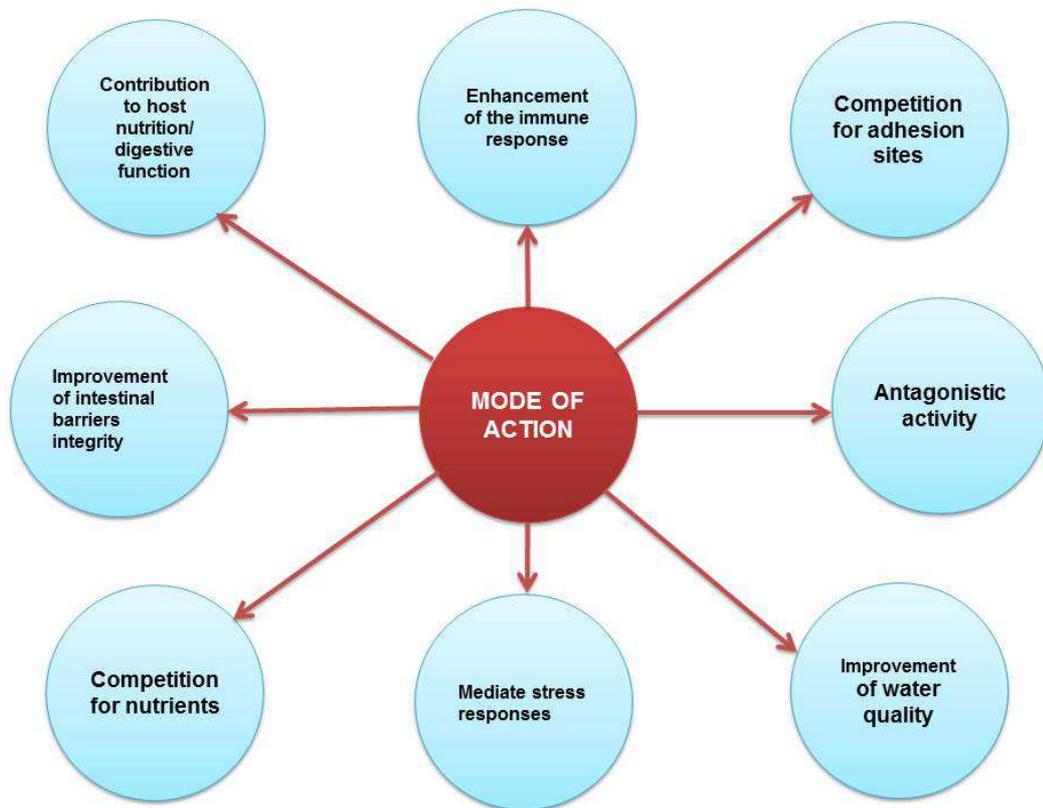


Figure 1.4 The general mode of action of probiotics.

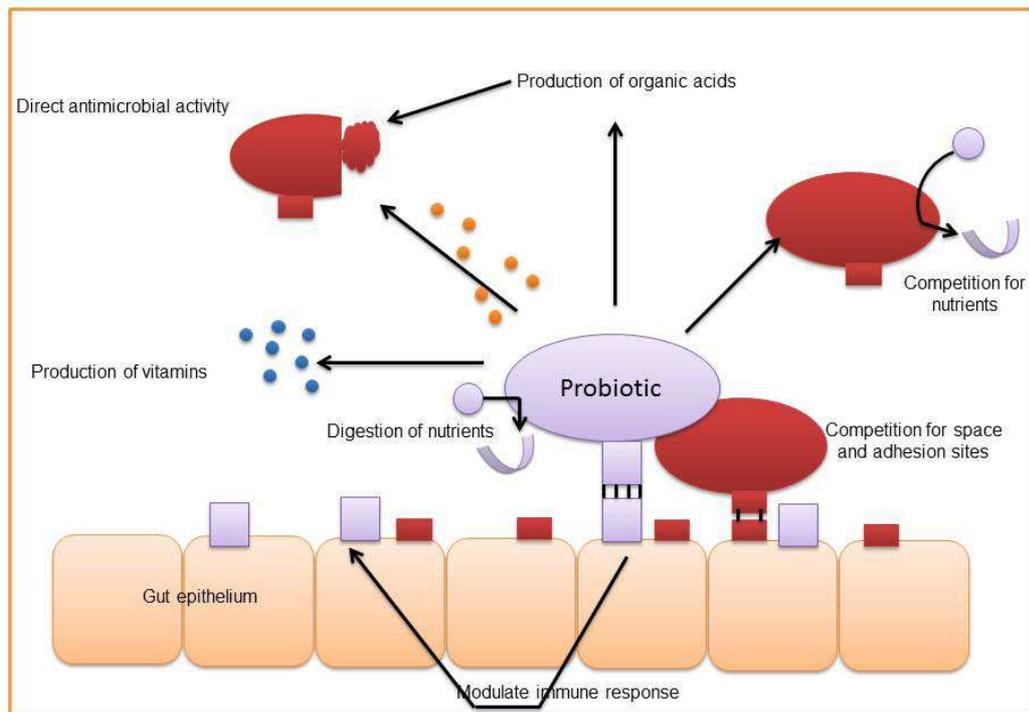


Figure 1.5 The activities of commensals bacteria including probiotic bacteria in the intestinal tract. Adapted from Snel *et al.* (2002).

1.6.4.1 Antagonistic activity against potential pathogens

Several reports have demonstrated that probiotic bacteria tend to be antagonistic towards harmful bacteria both *in vitro* and *in vivo* (Balcázar *et al.*, 2007c; Ai *et al.*, 2011; Pérez-Sánchez *et al.*, 2011a; Pérez-Sánchez *et al.*, 2011b; Boutin *et al.*, 2012). Antagonistic compounds are defined “as chemical substances produced by bacteria that are toxic or inhibitory towards other microorganisms”(Denev *et al.*, 2009), which may be produced as either primary or secondary metabolites and therefore have different modes of inhibitory action.

LAB are able to colonize the GIT of fish and produce several inhibitory compounds, whether singly or in combination, (Leroy, 2007; Arauz *et al.*, 2009; Harper *et al.*, 2011), which can be divided into two groups: high molecular mass substances >1000 Da (i.e bacteriocins) and low molecular

mass substances <1000 Da (including non-bacteriocins AMP) (Leroy, 2007; Šušković *et al.*, 2010). The production of these antagonistic compounds is governed by the exact species of LAB and under nutrients available *in situ*.

Competitive adhesion and production of antagonistic compounds against some fish pathogens by five potentially probiotic LAB were studied by Balcázar *et al.* (2007c). Following studies of mucus adhesion, competitive exclusion, and the suppression via inhibitory substances of fish pathogen growth, the authors concluded that the selected LAB strains were a promising alternative to conventional treatments in the fish farming industry.

Pérez-Sánchez *et al.* (2011a) demonstrated that members of the endogenous rainbow trout microbiota revealed antagonistic activity against pathogenic *La. garvieae*. A total of 335 bacterial isolates from rainbow trout were screened for antagonistic activity against *La. garvieae*. The study identified 11 species/ strains, including *Lactobacillus*, *Lactococcus* and *Leuconostoc* which revealed antagonistic activity against *La. garvieae* due to the production of bacteriocins.

In addition, in previous work undertaken by Burbank *et al.* (2011), rainbow trout were fed a commercial diet dressed with menhaden oil (10%) with a selection of individually-tested candidate probiotic bacteria at 10^6 - 10^8 CFU g^{-1} prior to, and after, infection with *F. psychrophilum*. Following the feeding trial, *Enterobacter amnigenus* or *Enterobacter* strain PIC15 significantly reduced mortality compared to the control group. Based on their findings, the above authors observed that these bacteria revealed potential protective roles in rainbow trout from *F. psychrophilum* infections.

1.6.4.2 Production of organic acids

Fermentation of carbohydrates by LAB is characterized by the accumulation of organic acids, mainly lactic and acetic acids, which are considered to be their primary antimicrobial compounds (Arauz *et al.*, 2009; Šušković *et al.*, 2010). Production of these organic acids leads to a reduction in pH, thereby inhibiting growth of some pathogenic bacteria and thus decreasing the absorption of potential pathogenic bacteria and their toxins by the aquatic animals (Dicks and Botes, 2010; De *et al.*, 2014).

It has been demonstrated that the mode of action of organic acids in the digestive tract is via two mechanisms. Firstly, via a decrease in pH level through delivery of H⁺ ions; secondly, the growth of Gram-negative bacteria can be prevented by acids via creation of anions inside bacterial cells (Luckstadt, 2008), where the inhibitory effect of organic acids is due to their ability in diffusing through the cell membrane to the more alkaline cytosol and interrupting vital metabolic functions (Servin, 2004).

The effect of nine potential probiotics including *Lactobacillus* spp., *Leuconostoc mesenteroides* and *P. acidilactici* cultures on four common bacterial pathogens of turbot including *Carnobacterium piscicola*, *Vibrio alginolyticus*, *Vibrio pelagius* and *Vibrio splendidus* has been investigated and the results showed that LAB-derived inhibitory substances (lactic and acetic acids) were behind the inhibition of *Vibriosis* in this case (Vázquez *et al.*, 2005). LAB could be beneficial in preventing Gram negative pathogen growth in the GIT as a result of their ability to produce lactic acid in the microenvironments of the gut and/or prohibition of detrimental Gram-negative

genera by the combination of activity of lactic acid and bile salts (Alakomi *et al.*, 2000).

1.6.4.3 Hydrogen peroxide production

Production of hydrogen peroxide (H_2O_2) occurs in LAB in the presence of oxygen through the action of NADH oxidase (Beutler, 2004), and the amount of H_2O_2 accumulated varies depending on the species/ strain and the availability of oxygen (Helander *et al.*, 1997). H_2O_2 has an inhibitory effect on a wide range of Gram positive and Gram negative bacteria, due to its powerful oxidizing effect on the cell wall and to the disruption of fundamental molecular structures of cell proteins (López-Boado *et al.*, 2000; De *et al.*, 2014).

Hydrogen peroxide can react with superoxide anion (O_2^-) to form a hydroxyl radical (OH^\cdot) which is also an inhibitory compound (Beutler, 2004). As this reaction requires environmental oxygen, its action is therefore most commonly observed in the mouth and upper regions of the GIT in the host and the bacterial cell and sulfhydryl groups of cell proteins have been found to be significantly influenced by strong the oxidising of H_2O_2 (Dicks and Botes, 2010).

1.6.4.4 Production of bacteriocins

Bacteriocins are peptides or proteins, with bactericidal activity, which are ribosomally synthesized by several Gram-positive and Gram-negative bacteria (Šušćković *et al.*, 2010). Bacteriocins can differ widely in molecular mass, biochemical properties and genetic origin (Abee *et al.*, 1995), and are

effective against closely related bacteria, which are killed by numerous mechanisms, including inhibition of cell wall biosynthesis, permeabilization of the target cell membrane and enzyme activity modulation (Cleveland *et al.*, 2001; Šušković *et al.*, 2010). Some bacteriocins have also been reported to form of pores in bacterial cell membranes due to interactions between bacteriocins with anionic lipids, which exist in abundance in the membranes of susceptible cells (Chen and Hoover, 2003). Furthermore, the sensitivity of bacteriocins to digestive enzymes or proteases activity are reported to be a major benefit of their use (Boutin *et al.*, 2012), and several LAB species, including *Lactobacillus* spp., *Enterococcus faecium*, *Leuconostoc* spp. and *P. acidilactici* are able to produce bacteriocins (De Vuyst *et al.*, 1996; Tahara and Kanatani, 1997; Hemme and Foucaud-Scheunemann, 2004; Tomé *et al.*, 2009).

Nisin, is a natural inhibitory peptide, or 'bacteriocin' produced by some species of LAB such as *Lactococcus lactis* subsp. *lactis* (Arauz *et al.*, 2009), and inhibits the growth of many Gram-positive bacteria (Sahl *et al.*, 1995). On the other hand, some Gram-positive bacteria have developed resistance to nisin by producing the enzyme nisinase (Abee *et al.*, 1995), whereas, Gram-negative bacteria are able to resist nisin due to the lipopolysaccharidic (LPS) composition of their outer membrane which represents a barrier to nisin action (Boutin *et al.*, 2012).

Nisin interacts with the phospholipid components of the plasma membrane of susceptible bacteria and interferes with the cell wall biosynthesis (Arauz *et al.*, 2009); as a result of this action the membrane becomes permeable for

ions, resulting in dissipation of both the membrane potential and the pH gradient (Guo *et al.*, 2009). The production of nisin has been reported to be influenced by various factors, including the producer bacteria, content of culture media, pH, temperature and oxygen availability (Parente and Ricciardi, 1999). The impact of nisin in vegetative cells of bacteria is likely to be at the cytoplasmic membranes by disruption the proton motive force and the hydrogen peroxide homeostasis leading to leakage of ions and hydrolysis of ATP resulting in cell death (Arauz *et al.*, 2009). Other bacteriocin types include pediocin PA-1 which is produced by *P. acidilactici* strains (Gonzalez and Kunka, 1987), pediocin AcH which is produced by *P. acidilactici* (Bhunja *et al.*, 1988), leucocin A-UAL 187 which is produced by *Leuconostoc gelidurn* (Hastings *et al.*, 1991), sakacin P and curvacin A which are produced by *Lactobacillus curuatus* (Tichaczek *et al.*, 1992).

1.6.4.5 Antiviral activity

Upon viral infection, the production of interferon I (IFNI) and tumor necrosis factor (TNF) are elevated and a cascade of subsequent molecules activate natural killer cells and macrophages, which are the primary effector cells against viral diseases (Khani *et al.*, 2012).

Several bacteria have been used as candidate probiotics on the basis of their antiviral properties (Balcázar *et al.*, 2006). It has been demonstrated that strains of *Pseudomonas*, *Vibrio*, *Aeromonas* and groups of *Coryneforms* isolated from salmonid hatcheries can exhibit antiviral activity against hematopoietic necrosis virus (IHNV) outbreaks (Kamei *et al.*, 1988).

It is well documented that several genera of LAB have an ability to protect fish species against viral diseases. For example, it was reported that two particular species of intestinal fish bacteria, *Aeromonas* and *Vibrio* spp. gave protection against hematopoietic necrosis virus (IHNV) when mixed with food pellets and fed to rainbow trout and masu salmon (*Oncorhynchus masou*), (Yoshimizu and Ezura, 1999). Additionally, Khani *et al.* (2012) tested *in vitro* the ability of probiotic (*Lb. rhamnosus*) in comparison with non-probiotic bacteria (*Escherichia coli*) to antagonize against Herpes simplex virus-1 (HSV-1) and investigated their effect on the activation of macrophages. Mechanisms, including competition with viruses for attachment sites, and increasing macrophage viability following stimulation of cytokines, were suggested by the authors to be behind the ability of *Lb. rhamnosus* to produce a number of antiviral effects against HSV-1.

1.6.4.6 Competition for nutrients

The resident microbial ecosystem in the GIT of aquatic animals, including fish, are dominated by heterotrophic bacteria competing for organic substrates, some of which cannot be digested by the host, as both carbon and energy sources, which could be utilised by potentially pathogenic bacteria (Verschuere *et al.*, 2000). Under specific conditions, microbiota in the GIT compete with each other for limiting organic, inorganic substances and energy sources, thereby resulting in alteration of the composition of the microbial community and creation a competition for iron and ferric siderophores between the pathogenic bacteria and animal host (Ringø and Gatesoupe, 1998; De *et al.*, 2014).

Probiotic bacteria with an ability to produce siderophores can compete and deprive pathogens which ferric iron Fe^{3+} metabolism plays an exceptionally important role in bacterial infections in fish (Verschuere *et al.*, 2000). Siderophore production is a kind of virulence factors in some pathogens (Denev *et al.*, 2009). Consequently, a siderophore-producing probiotic could deprive potential pathogens of iron under iron-limiting conditions. A culture supernatant of *Pseudomonas sp.* M147 grown in iron-limited conditions inhibited growth of *F. psychrophilum* (Korkea-aho *et al.*, 2011). Siderophores are low-molecular-weight produced and secreted by many bacteria, yeasts, fungi and plants which can dissolve precipitated iron and make it available for microbial growth (Verschuere *et al.*, 2000).

1.6.4.7 Adhesion and colonization

Adhesion is the preliminary step for colonisation of epithelial surfaces in the gut by bacteria and could be a criterion for selecting host microbiota to find the bacteria that are suitable as probiotic in fish farming (Grzeškowiak *et al.*, 2011). It is commonly accepted that the development stage of the fish, the additive feed, the ambient water and the GIT structure are likely to affect the bacterial colonization processes in fish (Ringø and Birkbeck, 1999; Nayak, 2010b). Microbes are more likely to colonize the mucosal epithelia when they have an ability to survive there for a long period, and by having a reproduction rate, which is higher than their exclusion rate (Ringø *et al.*, 2003). Additionally, some components of cell membrane such as teichoic acids, lipoteichoic acids and S-layer surface protein have been reported to be implicated in probiotic adhesion (Šušković *et al.*, 2010).

Recent studies have demonstrated that probiotic bacteria have an ability to adhere to the GI mucosa (Dicks and Botes, 2010; Merrifield *et al.*, 2010a). Bacterial colonization is influenced by a variety of factors, which not only relate to the host, such as body temperature, enzymes, bile salts, gastric acidity, digestive enzymes, immune parameters, but also relate to (inhibitory) compounds produced by autochthonous microbes, such as proteases, bacteriocins, lysozymes, ammonia, hydrogen peroxide and organic acids that lead to changes in pH (Balcázar *et al.*, 2006). In general, probiotic bacteria do not truly colonize the host and they have to be supplied repeatedly. Table 1.3 shows the persistence duration of probiotic bacteria after the cessation of probiotic provision. Colonizing bacteria can assist in the release of groups of cytokines that represent defence mechanisms against the invasion of pathogenic bacteria (Balcázar *et al.*, 2006).

Lazado *et al.* (2011) tested the action of two candidate probiotic bacteria (GP21 and GP12), which were isolated from the GIT of Atlantic cod and both exhibited good adhesion to primary cultures of the epithelial cells from the different regions of the gut, and thereafter effectively reduced the adhesion of two pathogens, *V. anguillarum* and *Aeromonas salmonicida* subsp. *salmonicida*. The results of the above study showed that the adhesion of the candidate probiotics and their interference with the adhesion of pathogens was influenced by both the source of the epithelial cells and the mechanism of action of these bacteria. Additionally, Vine *et al.* (2004b) demonstrated a competitive adhesion impact with five probiotics versus two pathogens on fish intestinal mucus. Authors found that the presence of one of the probiotics on the mucus inhibited the attachment of one of the pathogens tested.

However, the general trend from their study showed that post- treatment with the probiotics displaced the pathogen.

Table 1.3 Probiotics persistence in the GIT of fish after the cessation of probiotic dietary provision.

Fish species	Probiotic	Persistence duration	References
Rainbow trout	<i>Carnobacterium divergens</i>	Up to 3 weeks	Kim and Austin (2006b)
Rainbow trout	<i>Carnobacterium maltaromaticum</i>	Up to 3 weeks	Kim and Austin (2006a)
Rainbow trout	<i>Carnobacterium</i> sp.	Up to 4 days in fingerlings	Robertson <i>et al.</i> (2000)
Atlantic salmon		Up to 10 days in fry	
Brown trout	<i>Lactobacillus sakei</i> <i>Lactobacillus plantarum</i> <i>Leuconostoc mesenteroides</i>	Up to 2 weeks	Balcazar <i>et al.</i> (2007)
Rainbow trout	<i>Lactobacillus rhamnosus</i>	For two weeks	Panigrahi <i>et al.</i> (2005a)
Tilapia	<i>Pediococcus acidilactici</i>	For three weeks	Ferguson <i>et al.</i> (2010)
Tilapia	<i>Lactobacillus plantarum</i>	For three days	Bucio Galindo <i>et al.</i> (2009)
Rainbow trout	<i>Pseudomonas</i> sp. M174	For two weeks	Korkea-aho <i>et al.</i> (2011)
Rainbow trout	<i>Pseudomonas</i> M162	For one week	Korkea-aho <i>et al.</i> (2012)
Tilapia	<i>Lactococcus</i> sp.	A few days	Ridha and Azad (2012)

1.6.4.8 Probiotics stimulation of the immune system

Probiotic bacteria, as components of the intestinal microbiota, can also improve the host's defences by stimulating the immune system. Mono-species and multi-species probiotic supplementations have led to an increase in innate immune responses, including phagocytic activity, complement activity, respiratory burst activity, serum lysozyme activity, can modulate the expression of range of cytokines in fish and stimulate disease resistance (Robertson *et al.*, 2000; Irianto and Austin, 2002b; Balcázar *et al.*, 2007a; Sharifuzzaman and Austin, 2009; Nayak, 2010a; Ai *et al.*, 2011; Pérez-

Sánchez *et al.*, 2011a; Standen *et al.*, 2013). The immuno-modulatory actions of probiotics can be affected by different factors relating to the probiotics such as their type and source, dosage level and the feeding period (Nayak, 2010a).

Several studies have demonstrated that the activity of innate immune parameters may be affected by using LAB probiotics in rainbow trout. Panigrahi *et al.* (2007) report enhanced superoxide anion production, serum alternative complement activity and the expressions of IL-1 β 1, IL-1 β 2, TNF-1, TNF-2 and TGF- β in the spleen and head kidney when administering three freeze-dried probionts (*Lb. rhamnosus*, *E. faecium* and *Bacillus subtilis*) to rainbow trout for 45 days at a density of 10^9 CFU g⁻¹. Standen *et al.* (2013) demonstrated that Nile tilapia immune parameters were enhanced by using *P. acidilactici* including IELs, TNF- α mRNA levels and peripheral neutrophils and monocytes ratios. Rainbow trout treated with *La. lactis*, *Lactobacillus sakei* and *Leu. mesenteroides* at 10^6 CFU g⁻¹ for 2 weeks demonstrated an increase phagocytic activity of head kidney leucocytes and serum alternative complement activity in the probiotic fed fish groups compared to the control group (Balcázar *et al.*, 2007a). Lysozyme activity has been widely used as immune response marker to assess the efficacy of probiotic microorganisms to modulate the health of the host, as indicated in Table 1.4.

Table 1.4 Overview of some relevant studies using probiotic bacteria to modulate the immune response of salmonids.

Probiotics	Fish species	Effect on the immune system	References
<i>Lactobacillus plantarum</i> , <i>Leuconostoc mesenteroides</i>	Rainbow trout	↓ Fish mortality	Vendrell <i>et al.</i> (2008)
<i>Leuconostoc mesenteroides</i> , <i>Lactococcus Lactis</i> , <i>Lactobacillus sakei</i>	Rainbow trout	↑ HK leucocyte, alternative complement activity, ↓ lysozyme activity and fish mortality	Balcázar <i>et al.</i> (2007)
<i>Bacillus subtilis</i>	Rainbow trout	↑ Leucocytes, phagocytotic activity, respiratory burist, gut mucus and serum lysozyme, α1-antriptease, perioxidase assay ↓ Fish mortality, → complement activity, α2-macrogolobulin	Newaj Fyzul <i>et al.</i> (2007)
<i>Bacillus subtilis</i> + <i>Bacillus licheniformis</i> , <i>Enterococcus faecium</i>	Rainbow trout	↑ Lysozyme activity and lower in <i>E. faecium</i>	Merrifield <i>et al.</i> (2010b)
<i>Bacillus subtilis</i> + <i>Bacillus licheniformis</i> , <i>Enterococcus faecium</i>	Rainbow trout	→ Lysozyme activity	Merrifield <i>et al.</i> (2010a)
<i>Pediococcus acidilactici</i>	Rainbow trout	→ Lysozyme activity	Merrifield <i>et al.</i> (2011)
<i>Pediococcus acidilactici</i> , <i>Saccharomyces cerevisiae</i>	Rainbow trout	↓ The vertebral column compression syndrome	Aubin <i>et al.</i> (2005)
<i>Carnobacterium maltaromaticum</i> , <i>Carnobacterium divergens</i>	Rainbow trout	↑ Gut mucus and seum lysozyme activity, phagostic activity, resipratory burist → Gut mucus and seum lysozyme activity, leucocytes, erthrocytes	Kim and Austin (2006b)
<i>Carnobacterium maltaromaticum</i> , <i>Carnobacterium divergens</i>	Rainbow trout	↑ IL-β, TNF-α	Kim and Austin (2006a)

<i>Lactobacillus rhamnosus</i>	Rainbow trout	↑ Seum lysozyme activity, complement activity, phagocytic activity of HK leucocytes	Panigrahi <i>et al.</i> (2004)
<i>Carnobacterium sp.</i>	Atlantic salmon	↑ Resistance disease	Robertson <i>et al.</i> (2000)
<i>Lactobacillus lactis</i> , <i>Lactobacillus sakei</i> , <i>Leuconostoc mesenteroides</i>	brown trout (<i>Salmo trutta</i>)	↑ Alternative complement activity, serum lysozyme activity, serum immunoglobulin	Balcazar <i>et al.</i> (2007)
<i>Lactococcus Lactis</i> , <i>Leuconostoc mesenteroides</i>	brown trout	↑ Phagocytic activity of HK leucocytes, resistance against pathogenic bacteria	Balcázar <i>et al.</i> (2009)
<i>Carnobacterium piscicola</i>	Atlantic salmon	↑ Inhibition a pathogen bacteria	Ringø <i>et al.</i> (2000)
<i>Vibrio alginolyticus</i>	Atlantic salmon	↓ Fish mortality	Austin <i>et al.</i> (1995)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the probiotics relative to the control

1.6.4.9 Probiotics aid host digestion function

Several genera, including *Bacillus* spp., members of Enterobacteriaceae and *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Photobacterium*, *Pseudomonas*, *Vibrio*, *Microbacterium*, *Micrococcus*, *Staphylococcus* and yeast are considered to be digestive enzyme producers (Ray *et al.*, 2012; Raggi *et al.*, 2014). However, it is well documented that the enzyme production tends to be influenced by factors such as the morphology of the gut, developmental stages and rearing temperatures of fish (Miegel *et al.*, 2010), therefore, it is important to note that maintaining gut microbiota and improved gut morphology can contribute to reinforcing the host's nutrition status (Gatesoupe, 1999).

Dietary fibres can be fermented by probiotic bacteria to produce short chain fatty acid (SCFAs) in the gut. SCFAs can be absorbed by fish gut epithelial cells (Mountfort *et al.*, 2002) and may play an important role in increasing the villus height (Pelicano *et al.*, 2005) and decreasing the level of blood lipids by inhibiting hepatic cholesterol synthesis from the plasma to liver (Soccol *et al.*, 2010). In addition, it has also been reported that probiotics may improve intestinal epithelial structure, for example elevating microvilli height (Rodrig  nez S  enz de *et al.*, 2009; Merrifield *et al.*, 2010d), and potentially increase absorption by improving enterocyte endocytic activity (Merrifield *et al.*, 2010). Probiotics therefore have the potential to improve digestive function and feed utilisation.

Indeed, it has been demonstrated that the application of probiotics can improve growth parameters of fish species, including salmonids (Bagheri *et*

al., 2008; Merrifield *et al.*, 2010a; Ai *et al.*, 2011; Pirarat *et al.*, 2011). For example, Merrifield *et al.* (2010a) reported that rainbow trout fed with *B. subtilis* + *Bacillus licheniformis* for 10 weeks showed improved feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER). Furthermore, Bagheri *et al.* (2008) demonstrated that *B. subtilis* and *B. licheniformis* fed rainbow trout for two months showed increased FCR, SGR, K-factor, PER and carcass composition.

1.7 Prebiotics

In spite of the aforementioned advantages of probiotics, limitations in their use have been reported; these include viability (during production, transport and storage), survivability through the GIT and issues relating to regulatory authorisation for the using live microbes in several important markets, such as the EU. Significant attention has recently been focused on exploring alternatives methods to improve GIT microbial balance (Nayak, 2010a; Tacchi *et al.*, 2011; Boutin *et al.*, 2012; Kiron, 2012). According to the definition provided by Mansour *et al.* (2012) prebiotics are “nondigestible carbohydrates (NDCs) that selectively stimulate the growth and metabolism of health-promoting bacteria already present in the host gut”. Prebiotics exhibit various modes of action including stimulation of the immune system, protection against disease, improvement of growth parameters and modulation of the microbial community (Denev *et al.*, 2009). Prebiotics are fermented by the microbiota of the GIT which in turn could promote the growth of the commensals bacteria that act to keep the host in health status (De *et al.*, 2014).

A growing body of literature is available on the application of prebiotics to fish species, including salmonids (Grisdale-Helland *et al.*, 2008; Dimitroglou *et al.*, 2009; Dimitroglou *et al.*, 2011b; Ortiz *et al.*, 2013). Fructan based prebiotics, such as inulin, oligofructose and fructooligosachharides (FOS) are amongst the commonly studied prebiotics. A variety of plants including leeks, onions, garlic and chicory and in some bacteria and fungi are sources of inulin and oligofructose. Inulin type fructans are composed of β -D-fructofuranoses attached by β -2-1 linkages (Ringø *et al.*, 2010b).

Oligofructose is an inulin-type prebiotic which can be synthesized from sucrose by transfructosylation via β -fructofuranosidase that links additional fructose monomers to the sucrose molecule (Niness, 1999). Natural Oligofructose (chicory) contains both fructose chains and fructose chains with terminal glucose units, while synthesized oligofructose contains only fructose chains with glucose end units (Roberfroid, 1999). FOS are short and medium chains of β -D-fructans in which fructosyl units are linked by β -(2-1) glycosidic bounds and attached to a terminal glucose unit (Ringø *et al.*, 2010b). The term short-chain FOS (scFOS) is used to describe short chains of FOS with a degree of polymerisation of less than 5. Examples of fructan type prebiotics in fish, and their beneficial effects, are listed in Table 1.5.

Table 1.5 Overview of some prebiotics and examples of relevant studies, reported to affect fish health status.

Potential prebiotic	Fish species	General health benefits	References
Inulin (150 g Kg ⁻¹)	Arctic charr (<i>Salvelinus alpinus</i> L.)	↓ Adherent bacteria	Ringø <i>et al.</i> (2006c)
Inulin (5 or 10 g Kg ⁻¹)	Gilthead seabream (<i>Sparus aurata</i> L.)	Significant inhibition in phagocytosis and respiratory burst in leucocytes	Cerezuela <i>et al.</i> (2008)
Inulin (5 g Kg ⁻¹)	Nile tilapia (<i>Oreochromis niloticus</i>)	↑ WG, SGR, survival rate, nitroblue tetrazolium, resistance to pathogenic bacteria → Haematocrit	Ibrahim <i>et al.</i> (2010)
FOS (0.1, 0.2 ,0.3 g Kg ⁻¹)	Caspian roach (<i>Rutilus rutilus</i>)	↑ SGR, FCR, final weight, Ig levels, lysozyme and alternative complement activities, digestive enzyme → Feed intake, growth or digestibility	Soleimani <i>et al.</i> (2012)
FOS (10 g Kg ⁻¹)	Atlantic salmon	→ Feed intake, growth or digestibility	Grisdale-Helland <i>et al.</i> (2008)
FOS (5, 10 g Kg ⁻¹)	Rainbow trout	↑ WG, gross energy, Ca content ↓ Crude protein content	Ortiz <i>et al.</i> (2013)
scFOS (0.8 , 1.2 g Kg ⁻¹)	Hybrid tilapia (<i>Oreochromis aureus</i> ♂ x <i>O. niloticus</i> ♀)	↑ Final weight, SGR, ↓ FCR, hepatopancreasomatic → Survival rate, K- factor	Zhi-gang <i>et al.</i> (2007)
scFOS (1 g Kg ⁻¹)	Hybrid tilapia	Obvious effects on the intestinal communities	Zhou <i>et al.</i> (2009)
Oligofructose (Raftilose P95, 0.2 g Kg ⁻¹)	Turbot (<i>Psetta maxima</i>)	↑ Final mean weight, <i>Bacillus</i> spp. were isolated from Raftilose fed fish only	Mahious <i>et al.</i> (2006)
Oligofructose (0.1,0.2, 0.3 g Kg ⁻¹)	Beluga (<i>Huso huso</i>)	↑ Haemoglobin concentration, leucocyte, lymphocyte levels, haematocrit → Serum glucose, total protein ↓ Serum cholesterol	Hoseinifar <i>et al.</i> (2011)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the prebiotic relative to the control

1.8 Synbiotics

The term 'synbiotic' describes a mixture of probiotics and prebiotics forming part of nutritional ingredients, which increase the beneficial impacts on the host and is likely to influence the host by enhancing the persistence period and implantation of live microbial dietary supplements in the GIT by improving the growth of selective beneficial bacteria, thereby promoting the health of the host (Cerezuela *et al.*, 2011). The same authors reported that the activity of synbiotics could be influenced by fish species, time of feeding treatment, supplement dose and the different kinds of prebiotics and probiotics. The synbiotic combination of *E. faecium* and FOS was reported to exhibit several benefits such as improved final mean weight, weight gain percentage, SGR, K- factor, feed conversion ratio (FCR) and survival rate in rainbow trout (Mehrabi *et al.*, 2012). Similarly, positive synergistic effects in improvement of European lobster larvae weight gain, survival rate, SGR and FCR were found by feeding a combination of *Bacillus* sp. and MOS (Daniels *et al.*, 2010). Examples of synbiotic studies and their beneficial effects on fish health status are listed in Table 1.6.

Table 1.6 Overview of some synbiotic and examples of relevant studies, reported to affect fish health status.

Potential synbiotic	Fish species	General health benefits	References
<i>Enterococcus faecalis</i> , mannan oligosaccharide, polyhydroxybutrate	Rainbow trout	↑ Growth performance and immune response	Rodriguez-Estrada <i>et al.</i> (2009)
<i>Carnobacterium divergens</i> +EWOS prebiosa®	Atlantic salmon	↓ Resistance to pathogenic bacteria	Kristiansen <i>et al.</i> (2011)
<i>Pediococcus acidilactici</i> + short chain fructooligosaccharides (scFOS)	Atlantic salmon	↑ Total bacterial levels in the anterior mucosa, villi length, epithelial leucocytes, IL-1 β , TNF- α , IL-8, TLR3, MX-1 , serum lysozyme activity	Abid <i>et al.</i> (2013)
<i>Enterococcus faecalis</i> + mannan oligosaccharide	Rainbow trout	↑ WG, SGR, FGR, PER, haematocrit value, phagocytic activity ↓ Fish mortality	Rodriguez-Estrada <i>et al.</i> (2013)
<i>Lactobacillus rhamnosus</i> + Mannan oligosaccharides	Rainbow trout	↑ LAB	Alak and Hisar (2012)
<i>Bacillus subtilis</i> + FOS	Juvenile ovate Pompano (<i>Trachinotus ovatus</i>)	↑ Respiratory burst, phagocytic, lysozyme activities ↓ Fish mortality	Zhang <i>et al.</i> (2014)
<i>Bacillus subtilis</i> + inulin	Gilthead seabream (<i>Sparus aurata</i>)	↑ The haemolytic complement activity → Other innate immune parameters	Cerezuela <i>et al.</i> (2012)
<i>Bacillus subtilis</i> + FOS	Juvenile large yellow croaker (<i>Larimichthys crocea</i>)	↑ SGR, FER, serum lysozyme, serum superoxide dismutase activities	Ai <i>et al.</i> (2011)
<i>Bacillus</i> TC22 + FOS	Sea cucumber (<i>Apostichopus japonicas</i>)	↑ Phagocytosis, respiratory burst, phenoloxidase activities, resistance to pathogenic bacteria	Zhao <i>et al.</i> (2011)

Symbols represent an increase (↑), no effect (→) or decrease (↓) in the parameter of the synbiotic relative to the control

1.9 Conclusions

Farming of aquatic animals continues to be one of the fastest growing food animal producing sectors world-wide. Traditionally, disease outbreaks have been treated with disinfectants, antibiotic drugs and vaccinations. However, these approaches have raised further problems or have limited efficacy. Consequently, probiotics, prebiotics, immunostimulants and vitamins are considered to be environmentally friendly alternatives to vaccines and antibiotics. Recently, a combination of pre- and probiotic applications, termed a synbiotic, has been developed to promote the health of the host by improving GIT microbial balance. Fish possess a complex microbial community in their GIT which is mainly composed from aerobic, facultative anaerobic and possibly obligate anaerobic bacteria. Several important biological functions such as physiological, nutritional and immunological processes have been reported to be regulated and influenced by the microbial community as demonstrated in gnotobiotic studies. *In vitro*, *ex vivo* and *in vivo* test models have been used to provide important information to improve our understanding of the mechanisms of action of probiotics, thus *in vitro* models can provide a first step for the selection process of new probiotics. Candidate probiotics for use as biological control agents should be safe (i.e. non-pathogenic to the host and end consumer), able to adhere to the GIT mucosa and epithelium, show antagonistic properties towards pathogens and able to survive feed processing and storage conditions. The production of inhibitory compounds, competition with bacterial pathogens for nutrients and adhesion sites, stimulation of the immune response, enhancement of growth parameters, improvement of water quality, production of nutrients and enzymes which

contribute in digestion, modulation of the microbial equilibrium have been widely accepted as the mechanisms of probiotic action.

1.10 Aims and objectives

The overall aim of this research programme was to investigate the gut microbiota of salmonids (Atlantic salmon and rainbow trout due to their economic importance and brown trout in respect to their importance for recreational fishing and restocking-programmes) and the potential benefits of dietary feed additives (i.e. probiotics and synbiotics) on the microbiota, health and growth performance of salmonids. Additionally, the mechanisms of action, which may cause host benefits, were also investigated. These objectives were addressed by the following:

- Characterisation of the gut microbiota of brown trout to provide novel fundamental data on the autochthonous microbiota, and the attempt to isolate novel LAB, which if obtained, could be considered for applications in the latter experiments.
- Determination of the effects of probiotic *P. acidilactici* on rainbow trout intestinal microbial communities and the subsequent effects on intestinal health and systemic immune status.
- Evaluation of the application of a synbiotic on the gut microbiota, the innate immune system, and intestinal histology of Atlantic salmon.
- Evaluation of the efficacy of probiotics in soybean-rich diets with respect to the intestinal microbiology, growth performance and host mucosal immune response of rainbow trout.

Chapter 2

Chapter 2: General methods

2.1 Overview

All feeding experiments were carried out at the University of Plymouth's Aquaculture and Fish Nutrition Research Aquarium, in experimental system F in the west aquarium unless otherwise mentioned (Plate 2.1). The general procedures and analytical techniques, which were used in the present study, are listed in this chapter. Other techniques specific to particular trials (including diet formulation) are described in the relevant experimental chapters. All experimental work involving fish was conducted in accordance with the Plymouth University ethics committee, and where appropriate under the UK Home Office project licence (PPL 30/2644).

2.2 Experimental fish and husbandry

During the present research project, three different groups of fish were used to conduct the four separate experimental trials. The experimental fish used in this study were brown trout (*Salmo trutta*) for Chapter 3 and rainbow trout (*Oncorhynchus mykiss walbaum*) for Chapters 4 and 6 while Chapter 5 was conducted in using Atlantic salmon (*Salmo salar*). The methodological information on fish husbandry and dietary formulations provided in this chapter pertain to experiments 1, 2 and 4 (Chapters 3, 4 and 6), respectively.

All fish were left to acclimatize for an appropriate period which they were fed commercial trout diets (EWOS[®] Sigma 50; Bergen, Norway) until grading and random distribution into tanks prior to the start of the trials. The experimental holding unit consisted of 20 closed fibreglass tanks each with 80-L capacity (each measuring 94 cm × 50 cm × 40.5 cm). Each tank was provided with 98%

re-circulated-aerated freshwater at a rate of 100 L h⁻¹. The tanks were thoroughly cleaned when the fish were removed for weighing and partial water changes in the system were conducted if necessary. An automated photoperiod of 12h light and 12h dark system was used throughout the experimental trials. Plate 2.1 presents the design of the experimental system used in all experiments.



Plate 2.1 System F used in all experiments, at the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University.

2.3 Water quality

During the trials, water quality parameters such as temperature, dissolved oxygen (DO) and pH in the system were measured daily using HQ 40d multi parameter meter (HACH Company, Loveland, USA). The water temperature was maintained at a suitable temperature (14 - 17 °C) throughout the experiments with a thermostatically controlled chiller (Zodiac, France). The system pH was adjusted with sodium bicarbonate (NaHCO₃) as necessary to maintain the level within the desired range (pH 6.5 - 7.5) and the dissolved oxygen levels were maintained above 80% with additional aeration provided by a side supply of compressed air (Rietschle, UK).

Total ammonia, nitrite and nitrate were measured weekly by using an automatic analyser (HACH LANGE, DR 2800 Germany) and cuvettes for ammonia (Lange LCK 304), nitrite (Lange LCK 341) and nitrate (Lange LCK 340). The following levels of nitrogenous compounds were considered acceptable: ammonia (unionized) < 0.1 mg L⁻¹, nitrate < 50 mg L⁻¹ and nitrite < 1.0 mg L⁻¹. These levels were controlled with partial changes of water when necessary. Other undesired waste materials including faecal material, undigested feed were removed by a rotating drum screen filter (Aquasonic DF100, Aquasonic Ltd, Wauchop, Australia).

2.4 Feed formulation and rainbow trout trials dietary preparation

The experimental diets for each trial were prepared in 2 kg batches. Three different diets were formulated to meet the nutritional requirements of salmonids according to National Research Council (NRC). The diets for

Chapters 4 and 6 were prepared at Plymouth University (as described in Sections 4.2.1 and 6.2.1, respectively) and the diet for Chapter 5 was prepared by BioMar (as described in Section 5.2.1).

2.5 Determination of the viability of the probiotics in feed

To calculate the viability of *P. acidilactici* in the probiotic diets, 1 g of diet was put into a stomacher bag with 9 mL sterile of phosphate buffered saline (PBS; pH 7.3; Oxoid, UK), transferred into a stomacher (Bag Mixer[®] Interscience France) and stomached for 30 sec. This solution was then serially tenfold diluted to 10^{-7} with PBS and 100 μ L of each dilution was then spread on duplicate de Man, Rogosa and Sharpe (MRS; Oxoid, UK) agar plates and incubated aerobically at 37 °C for 24h (Ferguson *et al.*, 2010). The colony forming units were counted on all plates containing 30 - 300 CFU to determine the viable populations (expressed as CFU g⁻¹).

2.5.1 Polymerase Chain Reaction (PCR)

After DNA extractions, which are detailed in the relevant chapters, polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene to confirm the identity of a representative number of *P. acidilactici* colonies by using the primers 27F and 1491R. Each PCR reaction mix constituted of: 1 μ L 27F (50 pmol/ μ L) primer (5'-AGAGTTTGATCCTGGCTCA-3') and reverse primer 1492R (50 pmol/ μ L) (5'-GGTTACCTTGTTACGACTT-3'), 2 μ L DNA template, 25 μ L BioMix[™] Red Taq (Bioline, UK) and molecular grade water (MGW), (Promega, UK) yielding a total volume of 50 μ L. Thermal cycling was conducted in a GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA,

USA), under the following conditions: 94 °C for 10 min, then 35 cycles of 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 90 sec, with a final extension step of 7 min at 72 °C.

2.5.2 Agarose gel electrophoresis

In order to check the purity and molecular weight characteristics of PCR products, PCR products (6 µL) were loaded onto a 1.5% agarose gel (Lonza, Rockland ME, USA), made with 1x Tris-acetate-EDTA (TAE) buffer pre-stained with 4 µL of SYBR[®] Safe[™] DNA Gel Stain (Life Technologies[™] UK) per 100 mL of agarose (Fisher Scientific) and run with 1x TAE buffer in a Pharmacia electrophoresis tank at 90 volts for 60 min. Five µL of Hyper Ladder IV (Bioline) was run alongside the PCR products to assess the size of DNA products. Viewing of agarose gels was achieved under UV light using a Bio-Rad universal hood 11 (Bio-Rad laboratories, Italy).

2.5.3 Sequence analysis of pure colonies

The PCR products were cleaned using a Sure Clean Kit (Bioline, UK) according to the manufacturer's instructions and PCR yields (the concentration and purity of DNA) were checked using a Nanodrop[®] 1000 spectrophotometer. Protein purity (A260/A280) and humic acid purity (A260/A230) were checked. The PCR products were sequenced by GATC Biotech Ltd. (Germany).

The nucleotide sequences were compared with sequences from the National Center for Biotechnology Information, (NCBI, Bethesda, MD, USA) using the BLAST search in GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to detect the closest known alignment identities for the 16S rRNA gene sequences.

2.6 Chemical and proximate analysis

Diet samples were ground using a 1 mm screen (Knifetec™ 1095, Foss, UK) before analysing moisture, protein, lipid, ash, and gross energy. All chemical analyses were performed in triplicate according to AOAC (1995) protocols as described in the following sub sections.

2.6.1 Moisture content

Samples were dispensed evenly between three crucibles and dried in a fan-assisted oven (model VT6200 Thermo Scientific Heraeus, Germany) at 105 °C for 24h until a constant dry weight was achieved. The weight of the empty crucibles was previously also recorded. The moisture content (%) was calculated using the following formula:

$$\text{Moisture (\%)} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where: W_1 = initial weight of empty dish (g), W_2 =weight of dish and weight of sample before drying (g), W_3 =weight of dish and weight of sample after drying (g).

2.6.2 Protein content

Determination of total crude protein was carried out by the Kjeldahl method, which estimates crude protein content from the total nitrogen content of the sample. This value was then multiplied by a factor of 6.25 to calculate the crude protein content. Briefly, 100 mg of dried sample was weighed onto nitrogen-free paper and then transferred to borosilicate digestion tubes. A Kjeldahl catalyst tablet (3 g K_2SO_4 , 105 mg $CuSO_4 \cdot 5H_2O$ and 105 mg TiO_2 ; BDH Ltd UK), and 10 mL of concentrated H_2SO_4 (Sp. Gr. 1.84, BDH Ltd UK) were added to each tube.

Digestion was performed on a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 105 °C for 15 min. The temperature was then raised to 225 °C for a further 60 min and finally was raised to 380 °C for 45 min. After, the cooled samples were distilled using a Gerhardt Vapodest 40 distillation unit where the sample was diluted with distilled water and neutralized with 8.7M NaOH solution. The liberated ammonia in the sample was then trapped into 50 mL of 0.64M orthoboric acid (H₃BO₃ with '4.5' BDH indicator) by automatical steam distillation. Subsequently the distillate was back-titrated against 0.1M H₂SO₄ blanks and reference samples (material with known protein content for validation) were also run.

Crude protein was then determined as:

$$\text{Crude protein (\%)} = \frac{[(ST - BT) \times 0.2 \times 14.006 \times 6.25]}{SW} \times 100$$

Where 0.2 is the molarity of the acid, 14.006 the relative atomic mass of nitrogen, 6.25 is the constant relationship between N and animal protein, ST is sample titre (mL), BT is blank titre (mL) and SW is the initial sample weight (mg).

2.6.3 Lipid content

To determine the lipid content, the Soxhlet extraction method was used. Approximately 2 g of each diet was placed into a cellulose thimble, lightly plugged with cotton and inserted into the condensers (rinsing position) of a SoxTec extraction system (Tecator Systems, Högnäs, Sweden; model 1043 and service 1046). Pre-weighed cups containing 140 mL of petroleum ether (30 - 40 °C) were clamped into the condensers and the extraction knobs

were moved to the boiling position for 30 min. Afterwards the extraction knobs were then moved to the rinsing position for further 45 min. The cups containing extracted lipid were then transferred to a fume cupboard for 30 min before weighing. Lipid content was determined by using the following formula:

$$\text{Fat (\%)} = \frac{\text{Final weight of cup (g)} - \text{Initial weight of cup (g)}}{\text{Initial weight of sample (g)}} \times 100$$

2.6.4 Ash content

Ash (total mineral or inorganic content) was determined by adding a known weight of sample (~ 500 mg) to a pre-weighed crucible. Crucibles were then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550 °C for 12h. Then, all crucibles were placed in a dessicator to cool to room temperature. Percentage ash was determined from the sample residue using the following formula:

$$\text{Ash (\%)} = \frac{(\text{weight of crucible} + \text{residue}) - \text{weight of crucible (g)}}{\text{Sample weight (g)}} \times 100$$

2.6.5 Gross energy content

Gross energy was determined in triplicate with a Parr Adiabatic Bomb Calorimeter model 1356 (Parr Instrument Company, IL, USA). The method is based on measuring the liberated heat from a complete combustion of the sample by electrical ignition in an oxygen rich atmosphere (bomb). Briefly, ground feed was compressed into a ~1 g pellet and placed in a crucible. The

crucible was placed in the metal loop of the bomb. Fuse wire connected the anode and cathode of the bomb allowing the wire to touch the upper surface of the pellet. One mL of distilled water was pipetted into the bomb cylinder and the bomb head (electrodes) was placed into the bomb cylinder. The bomb was filled with oxygen to 30 bar pressure before ignition.

The calorimeter bucket was filled with 2000 g of distilled water at a temperature less than room temperature by at 2 - 3 °C sub-room temperature. The bucket was placed in the calorimeter chamber and the bomb was lowered down into the water bucket. The two ignition wires were connected into the terminal sockets which were located on the bomb head. The firing chamber was closed with the cover. The bomb was fired and the microprocessor automatically compared the temperature rise with a known thermal curve, and calculated the energy content (MJ kg^{-1}).

2.7 Microbiology

2.7.1 Fish dissection

Fish were euthanized by immersion in overdose (200 mg L^{-1} water for 15 min) of Tricaine Methane Sulfonate (MS222; Pharmaq, Fordingbridge, UK) and buffered with sodium bicarbonate, followed by a sharp blow to the cranium. To avoid possible external contamination while removing the intestine, the surface of each fish was cleaned using 70% Industrial Methylated Spirits (IMS). Under aseptic conditions, fish were dissected and the intestine was entirely excised. The intestinal tract was divided into two sampling regions, the anterior intestine (which was the region designated as the distance between the distal most pyloric caeca and proximal to the increase in

intestinal diameter which denotes the beginning of the posterior intestine) and the posterior intestine (which was designated as the region from the onset of the region in which the intestinal diameter increases to the anus).

After cutting at the proximal border between the two sections, digesta from the anterior and posterior regions was obtained by gentle squeezing the section with a sterile forceps into individual sterile 1.5 mL micro centrifuge tubes (MCT). Each mucosa section was aseptically opened longitudinally with a sterile scalpel and washed thoroughly three times with PBS. The intestinal sections were emptied into sterile 1.5 mL MCT and all samples were stored at -20 °C.

2.7.2 Culture-based enumeration of bacterial populations

One gram (wet weight) of the sample material (mucosa samples) was homogenized using sterile glass beads and a macerator (MSE, London, UK) for 30 sec and vortexed vigorously for five sec (Protamixer Deluxe Hook and Tucker LTD.) in 9 mL of PBS.

Samples were tenfold diluted with PBS and 100 µL of each dilution was spread onto duplicate tryptone soy agar (TSA) plates (Huber *et al.*, 2004) and MRS plates in order to enumerate aerobic colony count (ACC) and lactic acid bacteria (LAB) levels, respectively. ACC and LAB numbers in the samples were calculated by counting colonies (Gallenkamp colony counter) after plates were incubated aerobically at 15 °C and inspected regularly for up to 3 weeks. All media and components were purchased from Oxoid (Basingstoke, UK). Media, solutions and tips were sterilised in an autoclave (at 121 °C for 15 min at 15 pounds/sq inch psi).

2.7.3 PCR-based identification of pure cultures

Representative colonies were randomly picked from each plate containing 30 to 300 CFU (Kristiansen *et al.*, 2011) and sub-cultured onto TSA and MRS agar (as appropriate) repeatedly until pure cultures were obtained (Merrifield *et al.*, 2011). The isolates were stored at 4 °C.

2.7.3.1 DNA extraction

DNA from the aforementioned selected colonies was extracted as described in the relevant experimental chapters.

2.7.3.2 PCR for pure bacterial colonies

Each PCR tube mix constituted of 1 µL each of primers 27F and 1491R, 2 µL DNA template, 25 µL BioMix™ Red Taq (Bioline, UK) and MGW (Promega, UK) yielding a total volume of 50 µL. Negative and positive control containing all the components with exception of DNA templates replaced by MGW and other DNA template, respectively were also conducted.

Thermal cycling was conducted in a GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA, USA) under the following conditions: 94 °C for 10 min, then 35 cycles consisting of 94 °C for 30 sec, 53 °C for 30 sec and 72 °C for 90 sec, with a final extension step for 7 min at 72 °C. The PCR products were stored at 4 °C until used (Ringø *et al.*, 2006c). PCR products were loaded onto 1.5% agarose gel as described in Section 2.5.2. The PCR products were cleaned using a Bioline Sure Clean Kit according to the manufacturer's instructions as described in Section 2.5.3.

2.7.4 Denaturing gradient gel electrophoresis (DGGE) analysis

2.7.4.1 DNA extraction

DNA was extracted from the mucosa and digesta samples using a QIAamp® Stool Mini Kit (Qiagen) with minor modifications to the manufacturer's instructions, as described in Appendix 1.

2.7.4.2 Polymerase Chain Reaction (PCR)

PCR was conducted to amplify of the V3 region of the 16S rRNA gene using PCR with the forward primer P3 with a GC clamp on its 5'-end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and the reverse primer P2 (5'-ATTACCGCGGCTGCTGG-3') (Muyzer *et al.*, 1993). The following reagents were included in each PCR tube: 25 µL BioMix™ Red Taq (Bioline, UK), 1 µL of each primer (50 pmol/µL each MWG-Biotech AG, Germany), 5 µL DNA template and 18 µL sterile MGW yielding a total volume of 50 µL.

Touchdown thermal cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min as described by Muyzer *et al.* (1993). The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at 55 °C for the remaining cycles. PCR products were loaded onto 1.5% agarose gel as described in Section 2.5.2. The PCR products were stored at 4 °C until use.

2.7.4.3 DGGE

The resulting PCR products were used to obtain DNA fingerprints of the bacterial community present in the two gut sections by DGGE using a Bio-Rad DGGE system (DCode™ System, Italy).

DGGE was carried out by loading 15 µL of PCR products onto 10% acrylamide gels with a denaturing gradient of 40 - 60% (where the denaturants were 5.6M urea (Sigma, UK) and 40% formamide (Sigma, UK). Made using the following stock solutions; an 80% denaturant polyacrylamide solution consisted of 25 mL of 40% acrylamide mix (high purity acrylamide), 2 mL of 50x TAE buffer (pH 8.3), 32 mL of molecular grade formamide (Sigma, UK), 34 g of 5.6M ultrapure urea (Sigma, UK) and volume of MilliQ H₂O yielding a total volume of 100 mL. Stock 0% denaturant polyacrylamide solution consisted of 25 mL of 40% acrylamide mix (high purity acrylamide), 2 mL of 50x TAE buffer (pH 8.3) and 73 mL of MilliQ H₂O. One-hundred and fifty µL of 10% ammonium persulphate (APS, electrophoresis grade, Sigma, UK) and 17.5 mL of Tetramethylethyldiamine (TEMED) were added to the high and low denaturant solutions. Twenty one mL of each acrylamide solution was added to separate 30 mL syringes and these were mounted onto a Bio-Rad gradient delivery system (model 475, Bio-Rad laboratories). The major steps of DGGE are presented in Figure 2.1.

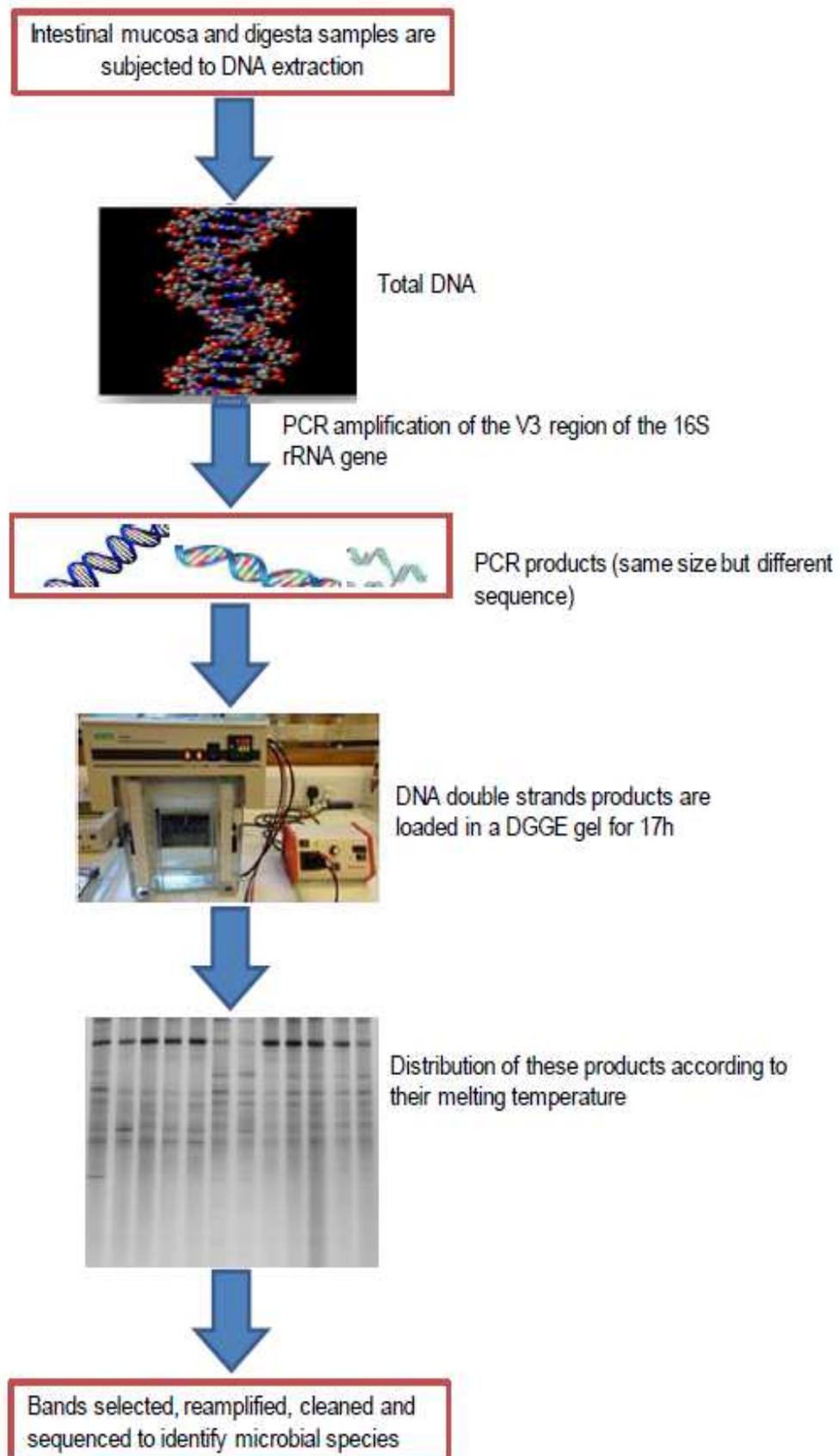


Figure 2.1 Schematic representation of the principle steps of the denaturation gradient gel electrophoresis (DGGE) process.

This was then used to pour the gel between gel plates and the gel was left to polymerize for two hours. Additionally, PCR products from *P. acidilactici* pure colonies were loaded to the gel as a reference species to aid probiotic identification. The gel was run at 65 V for 17h at 60 °C in 1 x TAE buffer.

Viewing of the DGGE bands was accomplished after SYBR® gold staining. Briefly, the gel was incubated for 20 min at room temperature in 200 mL tank buffer containing 20 µL of 10000x SYBR® gold nucleic acid gel stain (Invitrogen™, UK) with shaking on an IKA® VIBRAX VXR basic shaking platform at 100 rpm/ min. The gel was scanned in a Bio-Rad universal hood 11 (Bio-Rad Laboratories, Italy) and optimized for analyses by enhancing contrast and greyscale.

2.7.4.4 Excision of DGGE bands for sequence analysis

After DGGE, bands (or 'operational taxonomic units', OTU) of interest (those showing clear and consistent specialization either to intestinal regions or dietary treatments, or those clearly unaffected) were excised from the gel using sterile pipette tips and DNA was eluted overnight at 4 °C in 1.5 mL MCT containing 20 µL MGW. From the eluate, 5 µL was used as the template for reamplification using the forward primers P1 (5-CCTACGGGAGGCAGCAG-3; essentially P3 without the GC clamp at its 5' end) and the reverse primer P2 under the same conditions as previously described (Section 2.7.4.2). Six µL was loaded onto a pre-stained agarose gel (1.5%) to check the PCR product size. The PCR products were cleaned using a Bioline Sure Clean Kit according to the manufacturer's instructions as described in Section 2.5.3.

2.8 Histological examination

Samples for histology were taken from three fish per tank at the mid and end sampling points unless otherwise mentioned. Fish were dissected, and the appropriate intestinal sections were obtained, as described in Section 2.7.1.

2.8.1 Light microscopy (LM)

Intestine samples (1 cm) (the proximal most section, from the respective regions, was taken to ensure consistency between individual fish sampled) were immediately fixed in 4% formaldehyde solution for 48h. The fixative solution was substituted with 70% alcohol after 48h and samples were stored at 4 °C. Three small pieces from each gut sample were placed in the same cassette. These were passed through a dehydrating series of 50%, 70%, 90% and 100% ethanol (v/v in water) by immersion in the tissue processor (Leica, Germany). Dissected samples were cleared in three changes of xylene (1h for each change) to remove alcohol and to prepare the tissues for paraffin infiltration. Samples were then moved to the paraffin oven and embedded in melted paraffin to provide a matrix that could support the tissues during sectioning.

Blocks were then cut into 5 µm transverse sections using a Leica Microsystem microtome model RM2235 (Germany) and placed in 50 °C water for 2 min. Finally, slides were stained with Haematoxylin and Eosin (H & E) stain using a Leica Microsystem auto stainer XL, Germany). Haematoxylin has a blue colour and stains the nucleic acids (nucleus). Eosin is pink and stains protein in the cytoplasm and the extracellular matrix.

Stained sections were mounted with 22 mm coverslips using a polystyrene resin dissolved in xylene (DPX).

Additionally, replicate sections of the intestinal samples were stained with May-Grünwald/ Giemsa (MGG) stain (Sigma, UK) following standard methods with some modifications to the staining time to optimize results. The staining times were as follows: slides were cleared in twice in histolin for 2 min each, followed by rehydration in 2 changes of absolute alcohol and a series of water/alcohol solutions as following 90%, 70%, 50% and 30% for 2 min in each. Sections were washed in distilled water. Sections were then stained with May-Grünwald stain (diluted 1:1 in distilled water) at 37 °C for 10 - 15 min and then washed thoroughly with tap water. Sections were then stained with Giemsa stain (15 drops in 10 mL of distilled water) at 37 °C (10 - 15 min) and then washed with tap water. Sections were rapidly rinsed in acetic water (4-5 drops of acetic acid in 50 mL of distilled water) and then washed thoroughly with tap water. Sections were dehydrated in acetone and cleared in xylene. Finally, stained sections were mounted with coverslips using a polystyrene resin dissolved in xylene (DPX). Slides were examined by light microscopy using an Olympus Vanox-T microscope and images were taken with a digital camera (Olympus camedia C-2020 Z) at total magnifications of x100, x200 x400.

All images were analysed using Image J version 1.36 (National Institutes of Health, USA). Intestinal images from light microscopy (LM) were analysed to determine the length of villi and the number of goblet cells and intraepithelial leucocyte cells (defined as the region between the lamina propria and the

brushborder), across a standardized distance of 100 μm . The numbers of these cells were then calculated by averaging the cell numbers from all replicates (Ferguson *et al.*, 2010). For the villous height measurement, the 10 villi were randomly selected per section. The villous length was measured from the villous tip to the base. An average of these 10 villi per section was expressed as the mean villous height for each section.

2.9 Intestinal gene expression (chapters 4 and 5)

To evaluate whether probiotic treatment had an effect on intestinal health status, the expression of various genes, antiviral genes and genes involved with the regulation of cellular proliferation were examined using real time PCR. These genes are listed in the relevant experimental chapters. Samples (30 - 40 mg) were taken from the AM and PM of the intestine of fish and placed in 1.5 mL MCT contain RNA stabilization and protection reagent (RNA later[®] Sigma, UK) at 4 °C for 24h and then stored at -80 °C until used.

2.9.1 RNA extraction

Intestinal sample RNA was extracted using TRIzol[®] (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, with some modifications as previous described by Pérez-Sánchez *et al.* (2011b). Briefly, 40 mg of intestine tissue was homogenized in 1.5 mL MCT containing 1 mL of TRIzol for 15 sec at medium-speed by using Ultra-Turrax T18 Homogenizer (IKA), and 0.2 mL chloroform was added. After vigorous shaking and incubation at room temperature for 5 min, cell debris was removed by centrifugation at 10,000x *g* for 15 min. The upper aqueous phase containing the RNA was gently transferred to a fresh tube containing an equal volume of cold

isopropanol, while the lower phase and white protein inter-phase were discarded. Samples were vortexed for at least 20 sec and centrifuged at 12,000x *g* for 10 min. Supernatants were discarded and the precipitated RNA pellets were washed using 1 mL of 75% molecular grade ethanol (v/v in MGW). After the final wash, ethanol was removed and pellets were air-dried for 5 – 10 min then redissolved in 30 μ L MGW.

2.9.2 DNA digestion

To avoid contamination with genomic DNA, DNase, was used, following the manufacturer's instructions (10 UI at 37 °C for 10 min, MBI Fermentas). RNA quantity and purity was measured using a NanoDrop UV spectrometer (ND-1000) by measuring the absorbance at 230 nm. RNA integrity was verified by ethidium bromide staining onto a 1% agarose gel. RNA was stored at -80 °C until required.

2.9.3 Reverse transcription to obtain complementary DNA (cDNA)

Extracted RNA was used for cDNA synthesis, which was carried out using an iScript cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer's instructions. Briefly, cDNA was synthesised using 1 μ g of total RNA incubated with reverse transcriptase deoxynucleotide triphosphate (dNTP) mix along with random hexamers.

2.9.4 Real time PCR (RT-PCR)

RT-PCR was carried out with the SYBR® green method in a iQ5 iCycler thermal cycler (Bio-Rad). Duplicate PCR reactions were conducted for each

sample analyzed. The reactions were prepared on a 96-well plate by mixing 1 μL of diluted cDNA (1/20) with MGW, 5 μL of 2x SYBR® Green Supermix (Bio-Rad), containing SYBR® Green as a fluorescent intercalating agent, 0.3 μM forward primer and 0.3 μM of reverse primer in each well. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20 sec at 95 °C, 20 sec at 60 °C and 20 sec at 72 °C.

β -actin and 60S genes were used as reference genes in each sample in order to standardize the results by eliminating variations in mRNA and cDNA quantity and quality (Bustin *et al.*, 2009). The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) including Genex Macro iQ5 Conversion and Genex Macro iQ5 files.

The data was analysed based on the differences between the reference (control) and the treatment groups using a comparative Ct analyses, using the following equations:

$$\Delta\Delta\text{Ct} = \Delta\text{Ct sample} - \Delta\text{Ct reference control}$$

$$\text{Amount of target (RQ)} = 2^{-(\Delta\Delta\text{Ct})}$$

Where Ct is the threshold cycle

2.10 Haematological and immunological parameters

Fish were deprived of feed for 17h prior to sampling. Fish were randomly selected and sedated by transfer to an anaesthetic bath of 80 mg L⁻¹ (MS222) buffered with sodium bicarbonate. Fish were judged as suitably sedated for subsequent sampling by loss of equilibrium and handling time was less than 1 min in order to minimize stress effects. Blood was sampled from the caudal arch vein using a 25 gauge needle and 1 mL syringe.

Blood (ca. 500 μL) was placed in separate 1.5 mL MCTs containing 50 μL of heparin solution to determine the haematocrit values, conduct blood smears, determine haemoglobin and neutrophil respiratory burst activity. In order to separate serum, a further 500 μL of blood was transferred to separate 1.5 mL MCTs without the anticoagulant solution, and the blood was allowed to clot for at least 3h at room temperature prior for centrifugation. Serum was collected and stored at $-80\text{ }^{\circ}\text{C}$ until analysis of lysozyme activity. Any haemolysed, clotted or insufficient volume samples were discarded.

After sampling procedure, fish (12 fish per treatment) were placed in aerated water taken from the experimental system for a few minutes in order to recover and later fish were returned to the original tank in the experimental system.

2.10.1 Haematocrit

The haematocrit value expresses the corpuscular volume in relation to the total volume of blood. Heparinised haematocrit tubes were filled to three quarters of their total volume with heparinised blood and the ends of the tubes were sealed with Critoseal. Micro capillaries were centrifuged for 5 min at $6000\times g$ using a micro haematocrit centrifuge. Haematocrit values were determined as the total percentage packed cell (PCV) volume using a Hawksley haematocrit reader (Klontz, 1997).

2.10.2 Differential leucocyte counts

Blood smears were made as follows: 5 μL of blood was smeared on to microscope slides. The prepared smears were allowed to air dry at room temperature for an hour and were then fixed in 95% methanol for 5 min.

Slides were placed in May–Grünwald staining solution (Sigma, UK; 2 parts: 1 part pH 6.8 buffer solution) for 10 minutes, and slides were then stained with 20% Giemsa stain for 20 min and washed with distilled water. When thoroughly dried, slides were mounted with coverslips using DPX (BDH). The differential leucocyte counts (neutrophil, eosinophils, lymphocytes, monocytes) and thrombocytes were counted using a Kyowa MEDILUX-12 light microscope at 1000x magnification (e.g. Plate 2.2). Leucocytes were identified according for the descriptions of Rowley (1990).

A minimum of 200 cells per fish sample were counted (Page *et al.*, 1999) and the total number of each leucocyte class was expressed as a percentage of the total leucocyte populations. The images of selected leucocytes were taken using an Olympus C-2020Z digital camera under 1000x magnification on an Olympus AHBT-513 VANOX photomicroscope.

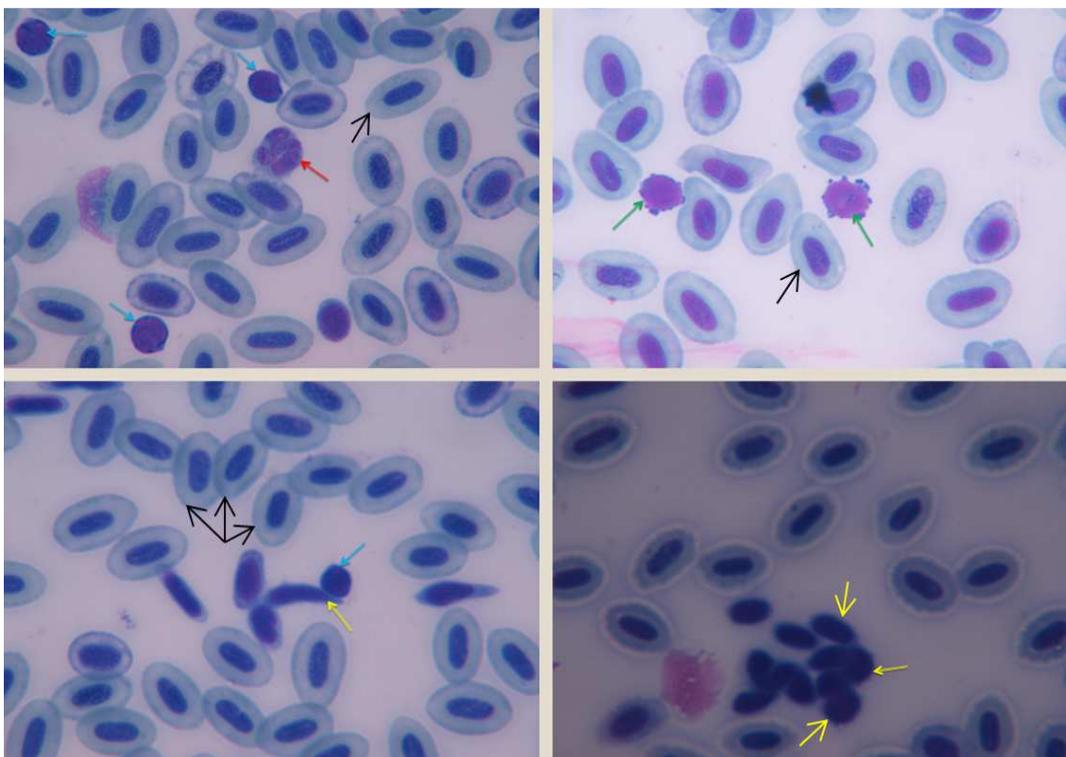


Plate 2.2 Leucocyte types: neutrophil (red arrow), thrombocyte (yellow arrow), lymphocytes (blue arrow) and monocytes (green arrow). Red blood cells are indicated by black arrows.

2.10.3 Circulatory leucocyte and erythrocyte levels

In order to enumerate total blood cell levels (total erythrocyte and leucocyte counts), 20 μL of blood sample was transferred into MCTs containing 980 μL of Dacies solution (10 mL of 40% formaldehyde, 31.3 g trisodium citrate, 1.0 g brilliant crystal blue dissolved in 1L of distilled water; filtered through a 0.45 μm syringe filter) and mixed gently to disperse the cells to give final concentration (1/50 dilution).

Samples were kept at 4 $^{\circ}\text{C}$ until cell numbers were calculated. Leucocyte and erythrocyte counts were carried out using a haemocytometer (400 x magnifications) and a Kyowa MEDILUX 12 light microscope.

Calculations of cell numbers were determined using the following equation:

$$\text{Cell count (x } 10^6 \text{ cells/mm}^3\text{)} = ((\text{average cell count/volume of square (mm}^3\text{)}) \times \text{dilution factor})/1000000$$

2.10.4 Haemoglobin (Hb)

The cyanohaemoglobin method was used for determination of haemoglobin. One mL of Drabkins reagent (Sigma, UK) was placed into each 1.5 mL MCT and 4 μL of heparinised blood was added. Drabkins reagent was used to zero the spectrophotometer UNICAM (set at 540 nm) and the absorbance of the samples was recorded. A standard curve was prepared using cyanmethemoglobin standard solutions of known haemoglobin concentrations verses OD absorbance.

The resulting absorbance values were then used to generate a standard curve. The haemoglobin content of the samples was determined from the standard curve and presented as g dL^{-1} .

2.10.5 Haematological indices

Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from RBC, Hct and Hb according to the following formulae: $MCV = (Hct (\%) \times 10) / RBC \text{ mm}^3$, $MCH = Hb \times 10 / RBC$ and $MCHC = (Hb \times 100) / Hct$ (Klontz, 1997).

2.10.6 Lysozyme activity

In order to analyse serum lysozyme activity, ca. 500 μL of blood was placed in each 1.5 mL MCT without anticoagulant solution and left to clot for 3h at room temperature prior to centrifugation at 2000x g for 5 min. Serum was pipetted into a new 1.5 mL MCT before further centrifugation at 2000x g for 5 min. The serum supernatant was stored at $-20 \text{ }^\circ\text{C}$ until used. Lysozyme activity was assessed using a turbidometric assay in a 96 well microplate according to Ellis (1990).

In summary, 190 μL of *Micrococcus lysodeikticus* (0.2 mg mL^{-1}) in 0.04 M Na_2HPO_4 buffer (pH 5.8 for trout and 6.3 for salmon) was pipetted into separate 96 well microplate wells. Two columns of wells, each containing 200 μL of 0.04 M Na_2HPO_4 (without bacteria) were used as controls. After that, 10 μL of serum was added to each of the *Micrococcus lysodeikticus*-containing wells. After mixing, the reduction in turbidity was measured at 540 nm at 0.5 min and at 4.5 min at $22 \text{ }^\circ\text{C}$ in a microplate reader (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA). Lysozyme activity was recorded as units of activity, where one unit is the amount of

enzyme causing a decrease in absorbance of 0.001 absorbance units per minute.

2.11 Growth parameters

Upon termination of the feeding trials, growth performance parameters, including percentage weight gain (WG), specific growth rate (SGR; per cent increase in body weight day⁻¹), thermal growth coefficient (TGC), feed conversion ratio (FCR) and condition factor (*K*) were calculated using the following formulae:-

$$WG = (FW - IW)$$

$$SGR = \frac{(\ln FW - \ln IW)}{t} \times 100$$

$$FCR = \frac{FI}{FW - IW}$$

$$K = (100 \times FW) / FL^3$$

$$TGC = ((FW^{1/3} - IW^{1/3}) / (\text{water temp. } (^\circ\text{C}) \times T)) \times 1000$$

Where FW is the final weight (g), IW is the initial weight (g), t is the duration of feeding (in days), FI is feed intake (g) and FL = final length (cm) and Ln = natural logarithm value.

2.12 Statistical analysis

Unless otherwise stated, all presented data are means \pm standard deviation (SD). DGGE banding patterns were transformed into presence/ absence matrices for similarity assessment between treatments using Quantity one software (Bio-Rad Laboratories, CA, USA) and PRIMER V.6 software. Band intensities were measured and were analysed using software Primer v6 to determine similarity percentage (SIMPER), species richness, species evenness and diversity according to the following formulae:

- Margalef's species richness: $d = (S-1)/\log(N)$
- Pielou's evenness: $J' = H'/\log(S)$
- Shannon's diversity index: $H' = - \sum (p_i \ln p_i)$

Where: N = total number of individuals (total intensity units), p_i = the proportion of the total number of individuals in the i th species, S = number of OTUs.

All other statistical analyses were carried out using MiniTab statistical software version 16, IBM (Pennsylvania, USA). Statistical analyses for growth parameters, haematology, gene expression and histology are explained in the relevant chapters.

Chapter 3

Chapter 3: Microbial community on the intestinal mucosa of brown trout (*Salmo trutta*)

Abstract

A study was conducted to characterise the autochthonous gut microbiota present in the pyloric caeca (PC), anterior mucosa (AM) and posterior mucosa (PM) of brown trout. Aerobic colony count (ACC) bacterial populations were enumerated using tryptone soy agar, lactic acid bacteria (LAB) levels were enumerated on de Man, Rogosa and Sharpe agar and PCR-DGGE was employed as a culture-independent method to assess the total communities.

No significant differences were observed between the different gut regions for ACC or LAB levels. 16S rRNA gene sequencing identified all LAB isolates as *Carnobacterium maltaromaticum*. In contrast, the ACC community was more diverse; Firmicutes and Bacteroidetes were present but all gut regions were dominated by Proteobacteria, accounting for 88.4 - 92.6% of the communities. *Citrobacter freundii* was the dominant species and accounted for 51.0 - 57.8% of the isolates.

Complex bacterial communities were observed by PCR-DGGE and a trend towards the reduction in the number of operational taxonomic units (OTUs), microbial richness and diversity was observed from the PC to the PM. The similarity between regions was low (52 - 68%) and cluster analysis revealed that the communities grouped into two distinct clusters; one dominated by the PM samples and the other contained the AM and PC samples. OTUs were identified as members of the phyla Proteobacteria and Firmicutes. Many OTUs were detected in all GIT regions, however, some OTUs showed regional specialization.

3.1 Introduction

The GIT of fish represents a primary point of contact between the external environment and the host body. The GIT has been reported to fulfil several functions including digestion, food uptake, water and electrolytic equilibrium, endocrine regulation of digestion, metabolism and immunity (Denev *et al.*, 2009). The mucosal epithelium of the intestine is directly in contact with the microbial community, thus it is considered the first line of defence due to existence of mucosal cells which produce and secrete mucus. Mucus contains numerous components including immunoglobulin, complement, lectins, pentraxins, lysozyme, complement proteins, proteolytic enzymes and antimicrobial peptides with biostatic or biocidal activities (Trichet, 2010), which act to bind, trap and prevent pathogenic bacteria and parasites from attaching to the mucosal surface (Ellis, 2001).

Fish possess a wide range of microbiota in their intestine, consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria (Ringø *et al.*, 2003). Bacteria by numbers are thought to be the largest component of the gut microbiota of fish and are the most studied (Huber *et al.*, 2004; Pond *et al.*, 2006; Hovda *et al.*, 2007). The microbiota of the intestine of fish is likely to be highly dependent on factors such as bacterial colonization during early development, dietary changes and environmental conditions such as temperature, salinity, level of dissolved oxygen and degree of pollution (Ringø *et al.*, 1995). The microbiota of fish can be helpful in the digestion process by the production of enzymes, amino acids and vitamins, they also modulate the immune system (Gómez and Balcázar, 2008), give resistance

against colonization and invasion of pathogenic bacteria (Nayak, 2010b), and improve water quality (Gatesoupe, 1999).

The brown trout (*Salmo trutta*) is distributed world-wide and is an important fish species in terms of recreational fisheries and angling; it is farmed in many countries for this purpose and for restocking programmes (FAO, 2013).

The microbial community of the GIT of brown trout has not been comprehensively studied. Balcázar *et al.* (2007b) characterised and identified LAB isolates from the whole gut contents of brown trout, and tested their efficacy as potential probiotics. More recently, the allochthonous microbial community of the GIT in brown trout was investigated using PCR-DGGE (Manzano *et al.*, 2012). However, this study investigated the microbial community in whole intestinal content but did not investigate autochthonous communities or the microbiota present in pyloric caeca. Most investigations in the field of the gut microbiota in fish have only used cultivation techniques to evaluate. These techniques commonly depend on the isolation of bacteria from intestinal samples on a wide range of selective and non-selective media under different incubation periods (Pond *et al.*, 2006). However, these methods are labour-intensive, time-consuming and are limited to providing information only on the small proportion of bacteria that are culturable (Spanggaard *et al.*, 2000; Huber *et al.*, 2004; Pond *et al.*, 2006; Romero and Navarrete, 2006; Navarrete *et al.*, 2009; Navarrete *et al.*, 2010).

To overcome the aforementioned problems, recent studies have shed light on numerous molecular techniques including DGGE (Hovda *et al.*, 2007; Merrifield *et al.*, 2009a), PCR-temperature gradient gel electrophoresis (PCR-

TGGE) (Navarrete *et al.*, 2010; Navarrete *et al.*, 2012), PCR-random amplified polymorphic DNA (RAPD) (Spanggaard *et al.*, 2000), fluorescence *in situ* hybridization (FISH) (Huber *et al.*, 2004) and clone libraries (Kim *et al.*, 2007). These approaches are useful in that they offer new opportunities for detection and identification of the microbiota, leading to a broader understanding of the microbial composition in the GIT of fish. Therefore, the aim of this study was to gain a better overall understanding about the GIT microbiota of brown trout (*Salmo trutta*) by using both culture-dependent and culture-independent techniques.

3.2 Materials and methods

3.2.1 Sampling and processing

Brown trout larvae were obtained from Torre fisheries (Watchet, Somerset, UK) and were fed a commercial standard diet (EWOS[®] Sigma 50; Bergen, Norway).

Fish were reared at 16 °C, with daily testing of basic water quality parameters as described elsewhere (Section 2.3). The pH, temperature and DO were maintained at 6.5 - 7.5, 16 - 18 °C and >85%, respectively. Ammonia, nitrite and nitrate levels were tested once a week (Section 2.3) and maintained at $0.07 \pm 0.06 \text{ mg L}^{-1}$, $0.03 \pm 0.06 \text{ mg L}^{-1}$ and $4.02 \pm 1.68 \text{ mg L}^{-1}$, respectively. A photoperiod of 12h light/12h dark was used throughout the entire trial period. Fish were maintained under these conditions for 8 months.

3.2.2 Fish dissection

Prior to commencing the study, six fish ($290.95 \pm 29.9 \text{ g}$ in weight, $28.47 \pm 1.3 \text{ cm}$ in length) were euthanized and dissected as described in Section 2.7.1. The intestinal mucosa and pyloric caeca were aseptically emptied into sterile 1.5 mL MCTs; in order to reduce variation the resulting materials from two fish were pooled into one sample (Hovda *et al.*, 2007; Merrifield *et al.*, 2011), thus yielding three samples in total.

3.2.3 Culture-dependent characterisation of the intestinal microbiota

For microbiological investigation, samples were processed and analysed as described in Section 2.7.2. ACC and LAB populations were counted after incubation at 15 °C for 7 days on TSA and 5 days on MRS, respectively

(Spanggaard *et al.*, 2000; Lamari *et al.*, 2013). Numbers were expressed as colony forming units per gram (CFU g⁻¹).

A total of 209 colonies were collected from TSA and MRS plates as follows: TSA (135 colonies in total) - 39 from the PC, 51 from the AM, 45 from the PM; MRS (74 colonies in total) - 25 colonies from the PC, 26 from the AM and 23 from the PM. Colonies were randomly picked irrespectively of colour and shape from each plate containing 30 to 300 CFU and sub-cultured on TSA and MRS agar (as appropriate) repeatedly until pure cultures were obtained (Merrifield *et al.*, 2011). The isolates were stored at 4 °C.

3.2.4 PCR-based identification of pure colonies

DNA from the aforementioned 209 selected colonies was extracted following the method described by Pitcher *et al.* (2008). Briefly, a pure colony was suspended in 50 µL MGW and subjected to 10 min boiling. The concentration and purity of DNA (ng/µL) was determined as described in Section 2.5.3. Amplification of 16S rRNA gene was carried out as described in Section 2.7.3.2. The amplified products were subsequently loaded onto a 1.5% agarose gel to assess the size of PCR products as described in Section 2.5.2. The PCR products were cleaned and sequenced either as described in Section 2.5.3 or as described by Ringø *et al.* (2006c).

3.2.5 Denaturing gradient gel electrophoresis (DGGE)

Mucosa samples and pyloric caeca samples from two fish were pooled into one sample and stored on ice prior to storage at -20 °C. DNA was extracted from three replicates of the pooled samples (from six fish) using a QIAamp®

Stool Mini Kit (Qiagen) with minor modification to the manufacturer's instructions, as described in Appendix 1. The variable V3 region of the 16S rRNA gene was amplified using PCR as described in Section 2.7.4.2. The amplified products were subsequently loaded onto 1.5% agarose gel to assess the size of PCR products as described in Section 2.5.2. The resulting PCR products were used to obtain DNA fingerprints of the bacterial community on a 40 - 60% DGGE as described in Section 2.7.4.3. After DGGE, OTUs of interest were excised from the gel and re-PCR'd as described in Section 2.7.4.4. Selected bands were purified and sequenced as described in Section 2.5.3.

3.3 Statistical analyses

The means and standard deviation (SD) were calculated for ACC and LAB numbers in each gut region. Data were tested for normality using Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) followed by Tukey's HSD multiple range *post hoc* testing were used to determine the significant differences between culturable ACC and LAB levels in the gut regions. DGGE fingerprints were analysed as described in Section 2.12. The level for accepted statistical significance was $P < 0.05$. Data management and analysis were carried out using MiniTab statistical software version 16, IBM (Pennsylvania, USA).

3.4 Results

3.4.1 Culture –based of bacterial population

The mean log counts of ACC and LAB populations associated with the brown trout intestine are displayed in Figure 3.1. Mean log values for ACC were to 3.89 ± 0.93 , 4.23 ± 1.40 , 4.90 ± 1.03 CFU g⁻¹ in the PC, AM and PM, respectively. LAB levels were log 3.60 ± 0.30 , 4.03 ± 1.40 and 4.44 ± 0.56 CFU g⁻¹ in the PC, AM and PM, respectively (Figure 3.1).

A one way ANOVA revealed no significant differences were observed between either LAB or ACC bacterial population in the gut regions. The levels of LAB constituted approximately 3.9%, 48.8% and 9.7% of the ACC levels in the PC, AM and PM, respectively Figure 3.2.

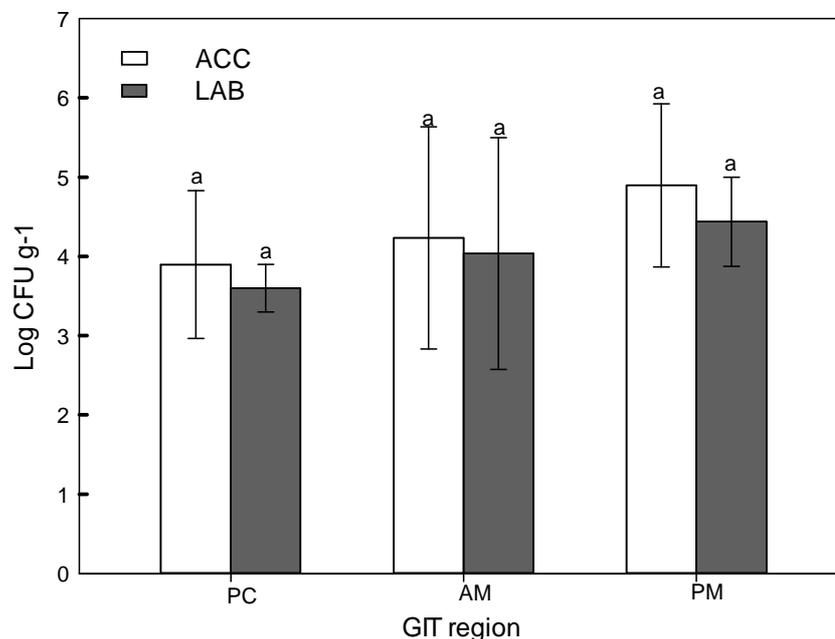


Figure 3.1 Number of ACC and LAB (log CFU g⁻¹) isolated from the anterior intestine (AM), posterior intestine (PM) and pyloric caeca (PC) of brown trout. Results are presented as mean log values \pm SD in each region of fish ($n = 3$). No significant differences between bacteria in each group were found among regions ($P > 0.05$).

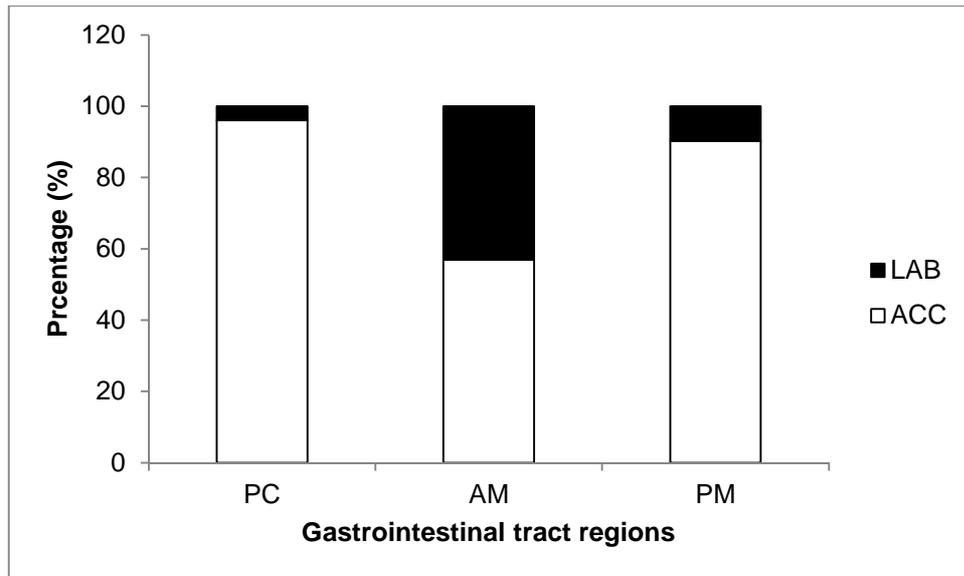


Figure 3.2 Proportion (%) of cultivable LAB of total counts (i.e. ACC+LAB) in the GIT of brown trout. AM (anterior mucosa), PM (posterior mucosa) and PC (pyloric caeca).

3.4.2 16S rRNA gene analysis of pure colonies

Examination of the ACC (i.e. total culturable bacteria) isolates by 16S rRNA gene sequencing from the NCBI using the BLAST showed that *Citrobacter freundii* was the dominant bacteria of the PC, AM and PM, comprising 56.4%, 51.0% and 58.0% of all sequenced isolates, respectively. A wide range of other bacterial species were also isolated from the PC, AM and PM (Figures 3.3.A - 3.5.A).

Representative gut isolates were classified into three phyla; Proteobacteria, Firmicutes and unidentified bacteria. In the PC, the relative percentages of these phyla were 84.6%, 5.1% and 10.3%, respectively (Figure 3.3.B), while in the AM, the relative percentages of these phyla were 90.2%, 1.9%, and 7.8%, respectively (Figure 3.4.B). In the PM, the relative percentages of

Proteobacteria and Firmicutes were 93.3% and 6.7%, respectively (Figure 3.5.B).

C. freundii and *Stenotrophomonas rhizophilla* were detected in all regions, while *Enterobacter hormaechei* and *Ochrobactrum pseudogrignonense* were detected in both PC and AM. In addition, *Stenotrophomonas maltophilia*, *Agrobacterium tumefaciens*, *Shigella flexneri* and *Aeromonas sobria* were identified only in the AM and PM (Figures 3.3.A – 3.5.A).

On the other hand, 16S rRNA gene genetic analysis results of pure cultures from TSA revealed that each region contained a variety of bacteria which were not present in the other regions.

In contrast to these findings, 16S rRNA gene genetic analysis results of pure culture from MRS plates demonstrated that *Ca. maltaromaticum* was the dominant component 100% representative of LAB isolates in all regions.

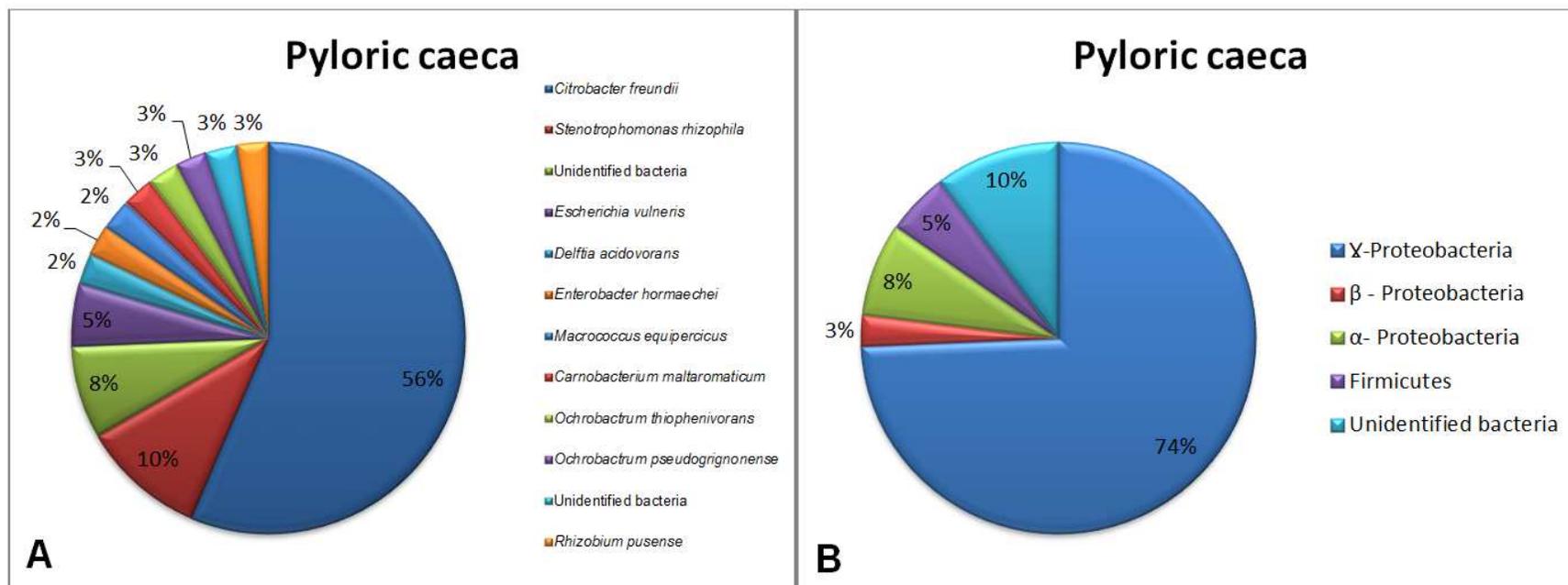


Figure 3.3 (A) Summary of BLAST search data arising from 16S rRNA gene analysis of pyloric caeca (PC) and (B) prevalence of the different bacterial phylotypes isolated from the same region in brown trout.

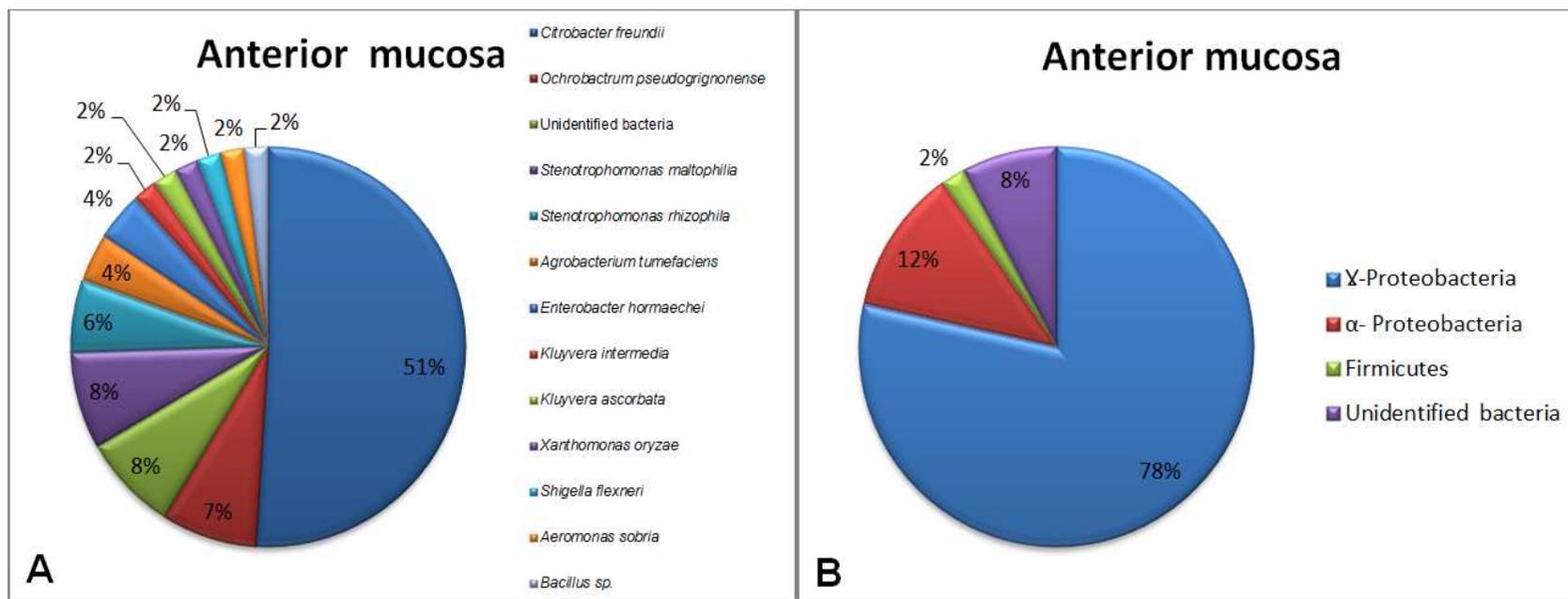


Figure 3.4 (A) Summary of BLAST search data arising from 16S rRNA gene analysis of anterior mucosa (AM) and (B) prevalence of the different bacterial phylotypes isolated from the same region in brown trout.

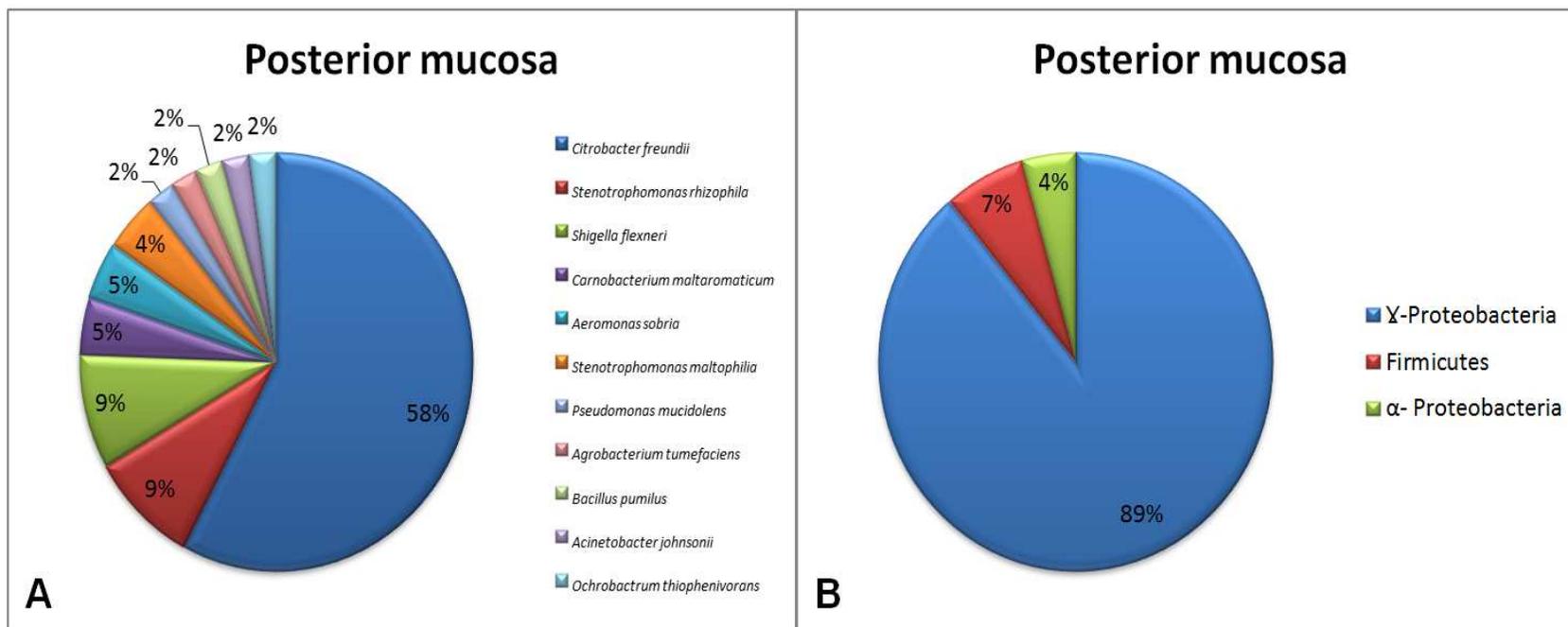


Figure 3.5 (A) Summary of BLAST search data arising from 16S rRNA gene analysis of posterior mucosa (PM) and (B) prevalence of the different bacterial phylotypes isolated from the same region in brown trout.

3.4.3 PCR- DGGE analysis

The bacterial community of the PC, AM and PM from three replicates (each replicate containing samples from two fish pooled) were analysed by PCR-DGGE (Figure 3.6).

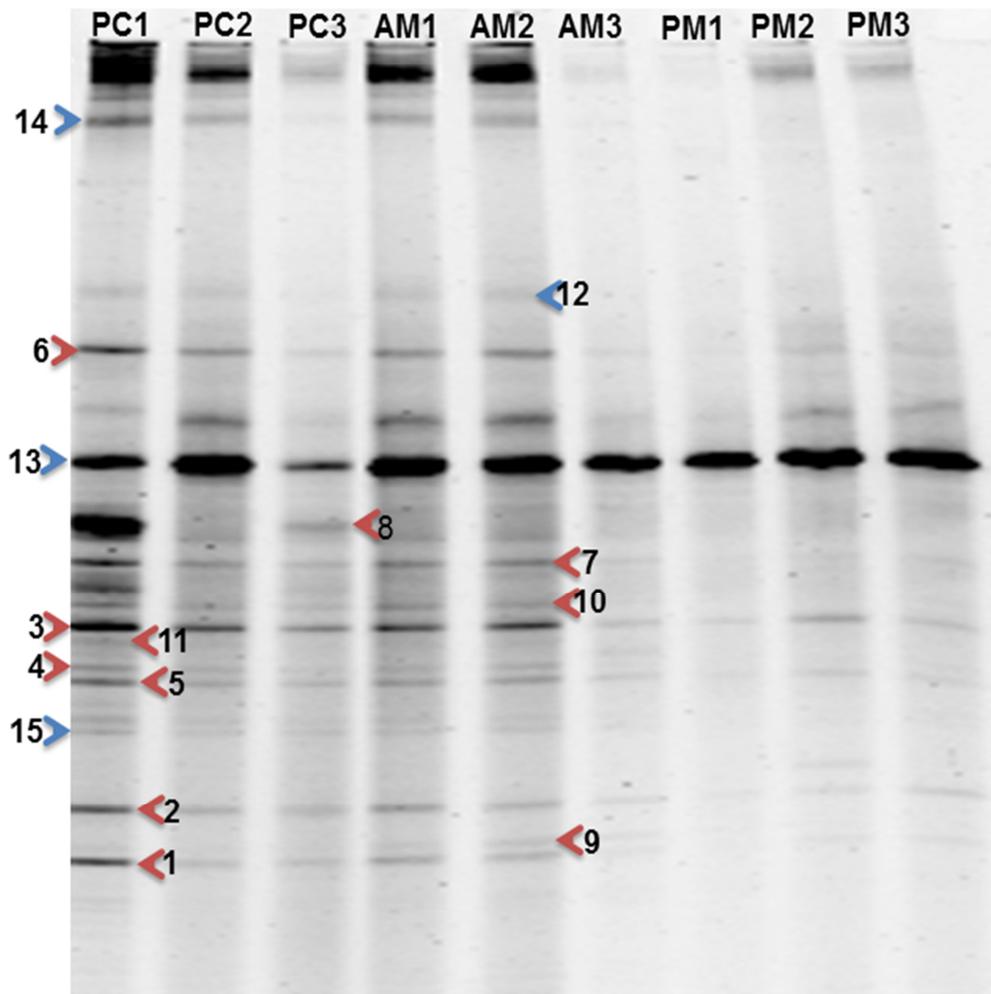


Figure 3.6 DGGE fingerprints of autochthonous microbiota in the pyloric caeca (PC), anterior mucosa (AM) and posterior mucosa (PM) in brown trout. Arrows represent the OTUs excised and sequenced. Bands which were not successfully sequenced are indicated by blue arrows.

Non-metric multidimensional scaling (nMDS) of DGGE fingerprints is presented in Figures 3.7. The nMDS gives a graphical representation of the sample replicates, where their relative positioning is a reflection of their

similarity to each other. The nMDS revealed that the communities grouped into two distinct clusters, each containing >60% similarity between samples. One cluster was dominated by the PM replicates (and included one AM replicate) and the other cluster contained the remaining AM replicates and all of the PC replicates. Significant differences were observed for the similarity of replicates within the gut regions; the similarity was significantly higher in the PC and AM ($68.00 \pm 10.9\%$) compared to its value between the groups PC and PM ($52.10 \pm 10.6\%$, $P = 0.022$). SIMPER (similarity percentage) values were calculated (Table 3.1) and revealed that no significant differences were observed between the regions. Further microbial community analysis parameters are also displayed in Table 3.1. In terms of OTUs numbers, species richness and diversity, no significant differences were observed between the regions (Table 3.1).

A total of fifteen OTUs were selected from the DGGE gel and are indicated on the gel image in Figure 3.6. Some OTUs were common to all groups and/or replicates and others showed regional specialization. The results of band sequence analysis are shown in Table 3.2. The percentage allocation of the selected OTUs to bacterial phyla was as follows: Firmicutes 54.5%, Proteobacteria 36.4%, and unidentified bacteria 9.1%. *Sphingopyxis* sp., *Shewanella* sp., *Escherichia fergusonii* and *Alcaligenes* sp. were the main representatives of Proteobacteria. The most frequently observed representatives of Firmicutes were *Streptococcus macedonicus*, *Streptococcus constellatus*, *Streptococcus gallolyticus*, *Weissella cibaria* and *La. lactis* subsp. *lactis*. Two OTUs (8 and 11) were not detected in the AM and PM, while they were detected in the PC and were most closely related to

St. gallolyticus and *St. constellatus*, respectively. Three OTUs (4, 9 and 10) were not detected in the PM, while they were detected in other regions and were most closely related to *Alcaligenes* sp., *Shewanella* sp. and uncultured bacterium. Six OTUs (1, 2, 3, 5, 6 and 7) appeared to be common to either all regions or all replicates, sequence analysis showed them to be most similar to *Sphingopyxis* sp., *Escherichia fergusonii*, *St. macedonicus*, *La. lactis* subsp. *lactis*, *W. cibaria* and uncultured bacterium. Overall, the DGGE fingerprints revealed a different bacterial community compared to culture based analysis. Four OTUs (12, 13, 14 and 15) failed to yield useful nucleotide sequence reads.

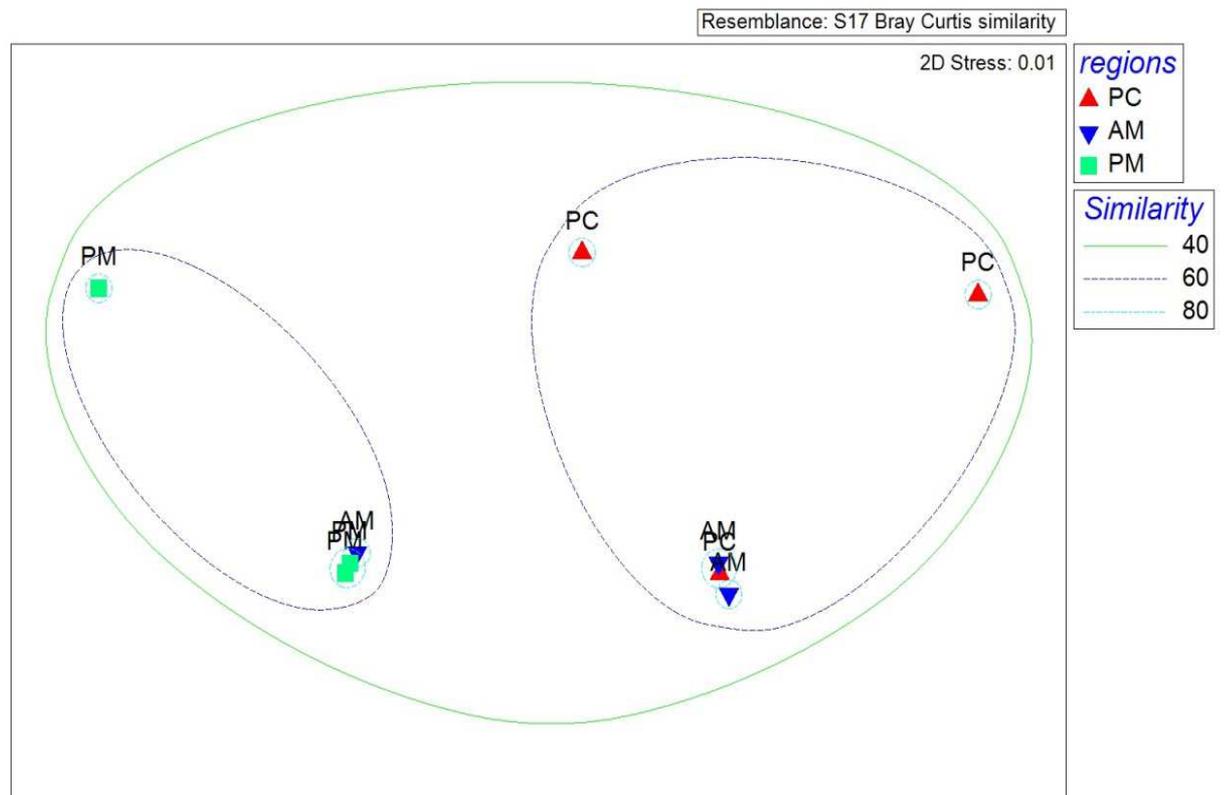


Figure 3.7 Non-metric multidimensional scaling (nMDS) of DGGE fingerprints incorporating similarity percentages of bacterial communities between regions ($n = 3$). Sample codes, AM- anterior mucosa, PM- posterior mucosa and PC- pyloric caeca.

Table 3.1 Autochthonous microbial community analysis from PCR-DGGE fingerprints of the microbiota in the GIT of brown trout.

	OTUs ¹	Richness ²	Evenness ³	Diversity ⁴	SIMPER (%)	ANOSIM		Similarity (%)
						R value	P value	
PC	29.0 ± 4.6	2.58 ± 0.39	0.99 ± 0.01	3.3 ± 0.1	65.00 ± 9.54			
AM	27.3 ± 8.0	2.34 ± 0.67	0.98 ± 0.00	3.2 ± 0.3	65.67 ± 10.02			
PM	18.7 ± 2.3	1.61 ± 0.20	0.98 ± 0.00	2.9 ± 0.1	70.33 ± 11.37			
PC v AM						-0.222	0.90	68.11 ± 10.98 ^a
PC v PM						0.778	0.10	52.22 ± 10.67 ^b
AM v PM						0.37	0.10	61.13 ± 12.03 ^{ab}

Results are presented as mean ± SD in each group of fish (n = 3). Means having different letters within the same region in the same column are significantly different ($P < 0.05$)

¹ Operational taxonomic unit.

² Margalef species richness: $d = (S - 1) / \log(N)$.

³ Pielou's evenness: $J' = H' / \log(S)$.

⁴ Shannons diversity index: $H' = -\sum(pi(\ln pi))$.

Table 3.2 Summary of the sequencing analysis results generated from OTUs excised from the DGGE gel. Numerical values represent the number of replicates (out of 3) that the OTUs were present in.

OTUs number	Phylum	NCBI blast matches	Max. Identity (%)	NCBI Accession number	Length of sequences	E value	PC	AM	PM
1	Proteobacteria	<i>Sphingopyxis</i> sp.	97	HM-484309.1	108	4e-49	3	2	2
2	Proteobacteria	<i>Escherichia fergusonii</i>	95	NR-074902.1	103	3e-40	3	3	3
9	Proteobacteria	<i>Shewanella</i> sp.	100	JQ670710.1	167	2e-45	1	2	0
4	Proteobacteria	<i>Alcaligenes</i> sp.	100	FN428756.1	172	2e-49	3	3	0
3	Firmicutes	<i>Streptococcus macedonicus</i>	100	NR-074404.1	138	4e-54	3	3	3
11	Firmicutes	<i>Streptococcus constellatus</i>	100	GU416005.1	130	2e-18	1	0	0
8	Firmicutes	<i>Streptococcus gallolyticus</i>	92	NR-074849.1	161	5e-18	2	0	0
6	Firmicutes	<i>Weissella cibaria</i>	100	JN851741.1	147	4e-21	3	3	2
5	Firmicutes	<i>Lactococcus lactis</i> subsp. <i>lactis</i>	93	JN851797.1	164	2e-54	3	3	3
7	Firmicutes	Uncultured bacterium	80	AY537010.0	135	4e-21	3	3	2
10	Unidentified	Uncultured bacterium	100	JQ475753.1	134	3e-21	3	2	0

Sample codes, AM- anterior mucosa, PM- posterior mucosa and PC- pyloric caeca.

3.5 Discussion

The present study revealed cultivable bacterial populations in the GIT of brown trout of up to $\log 5 \text{ CFU g}^{-1}$. Comparably high levels of adherent gut mucosal viable counts have been found in other salmonid species including Atlantic salmon (Ringø *et al.*, 2006a) and Arctic charr (Ringø *et al.*, 2006c). Several prior publications have investigated the microbial community in the different species of fish by means of both cultivation and molecular methods.

For example, Kim *et al.* (2007) investigated the microbial community of the intestine of rainbow trout by using culture based and the results revealed that the culturable microbiota levels were $\log 6.5 \text{ CFU g}^{-1}$ in the posterior mucosa of rainbow trout. Furthermore, the culturable microbiota in the range of $\log 4.77 \text{ CFU g}^{-1}$ have been found adhered with the anterior mucosa, whereas the levels of the culturable microbiota in the range of $\log 5.38 \text{ CFU g}^{-1}$ were attached to the posterior mucosa of rainbow trout (Merrifield *et al.*, 2009a). A study by Pond *et al.* (2006) reported the LAB and ACC counts associated with the intestinal mucosa of rainbow trout were $\log 2.3 \text{ CFU g}^{-1}$ and $\log 5.6 \text{ CFU g}^{-1}$, respectively. In 2007, Hovda and co-workers used cultivation techniques and molecular analysis of the 16S rDNA gene to assess the intestinal microbiota of farmed Atlantic salmon. The allochthonous ACC was reported to be $\log 3.9$, 3.7 and 4.8 CFU g^{-1} in the fore-, mid- and hind gut, respectively.

One of the aims of the present study was to assess the microbiota by identifying representative isolates by using 16S rRNA gene. Prior studies reported that genera of the phyla γ -Proteobacteria and Firmicutes were numerically the most predominant in the intestinal regions of numerous fish

species, among these bacteria Enterobacteriaceae, which have been commonly isolated from salmonids (Spanggaard *et al.*, 2000; Huber *et al.*, 2004; Pond *et al.*, 2006; Hovda *et al.*, 2007; Kim *et al.*, 2007; Manzano *et al.*, 2012; Ingerslev *et al.*, 2014).

In the study by Navarrete *et al.* (2012) the dominant bacteria in four unrelated families of rainbow trout were classified into the following five phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Fusobacteria. In accordance with aforementioned studies, the present study demonstrated that several species belong to Enterobacteriaceae were isolated from all GIT regions of brown trout.

In the current study, in common with most previous studies on salmonids, the microbial community was mainly composed by Proteobacteria followed by Firmicutes and unidentified bacteria, which was in the line with those reported in the literature (Spanggaard *et al.*, 2000; Hovda *et al.*, 2007; Svanevik and Lunestad, 2011; Navarrete *et al.*, 2012; Wu *et al.*, 2012).

The Enterobacteriaceae family is an important phylogenetic group within the γ - subclass of Proteobacteria, and members of this family, in particular *C. freundii*, were the most abundant isolates from TSA in the current study. Members of this family are Gram-negative, oxidase negative, facultatively anaerobic and are reported to infect humans in several diseases such as enteritis, typhoid fever and shigellosis (Bohnert *et al.*, 2000).

According to Austin and Austin (2007), some *C. freundii* strains can be regarded as opportunistic pathogens, and has been reported to cause several diseases in many species of fish particularly in salmonids in the UK

including enteritis in rainbow trout and hemorrhagic septicemia of cyprinids (Jeremić *et al.*, 2003). In this respect, Lü *et al.* (2011) demonstrated that wild zebrafish challenged with *C. freundii* displayed hemorrhagic septicemia. Recently, members of the genus *Citrobacter*, particularly *C. freundii*, have been commonly isolated from the gut of fish, including rainbow trout (Desai *et al.*, 2012), salmon (Holben *et al.*, 2002), carp (Lü *et al.*, 2011; Wu *et al.*, 2012), but their role in the GIT of fish is still unknown. Interestingly, supporting the findings of the present results, *C. freundii* has been isolated from the intestinal digesta in brown trout (Manzano *et al.*, 2012). However, *C. freundii* is not commonly considered as causal disease agent in healthy fish hosts (Lü *et al.*, 2011). Much more attention is needed to investigate the role of these bacteria as components of the microbiota of the GIT in brown trout.

In agreement with the current results, *Stenotrophomonas* spp. have previously been reported to be present in the intestine of rainbow trout (Heikkinen *et al.*, 2006; Desai *et al.*, 2012). According to the literature, the first isolation of *Stenotrophomonas maltophila* from the gut of a salmonid species was from the hindgut of Atlantic salmon fed krill supplemented diet (Ringø *et al.*, 2006a); *Stenotrophomonas maltophila* is Gram-negative with a tendency to be causative agent for a wide range of disease in plants and as an emerging opportunistic bacteria in humans with an ability to degrade xenobiotics (Schloter *et al.*, 2000).

Ochrobactrum, formerly called *Achromobacter* sp., is a soil bacterium belonging to the Brucellaceae family with an ability to colonize a wide range

of host and is recognized as opportunistic bacteria for humans (Schloter *et al.*, 2000). Two species of *Ochrobactrum* were identified in the present study. *A. sobria* was isolated in this study from the anterior and posterior mucosa. *Aeromonas* spp. are frequently dominant among culturable bacteria in the intestine of fish (Spanggaard *et al.*, 2000; Huber *et al.*, 2004; Hovda *et al.*, 2007; Kim *et al.*, 2007; Lin *et al.*, 2012; Wu *et al.*, 2012; Kühlwein *et al.*, 2013). Fish diseases, characterised by large ulcers and hemorrhagic septicemia, are caused by some species of *Aeromonas* including *A. sobria* (Austin and Austin, 2007). Wu *et al.* (2012) demonstrated that *Aeromonas* may also be beneficial, in terms of its ability to degrade cellulose which may be beneficial to the host. *Acinetobacter johnsonii* was only isolated from the PM in the present work, however these bacteria seem to be among the most frequently isolated cultivable bacteria among the microbiota of salmonids (Spanggaard *et al.*, 2000; Huber *et al.*, 2004; Hovda *et al.*, 2007; Merrifield *et al.*, 2009a; Merrifield *et al.*, 2009b).

The results of 16S rRNA gene analysis of pure colonies of LAB selected from MRS demonstrated that *Ca. maltaromaticum* was the only LAB dominant component in all regions of GIT of brown trout. *Ca. maltaromaticum* is Gram positive nonmotile, facultatively anaerobic rods and has no ability to produce H₂S, oxidase or catalase (Kim and Austin, 2008). *Ca. maltaromaticum* is commonly isolated from natural environments and feed; it can grow at low temperatures with tolerance to freezing/thawing and high pressure (Leisner *et al.*, 2007). The authors also reported that this microbe is able to produce antimicrobial peptides including bacteriocins. Numerous authors have reported that *Carnobacterium* spp. are part of the indigenous microbiota in a

variety of fish species. Several studies have revealed that among LAB, *Ca. maltaromaticum* (previously *Ca. piscicola*) has been frequently isolated from salmonids including rainbow trout (Spanggaard *et al.*, 2001; Huber *et al.*, 2004; Kim and Austin, 2006b; Pond *et al.*, 2006; Desai *et al.*, 2012), Atlantic salmon (Ringø *et al.*, 2006a; Bakke-McKellep *et al.*, 2007; Askarian *et al.*, 2012), brown trout (Balcázar *et al.*, 2007b), Arctic charr (Ringø *et al.*, 1997; Ringø *et al.*, 1998; Ringø *et al.*, 2006c) and *Carnobacterium* is reported to be also member of the human microbiota (Leisner *et al.*, 2007).

It is worth noting that some *Ca. maltaromaticum* strains are thought to cause several diseases in fish including septicaemia, exophthalmia, accumulation of ascitic fluid, haemorrhages and peritonitis (Leisner *et al.*, 2007). Also, it has been found to cause mortality in rainbow trout with clearly clinical signs including accumulation of ascitic fluid, haemorrhages and lesions in the liver, intestine and swim bladder muscle in addition to damaging eyes, kidney, liver, spleen, and pancreas (Toranzo *et al.*, 1993). However, in spite of this opportunistic pathogenicity, researchers have been successful in using *Ca. maltaromaticum* strains as a probiotic in aquaculture (Gram and Ringø, 2005; Kim and Austin, 2006b; Kim and Austin, 2006a).

It should be mentioned that only 3 - 50% of bacteria in the intestine of rainbow trout are reported to be cultivable on TSA after seven days at 15 °C (Spanggaard *et al.*, 2000; Huber *et al.*, 2004), whereas only 1% of Atlantic salmon bacteria were cultivable after 10 days at 17 °C (Navarrete *et al.*, 2009). Indeed, the diversity of bacteria that are able to be detected by these methods has been found to be low (Spanggaard *et al.*, 2000; Huber *et al.*,

2004; Navarrete *et al.*, 2009). Therefore it seemed logical to use molecular methods including PCR-DGGE direct from GIT materials to investigate the microbial community in the present study.

Therefore, in the present study DGGE was employed to characterise the bacterial community structure within the GIT of brown trout. Previous studies demonstrated that Proteobacteria phylum was dominant in fish (Spanggaard *et al.*, 2000; Huber *et al.*, 2004; Pond *et al.*, 2006; Ringø *et al.*, 2006c), which are not in accordance with the present study where most of the OTUs sequenced from the DGGE belonged to the Firmicutes phylum. For example, *W. cibaria*, a LAB from the Firmicutes phylum was identified in the present study. Research by Mourino *et al.* (2012) showed that *W. cibaria* has been successfully used as probiotic and showed that hybrid surubim (*Pseudoplatystoma* sp.) fed *W. cibaria* supplemented diet or *W. cibaria* used in conjunction with inulin elevated some immunological parameters and the level of pathogenic bacteria was significantly reduced in these groups compared to the control fed fish. However, *Weissella* sp. has been identified and isolated as an opportunistic pathogen from rainbow trout (Liu *et al.*, 2009). *Streptococcus* spp. are commonly reported to be components of the GIT communities of salmonids (Ringø *et al.*, 1998; Ringø *et al.*, 2000; Holben *et al.*, 2002; Ringø *et al.*, 2002; Navarrete *et al.*, 2012). Streptococci are Gram positive, non-motile, facultatively anaerobic, non-pigmented, cocci cells (Osawa *et al.*, 1995). In the present study three species belonging to *Streptococcus* genus were identified including *St. gallolyticus* (formerly *St. bovis* biotype 1 (Boleij *et al.*, 2011)), *St. macedonicus* and *St. constellatus*. *St. gallolyticus* was also only observed in the pyloric caeca. *Streptococcus* spp.

have been also isolated from the GIT of many mammalian animals including koalas (*Phascolarctos cinereus*), guinea pigs (*Cavia porcellus*), and brushtail possums (*Trichosurus arnhemensis*) (Osawa and Sly, 1992; Osawa *et al.*, 1995). Furthermore, *St. gallolyticus* are reported to be the main causative agent for some human diseases especially endocarditis associated with a colonic cancer and recently have been isolated from blood and faeces of humans (Köhler, 2007). However, there are no reports of this species causing disease in fish.

In the present study, a *Shewanella* sp. was isolated from the pyloric caeca and anterior mucosa, but not from the posterior mucosa. *Shewanella* spp. have been identified from the gills, skin and gut contents of Atlantic mackerel (*Scomber scombrus*) (Svanevik and Lunestad, 2011), the gut contents of rainbow trout (Spanggaard *et al.*, 2000; Mansfield *et al.*, 2010; Desai *et al.*, 2012) and from the stomach, pyloric caeca, and hindgut of the juvenile Atlantic Salmon (Navarrete *et al.*, 2009). *La. lactis* subsp. *lactis* is commonly reported to be components of the GIT communities of salmonids (Ringø *et al.*, 2000; Balcazar *et al.*, 2007; Navarrete *et al.*, 2010; Desai *et al.*, 2012; Hovda *et al.*, 2012) and is a well-documented probiotic species (Balcazar *et al.*, 2007; Balcázar *et al.*, 2007a; Balcázar *et al.*, 2009). In the current study LAB were only represented by *Weissella cibaria*, *La. lactis* subsp. *lactis* and *Streptococcus* sp. from DGGE and only by *Ca. maltaromaticum* from 16S rRNA gene. Despite being the dominant cultivable species, *C. freundii* and *Ca. maltaromaticum* was not detected as part of the dominant total microbiota from DGGE analyses, which illustrates the importance of utilising both culture and molecular-based approaches in such studies. Indeed, Vallaey *et al.*

(1997) demonstrated that single bands could yield several species (in some cases both Gram-negative and Gram-positive bacteria) with identical rDNA sequences under tighter denaturing gradient conditions. Another limitation in the present study is that only 15 bands were isolated for sequencing (with only 11 successfully sequenced), had all of the OTUs been sequenced a greater degree of confidence regarding the dominance of phyla might have been obtained. Further investigation using clone libraries and next generation sequencing (NGS) methods would provide more quantitative and robust information on the dominant genera. Additionally, quantitative molecular methods should also be conducted.

3.6 Conclusions

This study provides the first information of the autochthonous GIT of brown trout. This study reports for the first time the identification of some bacterial species as components of the GIT communities of brown trout and revealed some regional differences between the communities in the GIT regions. Results obtained from the current study suggest that intestinal bacteria of brown trout are mainly composed by γ , α and β subclasses of Proteobacteria followed by Firmicutes and Bacteroidetes. Moreover, the DGGE method was proven to be useful for identification of wide range of LAB in the GIT of fish. This study broadens our understanding of the microbiota of brown trout. In future investigations it might be possible to use clone libraries and NGS to improve the depth of coverage of the bacterial community investigated. In addition it would be interesting to compare the investigation of the allochthonous microbiota of brown trout with the present results. The present study provides fundamental information regarding the microbiota of GIT in

brown trout which can help to inform the selection of appropriate bacteria for future probiotic applications in brown trout.

Chapter 4

Chapter 4: Influence of dietary *Pediococcus acidilactici* on health and the microbial communities of rainbow trout (*Oncorhynchus mykiss walbaum*).

Abstract

A study was conducted to assess the probiotic potential of *P. acidilactici* on rainbow trout.

A total of 80 fish (310 ± 9.09 g) were randomly distributed into eight fibreglass tanks (80-L capacity), each group consisted of four replicates of 10 fish. Fish were fed either a diet containing *P. acidilactici* at 10^6 CFU g⁻¹ or a control diet for four weeks. Fish were fed at 1% of body weight twice daily.

The effect of *P. acidilactici* on the microbial community was assessed using 16S rRNA gene clone library analysis and PCR-DGGE, haematology, immunology parameters and mRNA levels of immune- and cell activity-related genes (IL-1 β , IL-8, IL-10, IgT, Tlr5, proliferating cell nuclear antigen (PCNA), heat shock protein 70 (HSP70) and casp-3) at week two and four were also assessed. High levels of *P. acidilactici* were isolated in the digesta samples, but not in the control group. Furthermore, these populations were able to persist for at least 24h after the cessation of probiotic feeding.

DGGE confirmed the presence of the probiotic but clone libraries indicated that these populations represented $\leq 1\%$ of the total bacterial populations. The dominant phylum present in all fish was Proteobacteria, accounting for ca. 92% of the total clones, followed by Bacteroidetes (ca. 4%), Firmicutes (ca. 3%) and Actinobacteria (ca. 1%). *P. acidilactici* was detected by nested PCR-DGGE analysis in both AD and PD (week 2) and the PD only at week 4. In contrast, *P. acidilactici* was detected in the PM at week 4 only, and not detected in any control samples.

LM analysis of the AM indicated a significantly ($P = 0.001$) higher density of the goblet cells in comparison to the control fed fish at week four. Additionally, expression of IL-1 β , IL-8 and IgT genes was up-regulated in the *P. acidilactici* fed fish in the anterior and posterior intestine at week 4, whilst PCNA, HSP70 and casp-3 genes were down-regulated in the probiotic group compared with the control group in all samples at week two and four, which may suggest better epithelial integrity.

At week four, serum lysozyme activity was observed to be significantly higher ($P = 0.045$) in the probiotic fed fish compared to the control group.

4.1 Introduction

Rainbow trout (*Oncorhynchus mykiss walbaum*) are industrially farmed fish of high economic importance globally, with production totalling 855,982, 000 tonnes in 2012 (FAO, 2014). Rainbow trout is the most common and popular salmonid fish species in aquaculture and has gained much attention for improving the farming industry in many countries including Iran, France, Italy, Denmark, the United States and Spain (Merrifield *et al.*, 2010c). In the past decades, there has been an increasing interest in using bio control agents in fish farming (in particular trout farming) to improve production. One particularly important outcome of this work to date has been that these agents can play an important role to keep fish healthy without using chemotherapeutics. Recent studies have focused on using probiotics in rainbow trout and concluded that these bacteria can compete against pathogenic bacteria and improve disease resistance, enhance the nonspecific immune system and haematology profiles (Sharifuzzaman and Austin, 2010; Burbank *et al.*, 2011). Despite these interesting observations there is distinct paucity information on the impact of probiotics on the GIT microbiota and the localised intestinal response.

P. acidilactici, is a Gram-positive, facultative anaerobic cocci grows in a wide range of pH, temperature and osmotic pressure, therefore being able to adhere to the GIT (Klaenhammer, 1993). *P. acidilactici* has been reported to produce a range of bacteriocins (pediocins), which combine with other antibacterial agents such as organic acids (lactic and acetic acid) to suppress and/or restrict the growth of pathogenic bacteria (Vázquez *et al.*, 2005). Furthermore, *P. acidilactici* has been shown to mitigate rainbow trout spinal

compression syndrome (Aubin *et al.*, 2005), to elevate blood leucocyte levels in rainbow trout (Merrifield *et al.*, 2010a), and to modulate the intestinal microbiota of red Nile tilapia and rainbow trout (Ferguson *et al.*, 2010; Ramos *et al.*, 2013). This bacterial species has also been reported by Lamari *et al.* (2013) to improve the growth of sea bass larvae *Dicentrarchus labrax* as well as to reduce spinal deformations. Therefore, the current chapter sought to investigate the ability of this probiotic species to modulate the gut microbiota of rainbow trout.

Many investigators have been used the detection of mRNA expression of cytokine genes in response to probiotic bacteria as a technique for measuring immune responses (Panigrahi *et al.*, 2007; Mansfield *et al.*, 2010). Pro-inflammatory cytokines including interleukin-1 β (IL-1 β), IL-8 and the anti-inflammatory cytokine IL-10 are secreted by macrophages and granulocytic cells; IL-1 β has profound effects on several immune cells and tissues and is concomitant with the stimulation of both the specific and non-specific immunity, inducing lymphocyte proliferation, granulocyte activation and migration (Verburg-van Kemenade *et al.*, 1995). IL-8 is also a commonly used pro-inflammatory cytokine immune biomarker which is produced in response to a variety of stimuli (e.g LPS, cytokines and viruses (Laing *et al.*, 2002) and has been reported to induce in the early stage of an immune response, attracting leucocytes, including neutrophils and T-lymphocytes, to the site of the infection (Kim and Austin, 2006a; Overturf and LaPatra, 2006; Gómez and Balcázar, 2008). In this respect, IL-10 is considered to be a multifunctional cytokine of which the main function seems to be regulation of the inflammatory response, thereby minimizing damage to the host induced

by an excessive response (Pérez-Sánchez *et al.*, 2011b; Swain *et al.*, 2012). Several studies have demonstrated that a number of probiotics can effectively modulate some cytokines including IL-1 β , TNF- α , IL-8 and IL-10 (Laing *et al.*, 2002; Mansfield *et al.*, 2010; Pérez-Sánchez *et al.*, 2011b). Therefore, the expression of these genes was also the subject of investigation in the present chapter.

In addition, the expression of toll like receptor (TLR5), the mucosal antibody IgT, Proliferation cell nuclear antigen (PCNA), caspase 3 (casp-3) and HSP70 genes were also investigated.

TLRs, as pattern recognition receptors (PRRs), have crucial roles in the inflammatory response in fish since they are able to recognise pathogen-associated molecular patterns (PAMPs), which are present on the cell wall of pathogenic microorganisms (Panigrahi *et al.*, 2007; Alvarez-Pellitero, 2008). TLR5, which recognises flagellin, plays an important role in the inflammatory response by activating tissue immune cells to produce inflammatory cytokines, including tumour-necrosis factor (TNF), IL-1 β and IL-6, consequently they are activated to produce acute-phase proteins (APPs), by hepatocytes, thus APPs play essential role in complement activation and phagocytosis (Mansfield *et al.*, 2010). IgT is a specialised polymeric immunoglobulin involved in the mucosal immunity, analogous to IgA in mammals; it exists as a membrane bound protein on the surface of B-cells and also as a secretory protein (Zhang *et al.*, 2010; Rauta *et al.*, 2012). It contains a J chain which allows it to be secreted into the GIT via PIgRs and contains a protective protein which inhibits degradation in the GIT. It is

therefore an important marker for assessing intestinal immune status. Members of the heat shock protein family (including HSP70) provide several functions and act as molecular chaperones that act under numerous stress conditions to maintain protein homeostasis of the cells and to suppress protein misfolding and aggregation (Garrido *et al.*, 2006; Qian *et al.*, 2006).

Proliferation cell nuclear antigen (PCNA) is a protein expressed in cells during DNA synthesis and repair which has been suggested as a sensitive biomarker for toxic exposures including anti nutritional/ antigenic components in (i.e soybean meal) (Sanden *et al.*, 2005; Bakke-McKellep *et al.*, 2007). The elevation of PCNA expression involved in cell proliferation and implicated in removing the damaged cells since several pathological cases is often caused as a result of a change in cell proliferation (Berntssen *et al.*, 2004). Caspases belong to a family of cysteinyl aspartate-specific proteases and are divided into initiators and executioners. The former cleaves inactive forms of the latter, which includes caspase 3 (casp-3) and participates in the cleavage of proteins involved mainly in apoptosis (Cols Vidal *et al.*, 2008).

Further the probiotic effect on intestinal morphology was assessed by light and electron microscopy and the effect on systemic health was monitored by assessing haemato-immunological parameters.

4.2 Materials and methods

4.2.1 Preparation of probiotic bacteria and diets

Preparation of *P. acidilactici* (MA 18/5 M) was carried out by culturing 100 mg of lyophilized Bactocell[®] (Lallemand Inc., Montreal, Canada) in conical flasks containing 50 mL of MRS broth. Cultures were incubated at 37 °C in a shaking water bath (Clifton, UK) for 22h. To determine bacterial levels within the broth culture, tenfold dilutions were made, using MRS broth as the diluent and 100 µL spread onto triplicate MRS plates. LAB numbers were calculated after plates were incubated aerobically at 37 °C for 22h. In order to produce a calibration curve, whereby bacterial level (CFU mL⁻¹) could be estimated against absorption (OD₅₉₀), a series of broth culture dilutions were made using MRS broth as the diluent (and the blank): 25 µL/975 µL (culture/diluent), 50 µL/950 µL, 75 µL/925 µL, 100 µL/900 µL, 200 µL/800 µL, 300 µL/700 µL, 400 µL/600 µL, 500 µL /500 µL, 600 µL/400 µL, 700 µL/300 µL, 800 µL/200 µL and 900 µL/100 µL. The absorbance of the dilutions in a spectrophotometer (UNICAM, UK) was recorded. Plate counts were measured by using a Gallenkamp colony counter and CFU mL⁻¹ calculated after 24 - 48h incubation. The absorbance values at optical density OD 590 nm and CFU mL⁻¹ values of dilutions were applied to construct a calibration curve (Vine *et al.*, 2004a), which had an R² value of 0.9195 in the range of 6.75 x 10⁸ – 4.83 x 10¹⁰ CFU mL⁻¹ (Figure 4.1).

P. acidilactici cultures were then prepared by adding 100 mg of Bactocell[®] as lyophilized cells in each of six conical flasks containing 50 mL MRS, which were then incubated at 37 °C in a shaking water bath for 22h. After incubation, 1 mL aliquots from each conical flask were centrifuged (2000x g

for 5 min); in order to ensure the density of the *P. acidilactici* in the feed was 10^6 CFU g⁻¹, which was the manufactures recommendation dose.

Pellets then were washed once with PBS and re-suspended in 120 mL fish oil (Seven Seas Ltd. UK). The resulting probiotic/oil mixture was top-dressed onto the basal diet (EWOS® Sigma 50; Bergen, Norway) to produce the probiotic feed by mixing using a Hobart mixer (Beater Co. Ltd Hobart House London UK). The same volume of fish oil was added to the same amount of control diet in order to produce identical diets with the exception of the presence of the probiotic. The basal diet used in this study had a declared nutritional profile of crude protein 45% and lipid 23%. The diets were air-dried at 25 °C for 24h and stored in plastic bags at 4 °C. New batches of diets were produced every two weeks to ensure that high levels of probiotics were maintained for the duration of the trial. The probiotic concentration in the experimental diet over the experimental period (refer to Figure 4.2) was checked by counting *P. acidilactici* colonies on MRS plates using serial dilution as described in Section 2.5. The mean level throughout the trial was 2.6×10^6 CFU g⁻¹. The control diet was checked for possible contamination by the probiotic strain. Diet samples were also analysed for the determination of moisture, protein, lipid, ash, and gross energy after two and four weeks. All protocols concerning analytical chemistry are described in Section 2.6. The proximate analyses results are presented in Table 4.1.

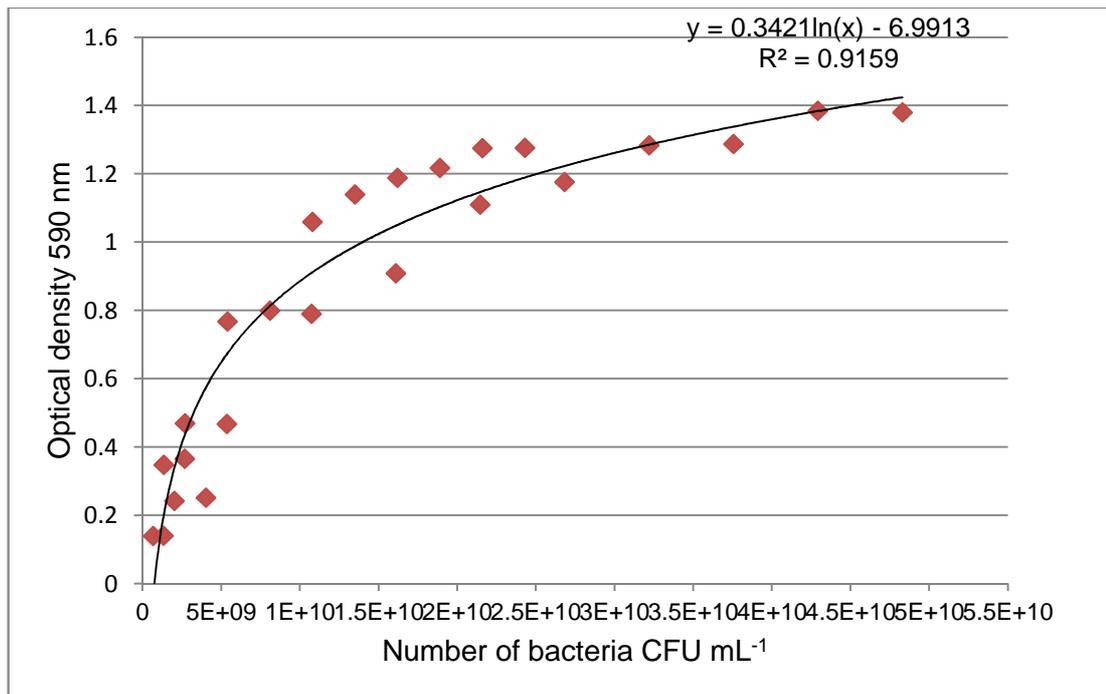


Figure 4.1 Standard curve for detection of the level of *P. acidilactici* in the diet.

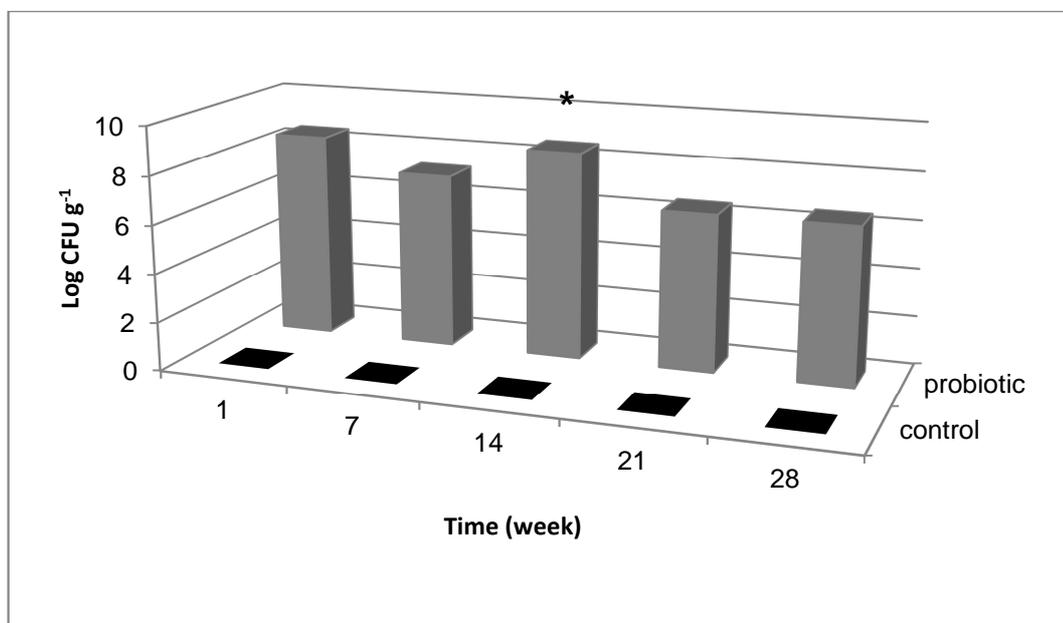


Figure 4.2 Viability of *P. acidilactici* (log CFU g⁻¹) in the probiotic and control diets during the trial period. Diets were stored at 4 °C (n = 3). *New diets prepared at day 14 of the study.

Table 4.1 Proximate analysis of experimental diets during the trial (mean \pm SD, n = 4).

proximate analysis	Batch 1 (two weeks)		Batch 2 (four weeks)	
	Control	<i>P. acidilactici</i>	Control	<i>P. acidilactici</i>
Moisture (%)	3.90 \pm 0.1	4.27 \pm 0.06	4.17 \pm 0.19	4.49 \pm 0.06
Crude protein (%)	46.66 \pm 0.43	45.72 \pm 0.14	44.72 \pm 0.65	44.40 \pm 0.64
Lipids (%)	24.11 \pm 0.3	24.71 \pm 0.25	25.05 \pm 0.86	25.04 \pm 0.39
Ash (%)	7.45 \pm 0.2	7.70 \pm 0.10	7.28 \pm 0.06	7.44 \pm 0.05
Gross energy (MJ kg ⁻¹)*	23.81 \pm 0.1	23.90 \pm 0.3	23.8 \pm 0.14	23.95 \pm 0.07

* (n = 3)

4.2.2 Experimental design

The study was carried out in the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK and lasted for four weeks. Rainbow trout were obtained from Torre fisheries, (Watchet, Somerset, UK) and acclimated for four weeks before commencement of the experiment. Fish were fed a commercial diet (EWOS[®]) during acclimation period. Upon commencement of the trial, a total of 80 fish (310 \pm 9.09 g) were randomly distributed in eight fibreglass tanks (80 L capacity), and each group consisted of four replicates of 10 fish. Full details of the rearing conditions are described in section 2.2 as appropriate.

4.2.3 Water quality

Water quality parameters such as temperature, DO and pH were measured as described in Section 2.3. Water temperature was maintained at 14.71 \pm 0.6 °C, pH was maintained at 6.5 \pm 0.8 and adjusted with NaHCO₃ as necessary to maintain pH 6.5 - 7.5. DO was maintained above 90% saturation. Additionally, ammonia, nitrite and nitrate were measured weekly

as described in Section 2.3 and maintained at $0.09 \pm 0.03 \text{ mg L}^{-1}$, $0.002 \pm 0.001 \text{ mg L}^{-1}$ and $3.6 \pm 0.4 \text{ mg L}^{-1}$, respectively.

4.2.4 Feeding and weighing

Fish were hand-fed 1% of biomass twice daily provided in equal rations at 09.00 and 17.00 for a period of four weeks. Daily feed was corrected on a fortnightly basis following batch weighing after a 24h starvation period.

4.2.5 Microbiology sampling

At the end of the trial, one fish per tank was sacrificed and dissected as described in Section 2.7.1. Digesta was squeezed out into Petri dishes by using sterile forceps. After, the anterior and posterior intestine were aseptically opened with a sterile scalpel and washed thoroughly three times with PBS. Each mucosa section was emptied into Petri dishes, thus four samples were obtained: anterior mucosa (AM), posterior mucosa (PM), anterior digesta (AD) and posterior digesta (PD). The samples were stored at $-20 \text{ }^{\circ}\text{C}$ for further use.

4.2.5.1 Culture-based enumeration of LAB populations

For bacteriological studies, at week four LAB numbers were calculated on MRS agar as described in Section 2.7.2.

4.2.5.2 Persistence of *P. acidilactici* in the GIT

After reverting the group of fish fed *P. acidilactici* back to the control diet at the end of the trial (week 4), three fish were dissected on the first and third day post change of diet, as described in Section 2.7.1, in order to assess the persistence of *P. acidilactici* within the intestine of the fish. The intestine was

divided into four regions (AD, PD, AM and PM) and LAB numbers were calculated on MRS agar as described in Section 2.7.2.

Using the primers 27F and 1491R, 16S rRNA gene sequence analysis was employed to confirm identification of a sub sample of presumptive *P. acidilactici* isolates, which were randomly selected as described in Section 2.5.1.

4.2.5.3 16S rRNA gene clone library analysis

Clone library construction was conducted at the Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China.

4.2.5.3.1 DNA extraction

Mucosa and digesta (~200 mg) for each sample was used for DNA extraction. Then 500 µL lysozyme solution (20 mg/mL, dissolved in TE buffer) was added into the sample in a 2 mL MCT. Tubes were shaken and vortexed, before incubating at 37 °C for 30 min. Cetyl trimethylammonium bromide (CTAB, 1000 µL) was added into the tubes before incubation at 65 °C for 5h with shaking every 30 min. Tubes were centrifuged at 2000x g for 10 min. Supernatant was transferred to a new 2 mL MCT and equal volume of chloroform was added, shaken for 15 sec. Tubes were centrifuged at 2000x g for 10 min. The supernatant was transferred to a new 2 mL MCT and an equal volume of isopropanol was added and mixed gently. Samples were kept at -20 °C for 30 min and centrifuged at 2000x g for 10 min. The supernatant was discarded and the precipitated DNA pellets were washed with 75% ethanol (1 mL). Tubes were centrifuged at 2000x g for 3 min. After

that, ethanol was removed and pellets were air-dried and resuspended in 100 μ L MGW. Finally, DNA was purified using TIANGEN DNA Purified Kit (Beijing, China).

4.2.5.3.2 PCR and clone library

Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene using the primers 27F and 1491R. PCR products were purified using TIANGEN Gel Extraction Kit (Beijing, China) and cloned into the Peasy-3 vector and transformed into *E. coli* Trans 1 (Trans Gene, China) according to the manufacturer's instructions. Blue/white colony selection was used for clone screening. For each sample, ~100 clones containing the correct inserts (~1400 bp) were randomly selected and verified by PCR amplification using the forward primer M13F (5'-GTAAAACGACGGCCAG-3'), and the reverse primer M13R (5'-CAGGAAACAGCTATGAC-3') and sequenced by Tsingke (Bing, China). Sequences that could not be classified into any known group were assigned as uncultured bacteria.

4.2.5.4 Denaturing gradient gel electrophoresis (DGGE)

Mucosa and digesta samples (200 mg) from three fish per treatment were collected to investigate the microbial community using DGGE at week two and four. DNA was extracted from the samples using a QIAamp[®] Stool Mini Kit (Qiagen) with minor modification to the manufacturer's instructions, as described in Appendix 1. The variable V3 region of the 16S rRNA gene was amplified using PCR as described in Section 2.7.4.2. The amplified products were subsequently loaded onto 1.5% agarose gel to assess the size and quality of PCR products as described in Section 2.5.2. The resulting PCR

products were used to obtain DNA fingerprints of the bacterial community on a 40 - 60% DGGE as described in Section 2.7.4.3.

4.2.5.4.1 DGGE analysis of LAB PCR amplicons generated from LAB

For LAB a nested PCR approach was conducted. A set of external and internal primer pairs (10 pmol/μL) were used; the external PCR was carried out using the forward primer S-D-Bact-0011-a-S-17 (5'-AGA GTT TGA TCA TGG CTC AG-3'), and the reverse primer S-G-Lab-0677-a-A-17 with a 40 bp GC clamp at the 5' end (5'-CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GCAC CGC TAC ACA TGG AG-3') (Heilig *et al.*, 2002). Thermal cycling was conducted under the following conditions: initial denaturation at 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 1 min, with a final extension of 7 min at 72 °C.

Using generated products from this amplification procedure as a DNA template, the internal PCR was conducted using the forward primer S-G-Lab-0159-a-S-20 (5'- GGA AAC AGA TGC TAA TAC CG-3') and the reverse primer Univ-0515-a-A-25 with a 40 bp GC clamp at the 5' end (5' -CGC CGG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC A-3'). Thermal cycling was conducted under the following conditions: 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 1 min, with a final extension 7 min at 72 °C. The DGGE was conducted as described in Section 2.7.4.3, except that a 30 – 60 % gradient was used.

In order to conduct DGGE analysis of *Aeromonas* populations, PCR was conducted using the *gyrB* forward primer (GAA GGC CAA GTC GGC CGC

CAG) and the *gyrB* reverse primer with a 43 bp GC clamp at the 5' end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCG GGA AGG CCA AGT CGG CCG CCAG -3') (Calhau *et al.*, 2010). The primers were used at 7.5 pmol/ μ L. Thermal cycling was conducted under the following conditions: 94 °C for 9 min, then 30 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 72 °C for 30 sec, with a final extension of 72 °C for 30 min.

4.2.5.4.2 Excision of DGGE bands for sequence analysis

After DGGE of universal PCR amplicons, OTUs of interest were excised from the gel and re-PCR'd as described in Section 2.7.7.4. Selected bands were purified and sequenced as described in Section 2.5.3. For LAB DGGE, 3 μ L of eluted DNA sample was used as the template for reamplification using the forward primer S-G-Lab-0159-a-aS-20 (5'-GGA AAC AGA TGC TAA TAC CG-3') and the reverse primer S-Univ-0515-a-A-24 (5'- ATC GTA TTA CCG CGG CTG CTG GCA-3') under the same thermal cycling conditions as previously described in Section 4.2.5.4.1. Selected bands were purified and sequenced as described in Section 2.5.3.

4.2.6 Histological examination

4.2.6.1 Light microscopy (LM)

Histology samples of the anterior and posterior intestine (the proximal most section, from the respective regions, was taken to ensure consistency between individual fish sampled), from six fish per treatment were studied at week two and four of the trial using light microscopy as described in Section 2.8.1.

4.2.6.2 Scanning electron microscopy (SEM)

Samples for SEM were taken from the anterior and posterior intestine of the intestine from six separate fish per treatment (two per tank). Intestinal samples (1 mm²) were washed with 1% S-carboxy methyl-L-cysteine (Sigma, UK), in order to remove epithelial mucus, prior to fixing in 2.5% glutaraldehyde (in 0.1 M sodium cacodylate). Fixative solution was expelled by rinsing samples in 0.1 M sodium cacodylate twice for 15 minutes each time. Later, samples were dehydrated with graded alcohol solutions of 30%, 50%, 70% and 90% and twice in 100 % for at least 15 min each. Samples were dried using a critical point dryer (EMITECH K850, Ashford, Kent, UK) with ethanol as the intermediate fluid and CO₂ as the transition fluid. Dried samples were mounted on aluminum stubs and coated with gold using a gold sputter coater (EMITECH K850, KENT, UK). Samples were screened with a Jeol JSM 5600 LV electron microscope at 15 kV (Jeol; Tokyo, Japan) and all the images were documented (5 images per region). High magnification (x 20,000) SEM images were analysed using image J 1.43 in order to measure the density of the microvilli of the enterocytes on top of the villi (Plate 4.1). Thus the ratio (arbitrary units, AU) between the microvilli covered area (A, foreground) to the background (B, background) was calculated using the formula: MD = A/B, after Dimitroglou *et al.* (2009).

4.2.7 Intestinal gene expression

Gene expression analyses were conducted at Dipartimento di Scienze della vita e dell'ambiente, Università Politecnica delle Marche, Ancona (Italia).

Samples (40 mg) were taken from the anterior and posterior intestine of six fish per treatment in order to investigate gene expression. At week two and

four of the feeding on the control and probiotic diets, mRNA levels of immune- and cell activity-related genes (IL-1 β , IL-8, IL-10, IgT, TLR5, PCNA, HSP70 and casp-3) in the AM and PM of rainbow trout were measured. Fish were dissected as described in Section 2.7.1 and samples were kept as described in Section 2.9. Total RNA extraction from the intestine tissue was conducted as described in Section 2.9.1. A DNA digestion method was carried out as described in Section 2.9.2. Extracted RNA was used for cDNA synthesis as described in Section 2.9.3. Primers used are listed in Table 4.2. In order to check the purity and molecular weight characteristics of PCR products, six μ L of each PCR product was loaded onto a 1% agarose gel as described in Section 2.5.2. RT-PCR was carried out with the SYBR® green method (Bio-Rad) as described in Section 2.9.4.

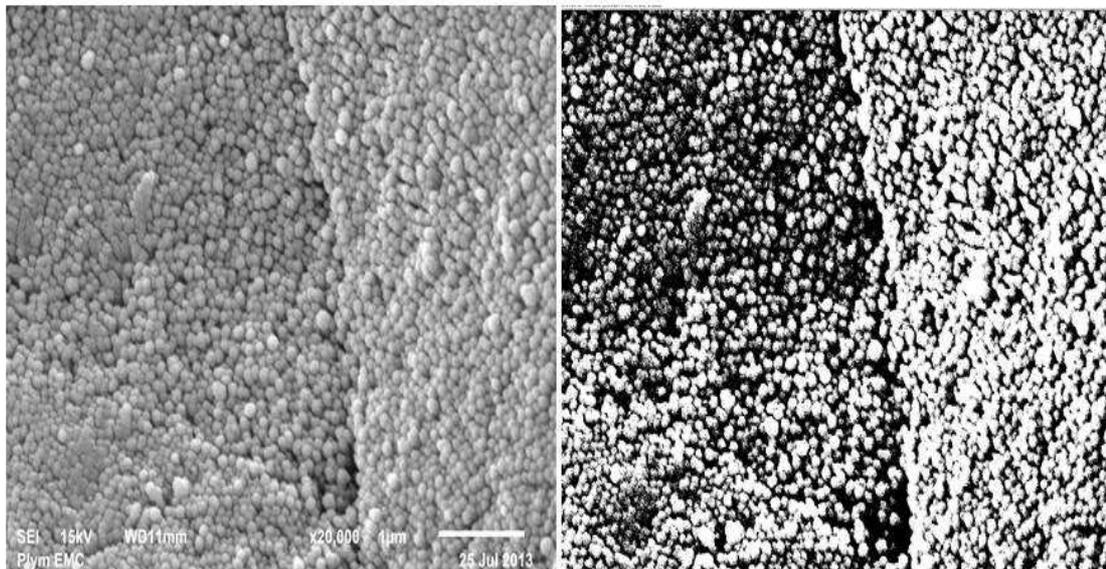


Plate 4.1 SEM micrographs were transformed to 8-bit and then after thresholding were converted to black and white. The microvilli density ratio (arbitrary units, AU) was calculated using the following formula: $MD = A/B$. Where A = the microvilli covered area (foreground, white area) to B = the background/spaces between microvilli (background, black area).

Table 4.2 Sequences of oligonucleotide primers used for detection of immune and cell relevant genes in rainbow trout by RT-PCR. Accession number refers to target gene sequence used.

Gene	Accession no.	Product size	Forward primer	Reverse primer
IL-1 β	AJ223954	91	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCTTG
IL-10	AB118099	70	CGACTTTAAATCTCCCATCGAC	GCATTGGACGATCTCTTTCTTC
IL-8	AJ279069	69	AGAATGTCAGCCAGCCTTGT	TCTCAGACTCATCCCCTCAGT
IgT	AY870265	72	AGCACCAGGGTGAAACCA	GCGGTGGGTTTCAGAGTCA
TLR5	AB091105	89	GGCATCAGCCTGTTGAATTT	ATGAAGAGCGAGAGCCTCAG
HSP70	AB062281.1	122	CGTCCTAGACAGGTCTCCGC	CAATGAGAGCGCAGCATTCC
Casp-3	NM001246335.1	131	TGTGGATGCTGGCTATGCAA	CTGACTGGCTGTGGTTGTCT
PCNA	KC747822.1	143	TATGGACTCGTCCCACGTCT	TGTCCTCATTCCCAGCACAC
β -actin	AJ438158	167	ACAGACTGTACCCATCCCAAAC	AAAAAGCGCCAAAATAACAGAA
60S	NM001165047	147	AGCCACCAGTATGCTAACCAGT	TGTGATTGCACATTGACAAAAA

4.2.8 Haematological and immunological parameters

At week two and four of the trial, four fish per tank (12 fish per treatment) were anaesthetized and blood was collected as described in Section 2.10. The haematocrit value was determined using heparinized capillary tubes as described in Section 2.10.1. The leucocyte differential counts were carried out as described in Section 2.10.2. Counts of the leucocytes and erythrocytes were calculated as described in Section 2.10.3. Total blood haemoglobin concentration was determined as described in Section 2.10.4. MCV, MCH and MCHC were calculated as described in Section 2.10.5. Serum lysozyme activity was determined using the turbidimetric method as described in Section 2.10.6.

4.2.8.1 Respiratory burst activity

In order to prepare cell monolayers, 100 μL of heparinised blood was placed into wells into a 96 well microtitre plates (Sigma, UK) and incubated at 22 °C for 2h to facilitate adhesion of cells. Then, the supernatant was removed and washed thrice with PBS. After washing, 50 $\mu\text{L well}^{-1}$ (three replicate wells were used per sample) of 0.2% nitroblue tetrazolium (NBT; Sigma, UK) and 1 $\mu\text{L mL}^{-1}$ of phorbol myristate acetate (PMA; Sigma, UK) was added and the plates which were then incubated for 1h at 22 °C. The reaction was stopped by fixing the cells with 100 $\mu\text{L well}^{-1}$ of 100% methanol for 3 min. Wells were washed thrice with 100 $\mu\text{L well}^{-1}$ of 70% methanol and were allowed to air dry. One hundred and twenty μL of 2M KOH (BDH 102104V) and 140 μL of dimethyl sulphoxide (DMSO, Sigma, UK) were added to each well to dissolve the blue formazan precipitate formed. The contents of each well were carefully mixed and the absorbance of the wells was measured at 610 nm

using a microplate reader (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA).

4.3 Statistical analyses

The mean and standard deviation (SD) of the replicates were calculated for each treatment by using conventional statistical methods. DGGE banding patterns were statistically analysed as described in Section 2.12. An independent two samples t-test was used to determine the significant differences in the immune response and histological parameters between the control and experimental groups. A series of one way ANOVA and two way ANOVA were used to compare between culturable LAB levels in the gut regions at the different time points. Tukey's HSD multiple range *post hoc* testing was used to determine significant differences between means. The accepted levels of significances were $P < 0.05$. All statistics were carried out using MiniTab statistical software version 16, IBM (Pennsylvania, USA).

4.4 Results

4.4.1 *P. acidilactici* population in the GIT

No LAB levels were detected in the control samples (i.e. total LAB levels <20 CFU g^{-1} at the lowest dilution). Figure 4.3 displays the mean numbers of potentially resident (i.e. on the AM and PM) and transient (i.e. in the AD and PD) LAB in the anterior and posterior intestine of rainbow trout fed the probiotic diet at the end of the trial (labelled as day 0 in Figure 4.3.), and after one and three days after reverting to the control diet. Culturable LAB levels in the probiotic fed fish at the end of the trial were $\log 4.9 \pm 0.2$, 5.3 ± 0.5 , 3.9 ± 0.2 and 3.6 ± 0.5 CFU g^{-1} in the AD, PD, AM and PM, respectively. On the first day after changing the probiotic diet back to the control diet the levels of LAB were \log of 4.6, \log of 5.5, \log of 3.9 and \log of 3.6 CFU g^{-1} which was approximately 93.8%, 100%, 82 % and 100% of their levels one day prior (i.e. at the end of the feeding trial, day 0), in the AD, PD, AM and PM, respectively. On the third day no culturable LAB were detected.

Two way ANOVA revealed that the number of LAB dropped significantly between day zero and day one in the AM region ($P = 0.016$). No significant differences regarding the numbers of LAB between the day zero and the day one were observed in any of the other intestinal regions investigated. However, levels of these bacteria in the digesta samples were found to be significantly higher compared with mucosa samples at day zero ($P < 0.001$), while the posterior digesta had significantly higher LAB levels compared with other regions at day one ($P < 0.001$). Presumptive *P. acidilactici* were identified and sequenced (97-100%).

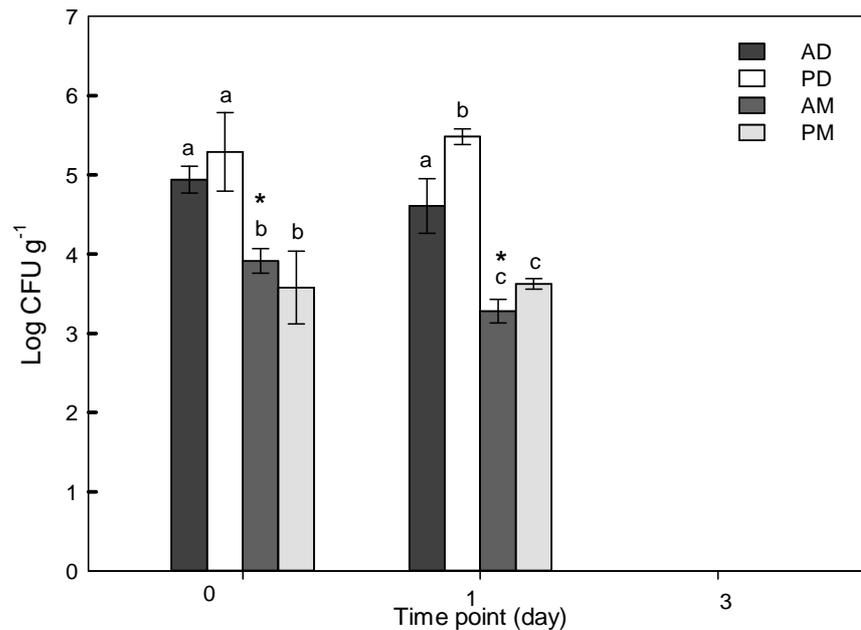


Figure 4.3 *P. acidilactici* recovery levels (log CFU g⁻¹) relating to the anterior and posterior intestine of rainbow trout fed probiotic diet at the end of the 4 week trial (day 0) and persistence of *P. acidilactici* after reverting fish to the non-probiotic supplemented control diet (days 1 and 3). Results are presented as mean log values \pm SD in each region of intestine (n = 3). Different letters within the same time point denote significant differences between gut regions ($P < 0.05$). Asterisks denote a significant difference between regions on different days ($P < 0.05$). Sample codes, AD- anterior digesta, PD- posterior digesta, AM- anterior mucosa, PM- posterior mucosa.

4.4.2 16S rRNA clone library analysis

A total of 1536 clones were obtained and sequenced. At week two 96 clones were sequenced for the control anterior mucosa (AMC), 100 for the probiotic anterior mucosa (AMP), 95 for the control posterior mucosa (PMC), 95 for the probiotic posterior mucosa (PMP), 96 for the control anterior digesta (ADC), 95 for the probiotic anterior digesta (ADP), 96 for the control posterior digesta (PDC) and 99 for the posterior digesta probiotic (PDP). At week four 92 clones were sequenced for the AMC, 96 for the AMP, 95 for the PMC, 97 for PMP, 99 for the ADC, 94 for the ADP, 99 for the PDC and 92 for the PDP.

Table 4.3 OTUs from clone libraries and their relative prevalence (%) within the intestinal tract of rainbow trout fed the control and probiotic diets at week two.

Phyla	NCBI BLAST matches	Control				Probiotic			
		AM	PM	AD	PD	AM	PM	AD	PD
β-Proteobacteria	<i>Alcaligenes faecalis</i>	92.7	64.2	6.25	27.1	86.7	70.5	14.7	34.3
β-Proteobacteria	<i>Alcaligenes</i> sp.	3.13	1.05		2.08		2.11		
β-Proteobacteria	<i>Achromobacter spanius</i>				1.04				
β-Proteobacteria	<i>Achromobacter xylosoxidans</i>				1.04				
β-Proteobacteria	<i>Achromobacter</i> sp.								1.01
β-Proteobacteria	<i>Delftia tsuruhatensis</i>		1.05						
β-Proteobacteria	<i>Delftia acidovorans</i>		2.11	1.04					
β-Proteobacteria	<i>Methylophilus</i> sp.							1.05	
β-Proteobacteria	<i>Dechloromonas</i> sp.		4.21						
β-Proteobacteria	<i>Comamonas aquatica</i>							1.05	
β-Proteobacteria	<i>Acidovorax temperans</i>							3.16	1.01
γ-Proteobacteria	<i>Acinetobacter johnsonii</i>	1.04	9.47	40.6	26			23.2	10.1
γ-Proteobacteria	<i>Acinetobacter junii</i>				7.29			6.32	
γ-Proteobacteria	<i>Acinetobacter parvus</i>					1.02			
γ-Proteobacteria	<i>Acinetobacter</i> sp.			6.25				3.16	3.03
γ-Proteobacteria	<i>Pseudomonas alcaligenes</i>	1.04							
γ-Proteobacteria	<i>Pseudomonas putida</i>		3.16	1.04				1.05	1.01
γ-Proteobacteria	<i>Pseudomonas mendocina</i>		1.05	7.29				5.26	2.02
γ-Proteobacteria	<i>Pseudomonas alcaliphila</i>			3.13					
γ-Proteobacteria	<i>Pseudomonas nitroreducens</i>			1.04				2.11	1.01
γ-Proteobacteria	<i>Pseudomonas lindanytica</i>			1.04					
γ-Proteobacteria	<i>Pseudomonas aeruginosa</i>				1.04			1.05	1.01
γ-Proteobacteria	<i>Pseudomonas</i> sp.				1.04			2.11	
γ-Proteobacteria	<i>Pseudomonas monteilii</i>							1.05	
γ-Proteobacteria	<i>Pseudomonas fluorescens</i>							1.05	
γ-Proteobacteria	<i>Pseudomonas saccharophila</i>					1.02			
γ-Proteobacteria	<i>Pseudoxanthomonas mexicana</i>		2.11			1.02			1.01
γ-Proteobacteria	<i>Aeromonas salmonicida</i>		2.11	1.04	2.08			1.05	
γ-Proteobacteria	<i>Aeromonas hydrophila</i>			1.04	7.29			2.11	
γ-Proteobacteria	<i>Aeromonas jandaei</i>						1.05		
γ-Proteobacteria	<i>Aeromonas bestiarum</i>							1.05	
γ-Proteobacteria	<i>Aeromonas media</i>			1.04					
γ-Proteobacteria	<i>Aeromonas punctata</i>			1.04				2.11	
γ-Proteobacteria	<i>Aeromonas aquariorum</i>				4.17				
γ-Proteobacteria	<i>Aeromonas veronii</i>						4.21		1.01
γ-Proteobacteria	<i>Aeromonas caviae</i>		2.11	2.08	1.04			2.11	
γ-Proteobacteria	<i>Aeromonas enteropelogenes</i>		2.11	9.38				1.05	
γ-Proteobacteria	<i>Aeromonas encheleia</i>		1.05		1.04				
γ-Proteobacteria	<i>Serratia grimesii</i>							1.05	
γ-Proteobacteria	<i>Serratia liquefaciens</i>			2.08	1.04				
γ-Proteobacteria	<i>Citrobacter freundii</i>			1.04				1.05	1.01
γ-Proteobacteria	<i>Erwinia amylovora</i>			2.08				1.05	
γ-Proteobacteria	<i>Escherichia coli</i>								28.3
γ-Proteobacteria	<i>Plesiomonas shigelloides</i>					2.04	10.5		
γ-Proteobacteria	<i>Stenotrophomonas maltophilia</i>		1.05	2.08	4.17			3.16	
γ-Proteobacteria	<i>Rubellimicrobium roseum</i>				1.04		3.16		
γ-Proteobacteria	Uncultured <i>Aeromonas</i> sp.								
γ-Proteobacteria	Uncultured <i>Acinetobacter</i> sp.				1.04			3.16	1.01
γ-Proteobacteria	γ-Proteobacterium				1.04				
α-Proteobacteria	<i>Ochrobactrum lupini</i>								1.01
α-Proteobacteria	<i>Ochrobactrum anthropi</i>		1.05		3.13			3.16	
α-Proteobacteria	<i>Paracoccus carotinifaciens</i>				1.04				
α-Proteobacteria	<i>Agrobacterium tumefaciens</i>							1.05	
α-Proteobacteria	<i>Methylobacterium zatmanii</i>					1.02			
α-Proteobacteria	<i>Sphingomonas melonis</i>					1.02			1.01
α-Proteobacteria	Uncultured <i>Paracoccus</i>								1.01
Proteobacteria	Uncultured Proteobacterium			1.04					
Firmicutes	<i>Veillonella parvula</i>					1.02			
Firmicutes	<i>Pediococcus acidilactici</i>								1.01
Firmicutes	<i>Staphylococcus pasteurii</i>			4.17	1.04				1.01
Firmicutes	<i>Staphylococcus saprophyticus</i>						1.05		
Firmicutes	<i>Lactobacillus crispatus</i>					1.02			
Bacteroidetes	<i>Flavobacteriaceae</i> bacterium							1.05	
Bacteroidetes	<i>Chryseobacterium hominis</i>							2.11	
Bacteroidetes	<i>Wautersiella falsenii</i>		1.05						
Unidentified bacteria	Uncultured bacteria	2.08	1.05	4.17	4.17	4.08	7.37	7.37	8.1

Table 4.4 OTUs from clone libraries and their relative prevalence (%) within the intestinal tract of rainbow trout fed the control and probiotic diets at week four.

Phyla	NCBI BLAST matches	Control				Probiotic			
		AM	PM	AD	PD	AM	PM	AD	PD
β-Proteobacteria	<i>Alcaligenes faecalis</i>	89.1	63.2	23.2	40.4	81.3	70.1	12.8	60.4
β-Proteobacteria	<i>Alcaligenes</i> sp.				1.01	2.08	1.03		
β-Proteobacteria	<i>Achromobacter xylosoxidans</i>							1.06	
β-Proteobacteria	<i>Comamonas testosteroni</i>	1.09							
β-Proteobacteria	<i>Comamonas aquatica</i>							3.19	
β-Proteobacteria	<i>Delftia tsuruhatensis</i>							2.13	1.10
β-Proteobacteria	<i>Acidovorax temperans</i>		1.05						
β-Proteobacteria	Uncultured <i>Acidovorax</i>			1.01					
β-Proteobacteria	Uncultured β-Proteobacteria			1.01					
γ-Proteobacteria	<i>Acinetobacter johnsonii</i>	17.9		18.2	6.06	4.17		25.5	
γ-Proteobacteria	<i>Acinetobacter junii</i>			2.02	1.01			1.06	
γ-Proteobacteria	<i>Acinetobacter</i> sp.			2.02				10.6	
γ-Proteobacteria	<i>Acinetobacter lwoffii</i>								4.40
γ-Proteobacteria	Uncultured <i>Acinetobacter</i> sp.			2.02		1.04			
γ-Proteobacteria	<i>Pseudomonas fluorescens</i>		7.4					1.06	
γ-Proteobacteria	<i>Pseudomonas mendocina</i>		1.05	7.07		1.04		1.06	
γ-Proteobacteria	<i>Pseudomonas alcaliphila</i>			1.01					
γ-Proteobacteria	<i>Pseudomonas pseudoalcaligenes</i>			1.01					
γ-Proteobacteria	<i>Pseudomonas alcaligenes</i>							1.06	
γ-Proteobacteria	<i>Pseudomonas aeruginosa</i>							2.13	
γ-Proteobacteria	<i>Pseudomonas</i> sp.							1.06	
γ-Proteobacteria	<i>Pseudomonas putida</i>							1.06	
γ-Proteobacteria	<i>Pseudoxanthomonas mexicana</i>				2.02			2.13	
γ-Proteobacteria	<i>Aeromonas salmonicida</i>	2.17	1.05	3.03	6.06			7.45	
γ-Proteobacteria	<i>Aeromonas</i> sp.		1.05						
γ-Proteobacteria	<i>Aeromonas hydrophila</i>			4.04	7.07			6.38	1.10
γ-Proteobacteria	<i>Aeromonas caviae</i>			2.02	4.04		2.10	3.19	
γ-Proteobacteria	<i>Aeromonas aquariorum</i>			2.02					
γ-Proteobacteria	<i>Aeromonas enteropelogenes</i>			1.01	1.01				
γ-Proteobacteria	<i>Aeromonas taiwanensis</i>				9.09				
γ-Proteobacteria	<i>Aeromonas punctata</i>				2.02			3.19	
γ-Proteobacteria	<i>Aeromonas veronii</i>						4.13		
γ-Proteobacteria	<i>Aeromonas tecta</i>					1.04			
γ-Proteobacteria	<i>Aeromonas sanarelli</i>								1.10
γ-Proteobacteria	<i>Serratia proteamaculans</i>				1.01				
γ-Proteobacteria	<i>Serratia grimesii</i>				1.01				
γ-Proteobacteria	<i>Citrobacter freundii</i>		1.05	6.06					
γ-Proteobacteria	<i>Enterobacter</i> sp.			1.01					
γ-Proteobacteria	<i>Escherichia coli</i>				1.01		2.10		8.79
γ-Proteobacteria	<i>Plesiomonas shigelloides</i>						18.6		3.30
γ-Proteobacteria	<i>Stenotrophomonas maltophilia</i>	1.09		2.02				1.06	
γ-Proteobacteria	<i>Raoultella ornithinolytica</i>				2.02				
γ-Proteobacteria	<i>Shewanella putrefaciens</i>							1.06	
γ-Proteobacteria	γ-Proteobacterium					1.04			
α-Proteobacteria	<i>Ochrobactrum anthropi</i>		1.05	7.07	6.06				
α-Proteobacteria	<i>Paracoccus marcusii</i>								1.10
α-Proteobacteria	<i>Paracoccus alkenifer</i>								1.10
α-Proteobacteria	<i>Agrobacterium tumefaciens</i>			1.01	2.02			1.06	
α-Proteobacteria	<i>Brevundimonas mediterranea</i>			1.01					
α-Proteobacteria	<i>Brevundimonas</i> sp.		1.05						
α-Proteobacteria	<i>Brevundimonas vesicularis</i>			1.01					
α-Proteobacteria	<i>Nitrobacteria hamadaniensis</i>			1.01					
α-Proteobacteria	<i>Rhizobium</i> sp.					1.04			
α-Proteobacteria	<i>Ensifer adhaerens</i>							3.19	
Proteobacteria	Unidentified Proteobacterium	2.17				1.04			
Firmicutes	<i>Geobacillus toebii</i>								3.30
Firmicutes	<i>Staphylococcus pasteurii</i>							1.06	
Firmicutes	<i>Streptococcus alactolyticus</i>						1.00		
Firmicutes	<i>Clostridium</i> sp.								2.20
Firmicutes	<i>Clostridium mayombeii</i>								1.10
Firmicutes	<i>Lactobacillus amylovorus</i>								1.10
Firmicutes	<i>Bacillus thermosphaericus</i>								1.10
Firmicutes	Uncultured <i>Clostridium</i> sp.								1.10
Firmicutes	Uncultured <i>Mycoplasma</i>					1.04			
Bacteroidetes	<i>Elizabethkingia miricola</i>								1.10
Bacteroidetes	<i>Sphingobacterium</i> sp.								1.10
Bacteroidetes	<i>Wautersiella falsenii</i>							1.06	
Bacteroidetes	Uncultured Bacteroidetes								2.20
Actinobacteria	<i>Propionibacterium acnes</i>								1.10
Unidentified bacteria	Uncultured bacterium	4.35	4.21	9.09	7.07	5.21	1.00	5.32	2.20

The results of blast search showed that the sequences from library were collected into four phyla: dominant Proteobacteria (α , γ and β), Firmicutes, Bacteroides and Actinobacteria (Figures 4.4 and 4.5). The dominant intestinal bacteria were identified as the genus *Alcaligenes* belonging to the β subclass of Proteobacteria which comprised 87% ~ 98% of the microbial community. The sequences were predominantly identified as *Alcaligenes faecalis*, which was abundant in the AM, PM and PD at both sampling points, representing 27.1% - 92.7% of the populations from these samples (Tables 4.3 and 4.4). The *Alcaligenes faecalis* abundance was considerably higher in the mucosa samples (78.5%-75.9%) than the digesta samples (20.5%-34.2%).

A considerably higher abundance of *Acinetobacter*, dominated by *Acinetobacter johnsonii*, was present in the digesta samples than the mucosa samples at both weeks two and four. At week two, the most abundant clones associated with the control samples were sequences relating to *Alcaligenes* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Aeromonas* spp. and *Staphylococcus* spp. These genera were dominant also in the probiotic samples, however, sequences relating to *Escherichia coli*, *Plesiomonas shigelloides* and uncultured bacteria were also abundant. On the other hand, the same trend was observed with regards to the dominant bacteria in both control and probiotic groups; *Ochrobactrum anthropi* was abundant only in the control group at week four.

Sequences relating to *Escherichia coli* were higher in abundance in the library of the PD of the probiotic fed fish, comprising 28.3% and 8.79% at week two and four, respectively. However, these bacteria were not detected

or very low ratio in the remaining samples. At week two, the library generated from fish fed the control diet was dominated by Proteobacteria, which accounted for 98%, 98%, 91.6% and 94.8% in the AMC, PMC, ADC and PDC, respectively, whereas in the probiotic libraries they accounted for 94%, 91.5%, 89.5% and 90% in AMP, PMP, ADP and PDP, respectively (Figure 4.4).

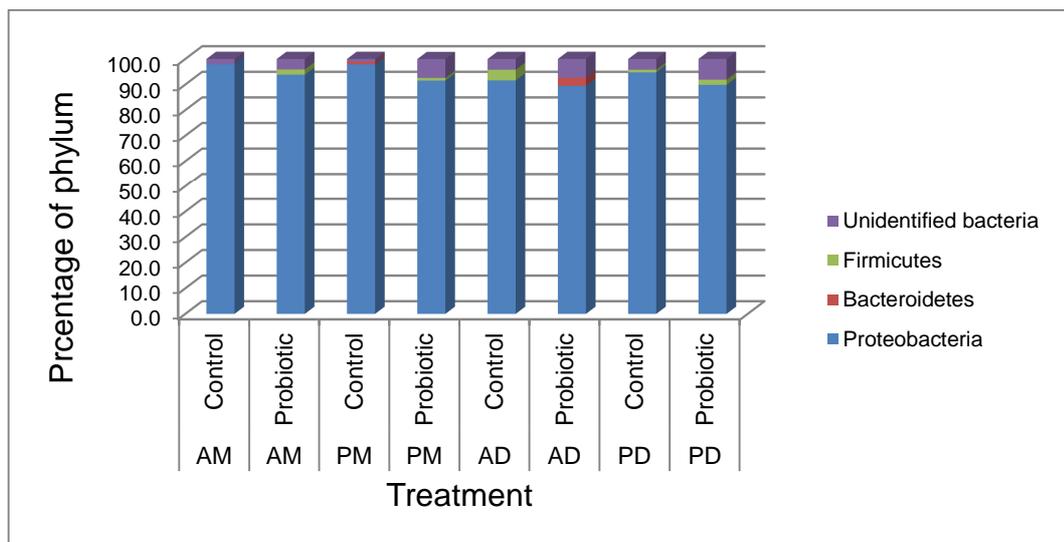


Figure 4.4 Bacterial phyla relative abundance within the different regions at week two. Sample codes, AM- anterior mucosa, PM- posterior mucosa, AD- anterior digesta, PD- posterior digesta.

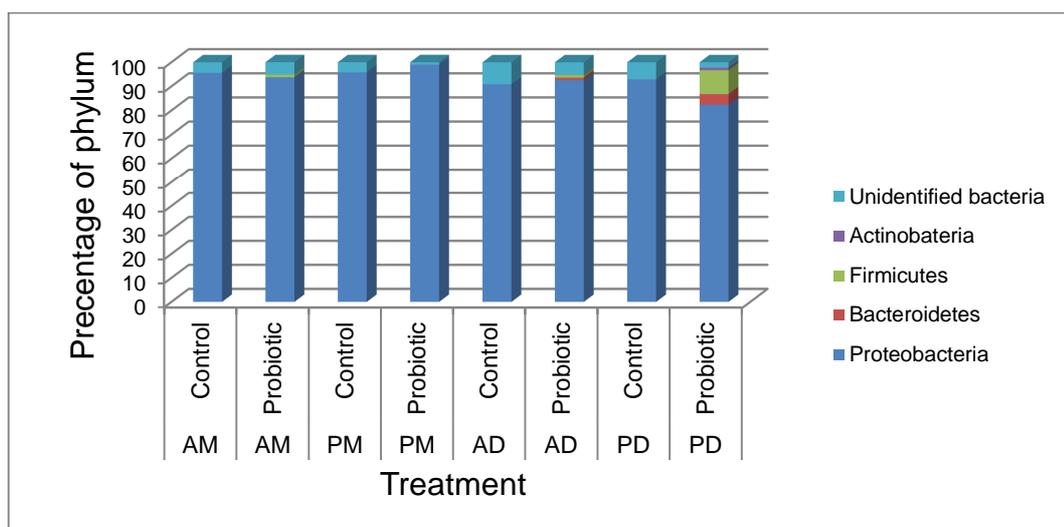


Figure 4.5 Bacterial phyla relative abundance within the different regions at week four. Sample codes, AM- anterior mucosa, PM- posterior mucosa, AD- anterior digesta, PD- posterior digesta.

At week four, the libraries were dominated by Proteobacteria which accounted for 95.62%, 95.8%, 90.89% and 92.92% of the clones in the AMC, PMC, ADC and PDC, respectively, whereas in the probiotic libraries they accounted for 92.9%, 93.8%, 98.95% and 82.4% in the AMP, PMP, ADP and PDP, respectively (Figure 4.5). However, other phyla, including Firmicutes, Bacteroides and Actinobacteria, were also identified and accounted for 9.9%, 4.4% and 1.1%, respectively in the PDP at week four.

At week two, the control group libraries were dominated by *Alcaligenes faecalis* (47.6%), *Acinetobacter* spp. (22.7%), *Aeromonas* spp. (9.6%), *Pseudomonas* spp. (5.7%) and members of family Enterobacteriaceae (1.5%), whereas in the probiotic group libraries these percentage changed to 51.6%, 11.7%, 3.9 %, 5.4% and 11.3%, respectively. On the other hand, *Alcaligenes faecalis* (54%), *Acinetobacter* spp. (12.0%), *Aeromonas* spp. (11.4%) *Pseudomonas* spp. (4.9%) and members of family Enterobacteriaceae (2.8%) were the dominant bacteria isolated from the control samples at week four, whereas in the probiotic group libraries these percentages changed to 56.1%, 12.2%, 7.4%, 3.2% and 8.7%, respectively.

Compared to the control, a reduction in the proportion of *Aeromonas* spp. in the AD and PD at week two and in the PD at week four was observed in fish fed the probiotic diet. *P. acidilactici* levels were below detectable levels (i.e. $\leq 1\%$) in the probiotic fed fish in most samples and time points with the exception of PD at week two, where one clone (representing 1.01% of the community) was detected. *P. acidilactici* was not detected in any control samples.

4.4.3 DGGE analysis

The resulting DGGE fingerprints are shown in Figures 4.6 and 4.9; excised OTUs (for sequencing analysis) are also indicated.

4.4.3.1 Week two DGGE analysis

Non-metric multidimensional scaling (nMDS) and cluster analysis of the week two DGGE fingerprint are presented in Figures 4.7 and 4.8, respectively. At this point it should be stated that digesta and mucosa regions could not be directly compared as samples were not run on the same gel. In the case of the week two mucosa region (Figure 4.8A), no clear patterns in terms of sample grouping (and thus similarity) were observed by either analysis method in the majority of replicates, regardless of region or treatment, although in the case of the AM, the majority of the control and probiotic replicates (2/3) were distinctly grouped. In addition, significant difference in SIMPER (similarity percentage) values was observed (Table 4.5). Further microbial community analysis parameters relating to the week two (mucosa) are also displayed in Table 4.5. Whilst the species evenness was observed to be significantly higher in the probiotic group compared to the control group in the AM ($P < 0.05$), no other significant differences in parameters relating to this region were observed. In the case of the week two digesta, the nMDS and cluster analysis (Figures 4.7B and 4.8B, respectively) showed that the anterior and posterior regions were, to some degree separated in terms of the similarity of their respective replicates. The greatest difference, in terms of spatial separation (Figure 4.7B) or clustering of replicates (Figure 4.8B), was observed between the AD control and probiotic treatments; this difference in similarity was not reflected by a significant difference in SIMPER (similarity percentage) value.

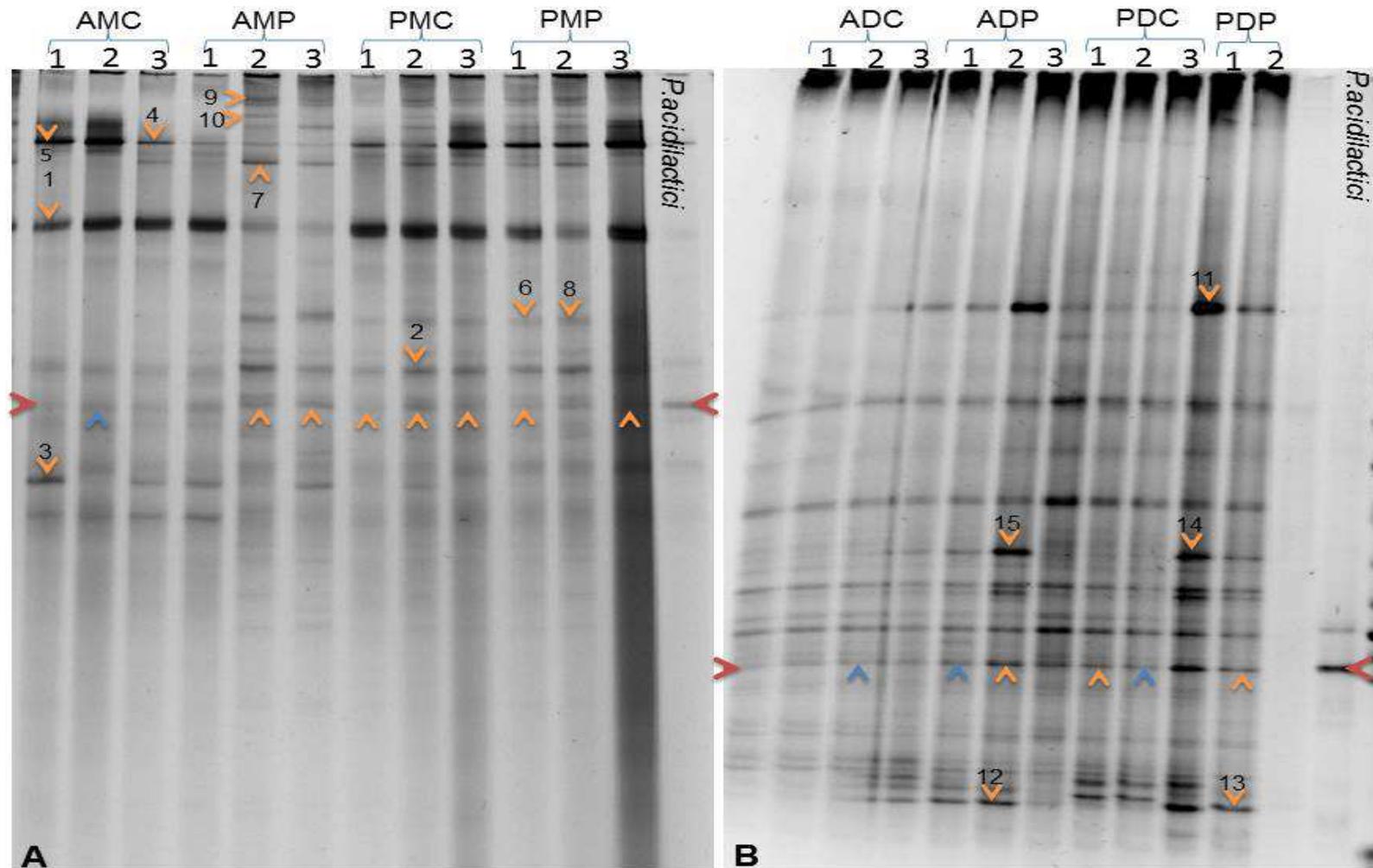


Figure 4.6 DGGE fingerprints from the mucosa (A) and digesta (B) of rainbow trout at 2 weeks feeding on the experimental diets. Arrows represent OTUs which were excised and sequenced. Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control, PMP- posterior mucosa probiotic, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic. Bands which were not successfully sequenced are indicated by blue arrows.

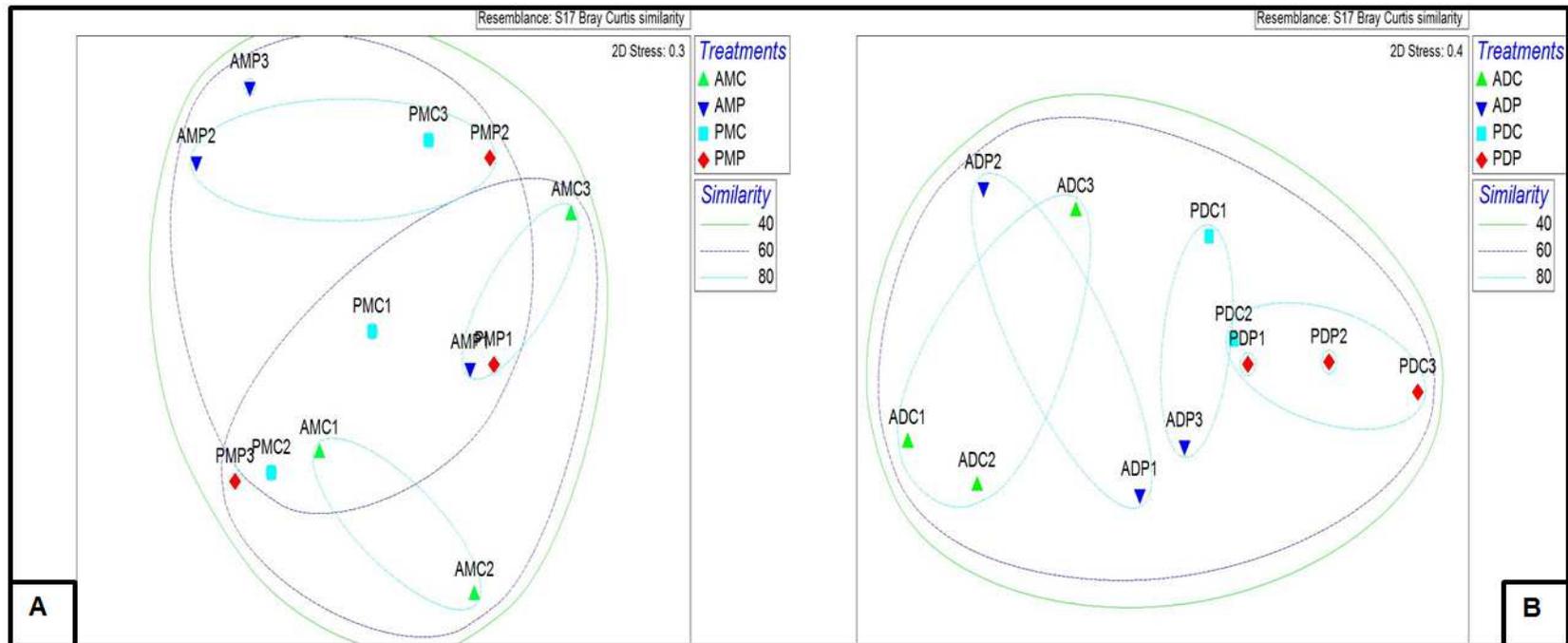


Figure 4.7 Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints incorporating similarity percentages (40, 60 and 80%) of bacterial communities between the control and probiotic groups at week two. (A) mucosa and (B) digesta (n = 3 for each region). Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control, PMP- posterior mucosa probiotic, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

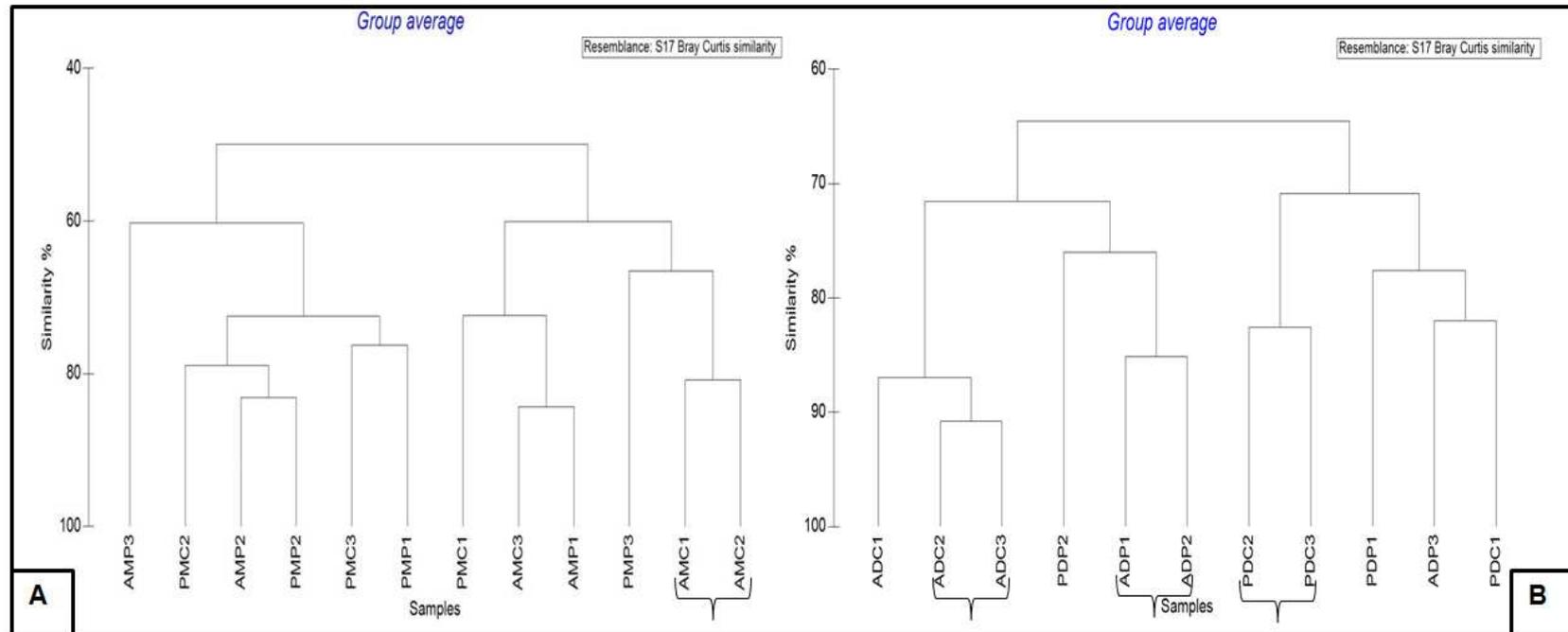


Figure 4.8 Cluster analysis of DGGE fingerprints incorporating similarity percentages of bacterial communities between the control and probiotic groups at week two. (A) mucosa and (B) digesta ($n = 3$ for each region). Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control, PMP- posterior mucosa probiotic, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

Indeed, no significant differences were observed when other analysis parameters were taken into account (Table 4.5), although values for these parameters (OTUs number, evenness etc.) were consistently higher for the control group than the probiotic group in both regions ($P > 0.05$). In both the AD and PD, similarities were also higher in the control compared to the probiotic group, but these were not significantly different ($P > 0.05$).

When the control and probiotic group replicates were directly compared (similarity percentage), the highest similarity was found between the groups of ADC and ADP ($70 \pm 9\%$), while the lowest similarity was found between the groups of AMC and AMP ($47 \pm 19\%$).

4.4.3.2 Band sequencing analyses

A total of fifteen OTUs were selected from the gel at week two, and are indicated on the gel images in Figure 4.6. Some bands were common to all groups and/or replicates. The results of band sequence analysis from week 2 gels (mucosa and digesta) are shown in Table 4.6. The percentage allocation of the selected OTUs to bacterial phyla was as follows: Firmicutes 73.4%, Proteobacteria 13.4%, Actinobacteria 6.7% and unidentified bacteria 6.7%. *Streptomyces cheonanensis* was the only representative of Actinobacteria, which was identified in all mucosa replicates of fish. Proteobacteria were represented by OTUs most similar to *Escherichia* sp. and unidentified Proteobacteria bacterium (OTUs 3 and 5). The most frequently observed representatives of Firmicutes were *Enterococcus* sp., *Lactococcus* sp., *Streptococcus* spp., *Lactobacillus* spp. and several Firmicutes which could not be identified to genus level. Four common OTUs (1, 4, 5 and 7) were

identified in all mucosa samples (although not all of the replicates); these were identified as *Streptomyces cheonanensis*, *Streptococcus* sp., two unidentified Proteobacteria and unidentified members of the Firmicutes, respectively. Five common OTUs (11, 12, 13, 14 and 15) were similarly identified in all of the digesta samples: these OTUs were identified as *Lactobacillus acidophilus*, uncultured *Lactobacillus* sp., *Streptococcus* sp., *Lactobacillus aviarius* subsp. *araffinosus*, and *Lactobacillus* sp. Five OTUs (2, 6, 8, 9 and 10) were not detected in the AMC, while they were detected in other regions and were most closely related to uncultured Firmicutes bacterium, an unidentified bacterium, *Streptococcus* sp., *Lactococcus* sp. and *Enterococcus* sp. Uncultured *Escherichia* sp. (OTU 3) was the only OTU that was not found in the PMP samples, but was detected in all other mucosa samples. The selected OTUs from the digesta samples appeared to be common to all replicates and all samples. *Lb. aviarius* subsp. *araffinosus* (OTU 14) and *Lactobacillus* sp. (OTU 15) were present in the digesta samples, but were not detected in any of the mucosa samples.

In addition, an OTU which aligned to same position in the gel as the known *P. acidilactici* was detected on the majority of the control samples and all of the probiotic samples (as indicated by the red arrows in Figure 4.6). Thirteen bands that had migrated to this point were excised and sequenced. None of the bands isolated from the control samples (4 OTUs) were identified as *Pediococcus*; these sequences were identified as *Lactococcus lactis* subsp. *cremoris*, two different uncultured bacteria, *Bacillus pumilus* and 4 sequences failed to yield useful nucleotide read OTUs (i.e. likely contained multiple sequences in the same band). From the 5 OTUs derived from the probiotic

samples, *Lactococcus lactis*, two *Lactococcus lactis* subsp. *lactis* and two different uncultured bacteria were identified.

Table 4.5 Microbial community analysis from PCR-DGGE fingerprints of the GIT of rainbow trout at week two.

	OTUs ¹	Richness ²	Evenness ³	Diversity ⁴	SIMPER ⁵ similarity (%)	Similarity%(control vs probiotic)
<u>Anterior mucosa</u>						
Control	10.3 ± 0.6	0.99 ± 0.08	0.91 ± 0.01 ^a	2.13 ± 0.07	73 ± 8 ^a	47 ± 19
Probiotic	18.0 ± 6.0	1.79 ± 0.61	0.97 ± 0.02 ^b	2.75 ± 0.42	48 ± 14 ^b	
<u>Posterior mucosa</u>						
Control	16.0 ± 6.6	1.53 ± 0.62	0.93 ± 0.03	2.55 ± 0.48	62 ± 6	68 ± 10
Probiotic	16.0 ± 7.0	1.54 ± 0.72	0.95 ± 0.02	2.54 ± 0.48	67 ± 12	
<u>Anterior digesta</u>						
Control	24.0 ± 1.0	2.21 ± 0.07	0.99 ± 0.00	3.16 ± 0.05	88 ± 4	70 ± 9
Probiotic	22.3 ± 1.5	1.99 ± 0.14	0.99 ± 0.00	3.08 ± 0.07	78 ± 7	
<u>Posterior digesta</u>						
Control	24.3 ± 2.9	2.14 ± 0.22	0.99 ± 0.00	3.16 ± 0.12	76 ± 10	67 ± 5
Probiotic	18.5 ± 2.1	1.63 ± 0.15	0.99 ± 0.00	2.89 ± 0.11	68 [*]	

Results expressed as mean ± SD in each group of fish (n = 3). Means having the different letters within the same region related to the same factor are significantly different ($P < 0.05$).

¹ Operational taxonomical unit.

² Margalef species richness: $d = (S - 1) / \log(N)$.

³ Pielou's evenness: $J' = H' / \log(S)$.

⁴ Shannon's diversity index: $H' = -\sum (p_i \ln p_i)$.

⁵ SIMPER, similarity percentage within group replicates.

* n = 2

Table 4.6 Summary of the sequencing analysis of OTUs from the week two DGGE gel. Numerical values represent the number of replicates (out of 3) that the OTUs were present in.

OTUs number	Phyla	Identity (%)	NCBI BLAST matches	NCBI Accession number	Length of sequences	E value	Anterior intestine		Posterior intestine	
							C	P	C	P
Mucosa										
4	Actinobacteria	100	<i>Streptomyces cheonanensis</i>	NR043208.1	109	1e-48	3	3	3	3
10	Firmicutes	98	<i>Enterococcus</i> sp.	AY751462.1	139	1e-11	0	1	2	2
9	Firmicutes	95	<i>Lactococcus</i> sp.	NR044358.1	133	9e-66	0	2	3	3
1	Firmicutes	91	Unidentified bacteria	NR074404.1	109	1e-48	3	3	3	3
8	Firmicutes	95	<i>Streptococcus</i> sp.	AB479549.2	161	3e-58	0	2	3	3
7	Firmicutes	88	Unidentified bacteria	NR029041.1	150	2e-41	1	3	3	3
2	Firmicutes	95	Uncultured bacterium	FJ893054.1	171	3e-58	0	2	3	3
5	Proteobacteria	89	Unidentified bacteria	JN975129.1	173	1e-11	3	3	3	3
3	Proteobacteria	94	<i>Escherichia</i> sp.	HQ877796.1	171	2e-45	2	2	1	0
6	Unidentified bacteria	100	Unidentified bacteria	NR026517.1	169	1e-04	0	2	3	3
Digesta										
14	Firmicutes	98	<i>Lactobacillus aviarius</i> subsp. <i>araffinosus</i>	JX986976.1	136	1e-56	3	3	3	2
15	Firmicutes	99	<i>Lactobacillus</i> sp.	HQ696436.1	164	6e-10	3	3	3	2
11	Firmicutes	98	<i>Lactobacillus acidophilus</i>	JQ962227.2	135	5e-60	3	3	3	2
12	Firmicutes	100	Uncultured <i>Lactobacillus</i> sp.	JQ284447.1	135	7e-63	1	3	3	2
13	Firmicutes	95	<i>Streptococcus</i> sp.	JQ461996.1	113	7e-63	1	3	3	2

Sample codes, C- control, P- probiotic.

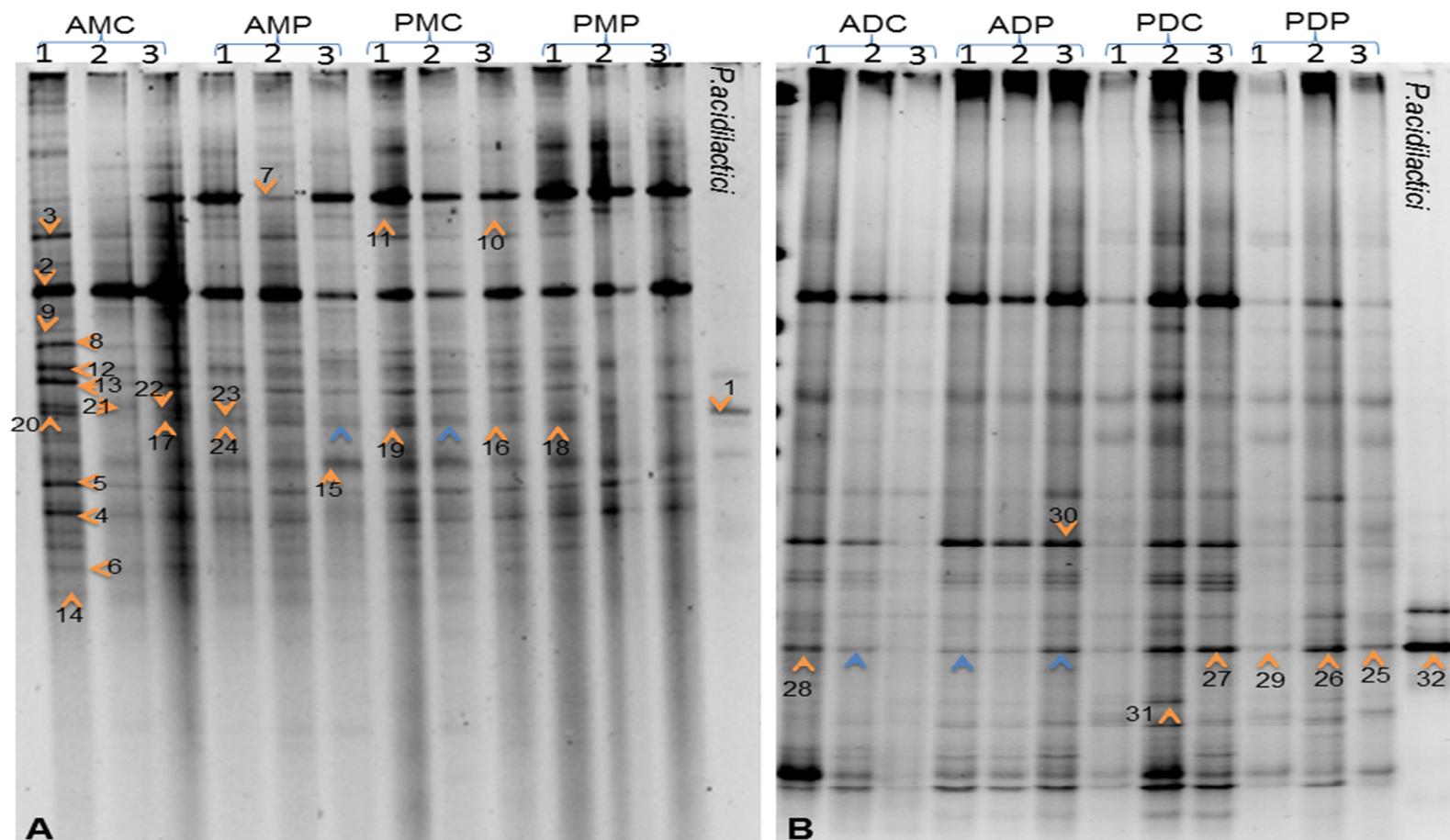


Figure 4.9 DGGE fingerprints from the mucosa (A) and digesta (B) of rainbow trout at 4 weeks feeding on the experimental diets. Arrows represent OTUs which were excised and sequenced. Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control, PMP- posterior mucosa probiotic, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic. Bands which were not successfully sequenced are indicated by blue arrows.

4.4.3.3 Week 4 DGGE analysis

Non-metric multidimensional scaling (nMDS) and cluster analysis of the week four DGGE fingerprints are presented in Figures 4.10 and 4.11, respectively.

In the case of the week four mucosa region, the nMDS (Figure 4.10A) showed that the anterior probiotic replicates were, to some degree separated in terms of the similarity of their respective treatments. The cluster analysis (Figure 4.11A) showed that in the case of the AMC, all replicates were distinctly clustered, whilst in the case of the majority of PMP replicates (2/3), clustering was again observed. The remaining replicates were grouped separately and no clear grouping patterns were observed in terms of either region or treatment in this case. No significant differences in the SIMPER analysis were observed.

Further microbial community analysis parameters relating to week four (mucosa) are also displayed in Table 4.7. In terms of OTU numbers, species richness and diversity, no significant differences were observed between the control group and probiotic groups in any regions (Table 4.7), although in both of the mucosa regions, marginally higher numbers were observed in the control than in the probiotic treatment ($P > 0.05$).

In the case of the week four digesta, both the nMDS and the cluster analysis (Figures 4.10B and 4.11B) showed that there few clear patterns in terms of replicate grouping. The three ADC replicates were shown to be clearly spatially separated in the nMDS analysis; this was also reflected in the cluster analysis. In slight contrast, the majority of ADP replicates (2/3) were

clearly clustered together, with SIMPER analysis indicating a higher (but not significant) level of similarity in this group than in the control. Overall, it was difficult to identify clear differences between the bacterial communities within the digesta at week four. In terms of OTU numbers, species richness and diversity, no significant differences were observed between the control group and probiotic groups in either region (Table 4.7), although their values were marginally higher in the control group compared to the probiotic group in the PD ($P > 0.05$). In the AD this pattern was reversed ($P > 0.05$).

When the control and probiotic group replicates were directly compared (similarity percentage), the highest similarity was found between the groups of ADC and ADP ($70 \pm 14\%$), while the lowest similarity was found between the groups of PDC and PDP ($51 \pm 15\%$).

4.4.3.4 Band sequencing analyses

A total of 19 OTUs were selected from the week four DGGE gel; some of them were common to all groups, whilst others were unique to the probiotic or the control groups (Figure 4.9). The results of band sequence analysis from week 4 gels (mucosa and digesta) are shown in Table 4.8. The percentage allocation of the selected OTUs to bacterial phyla was as follows: Firmicutes 31.6%, Proteobacteria 15.8%, Cyanobacteria 5.3%, Tenericutes 10.5% and unidentified bacteria 36.8%.

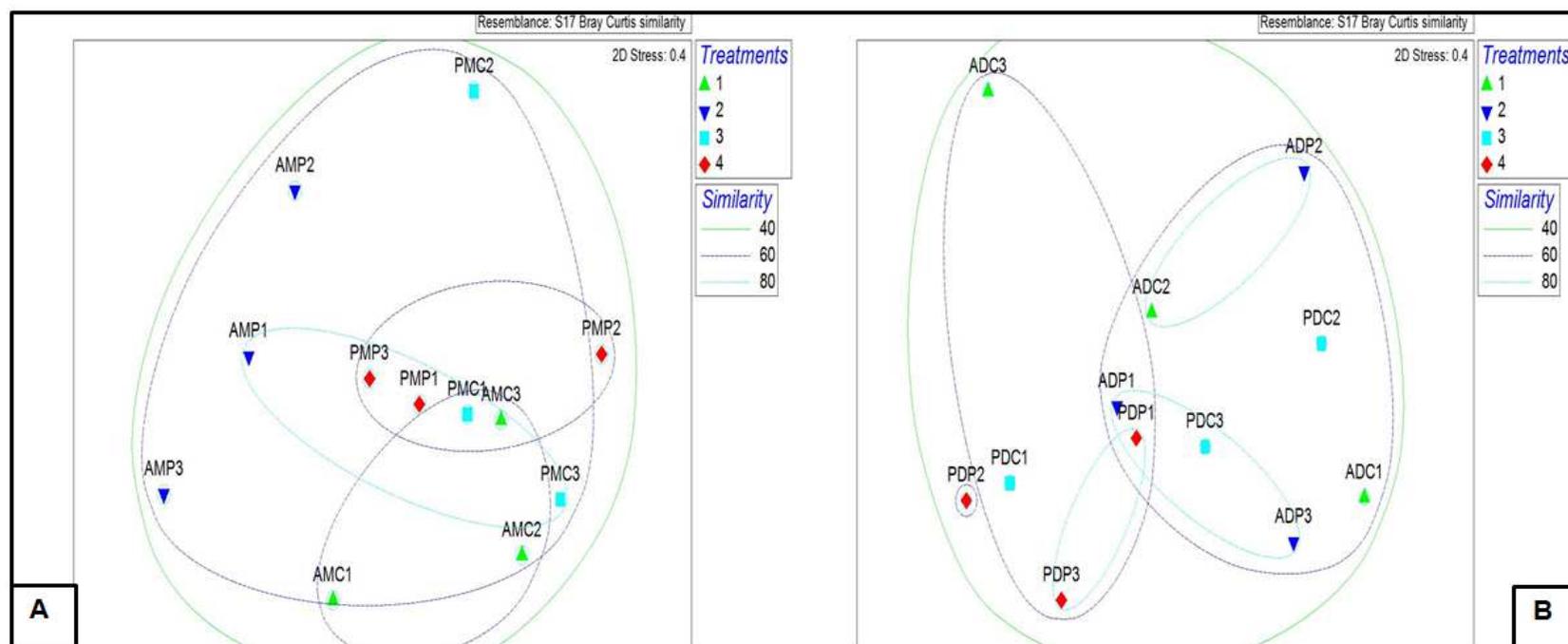


Figure 4.10 Non-metric multidimensional scaling (nMDS) analysis of DGGE fingerprints revealing similarity percentages (40, 60 and 80%) of bacterial communities between the control and probiotic groups at week four. (A) mucosa and (B) digesta (n = 3 for each region). Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control, PMP- posterior mucosa probiotic, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

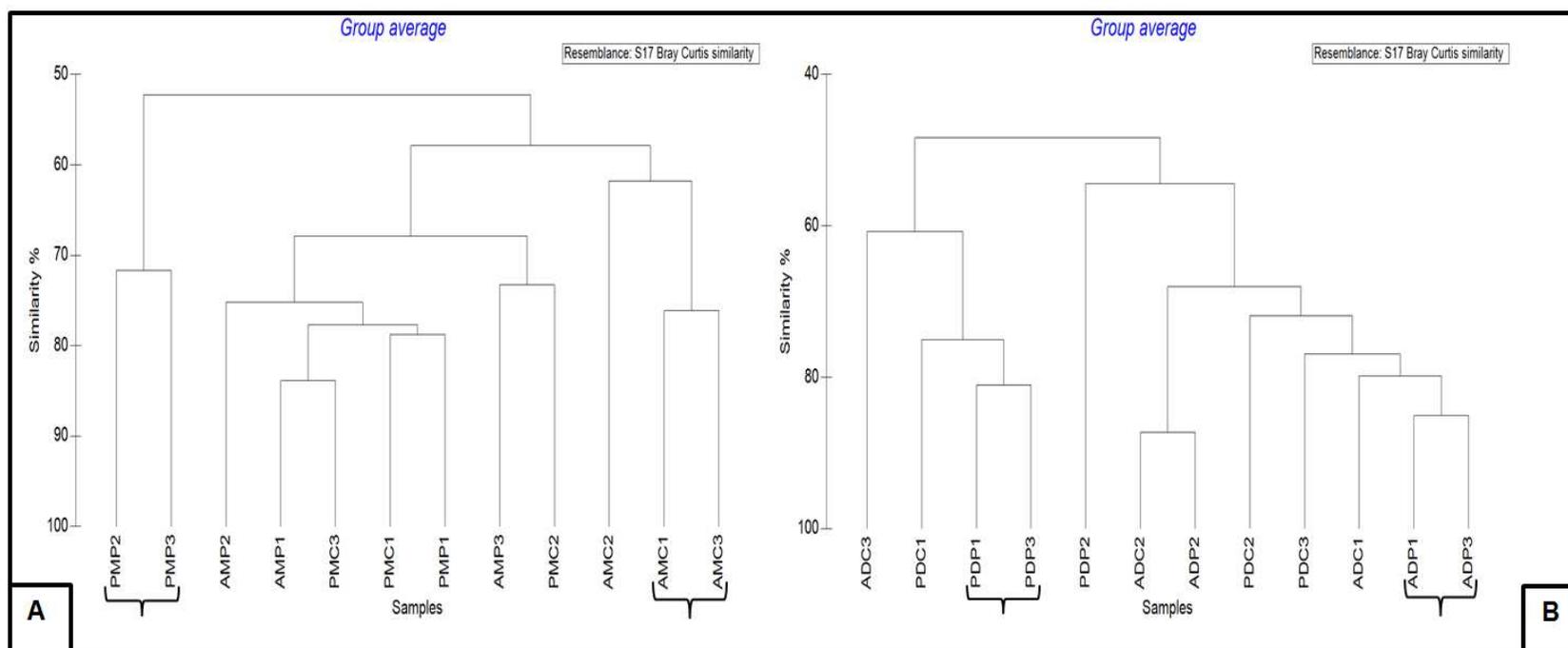


Figure 4.11 Cluster analyses of DGGE fingerprints incorporating similarity percentages of bacterial communities between control and probiotic groups at week two. (A) mucosa and (B) digesta ($n = 3$ for each region). Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control, PMP- posterior mucosa probiotic, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

Table 4.7 Microbial community analysis from PCR-DGGE fingerprints of the GIT of rainbow trout at week four.

	OTUs ¹	Richness ²	Evenness ³	Diversity ⁴	SIMPER ⁵ similarity (%)	Similarity%(control vs probiotic)
<u>Anterior mucosa</u>						
Control	25.3 ± 5.1	2.30 ± 0.40	0.97 ± 0.01	3.13 ± 0.20	66 ± 9	57 ± 9
Probiotic	23.0 ± 4.6	2.10 ± 0.40	0.97 ± 0.01	3.00 ± 0.21	70 ± 9	
<u>Posterior mucosa</u>						
Control	22.7 ± 2.1	2.07 ± 0.15	0.97 ± 0.01	3.03 ± 0.11	76 ± 6	59 ± 13
Probiotic	17.7 ± 5.7	1.61 ± 0.49	0.97 ± 0.01	2.75 ± 0.34	64 ± 8	
<u>Anterior digesta</u>						
Control	16.0 ± 5.6	1.44 ± 0.45	0.99 ± 0.00	2.71 ± 0.33	58 ± 12	70 ± 14
Probiotic	18.3 ± 3.8	1.64 ± 0.31	0.98 ± 0.01	2.84 ± 0.23	77 ± 9	
<u>Posterior digesta</u>						
Control	20.3 ± 2.6	1.77 ± 0.18	0.99 ± 0.00	2.96 ± 0.13	58 ± 13	51 ± 15
Probiotic	19.0 ± 2.6	1.75 ± 0.19	0.99 0.00	2.91 ± 0.14	59 ± 19	

Results expressed as mean ± SD in each group of fish (n = 3). Means having the different letters within the same region related to the same factor are significantly different ($P < 0.05$).

¹ Operational taxonomical unit.

² Margalef species richness: $d = (S - 1) / \log(N)$.

³ Pielou's evenness: $J' = H' / \log(S)$.

⁴ Shannon's diversity index: $H' = -\sum (p_i \ln p_i)$.

⁵ SIMPER, similarity percentage within group replicates.

OTUs 9 (89% alignment similarity to *Lactococcus fujiensis*) and 10 (91% similarity to *Mycoplasma hyorhinis*), were found in all regions and both treatments except the AMC samples. OTUs 3, 4, and 13 were identified as members of the Proteobacteria (with highest similarity to *Acinetobacter* sp. *Escherichia* sp. and an uncultured bacteria, respectively) and were detected in all intestinal regions. In the case of Firmicutes, the main representative bacterial genera/ species were *La. plantarum*, *Streptococcus fujiensis*, *W. cibaria* and *Leu. mesenteroides*. Twelve OTUs were common to either all regions or all replicates in the mucosa regions and sequence analysis showed them to be most similar to uncultured Cyanobacterium, *La. plantarum*, *St. fujiensis*, *Streptococcus lutetiensis*, *W. cibaria*, and eight different uncultured bacteria. In addition, three OTUs (15, 16 and 17) were not detected in the PM (either in the control or probiotic samples) and were shown to be most closely related to *Leu. mesenteroides* and two different uncultured bacteria. OTU 18 (uncultured bacterium) was common to all digesta replicates as was OTU 19 (uncultured bacterium), with the exception of ADC samples.

An OTU which aligned to same position in the gel as the known *P. acidilactici* was detected in the majority of the control samples and all of the probiotic samples (as indicated by the red arrows in Figure 4.9). Sixteen bands that had migrated to this point were excised and sequenced. None of the OTUs isolated from the control samples (8 bands) were identified as *Pediococcus*; these sequences were identified as uncultured bacterium, uncultured Actinobacteria, uncultured *Clostridium* sp., uncultured *Terribacillus* sp., *Enterococcus faecium* and *Bacillus licheniformis* and 2 bands failed to yield

useful nucleotide sequence reads. From the bands derived from the probiotic samples (8 bands), one was identified as *P. acidilactici*, one as *Pediococcus* sp., and the remainder as uncultured Oxalobacteraceae bacterium, *Bacillus licheniformis* and *Lactobacillus gasseri* and 3 bands failed to yield useful nucleotide sequence reads.

4.4.3.5 DGGE analysis of LAB

Figure 4.12 shows the results of agarose gel electrophoresis analysis of LAB PCR products of the mucosa samples. PCR products were obtained from mucosa from both regions in the probiotic and control groups at week two (Figure 4.12, top) and week four (Figure 4.12, bottom).

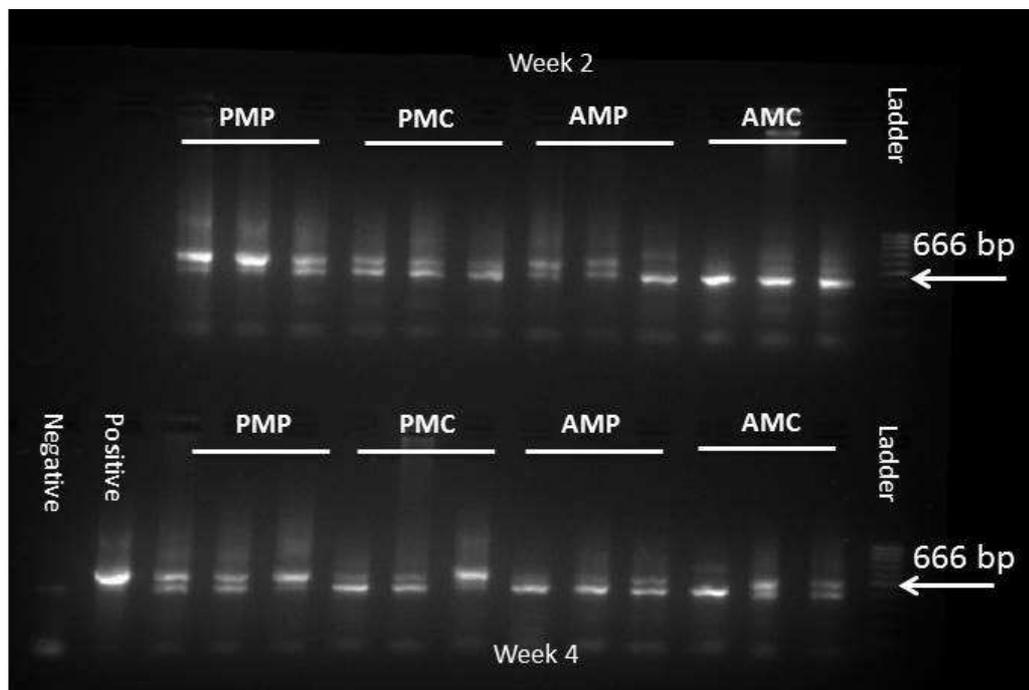


Figure 4.12 A 1.5 % agarose gel indicating that the PCR was successful in all mucosa samples. Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control and PMP- posterior mucosa probiotic.

Table 4.8 Summary of the sequencing analysis results generated from bands excised from week four DGGE gel. Numerical values represent the number of replicates (out of 3) that the OTUs were present in.

OUTs number	Phyla	Max. Identity (%)	NCBI blast matches	NCBI Accession number	Length of sequences	E value	Anterior intestine		Posterior intestine	
							C	P	C	P
<u>Mucosa</u>										
7	Cyanobacteria	100	Uncultured Cyanobacterium	EU882226.1	157	2e-05	3	3	3	1
9	Firmicutes	89	Unidentified bacterium	AB485959.1	159	2e-40	0	3	3	3
8	Firmicutes	99	<i>Lactococcus plantarum</i>	NR044358.1	163	5e-73	3	3	3	1
11	Firmicutes	98	<i>Streptococcus fujiensis</i>	GQ850525.1	166	4e-70	3	3	3	1
2	Firmicutes	100	<i>Weissella cibaria</i>	EU121684.1	167	6e-70	3	3	3	1
17	Firmicutes	97	<i>Leuconostoc mesenteroides</i>	JN792512.1	169	2e-21	3	2	0	0
12	Firmicutes	98	<i>Streptococcus lutetiensis</i>	KF245562.1	160	2e-60	2	3	3	1
13	Proteobacteria	100	Unidentified bacterium	FJ569790.1	161	5e-21	3	3	3	1
3	Proteobacteria	86	Unidentified bacterium	GU827529.1	142	4e-41	3	3	3	3
4	Proteobacteria	90	Unidentified bacterium	DQ129710.1	161	3e-43	3	3	3	3
10	Tenericutes	91	Unidentified bacterium	NR041845.1	158	2e-42	0	3	3	3
6	Tenericutes	92	Unidentified bacterium	KC686369.1	140	7e-54	3	3	3	3
15	Unidentified bacteria	100	Uncultured bacterium	FJ609998.1	161	4e-40	3	2	0	0
16	Unidentified bacteria	99	Uncultured bacterium	HM216400.1	140	4e-56	3	2	0	0
1	Unidentified bacteria	99	Uncultured bacterium	GU198326.1	139	3e-62	3	3	3	3
5	Unidentified bacteria	96	Uncultured bacterium	HM115905.1	165	7e-64	2	2	3	1
14	Unidentified bacteria	100	Uncultured bacterium	HM216375.1	141	8e-43	3	3	3	3
<u>Digesta</u>										
18	Unidentified bacteria	97	Uncultured bacterium	JQ284447.1	133	3e-52	2	3	2	2
19	Unidentified bacteria	100	Uncultured bacterium	KC700317.1	111	4e-50	0	2	1	1

Sample codes, C- control, P- probiotic.

These PCR products were run on a DGGE gel. The resulting DGGE fingerprints are shown in Figure 4.13; excised bands (for sequencing analysis) are also indicated.

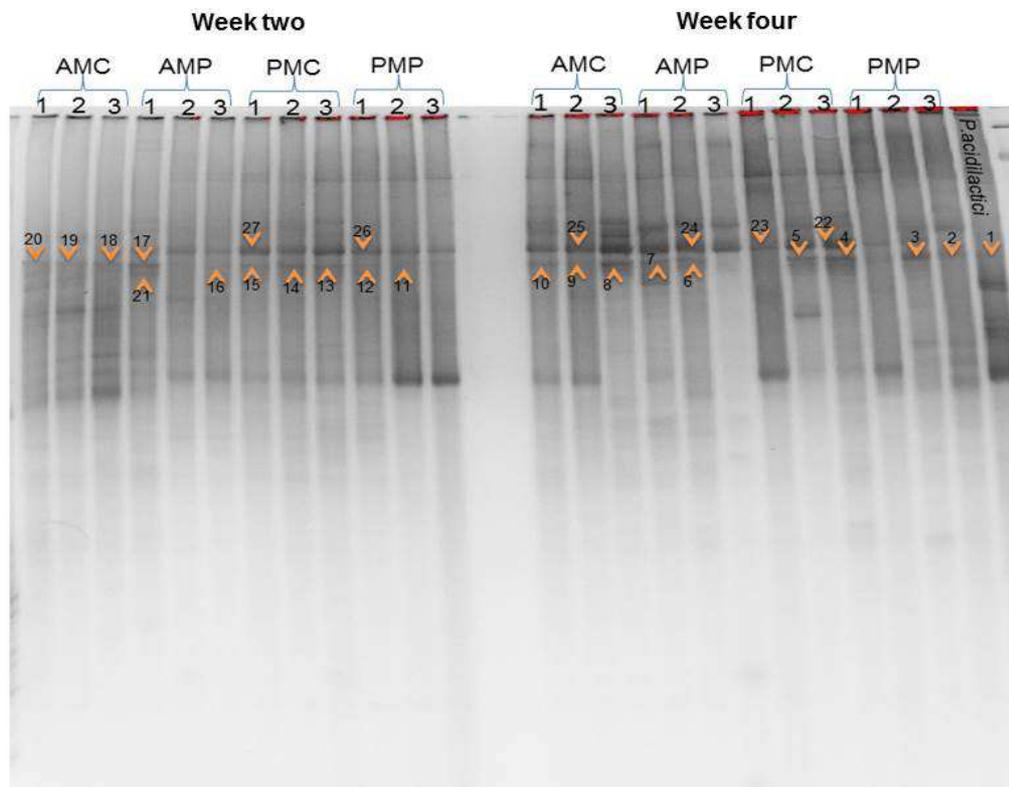


Figure 4.13 DGGE fingerprints of LAB amplicons from the AM and PM of the control and probiotic treated rainbow at week two and four. Arrows indicate bands which were excised and sequenced. Sample codes, AMC- anterior mucosa control, AMP- anterior mucosa probiotic, PMC- posterior mucosa control and PMP- posterior mucosa probiotic.

A total of twenty seven bands were selected from the mucosa gel, and are indicated on the gel images in Figure 4.13. Some bands were common to all groups and/or replicates. The results of band sequence analysis from mucosa gels are shown in Table 4.9. All bands were identified as LAB species. Only one band (3) was identified as *P. acidilactici*, which was present in the probiotic in the PM at week four. In addition, three bands (18, 19 and 20), which were identified as *Lb. plantarum*, were present in the AMC at week two and appeared to have migrated to the same position in the gel

as *P. acidilactici* in their respective different lanes, while bands 21 and 2 also related to *Lb. plantarum* and were observed in the AMP and PMP at week two and four, respectively. No bands isolated from the control samples were identified as *Pediococcus* spp. The predominating bacteria identified in the control samples were *Leuconostoc citreum* bands relating to this species again migrated to exactly the same point in the gel as bands confirmed to be *P. acidilactici* position. Six bands (22, 23, 24, 25, 26 and 27) corresponding to this bacterial species were identified in both control and probiotic samples.

Table 4.9 Summary of the 16S rRNA gene sequence analysis results generated from bands excised from week two and four DGGE gels of rainbow trout mucosa samples.

	NCBI BLAST matches	Max. Identity (%)	NCBI Accession number	Length of sequences	E value
1	<i>Pediococcus acidilactici</i>	99	KF511963.1	330	9e-166
2	<i>Lactobacillus plantarum</i>	99	KF545929.1	234	1e-112
3	<i>Pediococcus acidilactici</i>	96	KF511963.1	301	5e-143
4	<i>Leuconostoc citreum</i>	99	NR074694.1	321	7e-162
5	<i>Leuconostoc citreum</i>	99	KC417021.1	313	9e-156
6	<i>Leuconostoc citreum</i>	98	AB854223.1	174	8e-64
7	<i>Leuconostoc citreum</i>	96	AB854223.1	329	3e-155
8	<i>Leuconostoc citreum</i>	98	AB854223.1	323	1e-159
9	<i>Leuconostoc citreum</i>	98	AB854223.1	827	1e-56
10	<i>Leuconostoc citreum</i>	99	AB854218.1	554	3e-63
11	<i>Leuconostoc citreum</i>	99	AB854223.1	333	5e-168
12	<i>Leuconostoc citreum</i>	91	FJ040200.1	366	5e-134
13	<i>Leuconostoc citreum</i>	99	KC417013.1	317	2e-162
14	<i>Leuconostoc citreum</i>	99	KF150181.1	319	4e-159
15	<i>Leuconostoc citreum</i>	100	KF149651.1	366	6e-60
16	<i>Leuconostoc citreum</i>	98	AB854223.1	366	3e-58
17	<i>Leuconostoc citreum</i>	99	KF149712.1	128	3e-57
18	<i>Lactobacillus plantarum</i>	97	KF192886.1	362	2e-142
19	<i>Lactobacillus plantarum</i>	95	KF225698.1	368	7e-53
20	<i>Lactobacillus plantarum</i>	95	KF247233.1	192	1e-82
21	<i>Lactobacillus plantarum</i>	95	AB854180.1	562	3e-52
22	<i>Leuconostoc citreum</i>	99	KC417021.1	336	7e-172
23	<i>Leuconostoc citreum</i>	99	HF562952.1	253	7e-126
24	<i>Leuconostoc citreum</i>	94	KF149712.1	180	9e-74
25	<i>Leuconostoc citreum</i>	100	AB854223.1	555	1e-61
26	<i>Leuconostoc citreum</i>	96	AB854223.1	363	4e-100
27	<i>Leuconostoc citreum</i>	100	AB854223.1	165	2e-64

Figure 4.14 shows the results of agarose gel electrophoresis analysis of LAB PCR products from the digesta samples. At week two, PCR products were obtained from digesta from both regions in the probiotic group, whereas no products were observed in all, but one of the control replicate (ADC replicates 3; Figure 4.14, top). At week four, products were observed in all but two replicates (PDP1 and ADP2), regardless of the treatment (Figure 4.14, bottom).

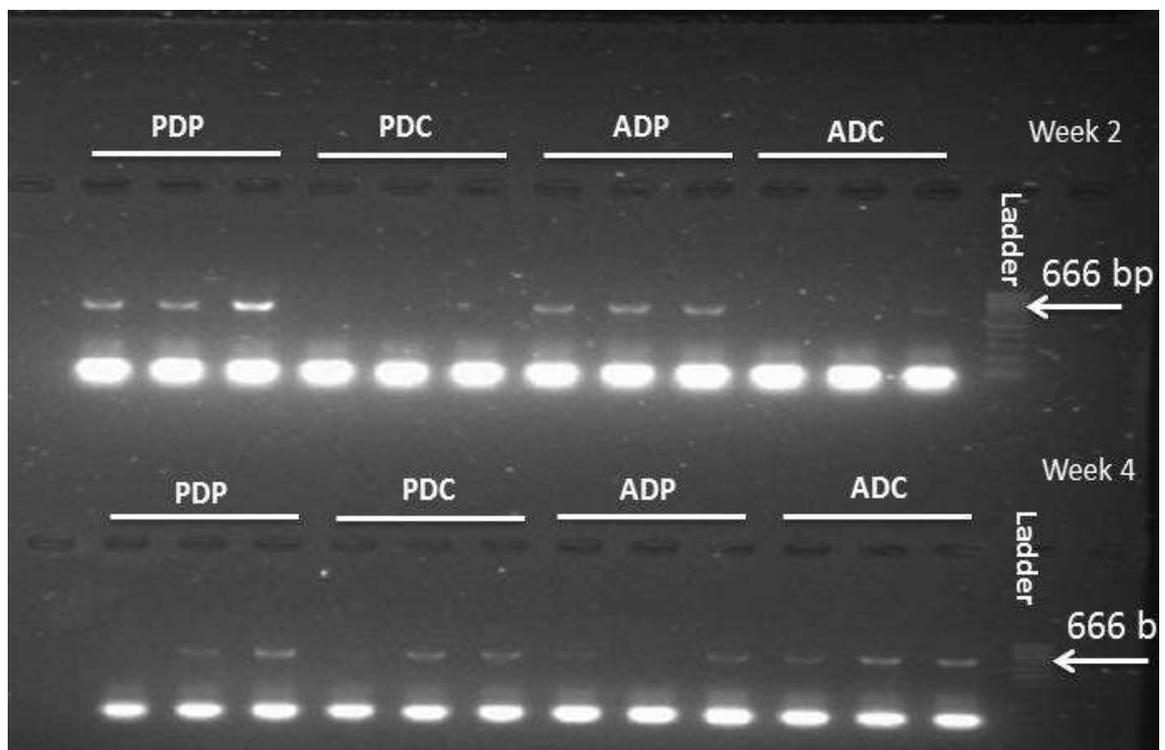


Figure 4.14 A 1.5 % agarose gel indicating that the PCR was successful in the majority of digesta samples; however there are no products in some samples. Sample codes, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

These PCR products were then run on a DGGE gel. The resulting DGGE fingerprints are show in Figure 4.15; excised bands (for sequencing analysis) are also indicated.

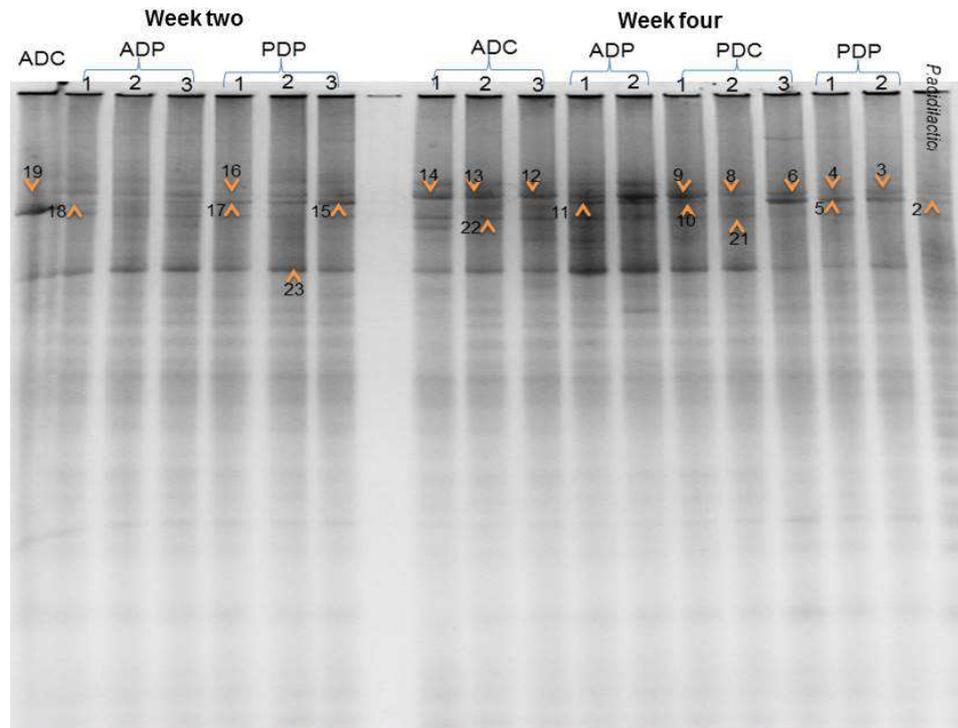


Figure 4.15 DGGE fingerprints of LAB amplicons from the AD and PD of the control and probiotic treated rainbow at week two and four. Arrows indicate bands which were excised and sequenced. Sample codes, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

A total of twenty bands were selected from the digesta gel and are indicated on the gel images in Figure 4.15. Some bands were common to all groups and/or replicates. The results of band sequence analysis from digesta gels are shown in Table 4.10. All sequences were identified as LAB species. Three bands (5) were identified as *P. acidilactici*, which was present in the PDP at week four, while bands 17 and 18 were present in the probiotic samples in PD and AD at week two, respectively. In addition, one band (15) was present in the PDP at week 2; due to the low length of the sequence this band could not be identified to species level but was most closely related to *Pediococcus* sp. Two bands, identified as *Lactobacillus* sp. (10) and *Lactobacillus gallinarum* (11), migrated to the same point in the gel as the *P.*

acidilactici; these were present in the PDC and ADP, respectively at week four. Additionally one band from the probiotic samples (3) was identified as *Leu. citreum*.

No bands isolated from the control samples were identified as *Pediococcus* spp. The majority of the bands (6, 9, 12, 13 and 14) isolated from the control samples were identified as *Leu. citreum* (but with differing maximum identity percentages). *Lb. rhamnosus* (bands 19 and 20) was also detected in the ADC at week two.

Table 4.10 Summary of the 16S rRNA gene sequence analysis results generated from bands excised from week two and four DGGE gel of rainbow trout digesta samples.

No.	NCBI BLAST matches	Max. Identity (%)	NCBI Accession number	Length of sequences	E value
2	<i>Pediococcus acidilactici</i>	97	AB841314.1	308	2e-102
3	<i>Leuconostoc citreum</i>	100	AM117165.1	331	3e-170
4	Uncultured <i>Leuconostoc</i> sp.	90	HQ897601.1	362	1e-115
5	<i>Pediococcus acidilactici</i>	99	AB841314.1	283	5e-143
6	<i>Leuconostoc citreum</i>	94	EU074846.1	364	8e-147
8	<i>Leuconostoc citreum</i>	99	AM117165.1	315	2e-161
9	<i>Leuconostoc citreum</i>	99	KF150181.1	318	7e-162
10	<i>Lactobacillus</i> sp.	83	AB810033.1	357	1e-49
11	<i>Lactobacillus gallinarum</i>	81	AJ242968.1	368	3e-51
12	<i>Leuconostoc citreum</i>	94	KF150181.1	363	2e-147
13	<i>Leuconostoc citreum</i>	99	KF150181.1	308	1e-154
14	<i>Leuconostoc citreum</i>	84	HQ897601.1	365	5e-89
15	<i>Pediococcus</i> sp.	75	AB550296.1	356	5e-25
16	<i>Leuconostoc citreum</i>	100	KF150181.1	314	8e-161
17	<i>Pediococcus acidilactici</i>	97	JQ927329.1	357	1e-119
18	<i>Pediococcus acidilactici</i>	90	KF198088.1	362	1e-89
19	<i>Lactobacillus rhamnosus</i>	80	GU425771.1	344	3e-41
21	<i>Lactobacillus rhamnosus</i>	88	JQ929645.1	365	3e-108
22	<i>Lactobacillus kefirgranum</i>	74	AJ575742.1	352	1e-35
23	<i>Lactobacillus acidophilus</i>	99	NR075049.1	322	1e-164

4.4.3.6 The analysis of *gyrB* *Aeromonas* fragments

The results from the *gyrB* primer set specific for *Aeromonas* spp. demonstrated no detectable PCR products in either mucosa or digesta samples at week 4 Figure 4.16.

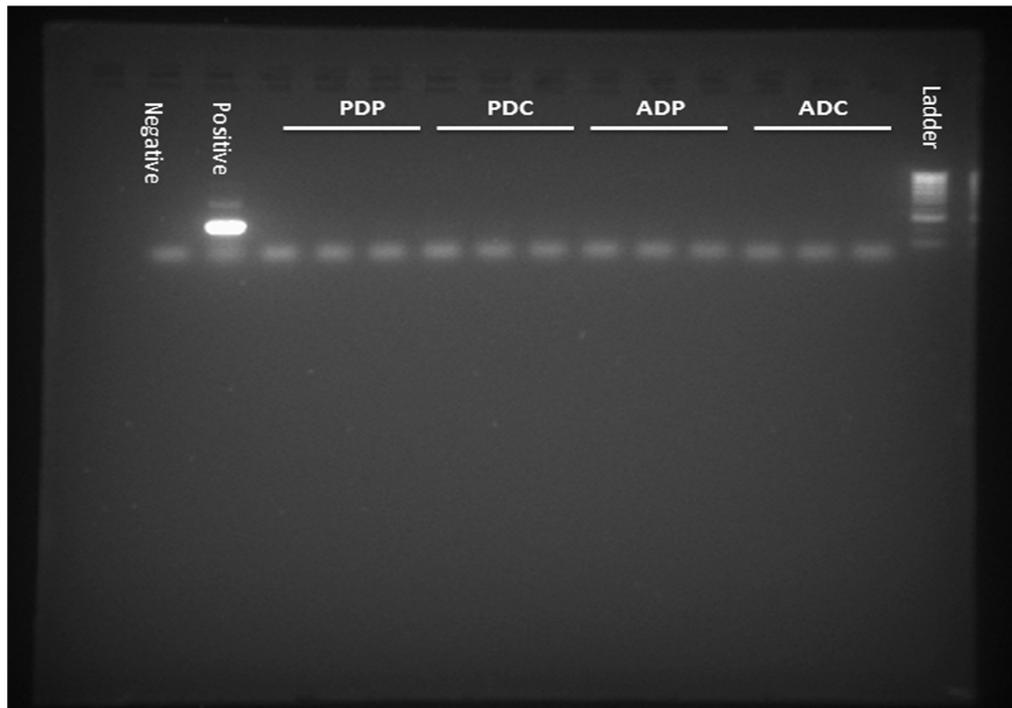


Figure 4.16 A 1.5 % agarose of gel indicating that no detectable of PCR products were observed. Sample codes, ADC- anterior digesta control, ADP- anterior digesta probiotic, PDC- posterior digesta control and PDP- posterior digesta probiotic.

4.4.4 Intestinal histology

The result of the LM analysis of villi length, IELs and goblet levels at two and four weeks of feeding periods are shown in Table 4.11.

LM from both the anterior and posterior intestine regions revealed that the intestine of rainbow trout fed both the probiotic and control diets displayed normal and intact epithelial barriers (Plates 4.2 and 4.3).

Data indicated that at two weeks, the length of villi in the anterior and posterior intestine of the probiotic treated fish did not significantly differ to that of the control group. In addition, the density of goblet cells and IELs residing between every 100 μm were not significantly affected by *P. acidilactici* even though their numbers were higher in the probiotic fed fish group compared to the control fed fish (Table 4.11).

At week four, LM analysis of the anterior intestine indicated that the probiotic group demonstrated a significantly higher density of goblet cells ($P = 0.001$) in comparison to the control fish, suggesting that *P. acidilactici* may stimulate the formation of goblet cells and elevate mucus secretion. Contrary to the current findings of the anterior intestine, the density of the goblet cells in the posterior intestine did not reveal any significant differences between the probiotic and control groups. Similarly, the *P. acidilactici* supplemented diet had no significant effect on villi length and IELs even their values were elevated in the probiotic treated fish compared to those in the control group. The lamina propria width was not affected by dietary treatment at either sampling point or either intestinal region. SEM revealed normal morphology of the mucosal folds and the enterocyte apical surface appeared intact with a high abundance of microvilli. SEM revealed that the microvilli density of the probiotic fed fish was not significantly different compared to the control treatments (Plates 4.4 and 4.5).

Table 4.11 Histological parameters of the rainbow trout intestine after feeding the probiotic and control diets at weeks two and four.

	Week two				Week four			
	Anterior intestine		Posterior intestine		Anterior intestine		Posterior intestine	
	Control	Probiotic	Control	Probiotic	Control	Probiotic	Control	Probiotic
Villi length (μm)	366.7 \pm 29.3	454.2 \pm 77.5	668.6 \pm 133.5	785.9 \pm 117.6	577.8 \pm 85.7	602.7 \pm 49.2	766.2 \pm 138.5	787.7 \pm 126.2
Microvilli density (AU)	1.2 \pm 0.1	1.1 \pm 0.1	1.3 \pm 0.1	1.5 \pm 0.3	1.3 \pm 0.1	1.2 \pm 0.1	1.1 \pm 0.1	1.4 \pm 0.2
Lamina propria width (μm)	19.5 \pm 5.6	16.5 \pm 4.4	8.5 \pm 2.7	10.6 \pm 3.2	18.3 \pm 4.1	20.7 \pm 1.8	11.9 \pm 2.5	9.5 \pm 2.2
Goblet cells (per 100 μm)	4.5 \pm 0.6	5.2 \pm 0.6	3.1 \pm 0.2	3.0 \pm 0.5	3.8 \pm 0.5 ^a	5.6 \pm 0.2 ^b	3.1 \pm 0.4	3.3 \pm 0.2
IELs (per 100 μm)	13.4 \pm 1.6	13.0 \pm 2.0	14.5 \pm 0.9	15.4 \pm 4.1	12.5 \pm 4.1	13.5 \pm 2.4	13.8 \pm 1.8	14.9 \pm 5.9

Results expressed as mean \pm SD in each group of fish (n = 6). Mean values with different letters within each intestinal region within the same row are significantly different ($P < 0.05$).

*Microvilli density = microvilli covered area foreground / microvilli covered area background - arbitrary units

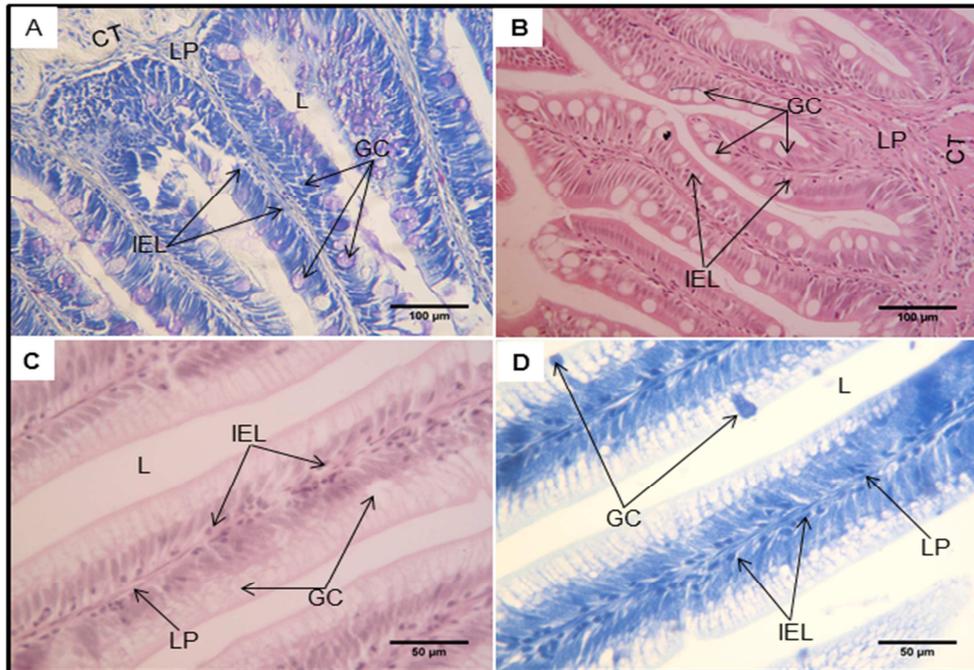


Plate 4.2 May-Grünwald-Giemsa (MGG; A and D) and haematoxylin and eosin (H&E; B and C) staining of the anterior (A and B) and posterior (C and D) intestine of rainbow trout at week two. Abundant intraepithelial leucocytes (IELs) and goblet cells (GC) are present in the epithelia of both the probiotic (A and C) and control (B and D) fed rainbow trout. Other abbreviations used, L- lumen, LP- lamina propria and CT- connective tissue.

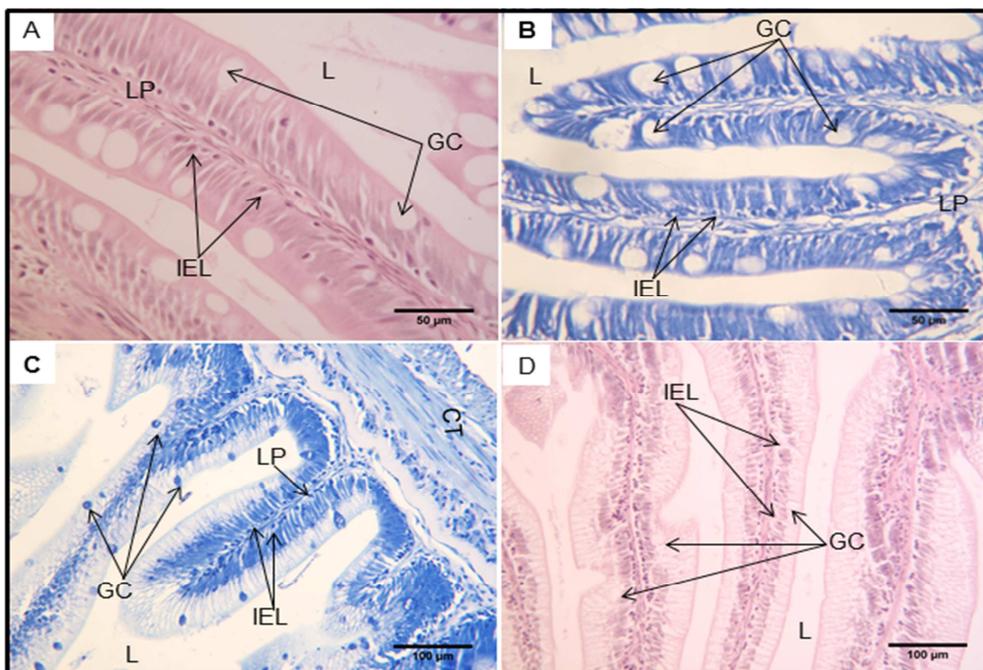


Plate 4.3 May-Grünwald-Giemsa (MGG; B and C) and haematoxylin and eosin (H&E; A and D) staining of the anterior (A and B) and posterior (C and D) intestine of rainbow trout at week four. Abundant intraepithelial leucocytes (IELs) and goblet cells (GC) are present in the epithelia of both the probiotic (A and B) and control (C and D) fed rainbow trout. Other abbreviations used, L- lumen, LP- lamina propria and CT- connective tissue.

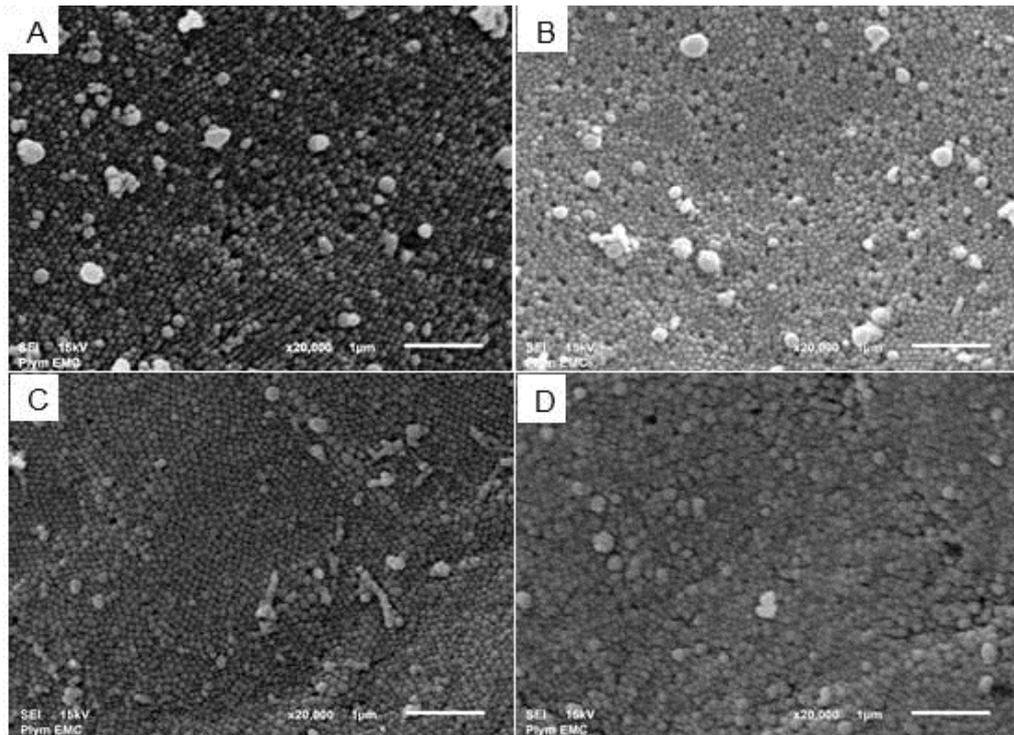


Plate 4.4 Representative scanning electron microscopy (SEM) images showing microvilli density of the anterior intestine of the control (A) and probiotic (B) and the posterior intestine of the control (C) and probiotic (D) at week 2.

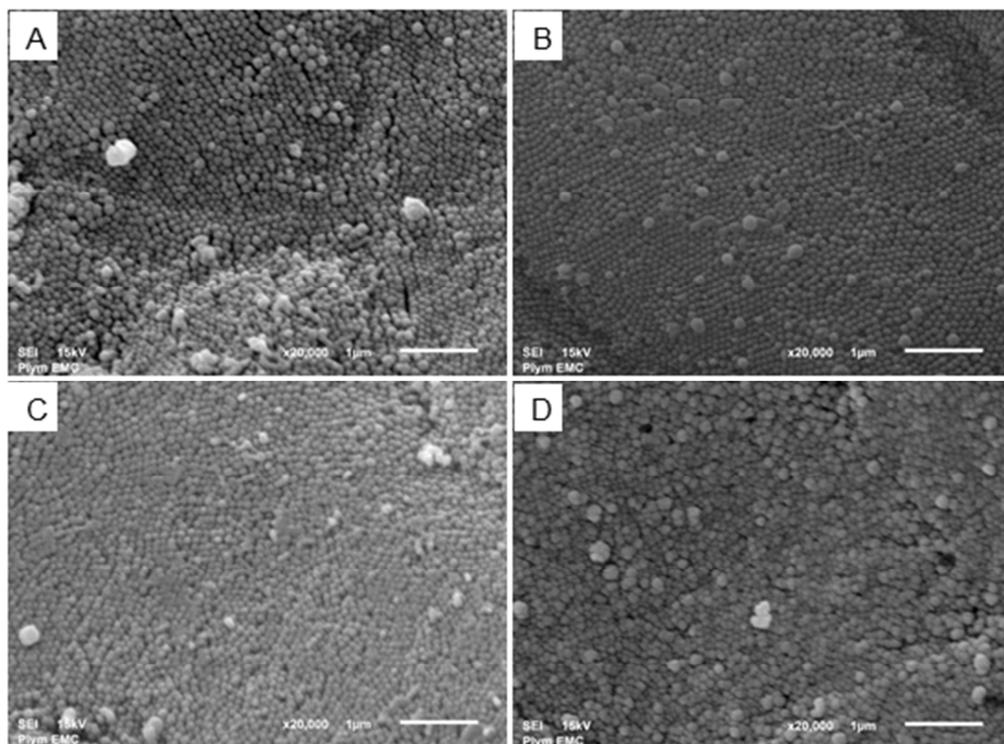


Plate 4.5 Representative scanning electron microscopy (SEM) images showing microvilli density of the anterior intestine of the control (A) and probiotic (B) and the posterior intestine of the control (C) and probiotic (D) at week 4.

4.4.5 Intestinal gene expression

The levels of relative expression of the immune-related genes from both of the intestinal regions in both control and probiotic groups of fish sampled at week two and four are presented in Figures 4.17 - 4.24.

Compared to the control treatment, the anterior intestine of probiotic treated fish displayed significant ($P < 0.001$) up-regulations of the pro-inflammatory cytokines IL-1 β and IL-8 at sampling week four, but not at week two (Figures 4.17 and 4.18). The mRNA levels of the anti-inflammatory cytokine IL-10 in the anterior intestine was not affected at week two, and was significantly lower in the probiotic fed fish compared to the control group at week four (Figure 4.19). On the other hand, the relative expression of IgT gene was significantly lower in the anterior intestine at week 2 ($P < 0.04$) and significantly higher at week 4 ($P < 0.001$) in the probiotic fed fish compared to the control group (Figure 4.20). The expression of TLR5 was not affected at week two but was significantly down regulated at week four in the anterior intestine of the probiotic fed fish, compared to the control fish ($P < 0.001$; Figure 4.21). The expression of casp-3 and PCNA were significantly lower in the anterior intestine of the probiotic fed fish, compared to the control fed fish, at both time points ($P < 0.001$; Figures 4.22 and 4.23). The expression of HSP70 was significantly lower at week 2 ($P < 0.001$) and numerically lower, although not significantly lower, at week 4 ($P = 0.078$; Figure 4.24).

In the posterior intestinal region, the probiotic treated fish displayed significant up-regulations of IL-1 β and IL-8 and down-regulation of IL-10 at both sampling points compared to the control fish ($P < 0.001$) (Figures 4.17, 4.18 and 4.19). IgT expression was also significantly up-regulated in the

posterior region at both time points ($P < 0.001$; Figure 4.20). The expression of TLR5 was not affected by probiotic treatment at either time point in the posterior intestine (Figure 4.21). Compared to the control fed fish significantly lower HSP70, PCNA and casp-3 gene expression levels were found at both time points in the posterior intestine of the of the probiotic fed fish ($P < 0.001$) (Figures 4.22, 4.23 and 4.24).

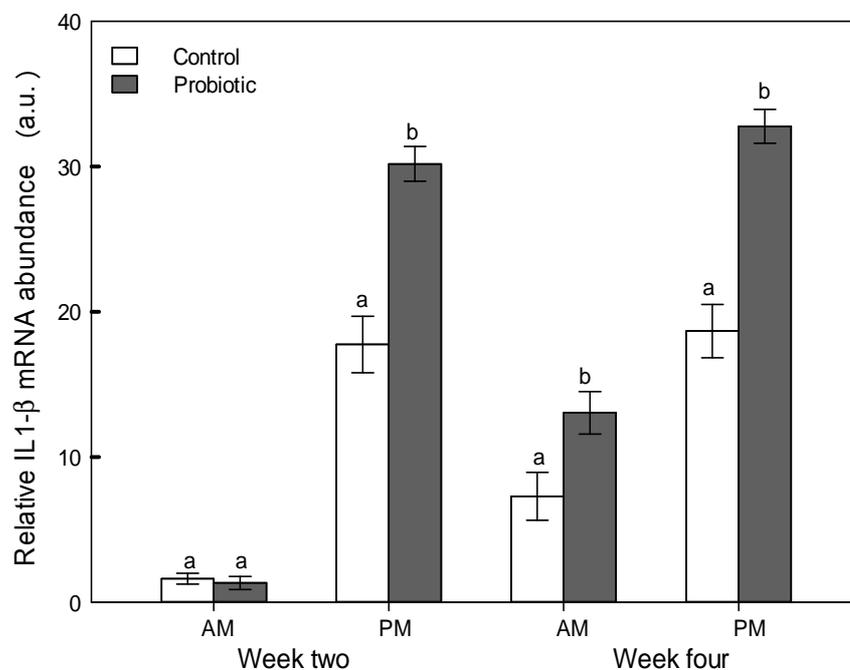


Figure 4.17 RT-PCR analysis of IL-1 β gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

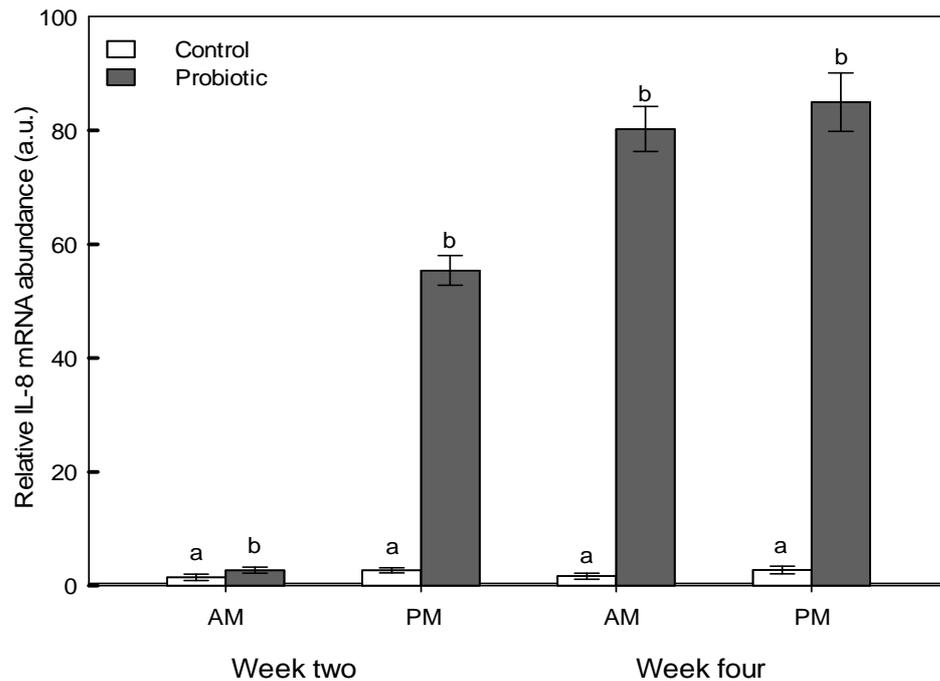


Figure 4.18 RT-PCR analysis of IL-8 gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

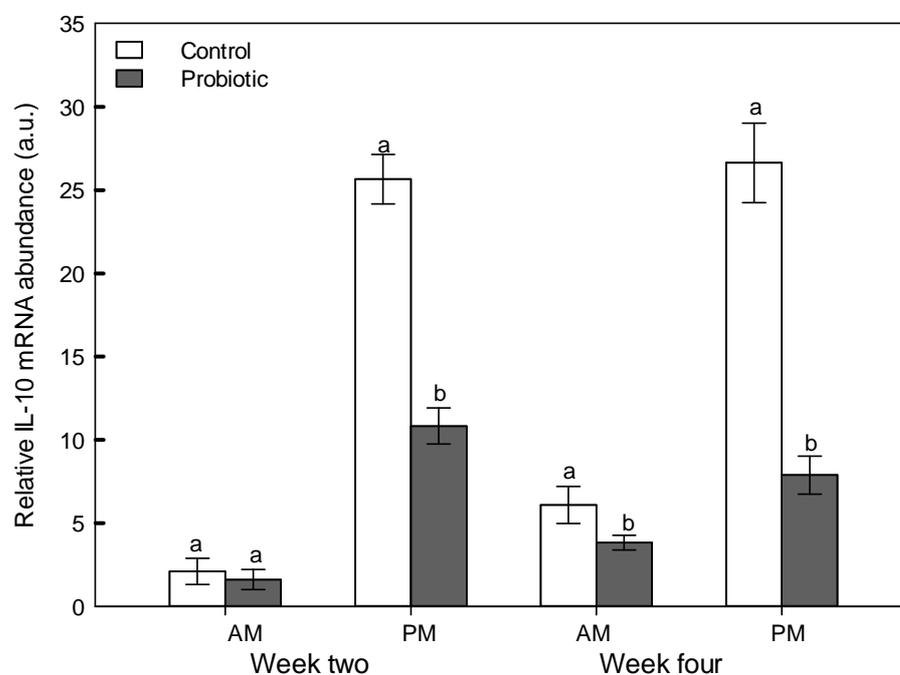


Figure 4.19 RT-PCR analysis of IL-10 gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

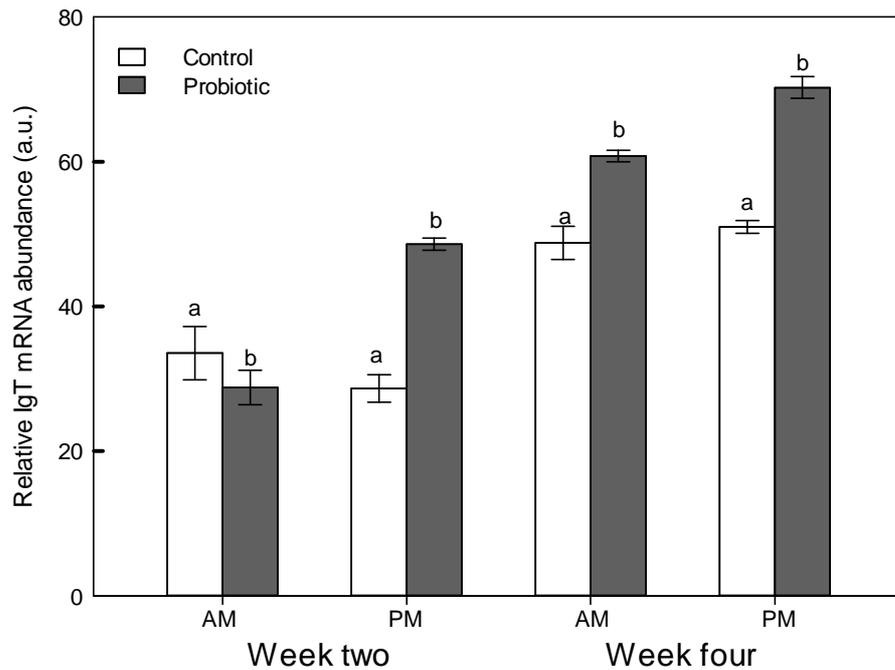


Figure 4.20 RT-PCR analysis of IgT gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

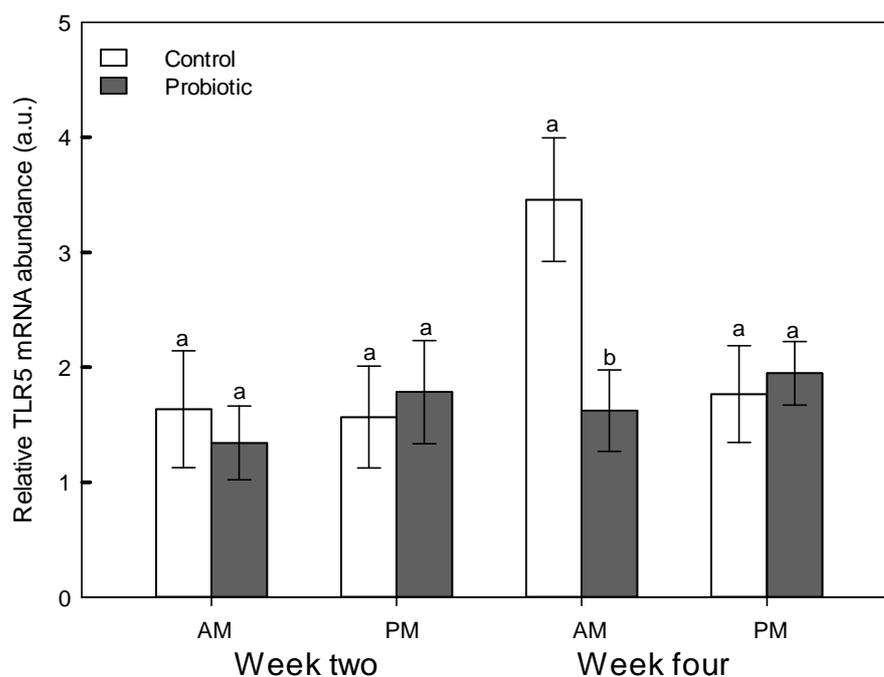


Figure 4.21 RT-PCR analysis of TLR5 gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

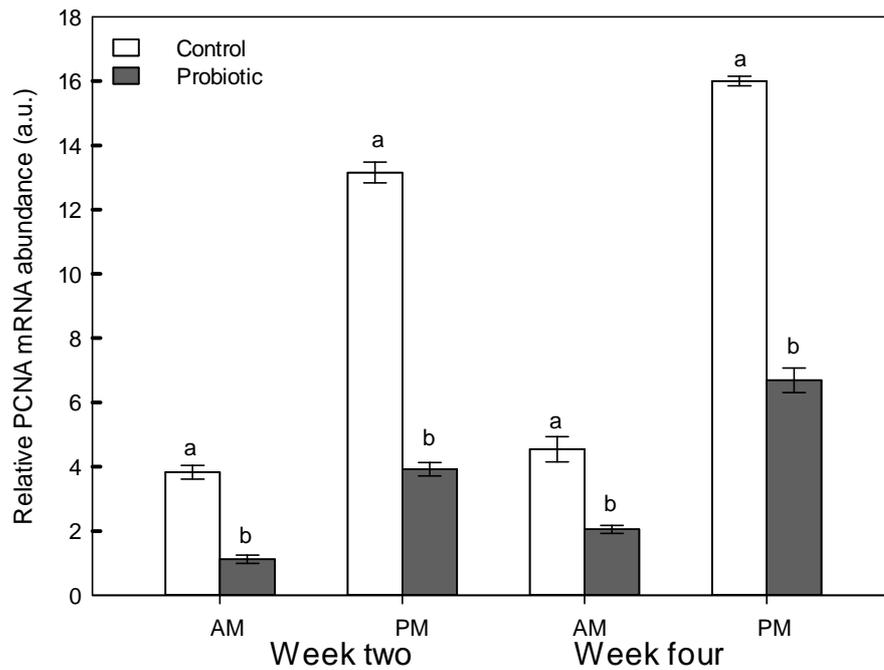


Figure 4.22 RT-PCR analysis of PCNA gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

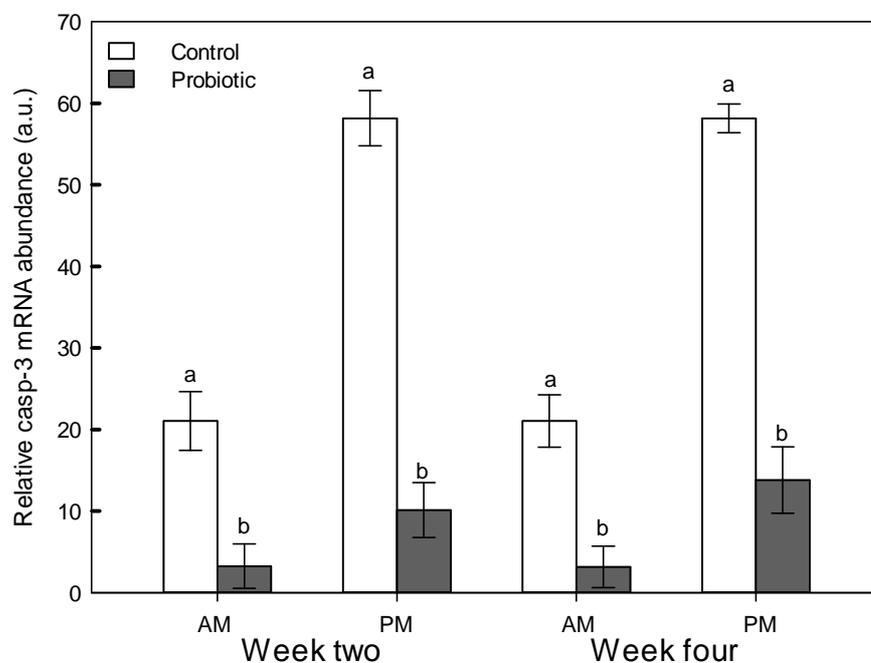


Figure 4.23 RT-PCR analysis of casp-3 gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

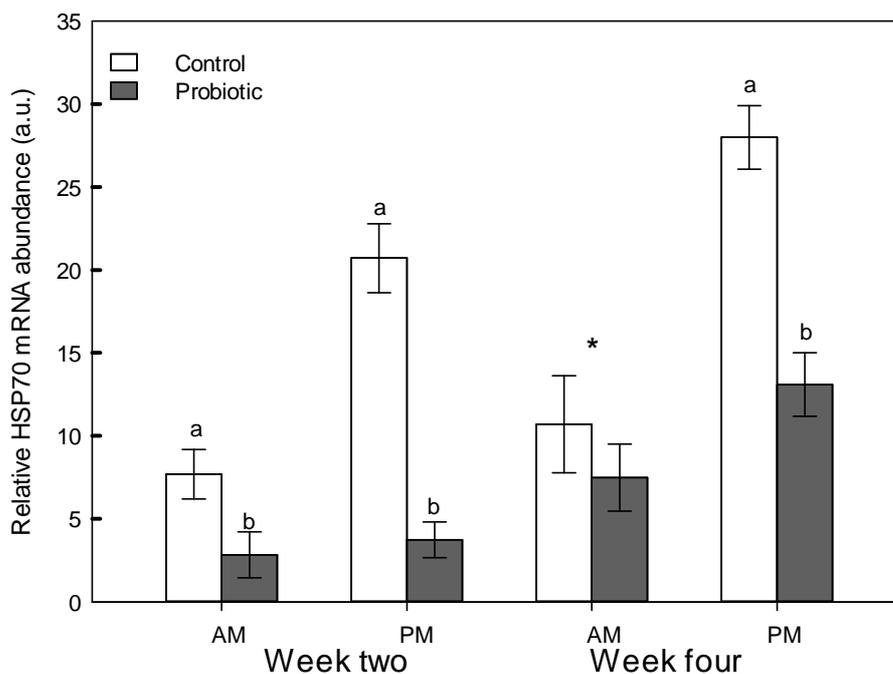


Figure 4.24 RT-PCR analysis of HSP70 gene expression in the intestine of rainbow trout fed the control and probiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Means having different letters in the same intestinal region and same time points are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

4.4.6 Haematological and immunological parameters

The haematological and immunological parameter measurements of rainbow trout fed the probiotic and control diets at two and four weeks are displayed in Table 4.12. During the present study, the most haematological parameters were not significantly affected by dietary *P. acidilactici* ($P > 0.05$). On the other hand, significant differences were found in MCV in the *P. acidilactici* group compared with the control group at week two ($P = 0.05$). In addition, serum lysozyme activity was slightly significantly higher in the probiotic group than in the control group at week four ($P = 0.045$).

Table 4.12 Rainbow trout haematological and immunological parameter measurements after feeding on the probiotic and control diets for four weeks.

Blood/ immunological parameters	Week two		Week four	
	Control	<i>P. acidilactici</i>	Control	<i>P. acidilactici</i>
Erythrocyte count ($\times 10^5 \text{ mm}^3$)	6.5 \pm 0.6	6.3 \pm 0.7	5.9 \pm 0.5	5.9 \pm 0.7
P.C.V (%)	42.5 \pm 3.8	44.5 \pm 2.7	40.8 \pm 4.0	41.1 \pm 4.9
Hb (g dL ⁻¹)	6.3 \pm 0.8	6.1 \pm 0.7	4.1 \pm 0.5	4.0 \pm 0.3
MCV(fL cell ⁻¹)	648 \pm 78 ^a	714 \pm 76 ^b	685 \pm 101.0	670 \pm 103.0
MCH (pg cell ⁻¹)	97.0 \pm 15	98.0 \pm 16.5	70.0 \pm 10.7	69.0 \pm 7.2
MCHC (g dL ⁻¹)	14.0 \pm 1.6	13.0 \pm 1.8	10.0 \pm 1.7	10 \pm 1.2
Lysozyme activity (Units mL ⁻¹)	414.8 \pm 42.9	403.8 \pm 50.6	362.3 \pm 134.9 ^a	503.4 \pm 169.2 ^b
Respiratory burst (OD _{610nm})	0.018 \pm 0.006	0.018 \pm 0.006	Nd	Nd
Leucocyte count ($\times 10^3 \text{ mm}^3$)	6.9 \pm 3.5	6.8 \pm 1.9	5.2 \pm 1.8	5.6 \pm 1.7
Lymphocytes (%)	88.0 \pm 5.1	89.0 \pm 3.4	86.1 \pm 6.3	83.3 \pm 6.0
Monocytes (%)	2.5 \pm 1.8	2.7 \pm 2.3	5.4 \pm 3.7	6.0 \pm 2.5
Neutrophil (%)	5.5 \pm 3.2	4.0 \pm 2.9	3.5 \pm 3.0	4.5 \pm 2.8
Eosinophils (%)	2.4 \pm 1.7	2.1 \pm 2.9	2.4 \pm 2.2	3.4 \pm 2.5
Thrombocytes (%)	1.5 \pm 1.2	1.7 \pm 1.6	2.8 \pm 2.1	2.6 \pm 2.1

Results expressed as mean \pm SD in each group of fish n = 12. Means having the different letter in the same at the same sampling point row are significantly different ($P < 0.05$).

4.5 Discussion

The present study demonstrated that *P. acidilactici* were detected as allochthonous bacteria through the GIT, where their numbers were observed in the digesta and mucosa at levels of $\log 3 - 5 \text{ CFU g}^{-1}$ which is in agreement with previous observations using this probiotic in rainbow trout (Merrifield *et al.*, 2010a; Merrifield *et al.*, 2011) and tilapia (Ferguson *et al.*, 2010; Standen *et al.*, 2013). Data from the current study demonstrated that high levels of LAB were present within the posterior intestine of probiotic fed fish, whose functions include food digestibility and immunity function, which is in agreement with most previous studies (Ringø *et al.*, 1998; Hovda *et al.*, 2007; Kim *et al.*, 2007; Merrifield *et al.*, 2009a; Askarian *et al.*, 2012).

After the cessation of feeding the probiotic to the fish, the probiotic levels remained high in the intestine after the first day but these bacteria were not detectable on the third day. The reason for this is not clear, but it seems possible that it might be related to the rearing temperature (15 °C) of rainbow trout, a cold water fish species. It could be because the optimum growth for *Pediococcus* spp. is typically 25 - 40 °C (Holt and Bergey, 1994) and so lower temperature applications are likely to reduce *Pediococcus* multiplication and metabolic activity which could reduce persistence of probiotic supplementation. A previous study by Ferguson *et al.* (2010) lends support to this hypothesis, although in this case tilapias were used. In tilapia reared at 25-26 °C the probiotic remained present in the GI tract for at least 17 days after the cessation of probiotic feeding.

In recent years, several studies have found that LAB have an ability to exist in the intestine of fish for varying periods depending on the circumstances of

the trial (Balcazar *et al.*, 2007; Newaj Fyzul *et al.*, 2007; Merrifield *et al.*, 2010a; Merrifield *et al.*, 2010b). The contradictions in the persistence duration of probiotics within the intestine of fish reported in different studies may be due to the type and term of adverse circumstances and on the fish species used. Furthermore, it is postulated that the establishment, persistence and subsequent induction of the immune responses in a host could affect the duration of probiotic persistence.

Supporting the culturable data, the presence of the probiotic was detected in the GIT by clone library and DGGE analyses. These different methodological approaches provide complimentary benefits; DGGE analysis provides a means to assess microbial community ecological parameters and can reveal the relatedness (i.e. similarity) of microbial profiles from different environmental samples, whereas clone libraries analysis allows for a high level identification based on near full length 16S rRNA sequence gene reads. Both approaches indicated that *P. acidilactici* was not amongst the most dominant bacterial species present.

In the present study the most abundant OTUs in rainbow trout at two and four weeks recovered from DGGE were related to Firmicutes; this was in contrast to most previous studies which showed that Proteobacteria were the most predominant phyla (Huber *et al.*, 2004; Hovda *et al.*, 2007; Kim *et al.*, 2007; Merrifield *et al.*, 2009a; Navarrete *et al.*, 2009). The Firmicutes identified from the DGGE in the present study were comprised of *Enterococcus* sp., *Lactococcus* sp., *Streptococcus* spp., *Weissella cibaria*, *Lactobacillus* spp., *Leuconostoc mesenteroides* and *Pediococcus pentosaceus*. With exception

to *Lb. aviarius* subsp. *araffinosus* and *St. fujiensis*, all other identified LAB have been isolated from salmonids in previous investigations (Ringø *et al.*, 2000; Balcazar *et al.*, 2007; Balcázar *et al.*, 2007b; Hovda *et al.*, 2007; Daniels *et al.*, 2010; Desai *et al.*, 2012). The probiotic, *P. acidilactici* was detected in the digesta samples of fish fed the probiotic supplemented diet, but not in the control samples. There was another band, relating to different bacteria, which had migrated to the identical position of *P. acidilactici*. Gafan and Spratt (2005) demonstrated that the co-migration of bands which generated from different taxa to the identical position in the different lanes within DGGE gels. In addition, Vallaey *et al.* (1997) demonstrated that single bands could yield several species (in some cases both Gram-negative and Gram-positive bacteria) with identical rDNA sequences under tighter denaturing gradient conditions. In addition, another possible explanation for some of these results may be attributed to the gel concentration that was used in this study or the accuracy of excision of bands for sequencing, influenced by the close proximity of bands.

In order to increase the chance of specifically identifying LAB species (particularly the probiotic), and to reduce the potential of co-location of non-probiotic bands to the same position, a nested PCR approach involving specific LAB primers was used. *P. acidilactici* was detected in the mucosa and digesta samples of fish fed the probiotic supplemented diet, but not in the control samples. However, in a number of samples, *Leu. citreum* co-migration to the same position as *P. acidilactici* which could be attributed to the fact that these bacteria are closely related to *P. acidilactici*, having fairly similar % G+C profiles. The 16S rDNA fragment analysed may not have

contained sufficient sequence differences, and therefore melting behaviours, to allow separation by DGGE (Muyzer *et al.*, 1993). However, *Leu. citreum* was the dominant bacteria isolated from the control samples. *Leuconostoc* sp. are Gram-positive, facultative anaerobic, non-sporulating cocci, present as pairs or short chains, non-motile and usually mesophilic (Hemme and Foucaud-Scheunemann, 2004). Although they are reported to be safe and have a major role in food industries including fermentation of carbohydrates, pickles etc., they are reported to cause numerous diseases in humans (Hemme and Foucaud-Scheunemann, 2004). *Leuconostoc* spp. have been isolated and identified in previous investigations (Ringø *et al.*, 1998; Ringø, 2004; Balcázar *et al.*, 2007a; Balcázar *et al.*, 2007b; Mansfield *et al.*, 2010; Pérez-Sánchez *et al.*, 2011a).

In addition to the Firmicutes identified by DGGE analyses, some OTUs were identified as belonging to the Actinobacteria, Proteobacteria and Tenericutes phylum, as well as Cyanobacteria and a number of unidentified bacteria.

Clone library analyses provided quantitative, and more robust sequence identification, approach in identifying the OTUs present in the rainbow trout GIT. Supporting the DGGE based analysis, clone libraries revealed that *P. acidilactici* was not amongst the most dominant bacterial groups in the GIT. Only one clone, derived from the posterior digesta, was identified as *P. acidilactici*. Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and unidentified bacteria were identified to populate the GIT of rainbow trout by clone library analysis. In contrast to the DGGE analysis, Proteobacteria was the predominant phylum isolated from rainbow trout using a 16S rRNA gene

clone library analysis which is in agreement with previous studies (Spanggaard *et al.*, 2000; Huber *et al.*, 2004; Pond *et al.*, 2006; Kim *et al.*, 2007; Merrifield *et al.*, 2009a). There are a number of possible reasons which may explain the variation in results between the two methods: 1] different primer sets were used for each technique, which makes comparisons between data sets difficult (Kim *et al.*, 2007), 2] the clone library analysis has limited sensitivity to detect species which constitute a low percentage of the population unless there are large numbers of clones (i.e. 1,000's per sample) (Muyzer *et al.*, 1993), 3] the DGGE method is only semi-quantitative, 4] not all of the OTUs derived from the DGGE gels were successfully sequenced, and 5] not all of the OTUs were isolated for sequencing. However, some culturable genera retrieved in this study have not been previously reported as part of the intestinal microbiota in rainbow trout.

In the present study, bacteria retrieved from samples of mucosa and digesta were evaluated, and demonstrated that the intestine contents harbour a comparatively larger bacterial diversity than the mucosa. The predominant family was Alacigenaceae and sequences similar to *Al. faecalis* were most frequent among all samples at week two and four. These results are not in agreement with those reported in the literature; this may be the first time that *Al. faecalis* has been identified from rainbow trout in the current study. *Al. faecalis* is Gram-negative rod-shaped which belongs to the family Comamonadaceae. *Alcaligenes* spp. are commonly found in soil, water, wastewater, treatment plants, vegetables and fruits (Nakano *et al.*, 2013). The authors mentioned that *Alcaligenes* spp. are opportunistic bacteria transferred from fruits and vegetables to human and animals through

consumption. *Alcaligenes* spp. were also detected in low levels as part of the autochthonous hind gut microbiota of Arctic charr (*Salvelinus alpinus*) (Ringø *et al.*, 2006a). *Alcaligenes* spp. were also identified in Chapter 3. The consistency of isolation of this species indicates that these could be common components of the GI microbiota of salmonids and are likely to be of importance to the host, but their role in the intestine of fish is not clear and further investigation is needed. Differences in the relative abundance of the microbial species identified were also observed using the clone library approach.

Sequences relating to Proteobacteria were frequently identified in high abundance in all intestine samples of fish fed both diets for two and four weeks, while Firmicutes were detected in low abundance in the anterior digesta and posterior digesta samples of fish fed the control diet at two weeks, with the most prominent species being *Staphylococcus pasteurii*. The first isolation of *Staphylococcus pasteurii* was from human, animal and food specimens (Chesneau *et al.*, 1993). It could be possible that *P. acidilactici* reduced these bacteria to an undetectable level, whereas this species was only detected in the posterior digesta in the probiotic group at week two. These findings indicate that *P. acidilactici* could exert competitive activity against pathogenic bacteria in the GIT of rainbow trout. At week four, *Staphylococcus pasteurii* and *Streptococcus alactolyticus* were found in the anterior digesta of the probiotic fed fish, while *Clostridium* spp., *Bacillus* spp. and *Lactobacillus amylovorus* were found in the posterior digesta of the probiotic group only. *Staphylococcus pasteurii* has been isolated from the distal intestine of Atlantic salmon fed fishmeal and soybean meal (Bakke-

McKellep *et al.*, 2007) and from Atlantic salmon fed diets with or without chitin (Askarian *et al.*, 2012).

Acinetobacter spp. were isolated from all control and probiotic samples at week two and four. *Acinetobacter* is a strictly aerobic, Gram-negative, nonmotile, oxidase-negative, glucose-non-fermentative and catalase positive bacteria, previously identified as a member of the microbiota in the human skin with a probability to cause opportunistic and nosocomial infections (Wagner *et al.*, 1994; Van Looveren and Goossens, 2004). Sequences related to *Acinetobacter johnsonii* were high in abundance in the posterior mucosa of fish fed the control diet, but were absent in the same region in the clone libraries of fish fed the probiotic diet. Also, a reduction in the proportion of *Acinetobacter* spp. in the anterior and the posterior digesta at week two and the posterior digesta at week four was observed. These findings indicated that probiotic *P. acidilactici* may possibly exert antagonistic activity against these bacteria in the GIT of rainbow trout; more studies are required to explain the competitive role between these bacteria and *P. acidilactici*. *Acinetobacter* spp. have been widely isolated from salmon (Holben *et al.*, 2002; Hovda *et al.*, 2007; Askarian *et al.*, 2012), rainbow trout (Huber *et al.*, 2004; Merrifield *et al.*, 2009a), Arctic charr (Ringø and Strøm, 1994).

Sequences similar to *Pseudomonas* spp. were frequent among all samples, particularly in the digesta samples at week two, with the exception of the PM of fish fed probiotic. However, a reduction in the frequency of these bacteria was found among all samples at week four. Some *Pseudomonas* spp., such as *Ps. fluorescens*, are potential pathogens (Zhang *et al.*, 2009) and their

reduction in the posterior mucosa at week 4 could be attributed to the competitive role of *P. acidilactici* against these bacteria. Indeed several studies have demonstrated that *P. acidilactici* could exert antagonistic effects against *Pseudomonas* spp. *in vitro* (Skyttä *et al.*, 1993; Giacometti *et al.*, 1999).

Aeromonas spp. were identified in this study. The frequent presence of *Aeromonas* in the mucosa samples is in agreement with the findings of Wu *et al.* (2012), indicating that the intestine might be the primary location for *Aeromonas* colonization. *Aeromonas* spp. are Gram-negative, motile, pigmented, rods, non-spore-forming, facultative anaerobic, oxidase positive, mesophilic bacteria with exception of *A. salmonicida* being able to grow at range of temperature (22 - 28 °C) (Beaz - Hidalgo and Figueras, 2013). *A. hydrophila* is an opportunistic pathogen and has been found to be causative agent of haemorrhagic septicemia disease and furunculosis in rainbow trout (Austin and Austin, 2007). *A. salmonicida* is a Gram-negative, non-motile rod, facultatively anaerobic, often indigenous to fish and causing both the acute and the chronic forms of furunculosis (Beaz - Hidalgo and Figueras, 2013). *Aeromonas caviae* causes bacterial enteritis and furunculosis (Austin and Austin, 2007). *Aeromonas* spp. have been found to be functional in fish health, other than as pathogenic bacteria (Irianto and Austin, 2002b) and detected in the normal intestinal mucosa from several fishes such as Arctic charr, rainbow trout, Atlantic salmon and Grass Carp (Pond *et al.*, 2006; Kim and Austin, 2008; Askarian *et al.*, 2012; Wu *et al.*, 2012; Li *et al.*, 2014).

Despite the fact that the probiotic was not a dominant component of the total microbiota, in terms of abundance, it was still able to exert some modulatory effect on the GIT microbiota. An example of such was clear with respect to the *Aeromonas* levels in the PD, particularly at week 4. At week 2, the probiotic application reduced the abundance of *A. hydrophila* in the PD from 7.29% in the control to non-detectable levels in the probiotic fed fish; similarly at week 4, the *A. hydrophila* levels were reduced from 7.07% to 1.10%. Further, *A. salmonicida* (6.06% in the control) and *A. taiwansis* (9.09% in the control) were reduced to non-detectable levels in the PD of the probiotic fed fish. To investigate this further, the *gyrB* gene, which encodes the RNA polymerase b subunit, was selected as an alternative phylogenetic marker. *gyrB* genes have been used as valuable phylogenetic markers for microorganisms, in particular *Aeromonas* (Calhau *et al.*, 2010), instead of the 16S rRNA gene which can yield multiple bands on denaturing gradient gels (Rantsiou *et al.*, 2004). Calhau *et al* (2010) demonstrated that using a *gyrB*-targeting primer set successfully generated products from a range of tested *Aeromonas* strains. In the present study however PCR using these primers did not produce *gyrB* PCR amplicons from the fish samples. The reason for this is not clear but may be due to the *Aeromonas* spp. template was too low or that there were PCR inhibitors in the samples. Indeed, it could be also possible that the amplification of *A. salmonicida* DNA was inhibited by high amount of fish tissue or high concentrations of fish DNA, as reported elsewhere (Byers *et al.*, 2002). In this respect, more investigations are needed to elucidate the reasons behind the failure of PCR in this case and also to determine the role of intestinal *Aeromonas* spp. in rainbow trout.

The Firmicutes identified by clone library analysis were predominantly LAB, with sequences related to *Lb. amylovorus*, *P. acidilactici*, *Lactobacillus crispatus*, but *Bacillus thermosphaericus* was also observed in low abundance. These observations are not in agreement to the results of previous studies which report that *Ca. maltaromaticum* were the most abundant LAB (Huber *et al.*, 2004; Mansfield *et al.*, 2010).

In addition to the abundance of taxa of interest, it is also recognised that the microbial community ecology contributes to the function of the GIT, potentially supporting positive adaptation to changing conditions, and therefore measures of microbial community ecology are also useful indicators of microbial community modulations (Nayak, 2010a). As well as the aforementioned reduction in *Aeromonas* spp. levels in the posterior digesta revealed by clone library analysis, DGGE revealed an elevation in the anterior mucosa species evenness and a reduction of intra-replicate similarity with probiotic application, at week 2. In previous studies, LAB probiotic applications have also been reported to influence GIT microbial ecology. *P. acidilactici* application was reported to reduce intestinal microbial diversity of red Nile tilapia (Ferguson *et al.*, 2010) but in contrast, increased the intestinal microbial diversity was reported when *P. acidilactici* was fed to rainbow trout for 56 days (Ramos *et al.*, 2013). On the contrary, and in keeping with the present study, Ingerslev *et al.* (2014) observed that *P. acidilactici* did not affect the intestinal microbial diversity of rainbow trout fed marine or diet containing reduced fishmeal (using pea meal for replacement), supplemented with or without *P. acidilactici*, after 26 and 49 days post first-feeding.

These probiotic induced microbial changes did not however affect the morphology of the intestine as observed by both light and electron microscopy. Confirming the findings of histology data in the present study, Ferguson *et al.* (2010) demonstrated that the number of IELs in red tilapia was not affected by *P. acidilactici* dietary inclusion. Contrary to these findings, Harper *et al.* (2011) found that IELs levels in the probiotic fed rainbow trout were significantly higher compared to the control group in the anterior mucosa. Similarly, IELs levels were increased in the intestine of tilapia fed *P. acidilactici* supplemented diet after six weeks (Standen *et al.*, 2013).

At week four of the present study the number of goblet cells was significantly higher in the *P. acidilactici* fed fish compared to the control diet fed fish in the anterior mucosa. Results of the present study are consistent with these observations that were obtained in the study of Harper *et al.* (2011). In the study of Standen *et al.* (2013) it was reported that the number of goblet cells in the intestine of tilapia was not affected by *P. acidilactici* after week three, but that their numbers were marginally significantly higher in the probiotic fed fish; the elevation of IELs and goblet cell levels could improve the host resistance against pathogenic microorganisms. The differences observed in these studies are difficult to explain and future research is required to provide a better understanding.

Despite no obvious signs of morphological changes in the GIT of the probiotic fed fish, the probiotic and/or modulated gut microbiome, induced changes to the intestinal gene expression profiles in the present study. IL-1 β and IL-8 gene expression was significantly up-regulated in the anterior and

posterior mucosa of *P. acidilactici* fed fish compared to the control group at two and four weeks. Compared to the control levels, IL-10 mRNA levels were significantly down-regulated in the posterior and both regions of the intestine in the probiotic fed fish compared to the control group at two and four weeks, respectively. Pérez-Sánchez *et al.* (2011b) demonstrated that the supplementation of *Lb. plantarum* increases the expression of IL-1 β , IL-10 and TNF- α in the head kidney prior to the infection with *La. garvieae* compared to the control group. Post-challenge with *La. garvieae*, IL-1 β and TNF- α level in the probiotic group were significantly lower than the control group. However, IL-10 mRNA expression was up-regulated in the probiotic group after *La. garvieae* infection; this contradicts the current results. In this same study, IL-8 levels were significantly up-regulated in the intestine of the pre-infection probiotic group, post-infection with *La. garvieae*.

IL-1 β is an early pro-inflammatory cytokine which is produced as the result of stimulating factors and contributes to the induction and proliferation of T and B lymphocytes, macrophages and vascular endothelial cells leading to inflammatory responses (Beutler, 2004; Raida and Buchmann, 2008). The pro-inflammatory cytokine IL-8 plays a major role in controlling the movement of immune cells, thus it attracts leucocytes including neutrophils and T-lymphocytes to the site of the infection (Overturf and LaPatra, 2006; Gómez and Balcázar, 2008).

Several previous studies report that non-pathogenic bacteria as well as pathogenic bacteria can induce cytokine responses in the epithelium. Such microbes are in direct contact with the intestinal mucosa and the initiation to

either tolerance or immune response is controlled by the ability of gut associated lymphoid tissue (GALT) to distinguish between these two types of microbes (Magnadóttir, 2006; Hardy *et al.*, 2013). Kim and Austin (2006a) demonstrated that *Yersinia ruckeri* elevated the expression of IL-8 as well as other cytokines in the intestine of rainbow trout after co-culturing for 6h with live probiotics. The mRNA level of IL-8 and TNF- α were found to be significantly higher in the head kidney of Nile tilapia fed *Lb. rhamnosus* (Pirarat *et al.*, 2011). Mulder *et al.* (2007) found that IL-1 β , IL-8 and TNF- α genes have been up-regulated in the posterior intestine of rainbow trout during challenge with *A. salmonicida*. Additionally, IL-1 β was found to be up-regulated in rainbow trout after probiotic feeding including *Lb. rhamnosus*, *E. faecium* and *B. subtilis* (Panigrahi *et al.*, 2007). The elevation of IgT in the present study in the probiotic fed fish suggests that the adaptive immune response can be positively affected by *P. acidilactici* in particular in the posterior intestine due to the fact that it plays a crucial immunogenic role in trout (Dorin *et al.*, 1993). In contrast to the present results, IgT expression was not affected in the intestine of rainbow trout fed *Lb. plantarum* prior to infection with *La. garvieae* compared to the control group (Perez-Sanchez *et al.* 2011). However, IgT gene expression was significantly higher in the probiotic group compared to the control group after infection with *La. garvieae*.

With the exception of the observed down-regulation in the anterior intestine at week four, the results of the present study demonstrated that TLR5 gene expression, which have crucial roles in the inflammatory response via the recognition of flagella (Pérez-Sánchez *et al.*, 2011b), in the intestine was

generally not affected by the probiotic. In accordance with the present study, Perez-Sanchez *et al.* (2011b) found that TLR5 gene expression in the *Lb. plantarum* fed rainbow trout group was not induced in the intestine, whether before or after infection with *La. garvieae*. *P. acidilactici* lacks flagella and this could be possible the reason that TLR5 gene expression was not affected in the present study. Although the high level of induced host immunity due to presence of the probiotic may succeed in the eradication of the pathogenic bacteria, an adverse reaction could occur if the inflammatory response is excessive; this could have deleterious causing inflammatory-mediated tissue injury (Hosoi *et al.*, 2003). However, no evidence of such injury was observed by microscopical analysis in the current study. Probiotics involved in modifying the immune response of the host by interacting with immune cells in the intestine epithelial tissue and induction of anti-inflammatory cytokine synthesis lead to reduce inflammation (Denev *et al.*, 2009). The down-regulation of IL-10 in the *P. acidilactici* fed fish compared to the control group indicating that the inflammatory response was not excessive or detrimental, as supported by the histological observations in the present study. Further, the mRNA levels of HSP70, PCNA and casp-3 (genes encoding for proteins important in defending against stress, and regulating cell turnover rates) were significantly lower in the group of fish fed *P. acidilactici* compared to the control group in both anterior and posterior mucosa during the trial. HSP70 can be divided into numerous families depending on their molecular weights confer three crucial biochemical functions: regulation of cellular redox state, regulation of protein turnover and molecular chaperoning (Sonna *et al.*, 2002). HSP70 is involved in programmed cell death and in defence against stress

which results in protein unfolding and protein aggregation; in this case the expression of HSP70 is increased to allow cells to fight with elevated concentrations of damaged proteins (Garrido *et al.*, 2006).

PCNA is a protein expressed in cells during DNA synthesis and repair which has been suggested as a sensitive biomarker for toxic exposures including anti nutritional/ antigenic components in (i.e soybean meal) (Sanden *et al.*, 2005; Bakke-McKellep *et al.*, 2007). The elevation of PCNA expression involved in cell proliferation and implicated in removing the damaged cells since several pathological cases is often caused as a result of a change in cell proliferation (Berntssen *et al.*, 2004). Caspases belong to a family of cysteine proteases which participate in the cleavage of several substrates including proteins involved in apoptosis, DNA metabolism and repair, and regulation of the cell cycle and proliferation (Fischer *et al.*, 2003).

Previous studies have demonstrated that expression of these genes increases in response to different stresses such as heat, irradiation and oxidative stress on the gut epithelium (Garrido *et al.*, 2006). In this respect, the findings of the current study are clearly supported by those of previous studies, with HSP70, casp-3 and PCNA contributing to cyto-protective effects, apoptosis or programmed cell death and cellular repair (Bakke-McKellep *et al.*, 2007; Mansfield *et al.*, 2010).

Supporting this observation, PCNA levels were observed to be significantly higher in the posterior intestinal region of salmon fed soybean than was observed in the control, as quantified using a monoclonal antibody raised against PCNA (Sanden *et al.*, 2005). Additionally, the expression of the

PCNA gene was significantly higher in rainbow trout fed soyabean meal (SBM) at an inclusion level of 30% compared to the control fish, indicating that this up-regulation could be associated with sub-acute intestinal damage (Mansfield *et al.*, 2010). Using immunohistochemistry, significant differences were observed regarding PCNA, HSP70 and casp-3 levels along the mucosal folds of SBM fed rainbow compared to control fish (Bakke-McKellep *et al.*, 2007). The present data clearly indicate that *P. acidilactici* may protect fish from possible potential deleterious effects and cellular damage within the intestine. This may therefore increase tolerance to stressful farming conditions in fish.

The haematological parameters of fish are reported to be influenced by a range of factors, including species, size, age, physiological status of fish, environmental conditions, dietary microbial supplementation such as probiotics and dietary regime (e.g. quality and quantity of food, protein sources, dietary ingredients, vitamins) (Osuigwe *et al.*, 2005).

In the present study, serum lysozyme activity was elevated in the probiotic fed fish at week four. Lysozyme is an important enzyme in the innate immunity, which has been found in mucus of fish, monocytes, neutrophil and macrophage that act as the valuable tool of defence to combat against microorganism invasion (Ellis, 1999). Supporting the present findings, Panigrahi *et al.* (2004) demonstrated that lysozyme activity was significantly higher in rainbow trout fed with *Lb. rhamnosus* at a concentration of 10^{11} CFU g^{-1} for 30 days compared to the control group. Although Panigrahi *et al.* (2005a) showed the slight increase in lysozyme activity in all groups fed

probiotics during the trial, no significant differences in lysozyme activity were observed between groups.

Furthermore, higher significant differences in lysozyme activity were found in red tilapia after 32 days feeding on *P. acidilactici* at 10^7 CFU g⁻¹ compared to the control group (Ferguson *et al.*, 2010), this is also in agreement with the current study. Previous studies have found that serum lysozyme activity in trout may be affected when using LAB probiotics (Merrifield *et al.*, 2010a; Merrifield *et al.*, 2010b; Merrifield *et al.*, 2011). However, the discrepancy in the results between previous studies can be attributed to differences in the selection of probiotic species, concentration, dosage, period of feed intake, and species and age or size of aquatic animals which can lead to a success or failure of probiotics supplementation to act positively in innate immune and/or the protection of aquatic animals against invasion of microorganism (Holzapfel and Naughton, 2005; Nayak, 2010b). This response appears to be time dependent and it would appear that feeding *P. acidilactici* for >2 weeks is necessary to induce elevated serum lysozyme activity in trout.

4.6 Summary and conclusions

P. acidilactici was capable of populating the GIT of rainbow trout and reduced the abundance of some pathogenic bacteria. The probiotic application enhanced the immune response in terms of immune gene expression and serum lysozyme activity. In addition, down-regulated expression of intestinal biomarkers of stress (HSP70), apoptosis (casp-3) and programmed cell death (PCNA) was observed. These observations indicate that *P. acidilactici* may increase rainbow trout robustness against pathogenic insult, perhaps particularly in the GIT.

These findings indicate, in previously unreported detail, the potential for *P. acidilactici* for use as a probiont and may assist in improving the way that it is administered. The current trial showed that *P. acidilactici* temporarily populated the digesta and mucosa of the GIT of rainbow trout, as these bacteria completely disappeared after reverting back to the control diet after three days. This suggests that this probiotic does not easily becomes an autochthonous member of the gut microbiota and that continuous regular administration may be required.

In addition, further research, such as disease challenge studies, would be useful to investigate the beneficial effects of *P. acidilactici* in trout and in other important species in aquaculture.

Chapter 5

Chapter 5: Dietary synbiotic modulates Atlantic salmon (*Salmo salar*) intestinal microbial communities and intestinal immunity

Abstract

A feeding trial was carried out to determine the efficacy of dietary administration of *P. acidilactici* and short chain fructooligosaccharide (scFOS) to modulate the GIT microbiota of Atlantic salmon. Fish (initial body weight 250 g) were randomly allocated into six pens (300 fish per pen) and triplicate groups were fed either a control diet or a treatment diet (control diet supplemented with *P. acidilactici* [Bactocell[®]] at 0.35 g Kg⁻¹+ 0.7 g Kg⁻¹ scFOS) for 132 days. The intestine microbiology, histology and expression of selected immune-related genes (IL-1 β , TNF- α , IL-8, TLR3 and MX-1) were investigated. Compared to the control group, the levels of total bacteria in all regions of the intestine, except anterior digesta (AD) were significantly lower in the synbiotic group at the mid sampling point (63 days). qPCR revealed good recovery (log 6 bacteria g⁻¹) of the probiotic in the intestinal digesta of the synbiotic fed fish. PCR-DGGE revealed that species richness, species diversity and the numbers of OTUs were significantly higher in the synbiotic group compared to the control group in the AD at the mid sampling point. These parameters were not affected at the end sampling point (132 days). *P. acidilactici* was detected in all replicates of the digesta in the synbiotic fed salmon at the end sampling point by DGGE. TEM demonstrated that synbiotic fed fish had marginally ($P = 0.053$) increased the microvilli length in the anterior mucosa (AM) compared to the control group, at the end sampling point. LM revealed that length of villi was significantly higher in the synbiotic group compared to the control group in the interior intestine at the end sampling point. IELs were found to be significantly higher in the synbiotic group compared to the control group in the posterior intestine at both sampling points. RT-PCR demonstrated that all investigated genes were significantly up-regulated in the synbiotic fed salmon compared to the control group in the both intestinal regions at both time points. Growth performance and feed utilisation were not significantly affected ($P > 0.05$). Results of both sampling points revealed that synbiotic supplementation significantly elevated lysozyme serum activity compared to the control group.

5.1 Introduction

Atlantic salmon are distributed in rivers from New England in the United States to Ungava Bay, Canada in the northwest of Atlantic Ocean (Øystein A. *et al.*, 2011). The Atlantic salmon has become an economically important aquaculture species and the production of Atlantic salmon has increased dramatically in recent years. According to FAO (2014) the total global aquaculture production of Atlantic salmon has risen from 895,808 metric tonnes with a total value of US \$ 2.8 billion in 2000 to 2.1 million metric tonnes with a total value of US \$ 10.1 billion in 2012. Atlantic salmon farming is a major component of the UK aquaculture industry with production of 162,604 tonnes worth US \$ 958 million in 2012. Therefore the present chapter sought to investigate the Atlantic salmon gut microbiota and the application of microbial feed additives.

As discussed in Chapter 4 (Section 4.1), pro-inflammatory cytokines, including interleukin-8 (IL-8), IL-1 β and tumor necrosis factor- α (TNF- α), are often used as an immune response indicator. These cytokines are thought to protect the host and to constitute defensive barriers against bacterial colonization or invasion (Pérez-Sánchez *et al.*, 2011b). TNF- α is a key cytokine in the specific immune system that stimulates immune indicators in response to the invasion of bacteria and tissue injury by activating lymphocytes, macrophages, NK cells or by activating the secretion of other cytokines (Mulder *et al.*, 2007; Panigrahi *et al.*, 2007). Therefore, the expression of these genes in addition to (Mx-1 and TLR3) was also the subject of investigation in the present chapter.

scFOS are produced by transfructosylation of sucrose, a molecule of glucose linked by β (2 \rightarrow 1) glycosidic bonds with up to 4 molecules of fructose which determines the final shape of scFOS (Ringø *et al.*, 2010b). It has been reported that bacteria producing β - fructosidase enzymes such as *Lactobacilli* and *Bifidobacteria* have an ability to ferment scFOS (Manning and Gibson, 2004). Therefore, scFOS supplemented diets have the potential role to selectively improve the growth and survival rate of certain bacteria in the GIT of animals (Ringø *et al.*, 2010b). Previous studies have demonstrated that dietary application of FOS can modulate the intestinal microbiota of fish and stimulate various non-specific immunological parameters (He *et al.*, 2003; Zhou *et al.*, 2009).

LAB such as Bactocell[®] *P. acidilactici* (Lallemand), are the most commonly used probiotics in aquaculture and has been licensed for use extensively as aquaculture commercial feed additives in European countries (Regulation(EC), 911/2009). Modulation the GIT microbiota of fish and stimulation various non-specific immunological parameters have been reported by application *Pediococcus acidilactici* MA18/5M (Ferguson *et al.*, 2010; Standen *et al.*, 2013).

The term “synbiotic” describes a mixture of probiotics and prebiotics forming part of nutritional ingredients which increase the beneficial impacts on the host (Cerezuela *et al.*, 2011). Merrifield *et al.* (2010c) suggested that the employment of synbiotics may be more advantageous than the application of probiotics or prebiotics synergistically. Synbiotics are likely to influence the host by enhancing the persistence period and implantation of live microbial

dietary supplements in the GIT by improving the growth of selective beneficial bacteria, thereby promoting the health of the host (Cerezuela *et al.*, 2011).

Previous studies have demonstrated that dietary application of synbiotics can provide multiple host benefits including the stimulation of various non-specific immunological parameters (Ai *et al.*, 2011; Zhang *et al.*, 2014), improve growth performance (Rodriguez-Estrada *et al.*, 2009; Rodriguez-Estrada *et al.*, 2013), and increase resistance against pathogenic bacteria (Kristiansen *et al.*, 2011; Zhang *et al.*, 2014). However, despite that, to date the use of synbiotics in fish farms has been poorly examined and the combination of *P. acidilactici* and scFOS has never been used in salmon culture. Further, the mechanisms in which synbiotics mediate host benefits has not been comprehensively elucidated. The aim of the current study was to determine the effect of a dietary synbiotic (*P. acidilactici* + scFOS) on localised intestinal immune status by assessing the expression of key immunological genes. In addition, the second aim of this study was to investigate the effects of synbiotic on the intestine microbial communities.

5.2 Materials and methods

5.2.1 Diet Preparation

The experimental diets were formulated to be iso-nitrogenous and iso-lipidic using the same basal ingredients (Table 5.1), which meet the known requirements for salmonids (NRC, 2011).

Diets were made by BioMar (Grangemouth, UK). A basal diet was formulated using fish meal, soybean concentrate and soybean meal as the main protein sources, and fish oil and rape seed oil as lipid sources (Table 5.1). The scFOS (Profeed, Tereos Syral, France) was added pre-extrusion and the probiotic *Pediococcus acidilactici* MA 18/5 M (Bactocell[®], Lallemand SAS, France) at the level of 3.03×10^6 CFU g⁻¹ was added post-extrusion. *P. acidilactici* viability was analysed by spread plating on MRS plates as described in Sections 2.5. Proximate analysis of the diets was conducted by BioMar (Grangemouth, UK) according to AOAC (1995) methodologies. The proximate analyses results are presented in Table 5.1.

Table 5.1 Dietary formulations and chemical composition for feeding phase 1 (4.5 mm pellet size; fish ca. 200-500 g) and feeding phase 2 (6.5 mm pellet size; fish ca. 500-1000 g).

Ingredients (%)	Feeding phase 1		Feeding phase 2	
	Control	Synbiotic	Control	Synbiotic
Fishmeal	47.7	47.7	41.3	41.3
Soybean meal	8	8	3	3
Soy protein concentrate	5.4	5.4	12.83	12.83
Sunflower	6.5	6.41	8	8
Wheat	7	7	7	7
Beans	7	7	7	7
Fish oil	9.25	9.25	12.34	12.34
Rape seed oil	9.15	9.15	10	10
Vitamin & mineral premix	0.98	0.98	0.43	0.43
Astaxanthin	0.055	0.055	0.055	0.055
Profeed Maxflow*	0	0.07	0	0.07
Bactocell®	0	0.035	0	0.035
Proximate composition (%)	Control	Synbiotic	Control	Synbiotic
Moisture	5.8	6.2	5.0	5.0
Lipid	23.7	23.2	28.0	28.0
Protein	46	46.1	43.7	43.7
Ash	9.2	9.5	7.6	7.6

*Profeed Maxflow, Tereos Syral, France

® Bactocell, *P. acidilactici*, Lallemand, France

The remaining ingredients were provided by (Biomar AS, Denmark).

Proximate composition values are mean \pm SD, n = 3

5.2.2 Sampling and processing

The feeding trial was conducted at the Ardnish Feed Trial Unit, Lochailort, Scotland (UK). Atlantic salmon were obtained from Loch Arkaig Smolt Unit, Marine Harvest Scotland, from Aquagen eggs. Fish (1800) were randomly distributed into 5 x 5 x 5 m³ sea pens (300 fish per pen average weight = 250 \pm 13 g) and each treatment was carried out in triplicate.

During the trial, water temperature, salinity and DO levels were monitored daily and remained within the normal range. The temperature at 2 m depth increased from 6.5 to 13.9 °C during the course of the trial, and was 10.7 °C

at the mid sampling point (at 63 d) and 13.9 °C at the end sampling point (132 d).

5.2.3 Feeding and weighing

In order to determine growth performance and feed utilization, all fish in each experimental pen were batch weighed at the commencement of the trial and fed the experimental diets to apparent satiation (two meals per day with automatic feeders or by hand). Feed waste was collected after each meal through a lift up system and the quantity estimated to facilitate calculation of feed intake and FCR. The feed rate was reduced to once per day (to apparent satiation) for the 4 days prior to the final sampling due to limited availability of feed.

5.2.4 Intestinal microbiology

At the mid sampling (day 63) and end sampling points (day 132), 9 fish per experimental treatment (3 fish per pen) were sampled to investigate the intestinal microbiota. Fish were euthanized, dissected and collected as described in Section 2.7.1.

5.2.4.1 Total direct counts

Digesta and mucosa samples were sent to Dr Jaime Romero (Universidad do Chile, Santiago) for total counts using the Acridine Orange method as described by Romero and Espejo (2001). Samples were stored and shipped in 100% molecular grade ethanol and were processed within six weeks. Samples were retained at 4 °C. Briefly, samples were filtered using a system which was composed of a membrane of mixed cellulose ester, 0.22 µm, 20 mm diameter (MFS A020A025A, white membrane) and above this filter, a

black nitrocellulose membrane, 0.22 μm , 20 mm diameter (Millipore GSWP0250, black membrane). Two mL of sterile PBS was filtered and then 1 mL of the sample was also filtered. Two mL of sterile PBS was filtered twice to wash the filter, and the process repeated.

Acridine Orange (0.5 mL of a 0.01% solution) was then added to the filter surface. After two minutes, 2 mL of sterile PBS was added and the sample filtered. The black membrane filter was placed on a microscope slide and a drop of mineral oil was added. A 100x objective on an epifluorescence microscope (Dremel DSM510) was used in order to observe the sample. All bacteria were calculated in at least 10 fields. The numbers of bacteria were calculated using the following formula:

$$\text{Number of bacteria mL}^{-1} = \text{number of bacteria} \times \text{factor} \times \text{dilution}$$

Factor was selected according to membrane diameter as displayed in Table 5.2.

Table 5.2 Multiplication factors used to calculate bacterial numbers in samples.

Membrane diameter	Factor (Bacteria mL ⁻¹)
Filter support: 20 mm	1.8 x 10 ⁴
Filter support: 10 mm	4.4 x10 ³

5.2.4.2 Quantitative PCR

Analysis of *P. acidilactici* recovery and bacterial community status was performed using qPCR on two fish per pen (n = 6) by Dr Jaime Romero (Universidad do Chile, Santiago). In brief, the intestinal homogenates were weighed and an equal weight of cold sterile TE buffer was added. DNA from intestinal homogenates was extracted as described previously (Navarrete *et al.*, 2012) and was amplified by PCR using a specific primer set designed to

detect and quantify the probiotic in the digesta and mucosa samples by using qPCR. Primers (see Table 5.3) were derived from the analysis of *gyrB* sequences using primer Premier Software (Biosoft, USA). qPCR reactions were performed using LightCycler® 480 SYBR Green I Master mix following the instructions of the manufacturer (Roche, USA). Primers were used at a final concentration of 20 pmol mL⁻¹ and the annealing temperature was 60 °C and cycling for the denaturing, annealing and extension steps were conducted at 95 °C for 8 sec, 60 °C for 10 sec and 72 °C for 10 sec, respectively. One µL of the extracted DNA was used in the qPCR reaction. A standard curve was prepared using fresh cultures of the probiotic; the bacterial concentration was measured initially by light microscopy using a Petroffe-Hausser counting chamber and the concentration was adjusted to 10⁸ bacteria mL⁻¹. Serial dilutions were prepared down to 10² bacteria mL⁻¹. Simultaneously, the culture was plated on MRS media to obtain viable counts. A standard curve was constructed based on the Ct values obtained from qPCR of the DNA extracted from the serially diluted probiotic samples. The standard curve had an *R*² value of 0.9799 for the probiotic in the range of 7.5 x10¹ - 7.5 x10⁶ CFU mL⁻¹. This standard curve was used to determine the number of probiotic cells in the samples.

5.2.4.3 Denaturing gradient gel electrophoresis (DGGE)

At the mid and end sampling points, three fish per treatment were sampled for the digesta and mucosa samples. It should be mentioned that the anterior digesta samples at the end sampling point were damaged during transportation from Scotland to Plymouth University, therefore they were not analysed. After dissecting aseptically as described in Section 2.7.1, the

intestine was divided into four samples: AM, PM, AD and PD. DNA was extracted from the mucosa and digesta samples using a QIAamp[®] Stool Mini Kit (Qiagen) with minor modification to the manufacturer's instructions, as described in Appendix 1. The variable V3 region of the 16S rRNA gene was amplified using PCR as described in Section 2.7.4.2. The amplified products were subsequently loaded onto 1.5% agarose gel to assess the size of PCR products as described in Section 2.5.2. The resulting PCR products were used to obtain DNA fingerprints of the bacterial community on a 40 - 60% DGGE as described in Section 2.7.4.3. After DGGE, bands or OTUs of interest were excised from the gel and re-PCR'd as described in Section 2.7.4.4. Selected bands were purified and sequenced as described in Section 2.5.3.

5.2.5 Histological examination

5.2.5.1 Light microscopy (LM)

Histological sections from the anterior and posterior intestinal the proximal most section, from the respective regions, was taken to ensure consistency between individual fish sampled) from three fish per pen in each treatment group at the mid and end sampling points of the trial (n = 9) were examined using LM as described in Section 2.8.1.

5.2.5.2 Transmission electron microscopy (TEM)

Samples for TEM were taken from three fish per treatment at the end sampling point. Intestinal samples from the anterior and posterior regions were fixed in 2.5% glutaraldehyde (pH 7.2) for at least 1h. Samples were then rinsed twice with 0.1 M sodium cacodylate buffer (0.1 M at pH 7.2) for

15 min each in order to remove fixative and post-fixed in 1% osmium tetroxide (OsO₄) buffer (pH 7.2) for 1h to provide contrast to the images. Afterwards, the alcohol was replaced by Agar low viscosity resin by placing it in increasing concentrations of resin (30% resin: 70% ethanol, 50:50% and 70:30% each change was left for at least 12h) until it was in 100% resin. The tissue was then placed in Beem capsules and embedded at 60 °C overnight. The resulting blocks were sectioned with a Leica Ultracut Eultra microtome using a diatome diamond knife. Ultrathin sections from each sample were placed on copper grids and stained with saturated uranyl acetate for 15 min, rinsed with distilled water and post-stained with Reynolds lead citrate (Lewis *et al.*, 1977) for 30 min. Final examinations of the ultrathin sections were made on a JEOL 1400 EX transmission electron microscope at 120 kV (Jeol, Tokyo, Japan). Images were analysed using Image J 1.43 (magnification x20 000) to calculate the length of microvilli. Ten well orientated individual microvilli were calculated per image, with typically three images per sample.

5.2.6 Intestinal gene expression

To evaluate whether probiotic treatment had an effect on cytokine mRNA expression, IL-1 β , IL-8, TNF- α , toll-like receptor 3 (TLR3) and myxovirus-resistant protein-1 (MX-1) genes were examined using a real time PCR method. At the mid and end sampling points of the trial, five fish were dissected as described in Section 2.7.1 and samples were kept as described in Section 2.9. Total RNA extraction from intestine tissue was conducted as described in Section 2.9.1. A DNA digestion method was carried out as described in Section 2.9.2. Extracted RNA was used for cDNA synthesis as

described in Section 2.9.3. Primers used are listed in Table 5.3. In order to check the purity and molecular weight characteristics of PCR products, six μL of each PCR product was loaded onto a 1% agarose gel as described in Section 2.5.2. RT-PCR was carried out with the SYBR® green method as described in Section 2.9.4.

5.2.7 Haematological and immunological parameters

Blood was taken from 10 fish per pen at the mid and end sampling points. Fish were sedated by transfer to an anaesthetic bath of 140 mg L^{-1} MS222. Blood was sampled from the caudal arch vein using a 21G \times 11/2" gauge needle and 2.7 mL non-heparinised syringes (S-Monovette Z, Sarstedt AG & Co, Nümbrecht, Germany).

Blood was left at room temperature for 2h to allow for coagulation and serum was separated by centrifugation (6750 g for 5 min at room temperature) and stored at $-80 \text{ }^\circ\text{C}$ until analysis. The leucocyte differential counts were carried out as described in Section 2.10.2. Counts of the leucocytes and erythrocytes were calculated as described in Section 2.10.3. Serum lysozyme activity was determined using the turbidometric method as described in Section 2.10.6.

5.2.8 Growth parameters

Throughout the experiment, the total fish biomass was weighed by staff at the Lochailort salmon farm, at each sampling, in order to determine the growth parameters and correct feeding rates.

Table 5.3 Sequences of oligonucleotide primers used for detection of immune relevant genes in Atlantic salmon by RT-PCR.

Gene name	Forward primer	Reverse primer	Amplicon size	Genbank number
gyrB	ACTTCAATGCCGTGTTTAG	ACCAAAGAATCTAACGGGA	280	AGKB01000007.1
IL-1 β	ACAAGTGCTGGGTCCTGATG	CGATTTGGAGCAGGACAGGT	188	NM-001123582.1
TNF- α	GGCGCTATTCGGACTCCATC	CACACCGAAGAAATTTTTGCC	224	AY848945.1
IL-8	GCTCTCTTCTCATAGCGGCA	TGACAAGGCTGGCTGACATT	201	NM-001140710.2
TLR3	CCCTCGCAGATTCACCACTT	TCAGGGTCTGGGAGATGGAG	135	BK008646.1
MX-1	CTGGAGGAACCAGCAGTCAA	TAAGGGTCGGTCGTCTTCCT	273	NM-001123693.1
β -Actin	TGGAAGATGAAATCGCCGCA	CCCATCCCAACCATCACTCC	142	NM-001123525.1

5.3 Statistical analysis

The mean and standard deviation (SD) were calculated for each treatment by using conventional statistical methods. DGGE banding patterns were statistically analysed as described in Section 2.12. An independent samples t-test was used to determine the significant variation in the immune response, histological parameters, bacterial numbers, numbers of OTUs, species richness and species diversity between the control and experimental group. The accepted levels of significance were $P < 0.05$. All statistics were carried out using MiniTab statistical software version 16, IBM (Pennsylvania, USA).

5.4 Results

5.4.1 Total direct counts

The mean total bacteria levels from the digesta and mucosa samples in the synbiotic and control regimes at the mid sampling point are shown in Figure 5.1. Bacteria levels were $\log 6.7 \pm 0.2$, 6.9 ± 0.1 , 7.8 ± 0.2 and 8.1 ± 0.3 bacteria g^{-1} in AM, PM, AD and PD regions, respectively of the control group of fish compared with 6.4 ± 0.2 , 6.4 ± 0.2 , 7.4 ± 0.6 and 7.1 ± 0.8 bacteria g^{-1} in the AM, PM, AD and PD, respectively of the synbiotic group. Total bacterial counts were significantly lower in the synbiotic group of salmon in the AM ($P = 0.046$), PM ($P = 0.006$) and PD ($P = 0.027$) compared to the control diet fed salmon. However, no significant differences were observed in the AD region ($P = 0.19$).

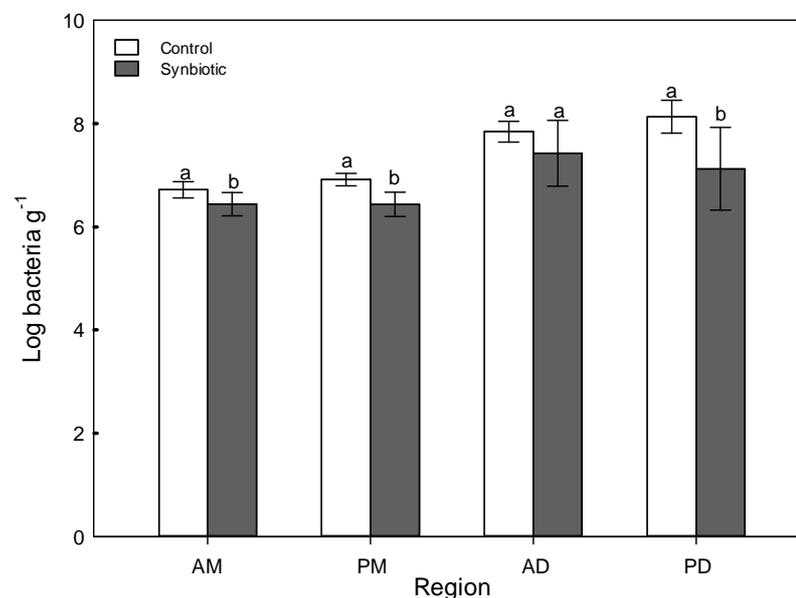


Figure 5.1 Total bacterial counts (log bacteria g^{-1}) in the intestinal tract of Atlantic salmon fed the control and synbiotic diets at the mid sampling point. Results are presented as mean log values \pm SD in each group of fish ($n = 4-6$). Columns having different letters in the same region at each sampling point are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa, PM- posterior mucosa, AD- anterior digesta, PD- posterior digesta.

Additionally, the mean total bacteria levels from the digesta and mucosa samples in the synbiotic and control diets at the end sampling point are shown in Figure 5.2. Bacteria levels were $\log 6.2 \pm 0.5$, 6.2 ± 0.8 , 7.4 ± 0.1 and 7.7 ± 0.1 bacteria g^{-1} in the AM, PM, AD and PD, respectively in the control group of fish compared with $\log 6.2 \pm 0.4$, 6.3 ± 0.2 , 7.6 ± 0.2 and 7.8 ± 0.3 bacteria g^{-1} in the AM, PM, AD and PD, respectively of the synbiotic fed fish. No significant differences were observed between treatments.

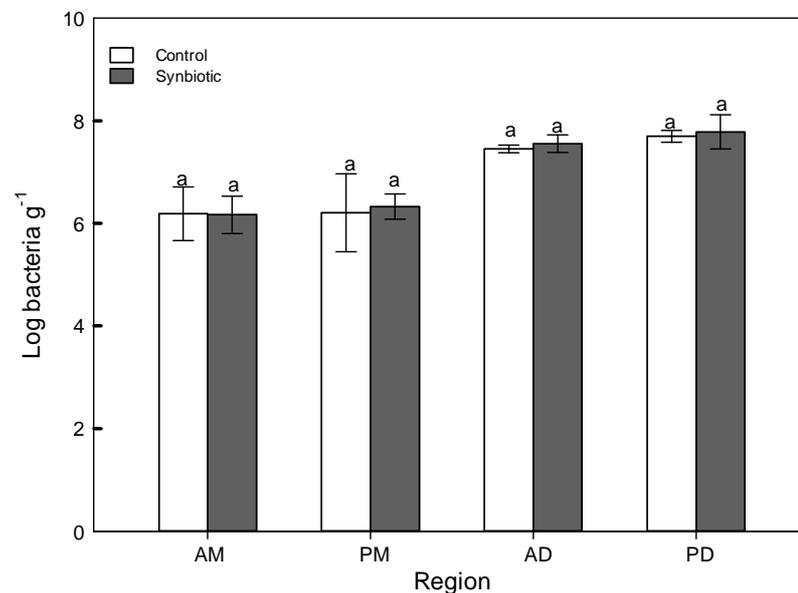


Figure 5.2 Total bacterial counts (log bacteria g^{-1}) in the intestinal tract of Atlantic salmon fed the control and synbiotic diets at the end sampling point. Results are presented as mean log values \pm SD in each group of fish ($n = 6$). No significant differences were present ($P > 0.05$). Sample codes, AM- anterior mucosa, PM- posterior mucosa, AD- anterior digesta, PD- posterior digesta.

5.4.2 Quantitative PCR

qPCR using specific primers for *P. acidilactici* revealed good recovery of the probiotic from the intestinal lumen at both intestinal regions; present in 100% (six from six replicates) of the anterior samples and 67% (four from six) of the posterior samples investigated. Mean *P. acidilactici* levels of $\log 5.98$ and 6.22 bacteria g^{-1} were detected in the anterior and posterior digesta,

respectively. The probiotic was less frequently detected in the mucosa samples with presence only above the detection threshold in 17% of the fish (one from six), in the anterior intestine, which was determined to be at log 4.78 bacteria g⁻¹. The probiotic was not detected in any of the control samples.

5.4.3 DGGE analysis

The resulting DGGE fingerprints are shown in Figures 5.3 and 5.6; the excised bands (for sequencing analysis) are also indicated.

5.4.3.1 Midpoint DGGE analysis

Non-metric multidimensional scaling (nMDS) and cluster analysis of the midpoint DGGE fingerprint are presented in Figures 5.4 and 5.5, respectively. The nMDS shown in Figures 5.4 gives a graphical representation of the sample replicates, where their relative positioning is a reflection of their similarity to each other. The cluster analysis shown in Figure 5.5 illustrates replicate 'groupings', such that samples within a cluster are more similar to each other than those in other clusters. In the case of the midpoint mucosa region, the nMDS (Figure 5.4A) showed the PMS2 was, to some degree separated in terms of the similarity of others replicates. Whilst, the AMS, AMC, PMS and PMC most (2/3) of the replicates were to some degree closely located in terms of the similarity. This observation were clearly reflected by the cluster analysis (Figure 5.5A), which showed clear patterns in terms of sample grouping (and thus similarity), with the majority (2/3) of the AMS, AMC, PMS and PMC replicates are being distinctly grouped. The

SIMPER analysis results revealed no significant differences between the treatments in both intestinal regions.

Further microbial community analysis parameters relating to the midpoint (mucosa) are also displayed in Table 5.4. In terms of OTU numbers, species richness and diversity, no significant differences were observed between the control group and synbiotic group in either region, although their values were marginally lower in the synbiotic group compared to the control group in the AM ($P > 0.05$). In the PM this pattern was reversed ($P > 0.05$).

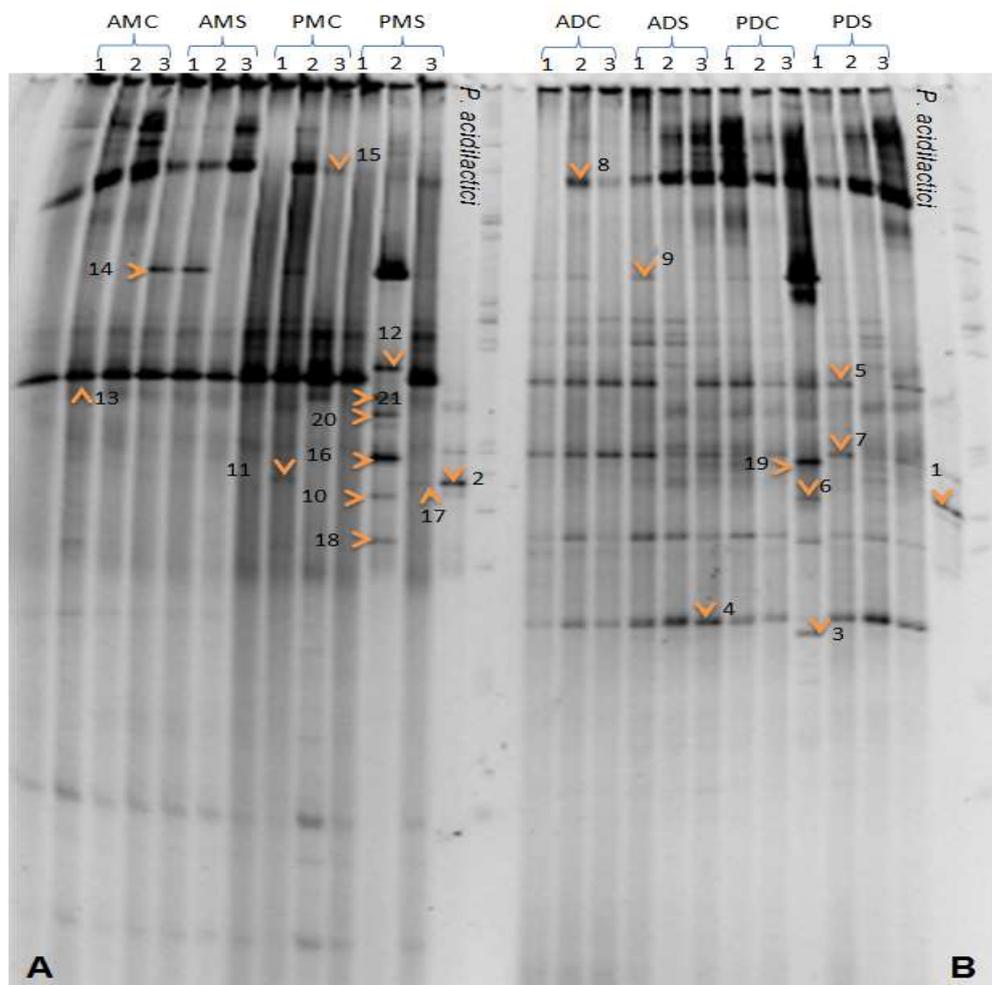


Figure 5.3 DGGE fingerprints from the mucosa (A) and digesta (B) of Atlantic salmon at the midpoint feeding on the experimental diets. Arrows represent OTUs which were excised and sequenced. Sample codes, AMC- anterior mucosa control, AMS- anterior mucosa synbiotic, PMC- posterior mucosa control, PMS- posterior mucosa synbiotic, ADC- anterior digesta control, ADS- anterior digesta synbiotic, PDC- posterior digesta control, PDS- posterior digesta synbiotic.

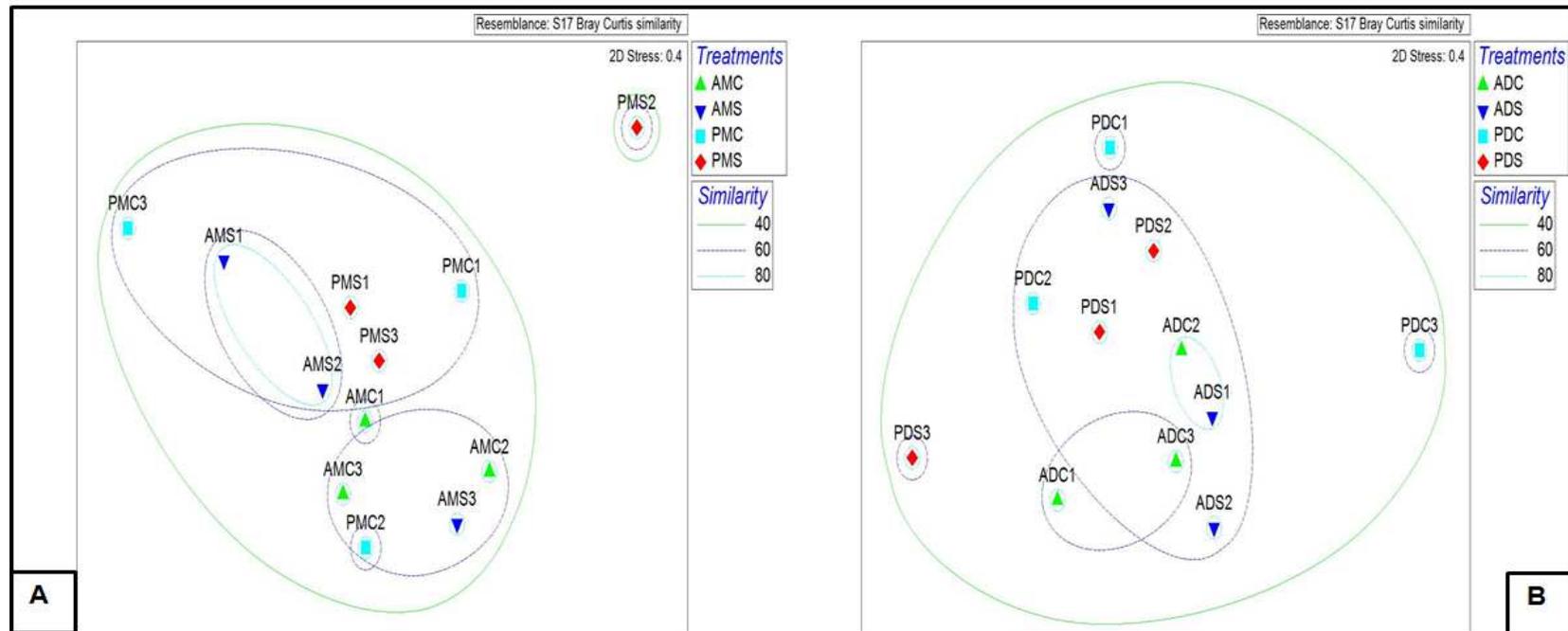


Figure 5.4 Non-metric multidimensional scaling (nMDS) of DGGE fingerprints with similarity percentages (40, 60 and 80%) of bacterial communities between the control and synbiotic groups at the mid sampling point, (A) mucosa and (B) digesta ($n = 3$ for each region). Sample codes, AMC- anterior mucosa control, AMS- anterior mucosa synbiotic, PMC- posterior mucosa control, PMS- posterior mucosa synbiotic, ADC- anterior digesta control, ADS- anterior digesta synbiotic, PDC- posterior digesta control and PDS- posterior digesta synbiotic.

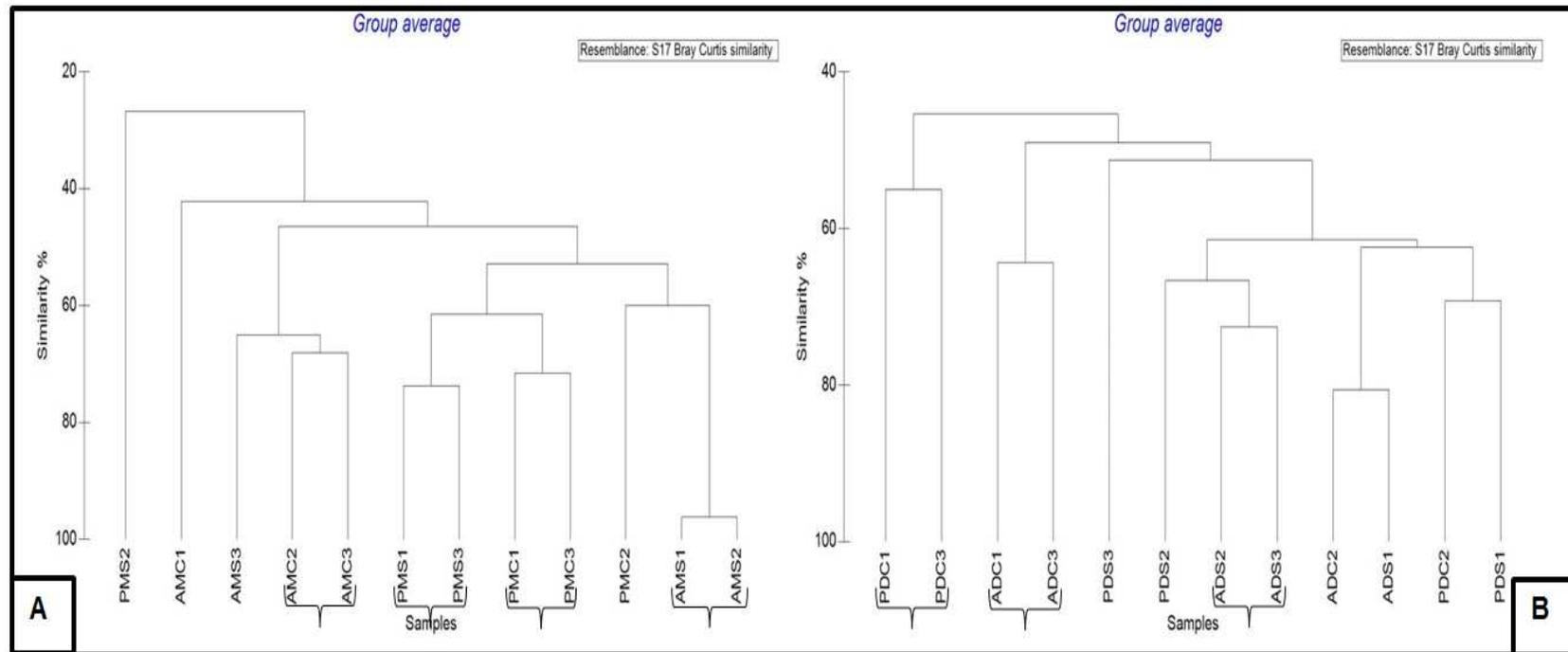


Figure 5.5 Cluster analysis of DGGE fingerprints incorporating similarity percentages of bacterial communities between the control and synbiotic groups at the mid sampling point, (A) mucosa and (B) digesta ($n = 3$ for each region). Sample codes, AMC- anterior mucosa control, AMS- anterior mucosa synbiotic, PMC- posterior mucosa control, PMS- posterior mucosa synbiotic, ADC- anterior digesta control, ADS- anterior digesta synbiotic, PDC- posterior digesta control and PDS- posterior digesta synbiotic.

Similarities between the synbiotic and the control in the AM and PM were not significantly different, with similarity values being slightly higher in the AMS ($P > 0.05$).

In the case of the midpoint digesta, the nMDS showed the PDC3 and PDS3 were, to some degree separated in terms of the similarity (Figure 5.4B). Additionally, the ADC and ADS most of the replicates (2/3) were to some degree closely located in terms of the similarity. The cluster analysis showed clear patterns in terms of sample grouping, with the majority of the ADS, ADC and PDC replicates (2/3) distinctly grouped (Figure 5.5B). The values SIMPER (similarity percentage) being higher in the synbiotics (but not significant differences were observed). The remaining replicates were grouped separately and no clear grouping patterns were observed in terms of either region or treatment in this case. Whilst the species evenness was not affected by the synbiotic in the AD ($P < 0.05$), the OTU numbers, species richness and species diversity were observed to be significantly higher in the synbiotic group compared to the control group in the AD ($P < 0.05$). In the posterior digesta, OTU numbers and species richness were also higher in the synbiotic group compared to the control group, but not significantly different ($P > 0.05$).

When the control and probiotic group replicates were directly compared (similarity percentage, Table 5.4), the highest similarity was observed between the groups of AMC and AMS ($53.4 \pm 7.1\%$), while the lowest similarity was found between the groups of PMC and PMS ($46.3 \pm 18.4\%$).

Table 5.4 Microbial community analysis from PCR-DGGE fingerprints of GIT of Atlantic salmon at the mid sampling point.

	OTUs ¹	Richness ²	Evenness ³	Diversity ⁴	SIMPER similarity (%)	Similarity % (control vs synbiotic)
<u>Anterior mucosa</u>						
Control	15.0 ± 5.0	1.3 ± 0.4	0.95 ± 0.02	2.5 ± 0.4	51 ± 15	53.4 ± 7.1
Synbiotic	13.0 ± 1.0	1.2 ± 0.1	0.95 ± 0.00	2.4 ± 0.1	73 ± 20	
<u>Posterior mucosa</u>						
Control	10.6 ± 2.3	0.9 ± 0.2	0.97 ± 0.02	2.3 ± 0.2	53 ± 17	46.3 ± 18.4
Synbiotic	13.0 ± 3.4	1.2 ± 0.3	0.97 ± 0.01	2.5 ± 0.0	42 ± 28	
<u>Anterior digesta</u>						
Control	14.6 ± 2.5 ^a	1.4 ± 0.2 ^a	0.98 ± 0.01	2.6 ± 0.2 ^a	62 ± 5	53.2 ± 14.9
Synbiotic	20.3 ± 0.6 ^b	1.8 ± 0.0 ^b	0.98 ± 0.00	2.9 ± 0.0 ^b	68 ± 6	
<u>Posterior digesta</u>						
Control	17.0 ± 2.6	1.6 ± 0.2	0.96 ± 0.00	2.7 ± 0.2	48 ± 8	49.1 ± 12.6
Synbiotic	18.0 ± 3.4	1.7 ± 0.4	0.95 ± 0.02	2.7 ± 0.2	60 ± 6	

Results expressed as mean ± SD in each group of fish (n = 3). Means having different letters within the same region in the same column are significantly different ($P < 0.05$).

¹ Operational taxonomical unit.

² Margalef species richness: $d = (S - 1) / \log(N)$.

³ Pielou's evenness: $J' = H' / \log(S)$.

⁴ Shannon 's diversity index: $H' = -\sum(pi / \ln pi)$.

5.4.3.2 Band sequencing analyses

A total of twenty OTUs were selected from the DGGE gel at the mid sampling point, and are indicated on the gel image in Figure 5.3. Some OTUs were common to all groups and/ or replicates. The results of sequence analysis from the mid sampling point gels (mucosa and digesta) are shown in Table 5.5. The percentage allocation of the selected OTUs to bacterial phyla was as follows: Proteobacteria 30%, Firmicutes 20%, Tenericutes 15%, Spirochetes 10%, Actinobacteria 5% and uncultured bacteria 20%.

A sequence similar to unidentified Spirochete (OTU 14) was present in all regions and treatments except the AMC. Two common OTUs (13, uncultured bacterium and 15, uncultured *Mycoplasma* sp.) were identified in all samples of the mucosa and five common OTUs (4, 5, 7, 8 and 9) were similarly identified in the digesta replicates: these were identified as *Geobacillus caldxylosilyticus*, uncultured bacterium, *Streptococcus* sp., *Mycoplasma* sp. and uncultured *Spirochaetales* bacterium, respectively. On the contrary, some OTUs were only present in limited replicates or sample regions: OTU 11 (an unidentified member of the Actinobacteria) was only observed in the one replicate of the PMC and uncultured *Weissella* sp. (OTU 3), uncultured bacteria (OTU 6) and *Mycoplasma* sp. (OTU 19) were only observed in the one replicate of the PDC. *Vibrio pelagius*, *Vibrio* sp., *Aliivibrio wodanis*, and *Aliivibrio* spp. were the main representatives of Proteobacteria, and were only detected in the one replicate of the PMS.

Table 5.5 Summary of the sequencing analysis results generated from bands excised from the mid sampling point DGGE gel. Numerical values represent the number of replicates (out of 3) that the OTUs were present in.

OTUs number	Phyla	Max. Identity (%)	NCBI BLAST matches	NCBI Accession number	Length of sequences	E value	Anterior intestine		Posterior intestine	
							C	S	C	S
<u>Mucosa</u>										
11	Actinobacteria	87	Unidentified bacteria	HQ465177.1	134	2e-16	0	0	1	0
10	Proteobacteria	98	<i>Aliivibrio wodanis</i>	JQ361726.1	132	2e-51	0	0	0	1
12	Proteobacteria	98	<i>Vibrio pelagius</i>	JX407164.1	137	6e-50	0	0	0	1
16	Proteobacteria	99	<i>Vibrio</i> sp.	JN618161.1	134	2e-53	0	0	0	1
18	Proteobacteria	98	<i>Aliivibrio wodanis</i>	JQ361730.1	127	1e-55	0	0	0	1
20	Proteobacteria	94	<i>Aliivibrio</i> sp.	HQ011252.1	131	2e-38	0	0	0	1
21	Proteobacteria	94	<i>Aliivibrio</i> sp.	JQ361730.1	141	2e-46	0	0	0	1
14	Spirochetes	83	Unidentified bacterium	AB672878.1	129	7e-32	0	2	1	1
15	Tenericutes	95	Uncultured <i>Mycoplasma</i> sp.	DQ340193.1	136	1e-30	3	3	2	2
13	Unidentified bacteria	100	Uncultured bacterium	AB649436.1	116	1e-50	3	3	3	2
17	Unidentified bacteria	99	Uncultured bacterium	HM216400.1	144	2e-32	0	0	0	1
<u>Digesta</u>										
1	Firmicutes	95	<i>Pediococcus acidilactici</i>	AB627837.1	163	6e-62	0	3	0	2
3	Firmicutes	100	Uncultured <i>Weissella</i> sp.	KC416975.1	192	3e-39	0	0	1	0
4	Firmicutes	100	<i>Geobacillus caldoxylosilyticus</i>	KC820650.1	128	2e-35	3	3	3	3
7	Firmicutes	94	<i>Streptococcus</i> sp.	AF323911.1	159	5e-30	3	3	2	2
9	Spirochetes	99	Uncultured <i>Spirochaetales</i> bacterium	AB672878.1	123	9e-13	2	1	2	2
19	Tenericutes	99	<i>Mycoplasma</i> sp.	HM489893.1	164	6e-15	0	0	1	0
8	Tenericutes	97	<i>Mycoplasma</i> sp.	FN808189.1	137	1e-35	2	3	3	3
6	Unidentified bacteria	89	Uncultured bacterium	FJ657899.1	156	1e-26	0	0	1	0
5	Unidentified bacteria	97	Uncultured bacterium	HM216405.1	157	5e-16	3	3	3	3

Sample codes, C- control, S- synbiotic

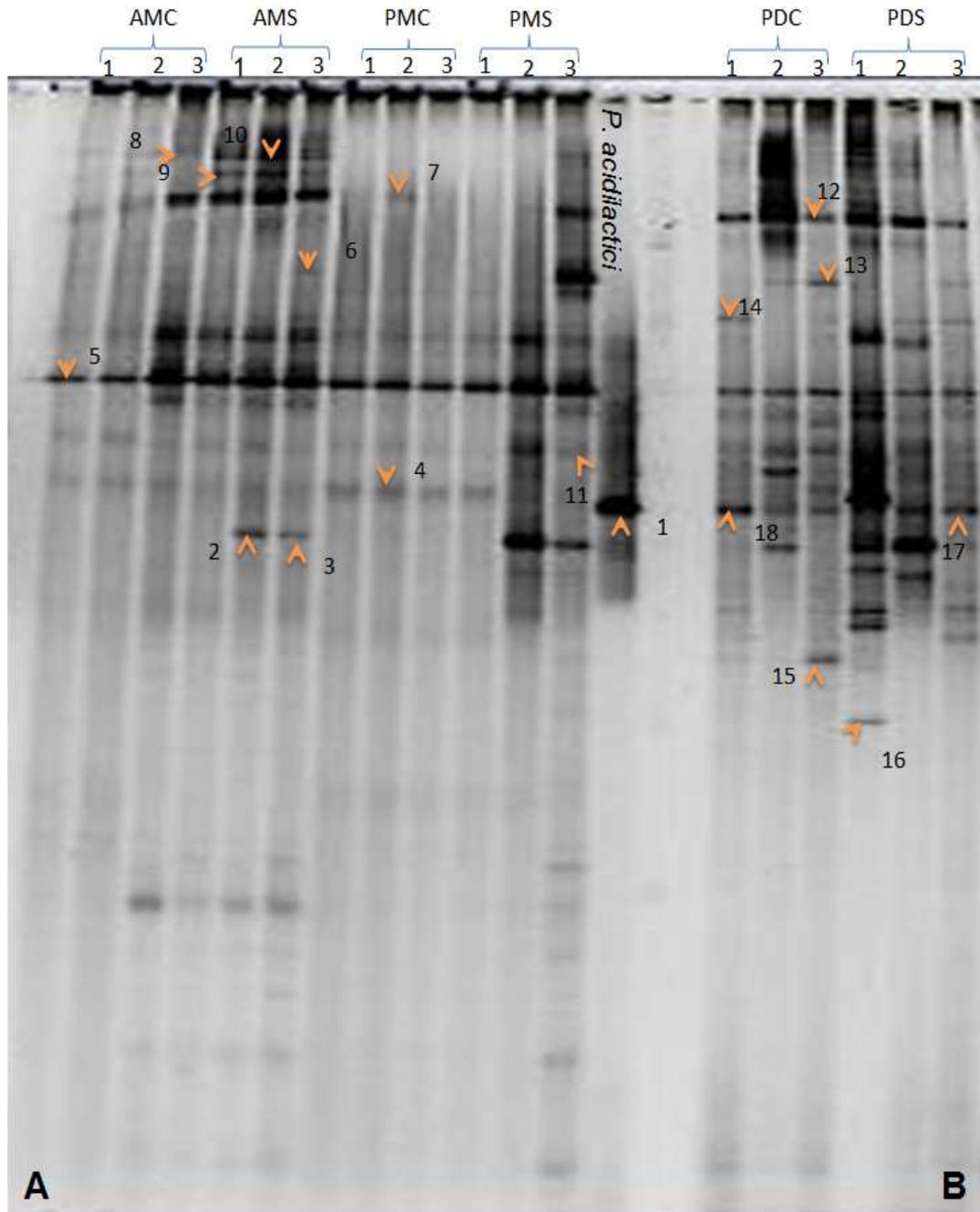


Figure 5.6 DGGE fingerprints from the mucosa (A) and digesta (B) of Atlantic salmon at the endpoint feeding on the experimental diets. Arrows represent OTUs which were excised and sequenced. Sample codes, AMC- anterior mucosa control, AMS- anterior mucosa synbiotic, PMC- posterior mucosa control, PMS- posterior mucosa synbiotic, PDC- posterior digesta control and PDS- posterior digesta synbiotic.

5.4.3.3 Endpoint DGGE analysis

Non-metric multidimensional scaling (nMDS) and cluster analysis of the endpoint DGGE fingerprint are indicated in Figures 5.7 and 5.8, respectively.

In the case of the endpoint mucosa region, the nMDS (Figure 5.7A) showed that the anterior and posterior synbiotic replicates (2/3) were, to some degree separated in terms of the similarity. The cluster analysis (Figure 5.8A) showed that clear patterns in terms of sample grouping were observed, with the majority of the AMS, AMC and PMC replicates (2/3) were distinctly grouped. SIMPER (similarity percentage) value revealed a significant difference in between the AMS and PMS than in their respective controls.

Further microbial community analysis parameters relating to the endpoint (mucosa) are also displayed in Table 5.6. In terms of OTU numbers, species richness and species diversity, no significant differences were observed between the control and synbiotic groups in any regions (Table 5.6), although in both of the mucosa regions, marginally higher numbers were observed in the synbiotic than in the control treatment ($P > 0.05$).

In the case of the endpoint digesta, the nMDS (Figure 5.7B) clearly showed that the three PDC replicates were clearly separated to their respective treatments. The cluster analysis (Figure 5.8B) showed that the majority of the PDC and PDS replicates (2/3) were distinctly grouped. SIMPER analysis indicating a higher (but not significant) level of similarity in PDC group than in the synbiotic.

In terms of OTU numbers, species richness and species diversity, no significant differences were observed between the control and synbiotic

groups in the PD (Table 5.6), although their values were marginally higher in the synbiotic group compared to the control group ($P > 0.05$).

When the control and synbiotic group replicates were directly compared (similarity percentage Table 5.6), the highest similarity was found between the groups of PDC and PDS ($54 \pm 14\%$), while the lowest similarity was found between the groups of AMC and AMS ($49 \pm 14\%$).

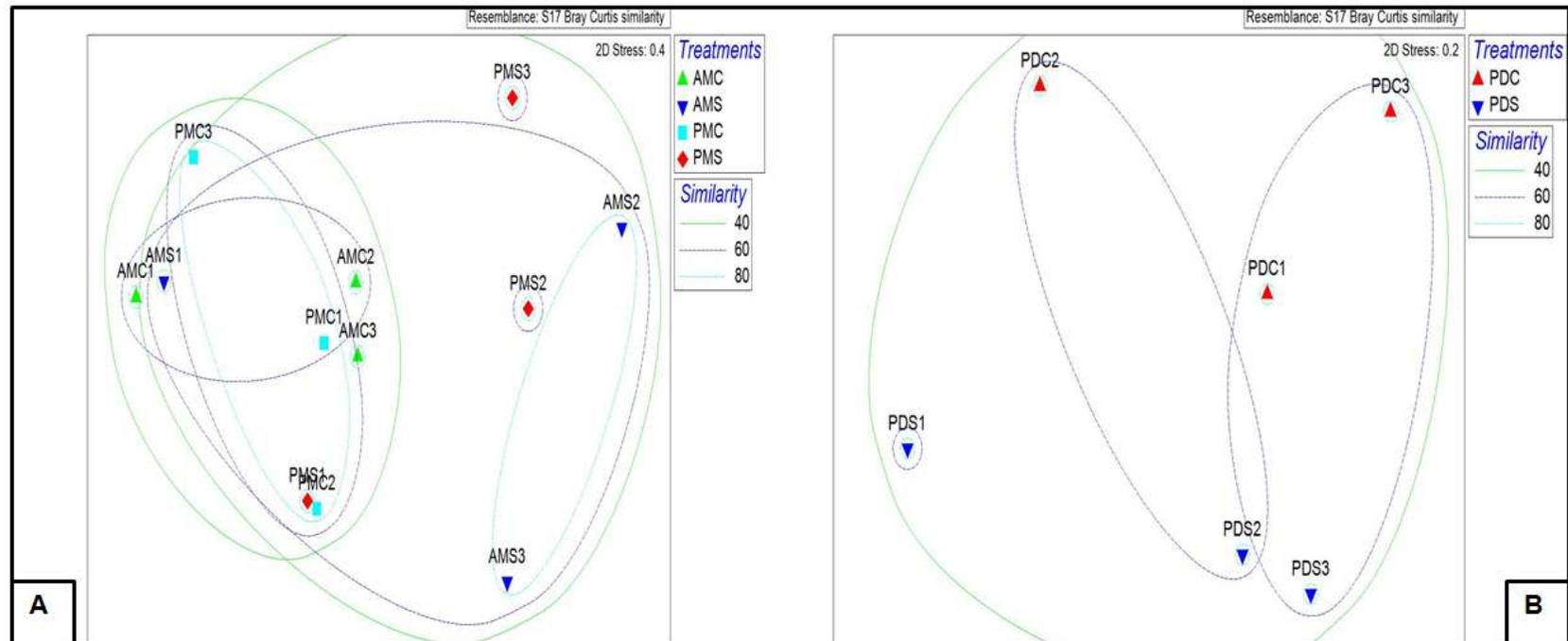


Figure 5.7 Non-metric multidimensional scaling (nMDS) of DGGE fingerprints revealing similarity percentages (40, 60 and 80%) of bacterial communities between the control and synbiotic groups at the end sampling point, (A) mucosa and (B) digesta ($n = 3$ for each region). Sample codes, AMC- anterior mucosa control, AMS- anterior mucosa synbiotic, PMC- posterior mucosa control, PMS- posterior mucosa synbiotic, ADC- anterior digesta control, ADS- anterior digesta synbiotic, PDC- posterior digesta control and PDS- posterior digesta synbiotic.

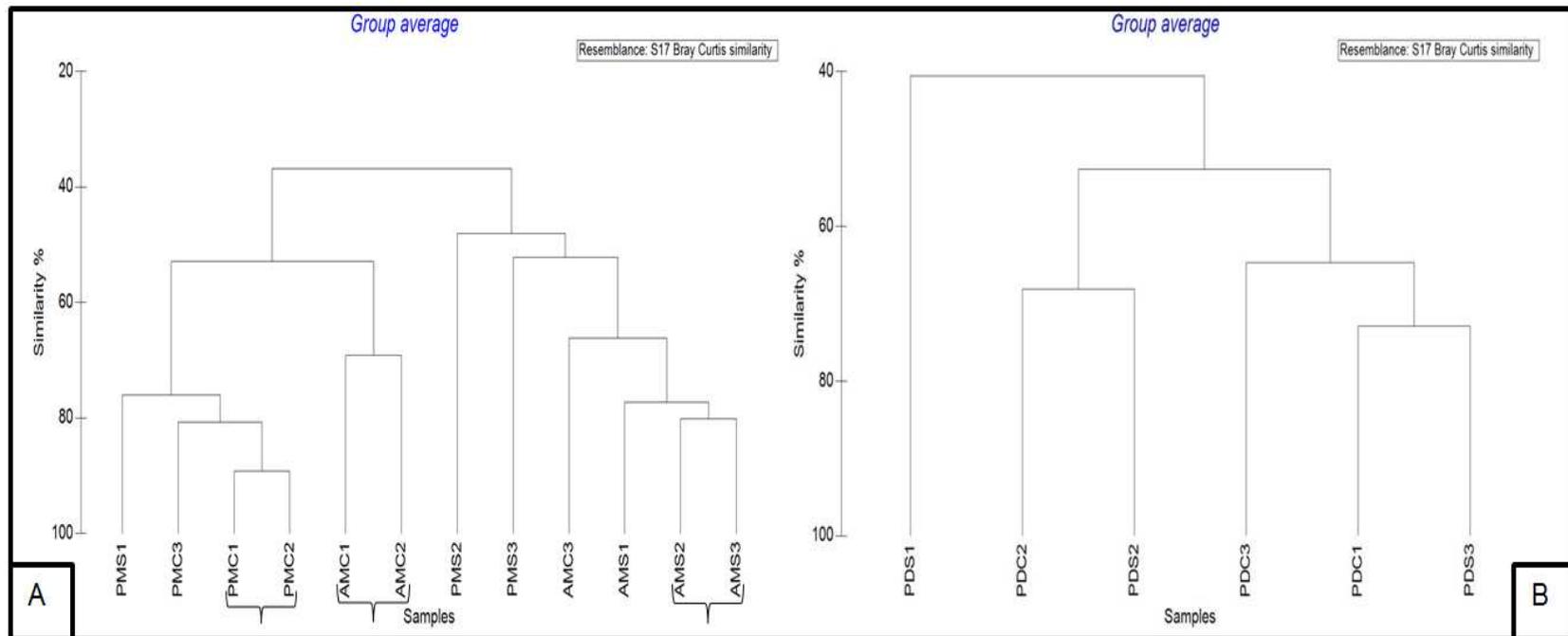


Figure 5.8 Cluster analysis of DGGE fingerprints incorporating similarity percentages of bacterial communities between the control and synbiotic groups at the end sampling point. (A) mucosa and (B) digesta ($n = 3$ for each region). Sample codes, AMC- anterior mucosa control, AMS- anterior mucosa synbiotic, PMC- posterior mucosa control, PMS- posterior mucosa synbiotic, ADC- anterior digesta control, ADS- anterior digesta synbiotic, PDC- posterior digesta control and PDS- posterior digesta synbiotic.

Table 5.6 Microbial community analysis from PCR-DGGE fingerprints of GIT of Atlantic salmon at the end sampling point.

	OTUs ¹	Richness ²	Evenness ³	Diversity ⁴	SIMPER similarity (%)	Similarity % (control vs synbiotic)
<u>Anterior mucosa</u>						
Control	14.0 ± 6.1	1.3 ± 0.5	0.95 ± 0.01	2.4 ± 0.4	48 ± 18 ^a	49 ± 14
Synbiotic	20 ± 4.6	1.8 ± 0.4	0.95 ± 0.00	2.8 ± 0.2	78 ± 2 ^b	
<u>Posterior mucosa</u>						
Control	9 ± 1.2	0.9 ± 0.11	0.94 ± 0.00	2.1 ± 0.1	84 ± 6 ^a	49 ± 20
Synbiotic	15 ± 7.2	1.4 ± 0.6	0.96 ± 0.03	2.5 ± 0.5	43 ± 8 ^b	
<u>Posterior digesta</u>						
Control	20 ± 5.2	1.8 ± 0.5	0.97 ± 0.01	2.9 ± 0.3	56 ± 8	54 ± 14
Synbiotic	21 ± 4.9	1.9 ± 0.4	0.98 ± 0.00	3.0 ± 0.2	45 ± 7	

Results are presented as mean ± SD in each group of fish (n = 3). Means having different letters within the same region in the same column are significantly different ($P < 0.05$).

¹ Operational taxonomical unit.

² Margalef species richness: $d = (S - 1) / \log(N)$.

³ Pielou's evenness: $J' = H' / \log(S)$.

⁴ Shannons diversity index: $H' = -\sum(pi(\ln pi))$.

5.4.3.4 Band sequencing analyses

A total of eighteen OTUs were selected from the endpoint DGGE gel; some of them were common to all groups, whilst others were unique to the synbiotic or the control groups (Figure 5.6). The results of band sequence analysis from the endpoint gels (mucosa and digesta) are shown in Table 5.7. The percentage allocation of the selected OTUs to bacterial phyla was as follows: Proteobacteria 22.3%, Tenericutes 22.3%, Firmicutes 22.3%, Cyanobacteria 5.6%, Spirochetes 5.6%, and uncultured bacteria 22.3%. Two OTUs (2 and 3) were detected in the AMS and PMS and were shown to be most closely related to uncultured cyanobacterium and *Alcaligenes faecalis*, respectively.

A sequence similar to *Serratia rubidaea* (OTU 11) was found in the PMS only. In addition, four OTUs (6, 8, 9 and 10) were not detected in the PMC, while they were detected in all other mucosa samples and were most closely related to uncultured γ -proteobacterium, two uncultured *Mycoplasma* sp. and uncultured bacterium. Two OTUs (4 and 5) appeared to be common to either all regions or all replicates in the mucosa regions and sequence analysis showed them to be most similar to uncultured bacterium. A sequence identified as *Enterovibrio calviensis* (OTU 16) was found in the PDS only. The reverse was observed in the case of uncultured *Weissella* sp. (OTU 15). Four common OTUs 12 (uncultured bacteria), 13 (unidentified Spirochete), 14 (*Mycoplasma* sp.) and 18 (unidentified Proteobacteria) were identified in the PDC and PDS replicates.

Table 5.7 Summary of the sequencing analysis results generated from bands excised from the end sampling point DGGE gel. Numerical values represent the number of replicates (out of 3) that the OTUs were present in.

OTUs number	Phyla	Max. Identity (%)	NCBI BLAST matches	NCBI Accession number	Length of sequences	E value	Anterior intestine		Posterior intestine	
							C	S	C	S
<u>Mucosa</u>										
1	Firmicutes	100	<i>Pediococcus acidilactici</i>	KC568555.1	138	4e-61	0	0	0	0
3	Proteobacteria	100	<i>Alcaligenes faecalis</i>	EF623834.1	161	5e-31	0	1	0	2
11	Proteobacteria	99	<i>Serratia rubidaea</i>	JQ045789.1	157	2e-19	0	0	0	2
6	Proteobacteria	99	Uncultured γ - proteobacterium	AY904518.1	158	4e-25	3	1	0	1
2	Cyanobacteria	100	Uncultured <i>cyanobacterium</i>	JX570954.1	157	2e-09	0	1	0	2
8	Tenericutes	95	Uncultured <i>Mycoplasma</i> sp.	DQ340195.1	161	3e-37	3	3	0	1
7	Tenericutes	96	Uncultured <i>Mycoplasma</i> sp.	DQ340194.1	147	3e-47	3	3	1	0
9	Tenericutes	94	Uncultured <i>Mycoplasma</i> sp.	KC169759.1	163	7e-45	1	3	0	1
4	Unidentified bacteria	95	Uncultured bacterium	EU697160.1	140	1e-41	3	3	3	1
5	Unidentified bacteria	99	Uncultured bacterium	EU697160.1	114	5e-49	3	3	3	3
10	Unidentified bacteria	94	Uncultured bacterium	AB672272.1	151	1e-04	3	3	0	1
<u>Digesta</u>										
14	Tenericutes	96	<i>Mycoplasma</i> sp.	JQ910955.1	164	3e-16			2	2
16	Firmicutes	100	<i>Enterovibrio calviensis</i>	NR041741.1	136	1e-60			0	1
17	Firmicutes	98	<i>Pediococcus acidilactici</i>	KC568555.1	137	1e-60			3	3
15	Firmicutes	97	Uncultured <i>Weissella</i> sp.	KC700317.1	100	1e-39			2	0
13	Spirochetes	87	Unidentified bacteria	JN540136.1	159	1e-24			2	2
18	Proteobacteria	98	Uncultured bacterium	AM179931.1	129	1e-49			3	2
12	Unidentified bacteria	99	Uncultured bacterium	FN808189.1	131	9e-57			3	3

Sample codes, C- control, S- synbiotic

5.4.4 Intestinal histology

The images of LM from both anterior and posterior intestine regions revealed that the intestine of Atlantic salmon fed the synbiotic and control diets had a normal and intact epithelial barrier. Abundant scattered lymphoid cells were observed in a simple epithelium and a lamina propria (LP), which considered as content of the intestinal mucosa in both diet groups (Plates 5.1 and 5.2).

Histological parameters at the mid and end sampling points are shown in Table 5.8. The results of the present study indicated that at the mid sampling point, length of villi in the anterior intestine was significantly increased ($P = 0.008$) in the synbiotic treated fish compared to the control group (Table 5.8).

In addition, the number of IELs in the posterior intestine of the synbiotic fed fish was significantly elevated compared to the control fed fish ($P = 0.034$ at the mid sampling and $P = 0.002$ at the end sampling) (Table 5.8). No significant differences were observed with respect to the level of IELs in the anterior intestine at both sampling points.

Likewise, at the mid sampling point, the width of the lamina propria was significantly smaller ($P = 0.003$) in the synbiotic group than the control group in the posterior intestine. Although, the density of the goblet cells was elevated in the synbiotic fed fish compared to the control group in all regions, no significant differences were observed Table 5.8.

Table 5.8 Histological parameters of the Atlantic salmon intestine after feeding the synbiotic and control diets at both sampling point.

	Mid sampling point				End sampling point			
	Anterior intestine		Posterior intestine		Anterior intestine		Posterior intestine	
	Control	Synbiotic	Control	Synbiotic	Control	Synbiotic	Control	Synbiotic
Villi length (μm)	549.7 \pm 31.7 ^a	586.2 \pm 22.8 ^b	640.9 \pm 86.1	631.3 \pm 76.4	724.4 \pm 138.5	727.0 \pm 126.2	650.5 \pm 161.7	659.7 \pm 160.7
Microvilli length (μm) [*]	Nd	Nd	Nd	Nd	2.3 \pm 0.4 ^a	2.8 \pm 0.4 ^b	1.8 \pm 0.3	1.7 \pm 0.3
Lamina propria width (μm)	12.9 \pm 2.2	13.4 \pm 2.6	11.0 \pm 1.8 ^a	8.5 \pm 2.0 ^b	14.9 \pm 1.9	13.6 \pm 3.3	11.6 \pm 2.5	9.5 \pm 2.4
Goblet cells (per 100 μm)	4.2 \pm 0.9	4.6 \pm 0.8	3.2 \pm 0.5	2.6 \pm 0.6	4.3 \pm 0.9	4.7 \pm 0.9	2.5 \pm 0.6	2.7 \pm 0.6
Intraepithelial leucocytes (per 100 μm)	8.6 \pm 1.0	8.1 \pm 0.8	6.2 \pm 0.8 ^a	7.2 \pm 1.1 ^b	8.1 \pm 1.5	8.9 \pm 1.1	7.9 \pm 1.1 ^a	9.6 \pm 1.1 ^b

Results are presented as mean \pm SD in each group of fish (n = 9). Means having different letters within the same region in the same row are significantly different ($P < 0.05$).

Nd = not determined

^{*}(n = 6)

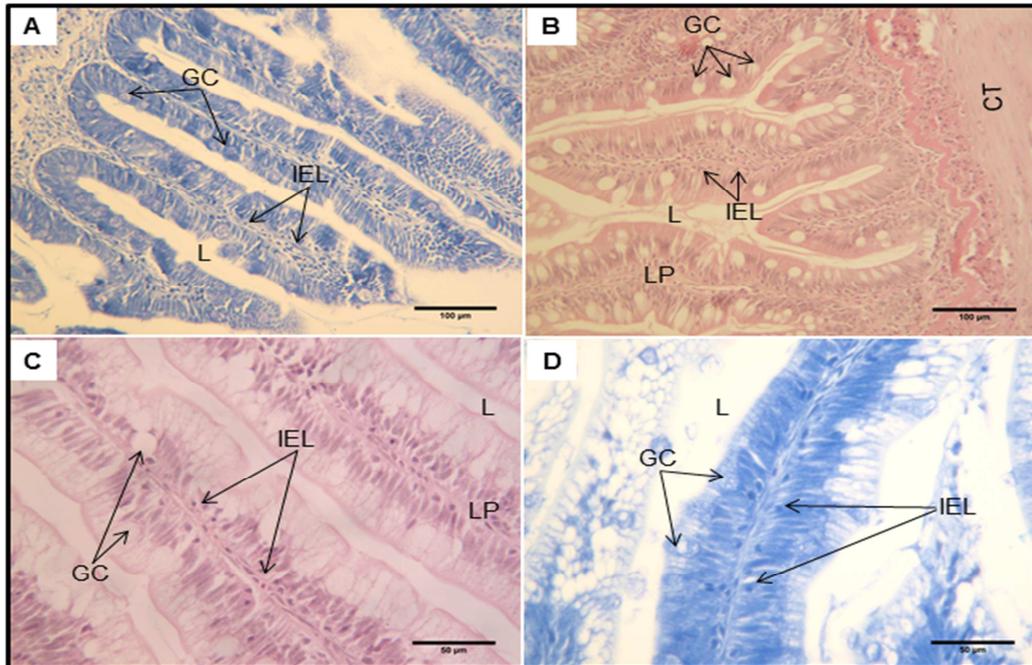


Plate 5.1 Haematoxylin and Eosin (HE) and May-Grünwald/Giemsa (MGG) staining of the anterior and posterior intestine of Atlantic salmon at the mid sampling point. (A) MGG and (C) HE staining of the anterior and posterior intestine, respectively (synbiotic group). (B) HE and (D) MGG staining of the anterior and posterior intestine, respectively (control group). Abbreviations used are GC- goblet cells, L- lumen, LP- lamina propria, IELs- intraepithelial leucocytes and CT- connective tissue.

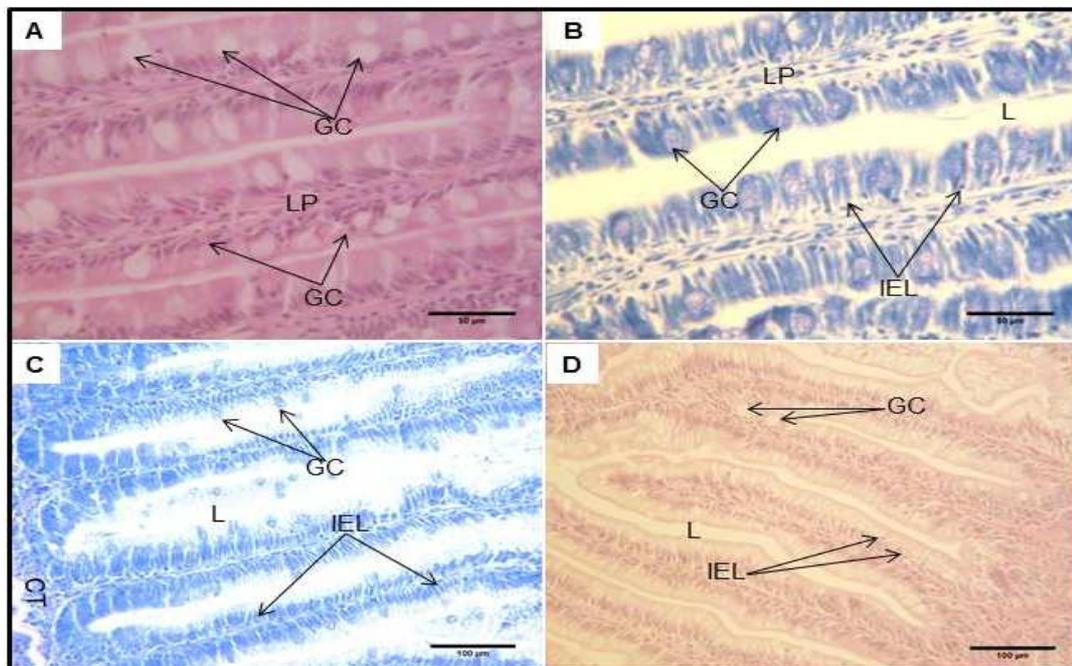


Plate 5.2 Haematoxylin and Eosin (HE) and May-Grünwald/Giemsa (MGG) staining of the anterior and posterior intestine of Atlantic salmon at the end sampling point. (A) HE and (C) MGG staining of the anterior and posterior intestine respectively (synbiotic group). (B) MGG and (D) HE staining of the anterior and posterior intestine, respectively (control group). Abbreviations used are GC- goblet cells, L- lumen, LP- lamina propria, IELs- intraepithelial leucocytes and CT- connective tissue.

Results from TEM investigations revealed that the samples of the end sampling point revealed that the apical brushborder and that the epithelial surface appeared to be intact and healthy in both intestinal regions for both treatments. TEM revealed no observable differences between the treatments with respect to signs of damage, cell debris and the amount of mucus in the lumen (Plate 5.3).

Microvilli height from the anterior intestine at the end sampling point was marginally higher ($P = 0.053$) in the synbiotic fed fish than the control fed fish (Table 5.8). There was no difference in microvilli height in the posterior intestine.

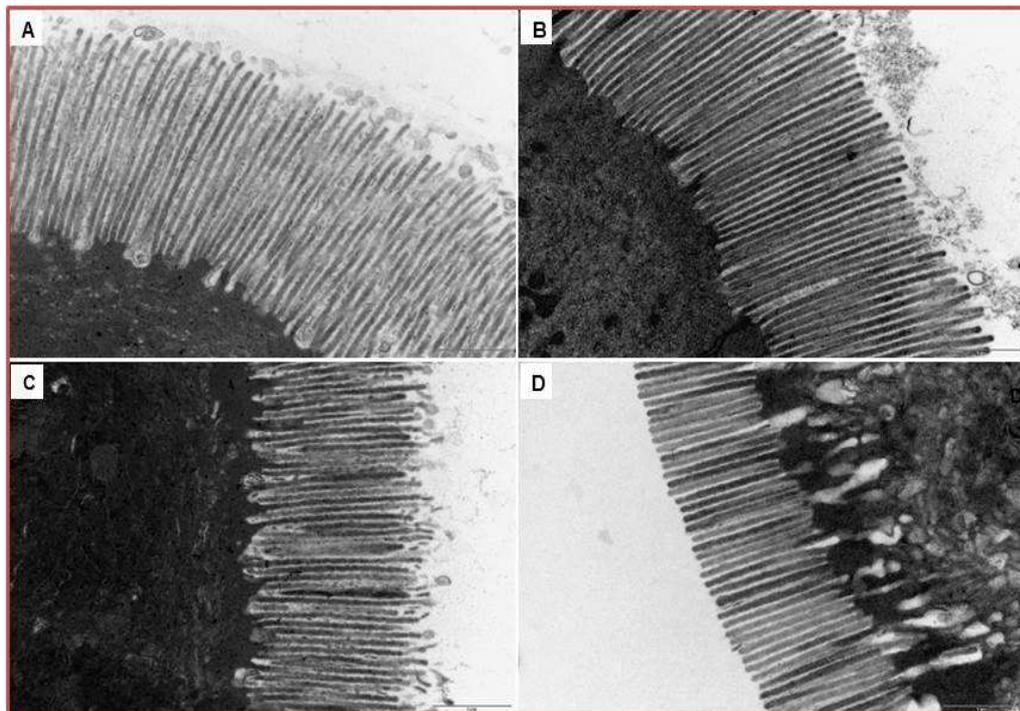


Plate 5.3 Comparative transmission electron microscopy (TEM) pictures of anterior and posterior region of the intestine in Atlantic salmon at the end sampling point. (A) anterior of the intestine in the control group, (B) anterior of the intestine in the synbiotic group, (C) posterior of the intestine in the control group, (D) posterior of the intestine in the synbiotic group. Although microvilli appear healthy in both treatments, they were significantly longer in the anterior region of synbiotic treated salmon ($P = 0.053$).

5.4.5 Intestinal gene expression

The relative levels of mRNA expression of the immune-related genes IL-1 β , IL-8, TNF- α , TLR3 and MX-1 from both parts of the intestine in both control and synbiotic groups of fish sampling at both sampling points are presented in Figures 5.9 - 5.13.

In both regions of the intestine at both sampling points pro-inflammatory cytokines IL-1 β , IL-8 and TNF- α in fish fed the synbiotic were significantly ($P < 0.001$) up-regulated compared to the control group (Figures 5.9 - 5.11). It was apparent that these three genes were found to be in high levels at the end sampling point compared to the mid sampling point.

TLR3 and MX-1 mRNA levels, which are relevant in viral responses, were significantly higher in the synbiotic fed fish compared to the control group at both sampling points, respectively ($P = 0.001$; Figures 5.12 and 5.13). These figures revealed that the expression of all these genes were higher in the AM compared to the PM at both sampling points.

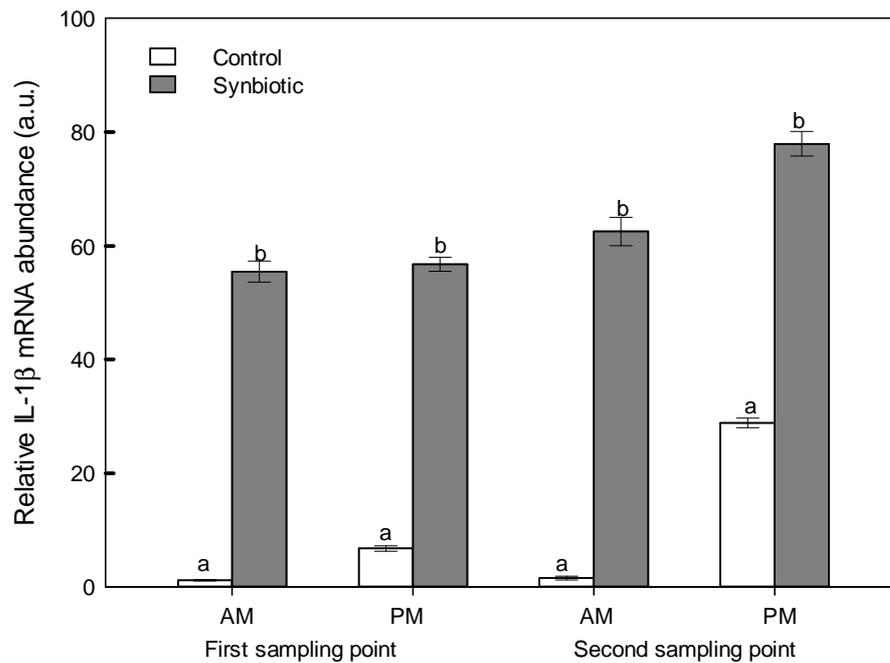


Figure 5.9 RT-PCR analysis of IL-1 β gene expression in the intestine of Atlantic salmon at the mid and end sampling points feeding of the control and synbiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Columns having different letters in the same region at each sampling point are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

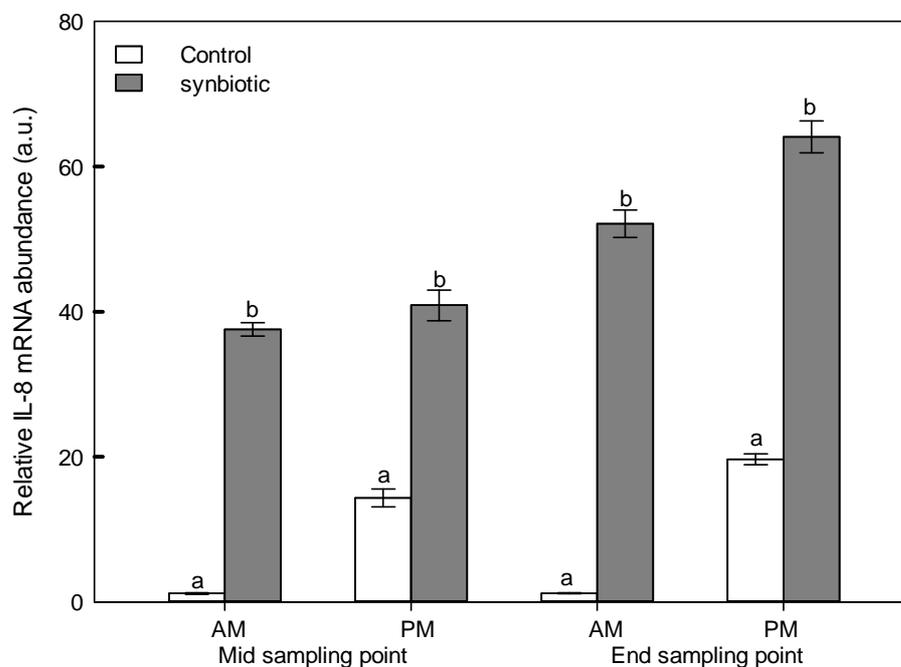


Figure 5.10 RT-PCR analysis of IL-8 gene expression in the intestine of Atlantic salmon at the mid and end sampling points feeding of the control and synbiotic diets. Results are presented as mean \pm SD in each group of fish ($n = 5$). Columns having different letters in the same region at each sampling point are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

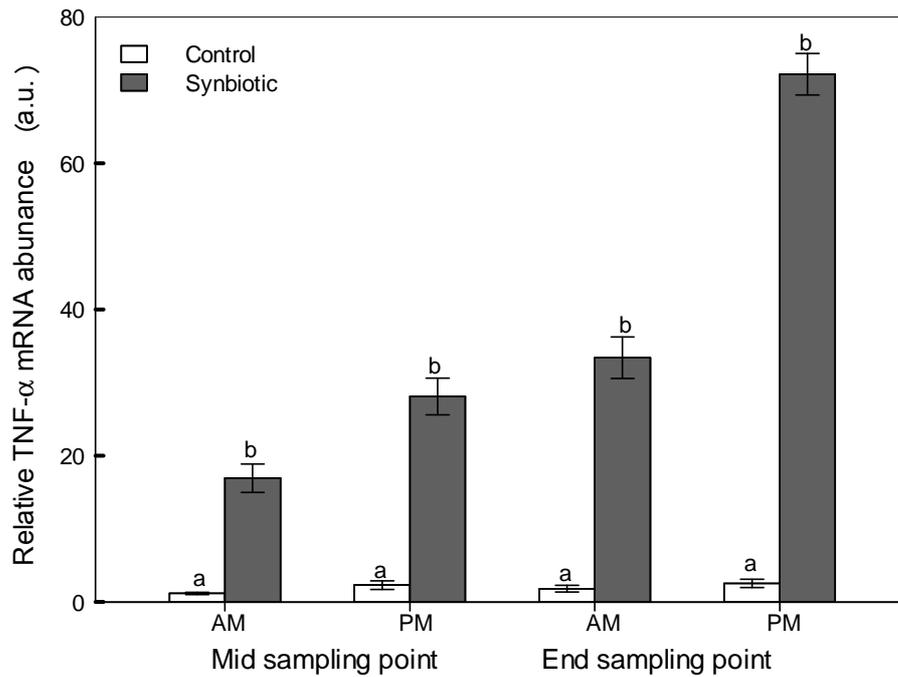


Figure 5.11 RT-PCR analysis of TNF- α gene expression in the intestine of Atlantic salmon at the mid and end sampling points feeding of the control and synbiotic diets. Results are present as mean \pm SD in each group of fish ($n = 5$). Columns having different letters in the same region at each sampling point are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

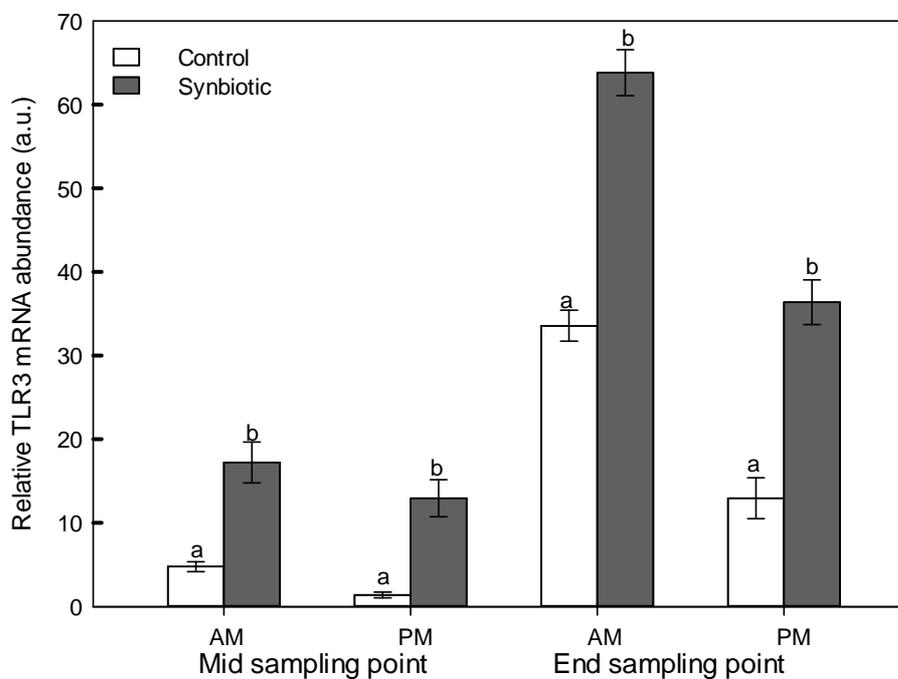


Figure 5.12 RT-PCR analysis of TLR3 gene expression in the intestine of Atlantic salmon at the mid and end sampling points feeding of the control and synbiotic diets. Results are present as mean \pm SD in each group of fish ($n = 5$). Columns having different letters in the same region at each sampling point are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

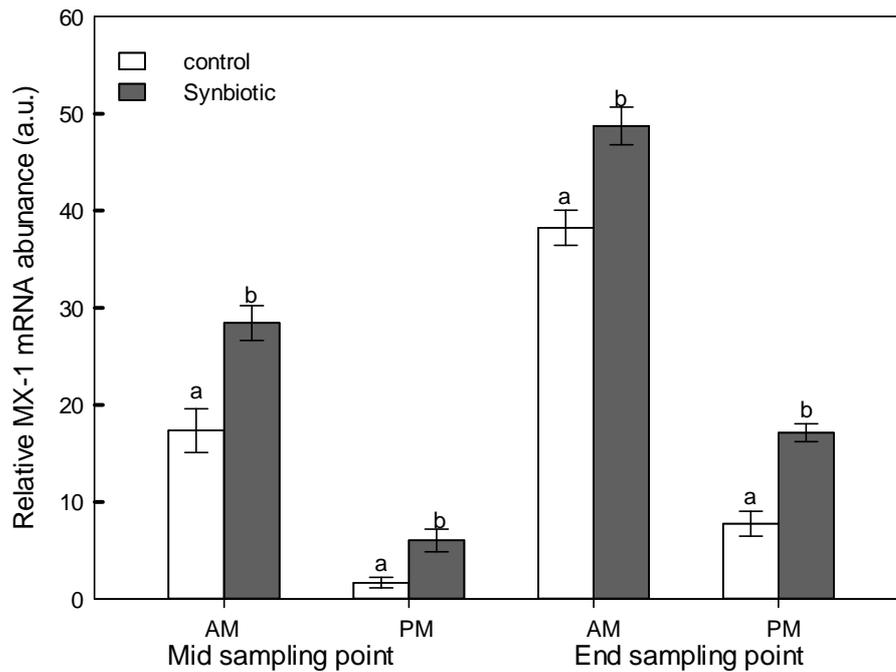


Figure 5.13 RT-PCR analysis of MX-1 gene expression in the intestine of Atlantic salmon at the mid and end sampling points feeding of the control and synbiotic diets. Results are present as mean \pm SD of replicates in each group of fish ($n = 5$). Columns having different letters in the same region at each sampling point are significantly different ($P < 0.05$). Sample codes, AM- anterior mucosa and PM- posterior mucosa.

5.4.6 Haemato-immunological profiles

The haematological and immunological parameters of salmon fed the synbiotic and control diets at the mid and end sampling points are displayed in Table 5.9. The results of present study indicated that the serum lysozyme activity was significantly higher in the synbiotic fed fish at the mid ($P = 0.005$) and end ($P = 0.001$) sampling points.

Additionally, the numbers of erythrocytes, leucocytes, lymphocytes, monocytes, thrombocytes, neutrophil and eosinophil revealed no significant differences between the treatments at either sampling points ($P > 0.05$).

Table 5.9 Atlantic salmon haematological and immunological parameters after feeding on the synbiotic and control diets for 63 days and 132 days.

	Midpoint sampling		Endpoint sampling	
	Control	Synbiotic	Control	Synbiotic
Lysozyme activity (U mL ⁻¹)	300.3 ± 46.0 ^a	334.0 ± 43.0 ^b	181.1 ± 44.1 ^a	216.5 ± 37.9 ^b
Erythrocyte count (×10 ⁵ mm ³)	-	-	9.8 ± 1.5	10.0 ± 1.5
Leucocyte count (×10 ⁴ mm ³)	-	-	1.2 ± 0.5	1.3 ± 0.2
Lymphocytes (%)	78.6 ± 6.4	74.3 ± 9.1	67.9 ± 7.7	69.1 ± 4.5
Monocytes (%)	2.8 ± 2.1	4.0 ± 1.2	4.6 ± 2.0	4.2 ± 1.7
Neutrophil (%)	11.5 ± 4.7	15.4 ± 6.5	19.9 ± 7.3	20.0 ± 3.4
Eosinophils (%)	4.4 ± 2.6	4.0 ± 2.8	6.1 ± 2.9	5.1 ± 2.7
Thrombocytes (%)	2.5 ± 0.5	2.2 ± 0.8	1.5 ± 0.3	1.4 ± 0.2

Results are presented as mean ± SD in each group of fish (n = 30). Means having different letters in the same row at each sampling point are significantly different ($P < 0.05$).

5.4.7 Growth performance

Results on the growth performance, including initial weight, final weight, SGR, FCR and TGC of fish fed the control and synbiotic diets are displayed in (Table 5.10). The dietary synbiotic application did not significantly affect the final weight gain, feed utilisation and K- factor of salmon. The weight gain was good however, both groups of fish had a good weight gain with >500% increase in biomass at 132 days feeding. Similarly, no effect of diet was observed on feed intake.

Table 5.10 Growth performance and feed utilisation performance of Atlantic salmon fed the synbiotic and control diets for 132 days. No significant differences were observed between treatments.

	Feeding phase 1		Feeding phase 2	
	Control	Synbiotic	Control	Synbiotic
Initial weight (g)	250 ± 13	250 ± 14	501 ± 5	501 ± 13
Final weight (g)	501 ± 4	501 ± 13	1207 ± 15	1215 ± 37
FCR	0.79 ± 0.04	0.81 ± 0.02	0.95 ± 0.01	0.95 ± 0.02
TGC	3.03 ± 0.16	3.04 ± 0.18	3.09 ± 0.04	3.12 ± 0.1
SGR (%)	1.11 ± 0.07	1.12 ± 0.07	1.27 ± 0.01	1.28 ± 0.04

5.5 Discussion

Investigation of the ecology of fish gut microbiota broadens our understanding regarding their roles in digestion, metabolism and in preventing the colonization of pathogens to the GIT (Romero and Espejo, 2001). The present study was undertaken to evaluate the intestinal microbiota of Atlantic salmon via direct microscopic counts (using acridine orange), DGGE and qPCR.

Bacterial numbers were significantly lower in the anterior mucosa, posterior mucosa and posterior digesta regions of the synbiotic group compared to the control group at the mid sampling point. It was observed that the levels of allochthonous bacteria in the present study revealed a marginal increase compared to the autochthonous bacteria approximately 1.5 log scale increasing. However, compared to the anterior digesta the number of allochthonous bacteria in the posterior digesta was also marginally increased. These findings are in accordance with results reported in Chapters 3 and 4. Previous studies which investigated the gut microbiota of salmon using direct counting found contradictory results. For instance, the dominant bacterial microbiota of the gut of juvenile farmed Atlantic salmon were investigated using cultivation techniques and DGGE, 16S rRNA gene and direct counting (Navarrete *et al.*, 2009). The results demonstrated that the number of bacteria detected by direct counting was slightly higher in the intestine in comparison to the others parts of the gut. In accordance with the results of Navarrete *et al.* (2009) the present study demonstrated that the levels of bacteria also were higher in the posterior digesta.

At the mid sampling point, qPCR revealed good recovery of the probiotic in the intestinal digesta of the synbiotic fed fish at levels of log 5.98 and 6.22 bacteria g⁻¹ in the anterior and posterior digesta, respectively. In terms of the total bacterial counts, the *P. acidilactici* therefore accounted for 1.48% and 4.17% of the total bacterial population in the anterior and posterior digesta, respectively. Previous studies have also shown that *P. acidilactici* can populate the GI tract of fish, both within the lumen (digesta) (Ferguson *et al.*, 2010; Merrifield *et al.*, 2011; Standen *et al.*, 2013) and also the mucosa (Merrifield *et al.*, 2010d; Merrifield *et al.*, 2011). Unfortunately, due to reduced feed availability during the last week of the trial (days 125 to 132), which may explain the lower levels of *P. acidilactici* recovery levels at the end of the trial.

The present study utilised banding pattern analysis to demonstrate the complexity of autochthonous and allochthonous microbiota associated with Atlantic salmon intestine, and to identify community changes in response to the dietary synbiotic. DGGE results indicated that in general, the synbiotic group exhibited higher numbers of bacterial species (DGGE gel band numbers) in all gut regions at the mid sampling point than control groups but no significant differences were found with the exception of the anterior digesta, where the number of bands was significantly higher. A possible explanation for this might be because of the addition of the synbiotic to the diet.

In the present study, the synbiotic did not affect the microbial diversity in the anterior mucosa, posterior mucosa and posterior digesta, while it was

significantly higher in the synbiotic fed group compared to the control group in the anterior digesta at the mid sampling point.

In contrast to the current finding, Ferguson *et al.* (2010) found that a *P. acidilactici* supplemented diet reduced the intestinal microbial diversity of red Nile tilapia after 32 days. In addition, in a study by Cerezuela *et al.* (2013) sea bream fed dietary *B. subtilis* and inulin revealed a significant reduction in the diversity of the intestinal microbiota compared to the control group. These findings demonstrated that adding the synbiotic increases the diversity of the microbial population in the anterior digesta and that it could possibly reduce the opportunity entry, or establishment of pathogens in the GIT. Similar findings have been observed in locust, where an inverse relationship was observed between the numbers of the pathogen *Serratia marcescens* and the overall bacterial diversity (Dillon *et al.*, 2005).

The increase in species richness produced by the synbiotic in the current study is not in agreement with above mentioned studies. Species richness was significantly higher in the treated group in comparison to the group fed the control diet in the anterior digesta at the mid sampling point.

At the mid sampling point, members of the Vibrionaceae (*Vibrio* and *Aliivibrio* spp.) were only detected in the synbiotic group. *Vibrio* spp. have been commonly reported in salmonids including rainbow trout (Huber *et al.*, 2004; Dimitroglou *et al.*, 2009), Atlantic salmon (Holben *et al.*, 2002; Hovda *et al.*, 2007) and Arctic charr (Ringø, 1993). *V. pelagius* was also detected in the synbiotic group only and has been previously isolated from Atlantic mackerel (*Scomber scombrus*) (Svanevik and Lunestad, 2011). These bacteria are

believed to have a useful nutritional role since the cellular lipids of these bacteria are composed of a variety of polyunsaturated fatty acids (Ringø and Vadstein, 1998). *Vibrio* bacteria are Gram negative, mesophilic, motile rods. Several strains of *Vibrio* sp. were used as probiotics for fish and shellfish (Verschuere *et al.*, 2000). *V. pelagius* was found to decrease the mortality of turbot larvae challenged with *Aeromonas caviae* (Ringø and Vadstein, 1998). *Vibrio wodanis* have been isolated from healthy Atlantic salmon and characterised using a range of established biochemical tests (Lunder *et al.*, 2000). The same authors also suggested that isolation of *A. wodanis* (formerly *Vibrio wodanis*; (Urbanczyk *et al.*, 2007)) from a group of healthy salmon, but not from a group with winter ulcer indicated that these bacteria have no pathogenic role. However, some species of *Vibrio* are disease causing agents in fish; these include, although are not limited to, *Vibrio (Listonella) anguillarum* and *Vibrio salmonicida* which cause vibriosis and coldwater vibriosis, respectively (Austin and Austin, 2007; Ringø *et al.*, 2010a). *Vibrio harveyi* and *Vibrio alginolyticus* have been also reported to cause some disease in fish include skin ulcers and infectious necrotising enteritis and the latter constitutes an opportunistic invader of fish (Austin and Austin, 2007).

Mycoplasma sp. were detected in both feeding groups at both sampling points. It is interesting to note that *Mycoplasma* was a common component of the microbiota within the differing gut regions, which is consistent with recovery of *Mycoplasma* in the salmon, Atlantic mackerel and rainbow trout GIT by Holben *et al.* (2002), Kim *et al.* (2007) and Svanevik and Lunestad (2011) suggesting that *Mycoplasma* may be more common in the intestinal

tract of fish than previously thought. It is evident from the literature that *Mycoplasma* sp. is deemed as pathogenic bacteria, which can cause a variety of diseases in numerous organisms. *Mycoplasmas* sp. are Gram negative bacteria, lack the cell wall and dominate among the microbiota members in humans, animals and plants (Razin, 1995).

Streptococcus sp. was observed in both groups. These are Gram positive, non-motile, facultatively anaerobic, non-pigmented, cocci cells (Osawa *et al.*, 1995). These bacteria were also isolated from brown trout in Chapter 3. *Al. faecalis* is Gram-negative, rod-shaped, and belongs to the family Comamonadaceae. These bacteria were also isolated from rainbow trout in Chapter 4.

A unique band was identified only in the posterior digesta of synbiotic fed salmon as *Enterovibrio calviensis*. These are Gram-negative bacteria, rod-shaped to slightly curved, motile and its identification in the synbiotic group could hint to some benefits, but further investigations are needed to address the role of these bacteria. Another bacteria stimulated by the synbiotic was *Serratia rubidaea*, which is a known lipase producing bacteria (Immanuel *et al.*, 2008). In the present study, some bands were detected in the same position of *P. acidilactici* but they corresponded to different species due to the fact that these bands had the same denaturing properties. Indeed, Vallaey (1997) demonstrated that one organism could present more than one bacterial bands due to multiple copies of heterogeneous rRNA and some bacterial DGGE bands could be identified in several bacterial species. These results suggested that a single DGGE band does not necessarily represent a

single bacterial strain and that the band which migrated to the same location in different lanes may contain different bacteria. In the line with the present study, Lamari *et al.* (2013) found that *P. acidilactici* was not present in the bacterial profiles obtained by DGGE from sea bass, although a band, which was confirmed by sequencing to correspond to another bacterial species, had migrated to the same position.

Similar to the present study, Pirarat *et al.* (2011) reported that dietary supplementation with *Lb. rhamnosus* revealed an obvious increase in the height of villi of tilapia in all parts of the intestine, but no significant differences were observed except in the anterior intestine. According to Ferguson *et al.* (2010), dietary *P. acidilactici* exerted no significant influence on the mucosal fold length of red tilapia and the results of that study were in opposition to the present results. In agreement with the findings of the current study, the length of villi in sea bream groups fed inulin, *B. subtilis* or inulin and *B. subtilis* was significantly higher compared to the control diet fed fish in the anterior intestine (Cerezuela *et al.*, 2013). There is little evidence in the literature regarding the use of the synbiotic combination used in this study having not been investigated previously, and therefore it would appear that the current study is the first to investigate scFOS and *P. acidilactici* in combination.

IELs are likely to perform immunological functions such as a defensive barrier against foreign antigens in the mucosal epithelium (Kiristioglu *et al.*, 2002). At two weeks after feeding of rainbow trout with a control diet or a probiotic diet (control diet supplemented with *P. acidilactici* (Bactocell®) at 10^7

CFU g⁻¹), the number of leucocytes was significantly elevated in the intestinal epithelium of given a diet supplemented with *P. acidilactici* compared to the control group (Harper *et al.*, 2011). The authors suggested that *P. acidilactici* supplementation could stimulate the migration of leucocytes from systemic sources to the epithelial brushborder.

In accordance with the results of the study of Harper *et al.* (2011), the results of the present study revealed a significant increase in the level of IELs in the intestinal epithelium of the synbiotic fed salmon compared to the control fed salmon in the posterior intestine at the mid sampling point. The same trend was observed in the posterior mucosa at the end sampling point. Similarly, IELs levels were increased in the intestine of tilapia fed diet supplemented with *P. acidilactici* at week six of the trial (Standen *et al.*, 2013). In contrast, the number of IELs was not affected in both intestinal regions at both sampling points (Chapter 4). Furthermore, Cerezuela *et al.* (2013) found that the number of IELs was lower in the combination of the inulin and *B. subtilis* fed sea bream compared with the control group. T-lymphocytes were found to be the main component of IELs and the IELs numbers seem to be associated with intestinal inflammation processes (Uran *et al.*, 2008; Rauta *et al.*, 2012). However, it seems possible that this significant increase in IELs population in the posterior intestine might be related to dietary *P. acidilactici* and/or scFOS that can stimulate the migration of IELs to the epithelial brushborder and affected intestinal immune cells. There is a distinct lack of information available on the effect of synbiotic application on intestinal parameters such as IEL and goblet cell abundance; there are however a number of previous studies that have demonstrated that goblet cell levels

were not affected by probiotic application (Cerezuela *et al.*, 2013; Standen *et al.*, 2013), whereas, in contrast other showed that the numbers of goblet cells were affected by probiotics application (Harper *et al.*, 2011); Chapter 4).

Microvilli are finger-like filaments (brush border) and their height varies with intestinal region, diet, ambient conditions and fish species (Buddington and Kuz'mina, 2000). They are located on the apical membrane of enterocytes which increase the absorptive surface. In contrast to the present findings no significant improvement in microvilli length was recorded in all treated fish compared with the control group (Cerezuela *et al.*, 2013). Additionally, the microvilli height of the intestine of tilapia was not affected by *P. acidilactici* compared to those in the control group (Standen *et al.*, 2013). In the present study, there was no evidence that the slight increase in the length of both villi and microvilli led to an improvement in the feed utilisation or growth performance. However, *P. acidilactici* acts to improve villi length thereby keeping mucosal epithelium in healthy status.

To evaluate whether synbiotic treatment had an effect on the expression of immune-related genes, three cytokines (IL-1 β , IL-8, TNF- α), TLR3 and MX-1 were investigated using RT-PCR. In response to the infection with *A. salmonicida*, IL-1 β , IL-8 and TNF- α were observed to be significantly higher in the infected group of rainbow trout in the anterior intestine but not in the posterior intestine (Mulder *et al.*, 2007). IL-1 β is an early pro-inflammatory cytokine, which is produced as the result of stimulating factors and contributes to the induction and proliferation of T and B lymphocytes, macrophages and vascular endothelial cells leading to inflammatory

responses (Beutler, 2004; Raida and Buchmann, 2008). It is widely reported that initial acquired responses are constituted by B cells and T cells and the latter carry different types of antigen specific receptors which can recognize almost all antigen by binding to a specific antibody which is produced by B cells (Alvarez-Pellitero, 2008; Kiron, 2012). The pro-inflammatory cytokine IL-8 attracts leucocytes including neutrophils and T-lymphocytes to site of the infection (Overturf and LaPatra, 2006; Gómez and Balcázar, 2008). IELs are mainly composed of T-cells which can be attracted by IL-1 β and IL-8 to infection sites as mentioned above, and this may explain the results of the present study, in which light microscopy revealed that IELs numbers were significantly higher in the treated group compared with the control group in the posterior mucosa at both sampling points.

Probiotics like *Lb. plantarum* has been reported to up-regulate the IL-1 β , IL-8 and TNF- α genes in the intestine and head kidney of rainbow trout (Pérez-Sánchez *et al.*, 2011b). Furthermore, the mRNA level of IL-8 and TNF- α have been found to be significantly higher in the head kidney of *Lb. rhamnosus* fed Nile tilapia (Pirarat *et al.*, 2011). Mulder *et al.* (2007) found that IL-1 β , IL-8, and TNF- α genes were up-regulated in the anterior intestine of rainbow trout during challenge with *A. salmonicida*.

TLRs, as pattern recognition receptors (PRRs), have crucial roles in the inflammatory response in fish since they are able to recognise pathogen-associated molecular patterns (PAMPs), which are present on the cell wall of pathogen microorganisms (Panigrahi *et al.*, 2007; Alvarez-Pellitero, 2008). TLRs act to activate the signal for the initiation inflammatory response by

recruiting and attracting immune cells to site of the infection (Trichet, 2010). The role of TLR3 is to recognise double-stranded RNA, which is released during the life cycles of many viruses and is responsible for the immune response to viral infection (Rodriguez *et al.*, 2005; Yang and Su, 2010). The activation of TLR3 signalling plays an important role in inducing both cytokine secretion and antiviral immunity (Strandskog *et al.*, 2008).

In accordance with the present findings, TLR3 gene in rainbow trout was found to be up-regulated after *in vitro* challenge with infectious hematopoietic necrosis virus (HNV) after three days (Rodriguez *et al.*, 2005). However, in the same study no significant up-regulation of the Tlr3 gene was found in the spleen and head kidney after infection with *Yersinia ruckeri*.

Another viral infection-related gene, MX-1, was investigated in the present study. MX-1, in conjunction with IL-8, has been reported to be effective against viral infections in vertebrates (Overturf and LaPatra, 2006). Mx proteins have been reported to be induced by IFN after viral infection and belong to the dynamin large GTPase family (Haller *et al.*, 2007). Mx is reported to protect against a number of viral agents including infectious pancreatic necrosis virus (IPNV), which is a serious viral disease of Atlantic salmon, by stimulating a general and non-specific protection particularly after sea water transfer (Das *et al.*, 2007).

In agreement with the current study, one research group investigated MX-1 in Atlantic salmon after challenging with two different doses of *Listonella anguillarum* lipopolysaccharide and chromosomal DNA, reported that MX-1 was up-regulated in challenged fish compared with the control group (Acosta

et al., 2004). In addition, MX-1 mRNA expression was shown to be significantly up-regulated in common carp *Cyprinus carpio*, challenged via intraperitoneal injection with grass carp *Ctenopharyngodon idella* reovirus (GCRV), after 24h compared to the control group, whilst after 96h MX-1 mRNA expression in treated group had recovered to normal levels (Yang and Su, 2010). Indeed, viruses are prominent disease causative agents in salmon including: infectious salmon anaemia virus, haemorrhagic kidney disease, infectious hematopoietic necrosis, infectious pancreatic necrosis (Lorenzen and LaPatra, 2005; Crane and Hyatt, 2011); therefore the up-regulation of Mx-1 and TLR3 in the present study could imply a the role of the synbiotic in immune stimulation against viral diseases. Given the scarcity of literature available it is not clear specifically how a probiotic, prebiotic or synbiotic can modulate viral associated immunological responses but it is clear from the available studies that a generic stimulation of the immune response can provide protection against both bacterial and viral challenges.

Modulation of the immune system is regarded as one of the most important benefits of probiotics, which interact with immune cells such as monocytes, macrophages and neutrophils to enhance nonspecific immune responses (Nayak, 2010a). Fish haematology profiles are reported to be useful tools in the rapid diagnosis of disease (Ferguson *et al.*, 2010). The results of the present study demonstrated that the synbiotic did not improve the haematological profiles in comparison to the control group. However, lysozyme enzyme activity was found to be significantly higher in the synbiotic group compared to the control group at both sampling points. Measurement of lysozyme activity is employed as an immunological indicator in fish.

Literature regarding the effect of scFOS + *P. acidilactici* on salmonid haematology and immunology is scarce. However, previous investigations in other fish species suggest that individual supplementation with scFOS or *P. acidilactici* improved the lysozyme enzyme activity of these fish. For example, these findings are in accordance to those reported in yellow croaker by Ai *et al.* (2011) who used a combination of *B. subtilis* and FOS in different levels, as a feeding for 10 weeks and the lysozyme activity was significantly higher in two groups of synbiotic compared to the group without *B. subtilis* supplementation.

In the study of Ye *et al.* (2011), eight experimental diets were formulated in order to investigate the effects of these diets on growth performance and immunological profiles, including lysozyme activity, on Japanese flounder (*Paralichthys olivaceus*). Lysozyme activity tended to be significantly elevated in flounder fed diets included *Bacillus clausii*, *B. clausii* and FOS, *B. clausii* and MOS as well as *B. clausii* with combination with FOS and MOS compared with those fed the control diet. The authors reported an apparent tendency towards elevation of lysozyme activity in the groups of flounder fed synbiotic diets compared to those groups fed diets supplemented with the prebiotics individually and a group of flounder fed *B. clausii* supplementation diet. In contrast, significant improvement in lysozyme activity was not shown in rainbow trout fed *P. acidilactici*, in spite of that the activity of rainbow trout fed both low and high vegetative of *P. acidilactici* was over 30% compared to those of the control fish (Merrifield *et al.*, 2011). Moreover, in agreement with the present study, lysozyme activity was significantly higher in red tilapia after 32 days *P. acidilactici* feeding compared to control fed fish (Ferguson *et al.*,

2010). However, the variation in the above results might be possibly due to such factors including species, exposure period, dosage of diet supplementation and the type of prebiotics and probiotics used (Cerezuela *et al.*, 2011).

In the present study, at 63 and 132 days dietary supplementation the synbiotic did not induce any significant differences in the growth performance. These results are in contrast with the results that have been reported by Meharabi *et al.* (2012) who demonstrated that a combination of *E. faecium* and FOS significantly increased final mean weight, WG, SGR, K-factor, FCR and survival rate compared to the control group in rainbow trout. Additionally, the same tendency was found in rainbow trout fed MOS and *E. faecium* (Rodriguez-Estrada *et al.*, 2009). The combination of *B. subtilis* and FOS significantly increased SGR, survival rate and FER of juvenile large yellow croaker compared to the control group (Ai *et al.*, 2011). However, the latter authors found that individual FOS supplemented diet did not significantly affect the growth performance, survival and feed utilization.

Similarly to the present findings, Grisdale-Helland *et al.* (2008) demonstrated that MOS, FOS or GOS had no beneficial effects on digestibility, feed intake or growth of Atlantic salmon. In addition, rainbow trout fed *P. acidilactici* for 10 weeks did not reveal any significant improvement in growth performance, feed utilization or carcass composition, while K-factor was significantly lower in the group of rainbow trout fed lyophilized diet compared to the control group of fish (Merrifield *et al.*, 2011). In accordance with these results, *P.*

acidilactici exerted no improvement in body weight gain of rainbow trout (Aubin *et al.*, 2005) or tilapia (Ferguson *et al.*, 2010; Standen *et al.*, 2013).

5.6 Conclusions

The present study demonstrated that *P. acidilactici* populated the digesta and mucosa of the GIT of Atlantic salmon, as these bacteria were identified by qPCR. The microbiota was modulated by the synbiotic composed of scFOS and *P. acidilactici* via increased OTUs, richness and diversity in the anterior digesta but not in other regions, and reduced the total number of bacteria present in the intestine at the mid sampling point. Some potentially beneficial bacteria were selectively stimulated, with one potential harmful species was likely reduced by the synbiotic. This might suggest that *P. acidilactici* performs a competitive role against harmful bacteria. The synbiotic had no effect on growth performance, while villi and microvilli length was significantly elevated by the synbiotic. In addition, it was observed from the current study that this synbiotic enhanced the immune response in terms of the expression of genes relating to the immunity in the both mucosa regions and some of the non-specific immunity.

Further research is needed to investigate the effects and potential benefits of this synbiotic on the GIT of Atlantic salmon. For example, diseases challenge to investigate the relationship between *P. acidilactici* and pathogenic bacteria in fish, clone libraries and NGS in order to give clear picture for microbial community in Atlantic salmon. Also further studies are needed to elucidate which feed additive (probiotic or prebiotic), was the predominant driving force behind the host benefits. Unfortunately, due to logistical constraints at the

fish farm, including limited availability of sea pens, it was not possible to include the singular use of the probiotic and prebiotic in separate treatments. This would also provide information as to whether a synbiotic or additive effect was achieved when using both additives simultaneously.

Chapter 6

Chapter 6: Efficacy of *P. acidilactici* on rainbow trout (*Oncorhynchus mykiss*) growth, health status and intestine microbiota when provided in different dietary formulations.

Abstract

Six groups of fish (initial body weight 48.9 ± 0.3 g) were randomly distributed into 18 tanks (20 fish per tank) and triplicate groups were fed either 1] a diet containing fishmeal (FM) as the crude protein source, 2] a diet containing 54.8% soybean meal (SBM), or 3] a diet containing a mixture of plant ingredients (PlantMix) for 12 weeks. In addition, three further groups were fed the aforementioned dietary regimes with the inclusion of 200 mg per kg lyophilized *P. acidilactici*.

The distal intestinal microbiota and the expression of selected immune-related genes (IL-8 and TNF- α) as well as cell activity-related genes (HSP70 and casp-3) were assessed at week five and twelve. Moreover, the haematological profiles, immunological profiles and growth performance were also assessed.

At both sampling points, with the exception of fish fed FM at week five and twelve, LAB levels were significantly higher in all groups of fish fed diets supplemented with *P. acidilactici*. LAB from the probiotic fed fish was confirmed as *P. acidilactici* by 16S rRNA gene sequence.

RT-PCR indicated that IL-8 and TNF- α mRNA levels were not affected by the probiotic treatment at either time point. In addition casp-3 mRNA levels were down-regulated in fish fed FM diet supplemented with *P. acidilactici* compared to the control fed fish at week five ($P = 0.001$).

Serum lysozyme activity was significantly higher ($P = 0.019$) in fish fed FM and SBM diets containing *P. acidilactici*, compared to the non-probiotic controls.

6.1 Introduction

The global supplies of fish meal (FM) and fish oil (FO) is limited and availability have been stagnant at about 6 million and 1 million metric tonnes, respectively, over the past decade (Tacon and Metian, 2009). These volumes will not be sufficient to meet the growing volumes of aquafeed required as aquaculture output increases; therefore the replacement of FM for fish diets is desperately needed. FM alternatives should not only meet favourable requirements such as the promotion of growth performance and health status but also the consumer's needs in terms of palatability (Uran *et al.*, 2008). SBM is deemed a suitable alternatives to FM because of its competitive price and long term availability compared to FM (Hardy, 2010). SBM has been commonly used in aquafeeds due to its relatively high protein content, well balanced amino acid profile and high nutrient digestibility (Gatlin *et al.*, 2007; Hardy, 2010).

However, it has been reported that plant protein sources, and particularly SBM, can cause pathological changes in the distal gut in several fish species including rainbow trout (Heikkinen *et al.*, 2006; Dimitroglou *et al.*, 2011a; Desai *et al.*, 2012), Atlantic salmon (Baeverfjord and Krogdahl, 1996; Bakke-McKellep *et al.*, 2007), Atlantic cod (Refstie *et al.*, 2006) and common carp (Uran *et al.*, 2008).

These changes, known as enteritis, include damaged enterocytes, microvilli and villi shortening, a reduction in both microvilli density and length, shortening of the enterocyte height and width, a swelling of the lamina propria and sub-mucosa, an increase in the number of goblet cells and inflammatory cells (Baeverfjord and Krogdahl, 1996; Merrifield *et al.*, 2009b;

Sahlmann *et al.*, 2013). At the systemic level this leads to an increased susceptibility to bacterial infection, reduced growth performance and nutrient digestion (Krogdahl *et al.*, 2000; Heikkinen *et al.*, 2006; Bakke-McKellep *et al.*, 2007; Merrifield *et al.*, 2009b; Sørensen *et al.*, 2011; Desai *et al.*, 2012).

It is widely accepted that this type of damage is due to the direct effects of anti-nutritional factors (ANFs) in SBM such as saponins, lectins, glucosinolates, phytate, proteinase inhibitors, tannins, oligosaccharides and non-starch polysaccharides, which also act to inhibit nutrient utilization and digestibility (Krogdahl *et al.*, 2010). The permeability of the intestinal membrane is altered by soy saponins which in conjunction with other ANFs induce an inflammatory response (Knudsen *et al.*, 2007).

Many studies have been conducted to investigate the effects of the partial or total replacement of FM by different soybean products which in turn could alter digestive physiology, health status and growth performance. For example, Atlantic salmon were fed a diet containing 20% extracted SBM for seven days and fish were scarified daily in order to investigate the signs of the enteritis and immune-related gene expression (Sahlmann *et al.*, 2013). Results revealed that the signs of the enteritis were observed on day five, whereas up-regulation in immune-related genes including GTPase IMAP, NF-kB-related genes and regulators of T cell and B cell function were initiated in the first five days.

The SBM inclusion in teleost fish feeds has been implicated in the disruption of the immune response as well. Regarding this, many studies demonstrated that SBM can induce the innate immune response via up-regulation of

immune-related genes and cell-related genes including PCNA or increasing the levels of macrophages, neutrophils and IgM (Uran *et al.*, 2008; Mansfield *et al.*, 2010; Sahlmann *et al.*, 2013). Confirming these gene expression studies, elevated levels of PCNA, HSP70 and casp-3 proteins have been detected in the mucosal epithelium of SBM-fed rainbow trout using immunohistochemistry (Bakke-McKellep *et al.*, 2007).

In addition, several studies have demonstrated that different soybean products induce alterations in the gut microbial community. The culturable bacteria levels were lowered in rainbow trout and Atlantic cod fed SBM compared to those fed FM diet (Heikkinen *et al.*, 2006; Ringø *et al.*, 2006d; Mansfield *et al.*, 2010). In contrast, Bakke-McKellep *et al.* (2007) observed that the levels of culturable bacteria were higher in the group of salmon fed diets containing 25% SBM (dehulled, extracted and toasted) compared to fish fed FM as the sole protein source. The autochthonous cultivable bacteria level was significantly reduced in the whole intestine of the SBM-fed Atlantic cod, whereas the allochthonous level was significantly increased in the pyloric and mid intestine compared to the FM fed group (Refstie *et al.*, 2006). Contrary to these findings, no differences, with respect to the culturable bacteria levels, were reported between two groups of rainbow trout fed SBM and FM (Merrifield *et al.*, 2009b). However, alterations in the gut microbial community have been also reported using culture-independent techniques including 16S rRNA gene analysis, PCR-DGGE, clone library analysis and length heterogeneity of PCR (Heikkinen *et al.*, 2006; Bakke-McKellep *et al.*, 2007; Mansfield *et al.*, 2010; Desai *et al.*, 2012).

Methods for alleviating these microbial changes and enteritis are currently unclear investigation. In addition, associated changes in the gut microbiota have been reported; these changes in bacterial abundance or community profiles are not always consistently observed and it is not yet known if they are a factor involved in inducing the inflammatory response or whether they occur due to the inflammatory response or changes in dietary nutrients (Heikkinen *et al.*, 2006; Bakke-McKellep *et al.*, 2007; Desai *et al.*, 2012).

Probiotics have been reported to reduce or mediate gastric disorders in mammals (Marteau *et al.*, 2001). In spite of a plethora of studies which have investigated the effects of SBM inclusion in health status of fish, limited reports are available on the use of a combination of probiotic and SBM products in fish. Sealey *et al.* (2009) used dietary inclusion of SBM at 0%, 10% and 20% supplemented with or without a multi-strain probiotic product which contained live *Saccharomyces cerevisiae*, *E. faecium*, *Lb. acidophilus*, *Lb. casei*, *Lb. plantarum* and *Lactobacillus brevis* and fed rainbow trout for 8 weeks. Data demonstrated that benefits included increase in the growth rate, whole body protein and higher energy retention as well as reduction of pathological changes in the intestine in the probiotic fed group was observed compared with the control group. The aim of the present study was to investigate the effects of three different dietary regimes with or without *P. acidilactici* on the posterior intestine microbiota and some immune and cell-related genes of rainbow trout.

6.2 Materials and methods

6.2.1 Diet preparation

Six experimental diets were formulated to be iso-nitrogenous (50.8%) and iso-lipidic (19.8%) containing different levels of different ingredients (Table 6.1).

Three standard diets were formulated as control or 'basal' diets (Table 6.1): a FM diet (FMC), where 100% of the protein was provided by fish meal as the sole protein source, a SBM diet (SBMC), where 25% and 54.8% of the protein was provided by FM and soybean, respectively, and a PlantMix control diet (PlantMixC) where 25% and 27% of the protein was provided by FM and soybean, respectively with the remainder of the protein provided by other vegetable meal (pea protein, glutalys and vital wheat gluten). All diets were supplemented with 100% fish oil (Seven Seas Ltd. UK) as the main source of oil and also contained corn starch, standard vitamin, mineral premix and carboxy methyl cellulose sodium salt.

In addition, three probiotic diets (FMP, SBMP, and PlantMixP) were formulated with the same basal formulations except *Pediococcus acidilactici* MA 18/5 M (Bactocell[®], Lallemand Inc., Canada), which was added at 200 mg per kg diet.

The dry ingredients were weighed and mixed for approximately 1h using a Hobart food mixer (model no: HL1400–10STDA; Hobart Food Equipment, Australia). After the initial mixing, FO was gradually added to the ingredients. After further mixing, warm water was added to produce a soft and slightly moist consistency. These represented the control diets.

For the probiotic diets, 200 mg of Bactocell® was gradually mixed with corn starch using a commercial blender and the remaining ingredients were added gradually with manual and electrical mixing to ensure that the level of probiotic remained approximately the same in all batches of the diet. This procedure was repeated with each 1 kg of the ingredients.

Dietary ingredients were then mixed in a Hobart food mixer (model no: HL1400–10STDA; Hobart Food Equipment, Australia) in order to produce identical diets with the exception of the probiont. After that the mixture was passed through an extruder (model P6; La Monferrina, Asti, Italy) to produce 4 mm pellets which were then spread out and dried using an oven at 45 °C for 36h. After drying, the diets were broken up and stored in airtight containers at 4 °C until use. New batches of diets were produced every four weeks to ensure that high levels of probiotics were maintained for the duration of the trial. The viability of *P. acidilactici* in the probiotic diets were determined by plate counts on MRS agar as described in Section 2.5. The viability of dietary *P. acidilactici* is shown in Figure 6.1. Diet samples from the feeding trial were analysed for the determination of moisture, protein, lipid, ash, and gross energy. All protocols concerning analytical chemistry are described in Section 2.6. The proximate analyses are presented in Table 6.1. Sequence analysis of 16S rRNA gene was conducted using the primers 27F and 1491R on a representative subset of LAB (a minimum of three isolates in each probiotic diet) to confirm identification of *P. acidilactici* isolates, as described in Section 2.5.1.

Table 6.1 Formulation of experimental diets and chemical composition.

Ingredients (%)	FMC	FMP	SBMC	SBMP	PlantMixC	PlantMixP
Fish meal LT94 [‡]	670.7	670.7	250	250	250	250
Soya HP 48 [¥]	-	-	398.4	398.4	189.7	189.7
Soya SPC 60 [¥]	-	-	150	150	80	80
Pea protein [‡]	-	-	-	-	80	80
Glutalys [‡]	-	-	-	-	60	60
Vital Wheat gluten [‡]	-	-	-	-	60	60
Fish oil [‡]	121	121	157.1	157.1	154.2	154.2
Corn starch [§]	183.4	183.4	19.5	19.5	101.1	101.1
Premix [‡]	20	20	20	20	20	20
Vitamin-mineral premix						
CMC-binder [§]	5	5	5	5	5	5
<i>P. acidilactici</i> *	-	0.200	-	0.200	-	0.200
Proximate composition (%)						
Moisture (%)	3.9 ± 0.3	2.5 ± 0.2	2.5 ± 0.8	2.3 ± 0.2	3.1 ± 0.7	1.9 ± 0.4
Solids (%)	96.1 ± 0.3	97.5 ± 0.2	97.5 ± 0.8	97.7 ± 0.2	96.9 ± 0.7	98.1 ± 0.4
Crude protein (%)	50.8 ± 1.1	51.8 ± 1.1	50.8 ± 0.8	50.0 ± 0.8	50.8 ± 0.8	49.0 ± 0.4
Lipids (%)	19.4 ± 2.5	20.6 ± 4.4	19.3 ± 0.3	20 ± 1	20.7 ± 1.8	18.8 ± 0.6
Ash (%)	9.3 ± 0.2	9.4 ± 0.2	7.5 ± 0.3	7.6 ± 0.1	6.7 ± 0.3	5.0 ± 1
Gross energy (MJ kg ⁻¹)*	21.9 ± 0.2	22.1 ± 0.1	22.1 ± 0.0	21.7 ± 0.1	22.7 ± 0.1	22.1 ± 0.0

Proximate composition data are mean ± SD, n = 3.

[‡]Herring meal LT94 – United Fish Products Ltd., Aberdeen, UK.

[¥] BioMar.

[§] Sigma, UK.

³ Premier nutrition vitamin/mineral premix: 121 g kg⁻¹ calcium, Vit A 1.0 µg kg⁻¹, Vit D3 0.1 µg kg⁻¹, Vit E (as alpha tocopherol acetate) 7.0 g kg⁻¹, Copper (as cupric sulphate) 250 mg kg⁻¹, Magnesium 15.6 g kg⁻¹, Phosphorous 5.2 g kg⁻¹.

**Pediococcus acidilactici* (CNCM MA 18/5 M), Bactocell[®] (Lallemand Inc., Canada).

⁵ Epanoil, Seven Seas Ltd, UK.
& Roquette Frères, France.

6.2.2 Experimental design

The study was carried out in the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK and lasted for 12 weeks. Rainbow trout were obtained from Torre fisheries, Watchet, Somerset, UK. Fish were allowed to adapt to the new conditions for three weeks prior to initiation of the feeding experiments. Fish were fed a commercial diet (Sigma[®] 50, EWOS; Bergen, Norway) during the acclimation interval. Prior to the experimental period, a total of 360 fish (48.9 ± 0.3 g) were randomly distributed over eighteen fibreglass tanks (80 L capacity), and each group consisted of three replicates at a stocking density of 20 fish per tank. Full details of the rearing conditions are described in Section 2.2 as appropriate.

6.2.3 Water quality

Water temperature, pH and DO were maintained at 15.1 ± 0.9 °C, 5.8 ± 0.6 and 81.2 ± 3.9 , respectively. Additionally, ammonia, nitrite and nitrate were measured weekly as described in Section 2.3 and maintained 0.1 ± 0.02 mg L⁻¹, 0.04 ± 0.01 mg L⁻¹ and 15.6 ± 5.9 mg L⁻¹, respectively.

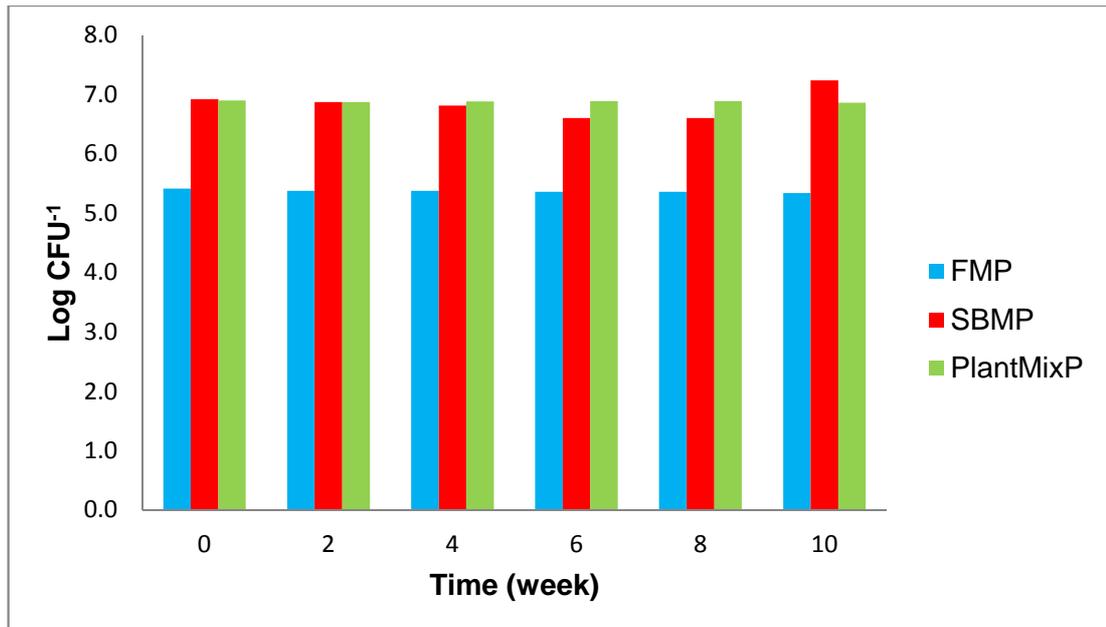


Figure 6.1 The viability of *P. acidilactici* (CFU g⁻¹) in the probiotic diets during the trial period. Sample codes, FMP- fishmeal probiotic, SBMP- soybean meal probiotic and PlantMixP- PlantMix probiotic. Diets were stored at 4 °C (n = 3). *New diets prepared every 4 weeks of the study.

6.2.4 Feeding and weighing

To determine the growth performance and feed utilization, all fish in each experimental tank were weighed at the start of the trial and the six different diets were fed to randomly assigned triplicate tanks (3 tanks per treatment) three times a day (0900, 1300 and 1700), seven days a week at a rate of 1.5% – 2.2% body weight in equal rations for a period of twelve weeks.

Throughout the experiment, fish were reweighed (in bulk, by tank) every two weeks and within this period feed input was adjusted daily based on a predicted FCR. Daily feed was corrected on a fortnightly basis following batch weighing after a 24h feed deprivation. At each weighing, the total fish biomass and number in each tank were determined to adjust the amount of food and to calculate the growth rate.

6.2.5 Microbiology sampling

At weeks five and twelve, four and three randomly selected fish, respectively, were removed and euthanized from each treatment (at least one fish from each replicate tank) and dissected as described in Section 2.7.1. The posterior intestinal mucosal was aseptically opened with a sterile scalpel and the digesta was emptied into 1.5 mL MCT and stored at -20 °C for further use.

6.2.5.1 Enumeration of intestinal bacterial populations

For bacteriological studies, digesta were tenfold diluted in sterile 0.9% PBS; ACC and LAB numbers were calculated on TSA and MRS agar, respectively as described in Section 2.7.2.

6.2.5.2 PCR-based identification of pure cultures

After enumeration, a representative selection of 60 colonies, for each probiotics group at each sampling points were randomly picked from each MRS plate containing 30 to 300 CFU and sub-cultured on MRS agar repeatedly until pure cultures were obtained; these isolates were stored at 4 °C.

For DNA extraction, isolates were inoculated into 15 mL Falcon tubes containing MRS broth and incubated at 37 °C for 48h. After incubation, 1 mL aliquots from each Falcon tube were placed into 1.5 mL MCTs and centrifuged (2000x g for 5 min). Pellets then were washed twice with PBS and DNA was extracted from these isolates as described in Appendix 1.

6.2.5.3 Fluorescence *in situ* hybridization (FISH)

At weeks five and twelve, digesta samples were serially diluted in 1.5 mL MCT to 10^{-3} and an equal volume of 4% freshly prepared paraformaldehyde (PFA; w/v PBS) solution was added.

Tubes were stored overnight at 4 °C. After that, tubes were centrifuged at 5000x g for 5 min and the pellets were washed with PBS twice to remove residual PFA and resuspended in 1:1 of ice-cold PBS and 96% ice-cold ethanol. Tubes were stored then at -20 °C until use. Dehydration was carried out by applying 30 - 60 μ L of fixed sample onto a microscopic slide. Slides were allowed to dry for 30 min at 46 °C. Slides were dehydrated for 1 min in each graded ethanol solutions of 50%, 80%, 90% (v/v in ddH₂O) and 96%. Then slides were dried at 46 °C for 2 min.

Dehydrated samples were hybridized by adding 1 μ L LAB 759 probe (50 pmol/ μ L, labelled with FITC fluorescent dye at the 5' end; CTA CCC ATR CTT TCG AGC C) which is specific to *Lactobacillus* spp. and *Pediococcus* spp. (Zijngel *et al.*, 2010), to 9 μ L of hybridization buffer containing (per mL: 180 μ L of 5M NaCl, 20 μ L of 1M Tris-HCl pH 8.0, 1 μ L of 10% SDS, 300 μ L of formamide and 499 μ L of ddH₂O). The mixture was then applied to the sample on the slides. Slides were covered with cover slips and horizontally placed into 50 mL Falcon tubes. Tissue paper pre-moistened with hybridization buffer was placed under the slides and tubes were closed. Tubes were placed horizontally onto a rack and incubated at 46 °C for 180 min. The tubes were then removed and the slides immediately washed with 50 mL of pre-warmed washing buffer (1 mL of 1M Tris pH 8.0, 1.02 mL of 5M NaCl and 0.5 mL of 0.5 M EDTA to 50 mL ddH₂O) at 48 °C for 1 min.

Washing buffer was then removed by tipping the tube and slides were left to dry in room temperature. After that, one drop of fluoroshield (Sigma) was added and slides were covered with a coverslip. Finally, slides were stored at -20 °C in the dark. The slides were examined with a Nikon 80i epifluorescence microscope (Tokyo, Japan).

6.2.6 Gene expression

Samples were taken from the posterior intestine at weeks five and twelve (n = 4 and 3, respectively) from the control and probiotic fed fish and kept as described in Section 2.9. The mRNA levels of immune (IL-8 and TNF- α) and cell activity-related genes (HSP70 and casp-3) were assessed.

6.2.6.1 RNA Extraction

Total RNA was extracted from the intestinal tissue (~30 mg) using GenElute Mammalian kit (Sigma Aldrich, UK) following the manufacturer's instructions. Briefly, intestine tissue was sonicated in 1.5 mL MCT containing 500 μ L of lysis buffer and 2.5 μ L of mercapto-ethanol at medium speed using a Microsone Ultrasonic Cell Disruptor, USA for 5 sec after which serial steps were carried out according to the manufacturer's instructions. Finally, RNA was collected in 2 mL MTCs pre-labelled tubes and stored at -80 °C until use.

6.2.6.2 DNA digestion

To avoid contamination with genomic DNA, the RNase-free DNase kit (Qiagen, UK) (10 μ L of DNase and 70 μ L of digestion buffer) was used after the first washing for 15 min in room temperature. RNA quantity and purity was measured using a NanoDrop UV spectrometer (ND-1000) by measuring the absorbance at 230 nm. RNA samples were stored at -80 °C until required.

6.2.6.3 Reverse transcription to obtain complementary DNA (cDNA)

A total amount of 2 µg of RNA was used for cDNA synthesis, which was conducted using an iScript cDNA Synthesis Kit (Sigma, Poole, UK). Briefly, cDNA was synthesised using 2 µg of total RNA incubated with reverse transcriptase dNTP mix along with specific primer random nonamers and to complete the volume to 10 µL, MGW was added. Tubes were incubated at 70 °C for 10 min (Gene Amp PCR system 9700) followed by incubation on ice for 5 min. A second mixture was prepared as following: 2 µL reverse transcriptase buffer, 1 µL reverse transcriptase enzyme and 7 µL MGW.

The two mixtures were mixed to give a final volume of 20 µL for each sample and all tubes were vortexed for 5 sec. Tubes then were incubated at 37 °C for 50 min and 95 °C for 10 min with a final hold at 4 °C. The resulting cDNA samples were stored at -20 °C until further analysis. The primers used are listed in Table 6.2. β-actin and 60S were used as reference genes in the samples in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality.

6.2.6.4 Conventional PCR

In order to check the product size of primers and to evaluate the PCR condition, conventional PCR was conducted. The following reagents were included in each PCR tube: 12.5µL BioMix™ Red Taq (Bioline, UK), 0.5 µL of each primer (forward and reverse) (10 pmol/µL MWG-Biotech AG, Germany), 1 µL DNA template and 10.5 µL MGW yielding a total volume of 25 µL.

Thermal cycling was conducted in a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 95 °C for 10 min, then 40

cycles of 95 °C for 15 sec, 60 °C for 1 min and 72 °C for 30 sec, with a final extension at 72 °C for 7 min and a final hold at 4 °C.

6.2.6.5 Agarose gel electrophoresis to check the purity of PCR products

In order to check the purity and molecular weight characteristics of PCR products, six µL of each PCR product was loaded onto a 1% agarose gel as described in section 2.5.2.

6.2.6.6 Real time PCR

RT-PCR was carried out using the SYBR[®] green method in Biosystem StepOne[™] PCR cycler and software V. 2.1. Triplicate PCR reactions were conducted for each sample for target, housekeeping genes and also for negative control.

The reactions were prepared on a 96-well plate by mixing, for each sample, 2 µL of cDNA, 12.5 µL of SYBR[®] Green JumpStart[™] Taq ReadyMix (Sigma , UK), 0.5 µL (10 pmol/µL) of each forward and reverse primers, 0.25 µL reference dye and 10.25 µL of MGW. The thermal profile for all reactions was 94°C for 2 min and then 40 cycles starting at 94°C for 15 sec, 60 °C for 1 min and at 72°C for 30 sec.

The data was analysed based on the differences between the reference (control) and the treatment groups using a comparative Ct analyses, using the following equations:

$$\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ reference control}$$

$$\text{Amount of target (RQ)} = 2^{-(\Delta\Delta Ct)}$$

Where Ct is the threshold cycle

Table 6.2 Sequences of oligonucleotide primers used for detection of immune and cell relevant genes in rainbow trout by RT-PCR. Accession number refers to target gene sequence used.

Gene name	Accession no.	Product size	Forward primer	Reverse primer
TNF- α	AJ277604	75	GGGGACAAACTGTGGACTGA	GAAGTTCTTGCCCTGCTCT
IL-8	AJ279069	69	AGAATGTCAGCCAGCCTTGT	TCTCAGACTCATCCCCTCAGT
HSP70	AB062281.1	122	CGTCCTAGACAGGTCTCCGC	CAATGAGAGCGCAGCATTCC
Casp-3	NM-001246335.1	131	TGTGGATGCTGGCTATGCAA	CTGACTGGCTGTGGTTGTCT
β -actin	AJ438158	167	ACAGACTGTACCCATCCCAAAC	AAAAAGCGCCAAAATAACAGAA
60S	NM001165047	147	AGCCACCAGTATGCTAACCAGT	TGTGATTGCACATTGACAAAAA

6.2.7 Haematological and immunological parameters

At week twelve, five fish per tank were randomly anaesthetized and blood collected as described in Section 2.10. The haematocrit value was determined using heparinized capillary tubes as described in Section 2.10.1. Counts of the leucocytes and erythrocytes were calculated as described in Section 2.10.3. Total blood haemoglobin concentration was determined as described in Section 2.10.4. MCV, MCH and MCHC were calculated as described in Section 2.10.5. Serum lysozyme activity was determined using the turbidometric method as described in Section 2.10.6.

6.2.8 Growth parameters

Percentage increase in weight gain (WG), SGR, FCR and K-factor were calculated as described in Section 2.11.

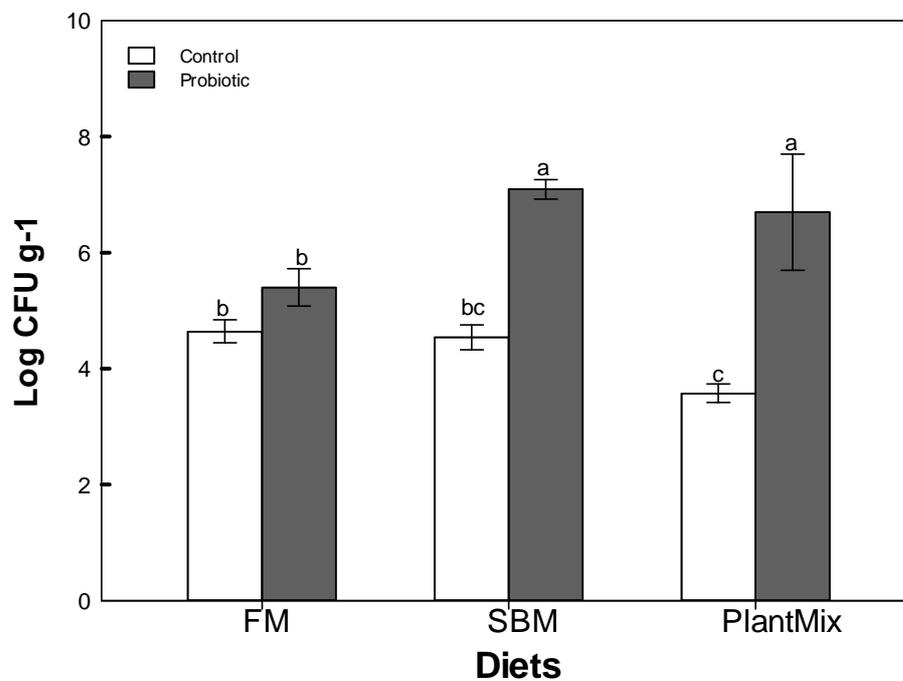
6.3 Statistical analyses

The mean and standard deviation (SD) were calculated for each parameter by using conventional statistical methods, whilst the mean and standard error (SE) were calculated for gene expression. Two way ANOVA was used to determine the significant variation among all parameters between diets, treatments and the interactions. Tukey's HSD multiple range *post hoc* testing was used to determine significant differences between means. The accepted levels of significance differences were ($P < 0.05$). All statistics was carried out using MiniTab statistical software version 16, IBM (Pennsylvania, USA).

6.4 Results

6.4.1 *P. acidilactici* colonization and effect on gut microbiota

The microbial community of the content of the posterior intestine in rainbow trout was assessed at weeks five and twelve for fish fed the control and probiotic supplementation. The numbers of transient LAB (allochthonous) in the posterior intestine of rainbow trout in the probiotic and control fed fish at week five of the trial are displayed in Figure 6.2.



Two-way ANOVA

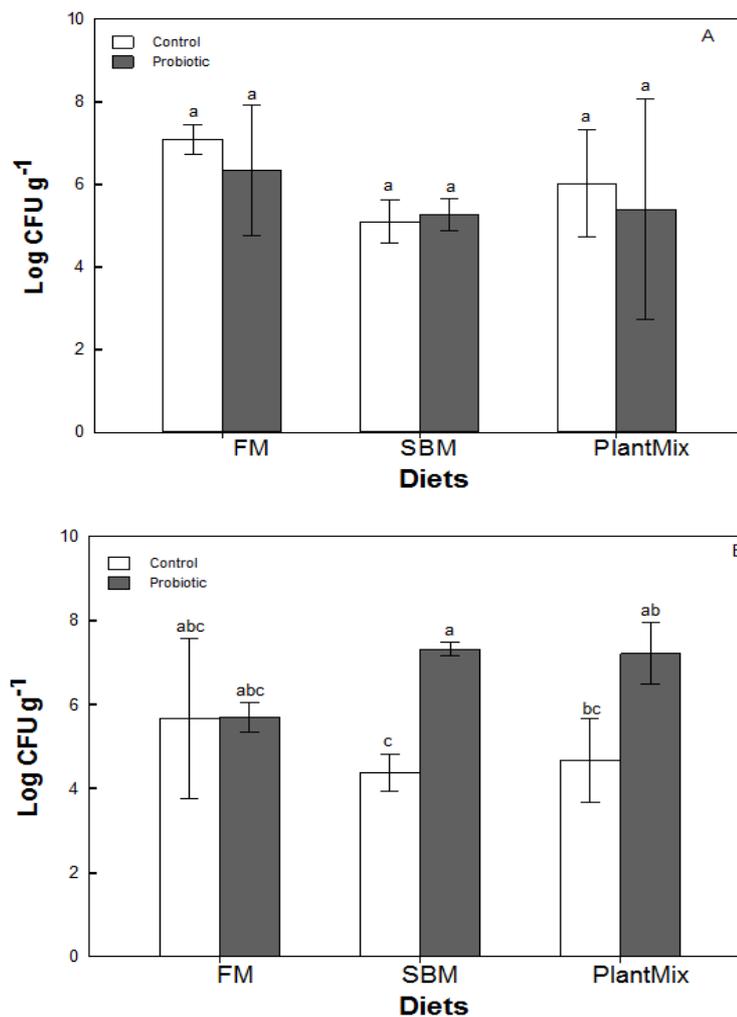
Diets	P value		Diets			Treatment	
	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
	0.005	<0.001	b	a	b	a	b

Figure 6.2 Number of LAB isolated from the digesta in the posterior intestine of rainbow trout fed the control and probiotic basal diets at week five. Results are presented as mean log values \pm SD in each region of fish ($n = 4$). Bars with different letters within each diet are significantly different ($P < 0.05$). The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FM- fishmeal and SBM- soybean meal.

Significant differences in LAB levels in the distal intestinal digesta were found between the control and probiotic treatment for fish fed SBM and PlantMix based diets at week five ($P < 0.001$). LAB levels were significantly lower in rainbow trout fed PlantMixC compared with the FMC group ($P = 0.005$). Additionally, LAB levels were significantly lower in rainbow trout fed FMP compared with other groups fed SBMP and PlantMixP ($P = 0.005$). There were also significant differences between the LAB levels in respect to diet and treatment and that there was an interactive effect (Figure 6.2).

The levels of LAB at week twelve were significantly higher in fish fed diet of SBMP compared with SBMC ($P = 0.002$). No differences were observed between FMP and FMC and although a numerical difference was observed, not significant difference was observed between PlantMixP and PlantMixC (Figure 6.3B). Overall LAB levels were not affected by diet, but were affected by treatment ($P = 0.002$), and an interactive effect ($P = 0.045$) was observed (Figure 6.3). It was observed that the levels of LAB were one log higher an order of magnitude at week twelve in FMC and both of the PlantMix diets, (control and probiotic), compared to the groups fed the same diets at week five. Representative pure isolates at week five and twelve from MRS plates (probiotic treatment groups) were sequenced and identified as *P. acidilactici*.

The levels of ACC at week twelve are illustrated in Figure 6.3A. Two way ANOVA showed that even though ACC levels were reduced in the probiotic groups in FMP and PlantMixP, (log 6.3 and log 5.4 respectively), compared with their respective control groups, (log 7.1 and log 6.1), no significant differences were found ($P > 0.05$) between the treatments.



Two-way ANOVA

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
ACC	0.147	0.668	0.701	a	a	a	a	a
LAB	0.895	0.002	0.045	a	a	a	a	b

Figure 6.3 Numbers of ACC (A) and LAB (B) isolated from the digesta of the posterior intestine of rainbow trout fed the control and probiotic basal diets at week twelve. Results are presented as mean log values \pm SD in each region of fish ($n = 3$). Bars with different letters within each diet are significantly different ($P < 0.05$). The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FM- fishmeal and SBM- soybean meal.

6.4.2 FISH analysis

P. acidilactici like cells were detected in fish fed probiotic diets using a specific probe as indicated in plate 6.1.A.

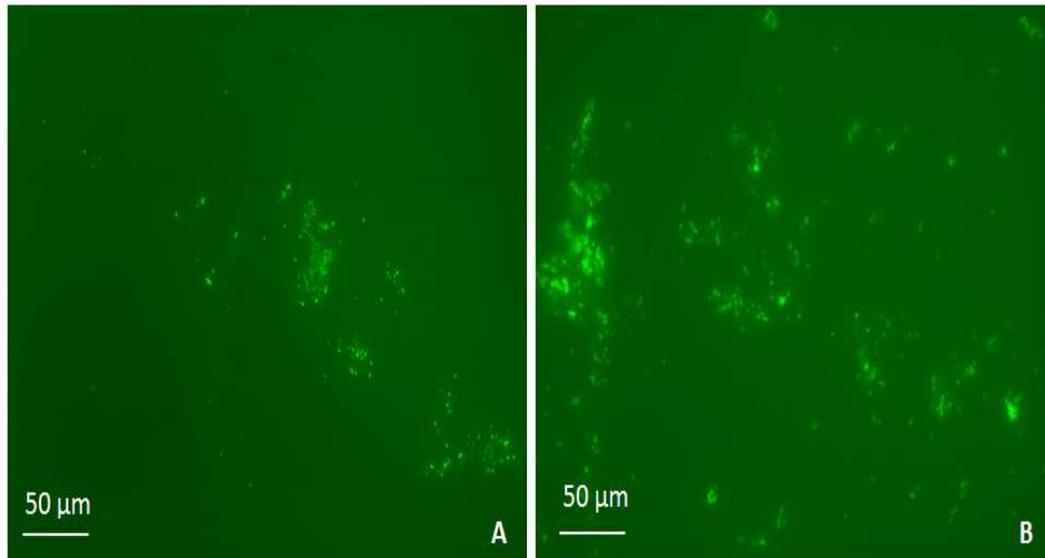
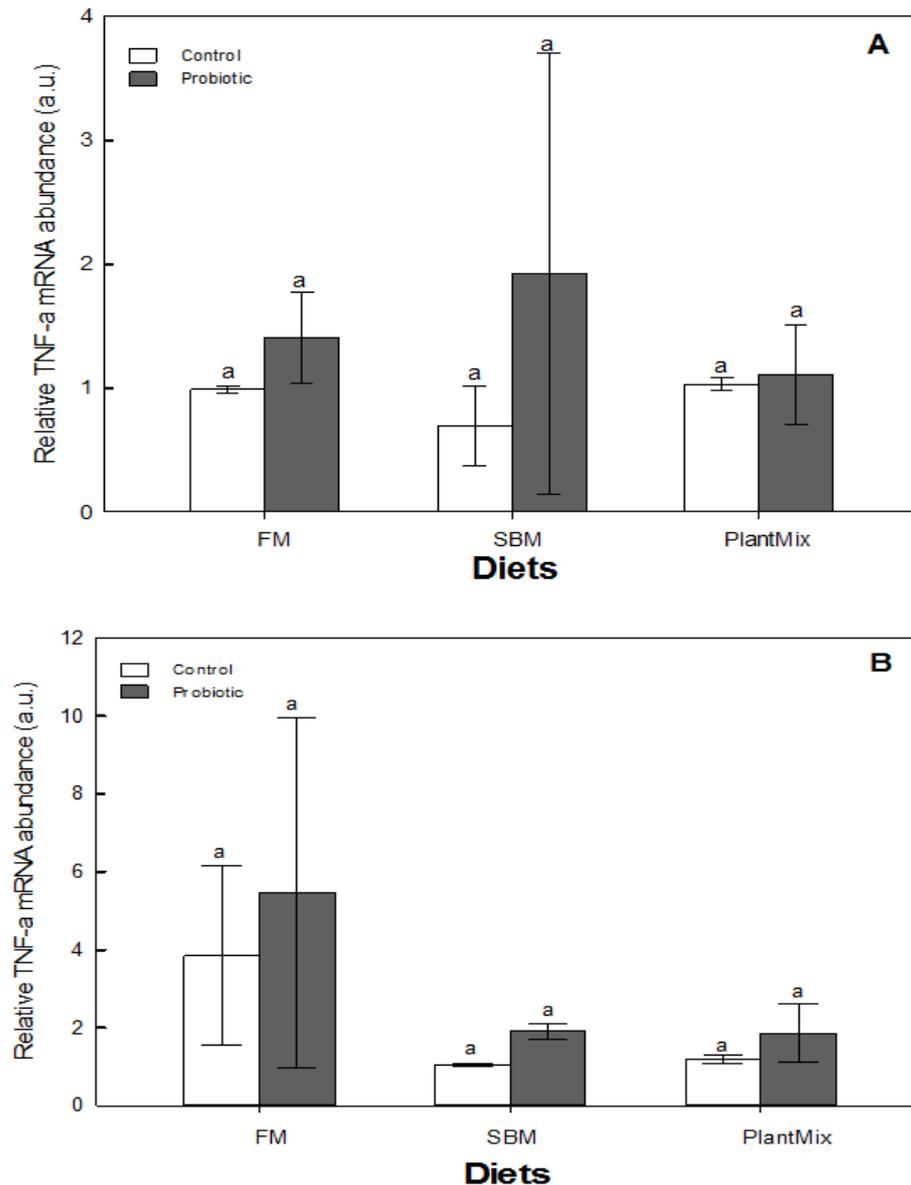


Plate 6.1 (A) FISH of digesta samples using the *Lactobacillus* sp. and *Pediococcus*-specific probe LAB759-FITC. (B) FISH performed on pure culture of *P. acidilactici* using the LAB759-FITC.

6.4.3 Gene expression results

The results of TNF- α , IL-8, HSP70 and casp-3 gene expression at weeks five and twelve are presented in Figures 6.4 - 6.7. The different cytokine genes measured at these time points and their expression relative to the most stable house-keeping gene, (60S or β -actin), were used at weeks five and twelve, respectively.

Although an increase in the pro-inflammatory cytokine TNF- α expression was observed in rainbow trout fed the FMC and SBM diets supplemented with *P. acidilactici* at week five, compared to fish fed the control diet, these differences were not significant, due to high variability in the data (Figure 6.4A). At week twelve, TNF- α expression was high in two fish out of three, but due to the large variation within the group, this was not significantly different in the FMP and PlantMixP groups compared to those in the control fed diets (Figure 6.4B). No treatment, diet or interactive effect was observed.



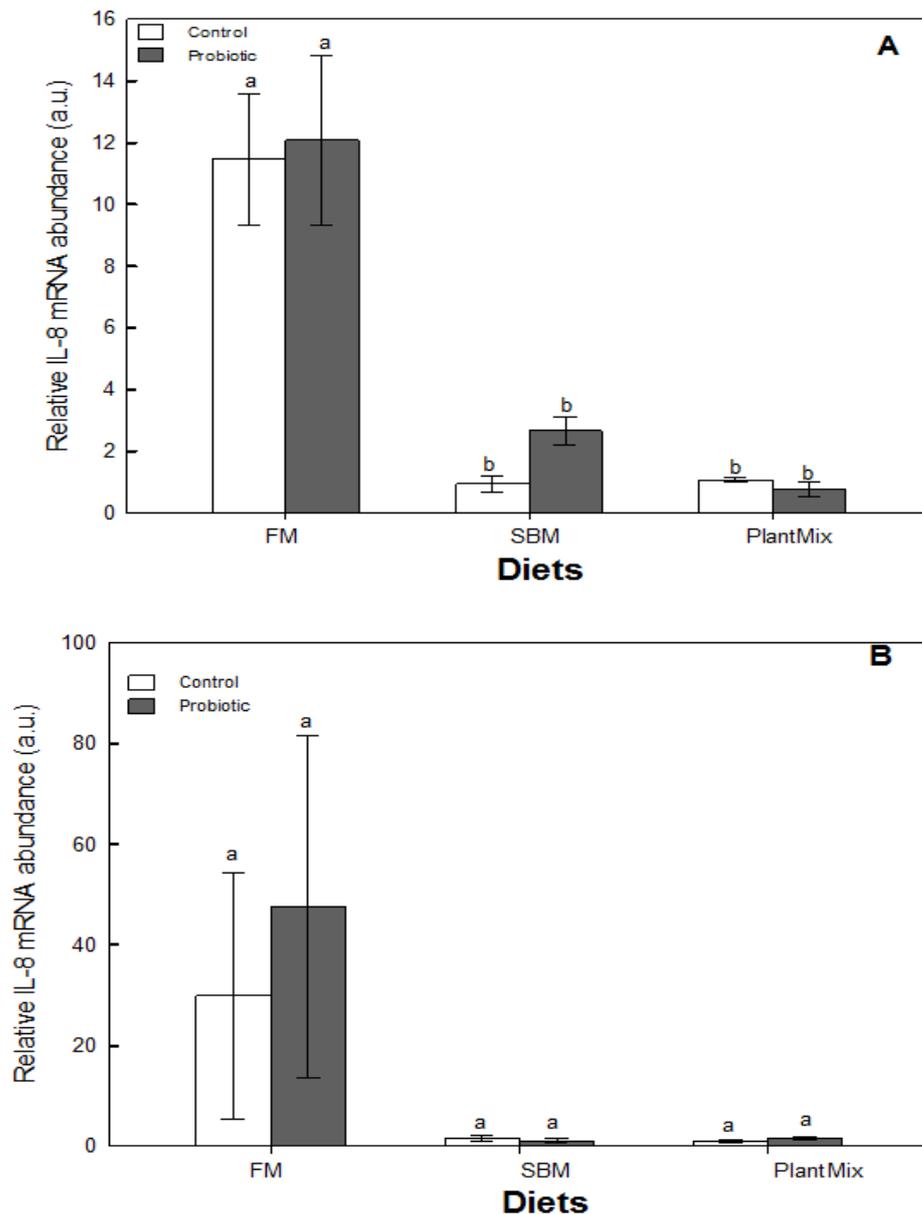
Two-way ANOVA

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
Mid	0.874	0.316	0.801	a	a	a	a	a
End	0.262	0.553	0.973	a	a	a	a	a

Figure 6.4 RT-PCR analysis of TNF- α gene expression in the posterior intestine of rainbow trout fed the control and probiotic basal diets at weeks five (A) and twelve (B) ($n = 4$ and 3 , respectively). Results are presented as mean \pm SE in each group of fish. The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FM- fishmeal and SBM- soybean meal.

At both sampling points, the level of IL-8 gene expression in fish fed *P. acidilactici* was not affected, even though a marginal increase was observed (Figures 6.5A and 6.5B, respectively). An effect of diet was observed, with

significance at week 5 ($P < 0.001$) and near significance at week 12 ($P = 0.076$). No effect of diet, or interaction with treatment, was observed.

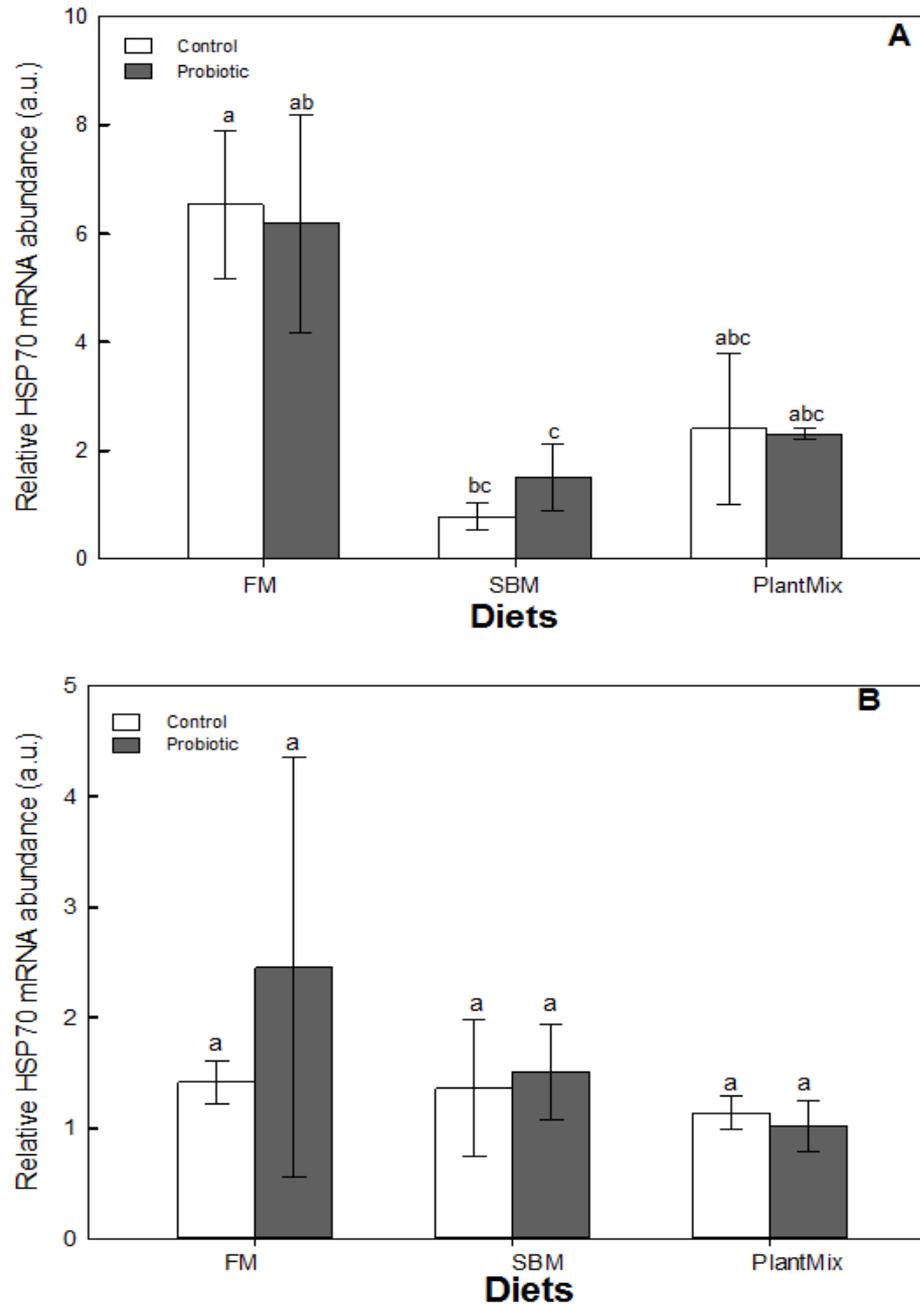


Two-way ANOVA

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
Mid	<0.001	0.568	0.785	a	b	b	a	a
End	0.076	0.677	0.839	a	a	a	a	a

Figure 6.5 RT-PCR analysis of IL-8 gene expression in the posterior intestine of rainbow trout fed the control and probiotic basal diets at week five (A) and twelve (B) ($n = 4$ and 3 , respectively). Results are presented as mean \pm SE in each group of fish. Bars with different letters are significantly different ($P < 0.05$). The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FM- fishmeal and SBM- soybean meal.

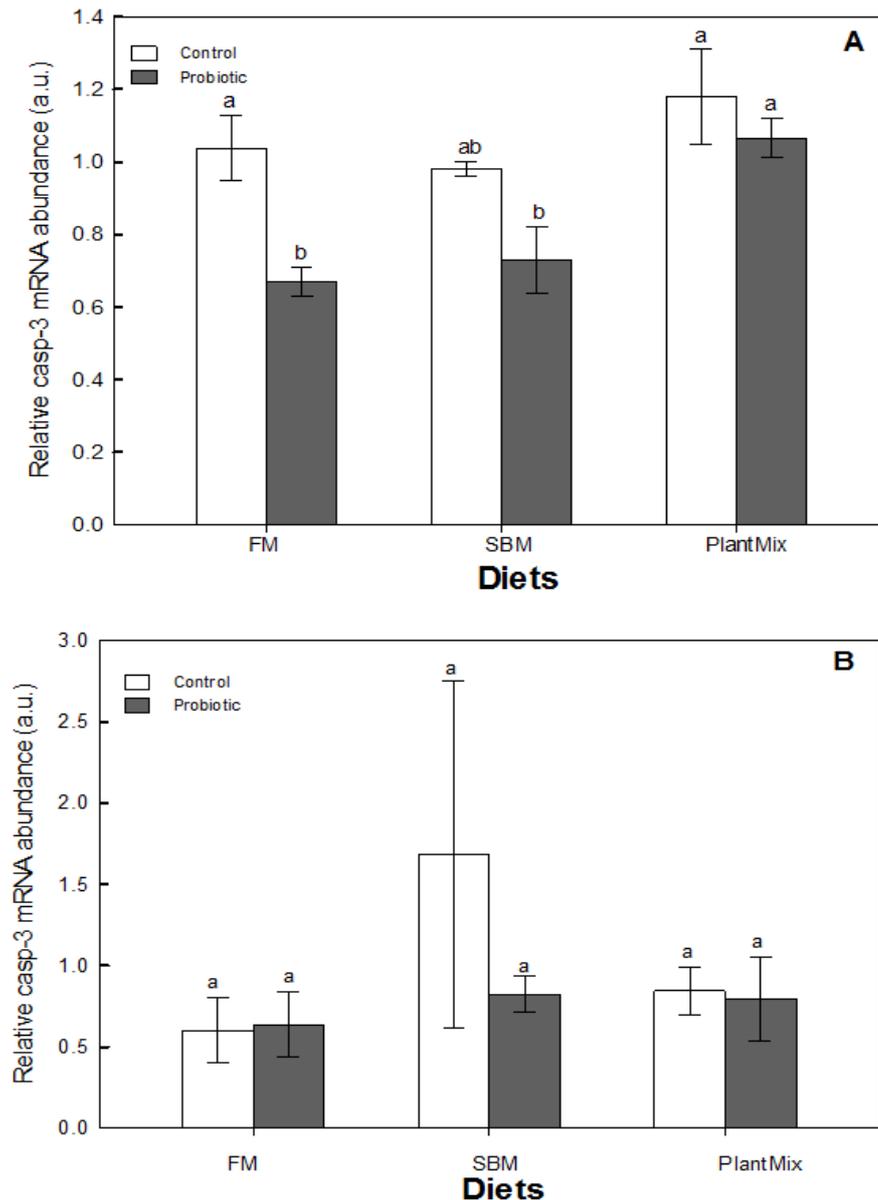
Additionally, no significant differences were found regarding the intestinal expression level of HSP70 between the treatments at both sampling points (Figures 6.6A and 6.6B). There was a dietary effect however at week 5, where the HSP70 expression in the FM fed fish was higher than the SBM fed fish ($P = 0.001$). The casp-3 mRNA level was significantly down-regulated at week 5 in the group of fish receiving the FMP ($P = 0.001$) diet compared with the FMC fed fish (Figure 6.7A). There was an effect of diet on the casp-3 expression, with significantly higher levels in the PlantMix treatment than the FM and SBM treatments ($P = 0.002$) and treatment, with casp-3 levels significantly lower in the probiotic treatment compared to the control treatment ($P = 0.001$). Casp-3 mRNA levels were not affected by treatment or diet at week twelve (Figure 6.7B).



Two-way ANOVA

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
Mid	0.001	0.853	0.992	a	b	b	a	a
End	0.606	0.608	0.777	a	a	a	a	a

Figure 6.6 RT-PCR analysis of HSP70 gene expression in the posterior intestine of rainbow trout fed the control and probiotic basal diets at week five (A) and twelve (B) ($n = 4$ and 3 , respectively). Results are presented as mean \pm SE in each group of fish. Bars with different letters are significantly different ($P < 0.05$). The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FM- fishmeal and SBM- soybean meal.



Two-way ANOVA

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
Mid	0.002	0.001	0.284	b	b	a	a	b
End	0.411	0.495	0.577	a	a	a	a	a

Figure 6.7 RT-PCR analysis of casp-3 gene expression in the posterior intestine of rainbow trout fed the control and probiotic basal diets at week five (A) and twelve (B) ($n = 4$ and 3 , respectively). Results are presented as mean \pm SE in each group of fish. Bars with the different letters within each diet are significantly different ($P < 0.05$). The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FM- fishmeal and SBM- soybean meal.

6.4.4 Haematological and immunological profiles

The haematological and immunological parameters of rainbow trout fed different diets supplemented with or without *P. acidilactici* at week twelve are presented in Table 6.3. Serum lysozyme activity was significantly affected by treatment ($P = 0.019$), with higher activity in fish fed FM and SBM diets containing *P. acidilactici* than that of fish fed the control diets. Further, diet was observed to have a significant effect on MCH ($P < 0.001$) and MCV ($P = 0.003$) with lower values observed in the SBM and PlantMix groups than the FM group. Further, the PlantMix treatment induced a significantly higher erythrocyte count than the fish fed the FM and SBM treatments ($P < 0.001$).

Table 6.3 Haematological and immunological parameters of groups of rainbow trout fed different diets supplemented with or without *P. acidilactici* at week twelve (n = 15).

Blood profiles	FMC	FMP	SBMC	SBMP	PlantMixC	PlantMixP
Erythrocyte count ($\times 10^5 \text{ mm}^3$)	6.7 \pm 0.9 ^b	7.0 \pm 1.6 ^{ab}	7.9 \pm 1.2 ^{ab}	8.3 \pm 1.5 ^a	8.1 \pm 1.1 ^a	8.1 \pm 0.8 ^a
P.C.V (%)	37.3 \pm 5.2 ^a	38.7 \pm 4.1 ^a	35.5 \pm 4.7 ^a	37.8 \pm 7.1 ^a	33.5 \pm 6.8 ^a	35.3 \pm 5.5 ^a
Hb (g dL ⁻¹)	7.5 \pm 2.8 ^a	8.4 \pm 1.0 ^a	7.3 \pm 1.4 ^a	7.0 \pm 1.5 ^a	6.0 \pm 1.1 ^a	7.0 \pm 4.9 ^a
MCV (fL cell ⁻¹)	557.5 \pm 113.2 ^a	554.0 \pm 111.9 ^a	461.8 \pm 129.2 ^a	510.5 \pm 118.9 ^a	440.0 \pm 88.0 ^a	439.0 \pm 62 ^a
MCH (pg cell ⁻¹)	110.9 \pm 40.7 ^{ab}	128.7 \pm 51.3 ^a	93.4 \pm 15.4 ^{bc}	85.6 \pm 16.9 ^{bc}	73.0 \pm 6.8 ^c	71.3 \pm 14.2 ^c
MCHC (g dL ⁻¹)	19.6 \pm 9.3 ^a	21.5 \pm 3.8 ^a	19.7 \pm 3.9 ^a	19.6 \pm 6.0 ^a	16.5 \pm 3.0 ^a	16.6 \pm 3.8 ^a
Lysozyme activity (Unit mL ⁻¹)	266.9 \pm 121.2 ^{bc}	406.2 \pm 116.3 ^a	225.7 \pm 69.2 ^c	388.5 \pm 161.1 ^{ab}	416.9 \pm 188.9 ^a	308.5 \pm 62.9 ^{abc}
Leucocyte count ($\times 10^4 \text{ mm}^3$)	1.3 \pm 0.5 ^a	1.2 \pm 0.1 ^a	1.4 \pm 0.03 ^a	1.4 \pm 0.03 ^a	1.5 \pm 0.3 ^a	1.3 \pm 0.6 ^a

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
Erythrocyte count ($\times 10^5 \text{ mm}^3$)	<0.001	0.397	0.841	a	a	b	a	a
P.C.V (%)	0.059	0.169	0.951	a	a	a	a	a
Hb (g dL ⁻¹)	0.096	0.303	0.504	a	a	a	a	a
MCV (fL cell ⁻¹)	0.003	0.568	0.785	a	ab	b	a	a
MCH (pg cell ⁻¹)	<0.001	0.655	0.213	a	b	b	a	a
MCHC (g dL ⁻¹)	0.217	0.610	0.768	a	a	a	a	a
Lysozyme activity (Unit mL ⁻¹)	0.249	0.019	<0.001	a	a	a	a	b
Leucocyte count ($\times 10^4 \text{ mm}^3$)	0.222	0.555	0.873	a	a	a	a	a

Values are present as means \pm SD. Control and probiotic values within a row, in the same basal diet, with different superscript letters are significantly different ($P < 0.05$). The table shows the two-way ANOVA analysis of diets, treatments and interactions. Sample codes, FMC- fishmeal control, FMP- fishmeal probiotic, SBMC- soybean meal control, SBMP- soybean meal probiotic, PlantMixC- PlantMix control and PlantMixP- Plantmix probiotic.

6.4.5 Growth performance

Data on the growth performances, including WG, SGR, FCR and K-factor of rainbow trout fed diets supplemented with or without *P. acidilactici* are summarized in Table 6.4. At week twelve, all growth performances parameters were not significantly affected by *P. acidilactici* supplementation compared to the control groups in all diet types. Fish fed SBMC had the highest FCR (0.90 ± 0.04) and the lowest SGR (1.56 ± 0.1) values while the group of fish fed FM supplemented with *P. acidilactici* had the lowest FCR (0.74 ± 0.02) and the highest SGR (1.85 ± 0.05). However, some growth performances parameters were significantly affected by diets. Even though there were no significant differences in terms of the final body weight between treatments and controls after 70 days of feeding, fish had grown well and their biomass increased by 150% - 200% in all groups (Table 6.4).

On the other hand, the highest WG (164.3 ± 7.7 g) was observed in FMP fed fish, whereas fish fed SBMP had the lowest WG (121.5 ± 13.6 g). The WG of rainbow trout fed different diets is presented in Figure 6.8. Although probiotic treatment did not affect these performance parameters, a clear effect of diet was observed, with fish fed the FM diet displaying significantly improved final mean weight ($P < 0.001$), WG ($P = 0.001$) and SGR ($P < 0.001$) compared to the SBM and PlantMix fed fish. FCR was also significantly improved in the FM fed fish compared to the SBM fed fish. The SBM fed fish performed significantly worse than both FM and PlantMix fed in respect to final mean weight ($P < 0.001$), SGR ($P < 0.001$) and FCR ($P = 0.001$).

Table 6.4 Growth and feed utilization performance of rainbow trout fed diets containing different ingredients supplemented with or without *P. acidilactici* over 12 weeks, (n = 3).

Blood profiles	FMC	FMP	SBMC	SBMP	PlantMixC	PlantMixP
Initial mean weight (g)	49.0 ± 0.04 ^a	48.5 ± 0.1 ^a	48.8 ± 0.3 ^a	48.9 ± 0.3 ^a	49.0 ± 0.3 ^a	48.9 ± 0.3 ^a
Final mean weight (g)	212 ± 8.9 ^a	213 ± 7.7 ^a	171 ± 8.7 ^b	170 ± 13.5 ^b	195 ± 10.8 ^{ab}	190 ± 7.2 ^{ab}
Weight gain (g)	162.7 ± 8.9 ^a	164 ± 7.7 ^a	122.0 ± 8.6 ^b	121.5 ± 13.6 ^b	145.9 ± 11.1 ^{ab}	141.2 ± 4.3 ^{ab}
SGR (% day ⁻¹)	1.83 ± 0.05 ^a	1.85 ± 0.05 ^a	1.56 ± 0.06 ^b	1.56 ± 0.10 ^b	1.72 ± 0.07 ^{ab}	1.70 ± 0.05 ^{ab}
FCR	0.76 ± 0.01 ^{bc}	0.74 ± 0.02 ^c	0.90 ± 0.04 ^a	0.86 ± 0.07 ^{ab}	0.80 ± 0.05 ^{abc}	0.81 ± 0.04 ^{abc}
K-factor	1.39 ± 0.08 ^a	1.45 ± 0.16 ^a	1.32 ± 0.07 ^a	1.42 ± 0.04 ^a	1.42 ± 0.12 ^a	1.45 ± 0.11 ^a

	P value			Diets			Treatment	
	Diets	Treatment	Interaction	FM	SBM	PlantMix	Control	Probiotic
Initial mean weight (g)	0.439	0.271	0.253	a	a	a	a	a
Final mean weight (g)	<0.001	0.772	0.866	a	c	b	a	a
Weight gain (g)	0.001	0.459	0.915	a	b	b	a	a
SGR (% day ⁻¹)	<0.001	0.862	0.845	a	c	b	a	a
FCR	0.001	0.380	0.665	b	a	b	a	a
K-factor*	0.286	0.074	0.710	a	a	a	a	a

Values are presented as means ± SD. Control and probiotic values within a row in the same basal diet with different superscript letters are significantly different $P < 0.05$. The table shows the two-way ANOVA analysis of diets, treatments and interactions. * n = 6. Sample codes, FMC- fishmeal control, FMP- fishmeal probiotic, SBMC- soybean meal control, SBMP- soybean meal probiotic, PlantMixC- PlantMix control and PlantMixP- Plantmix probiotic.

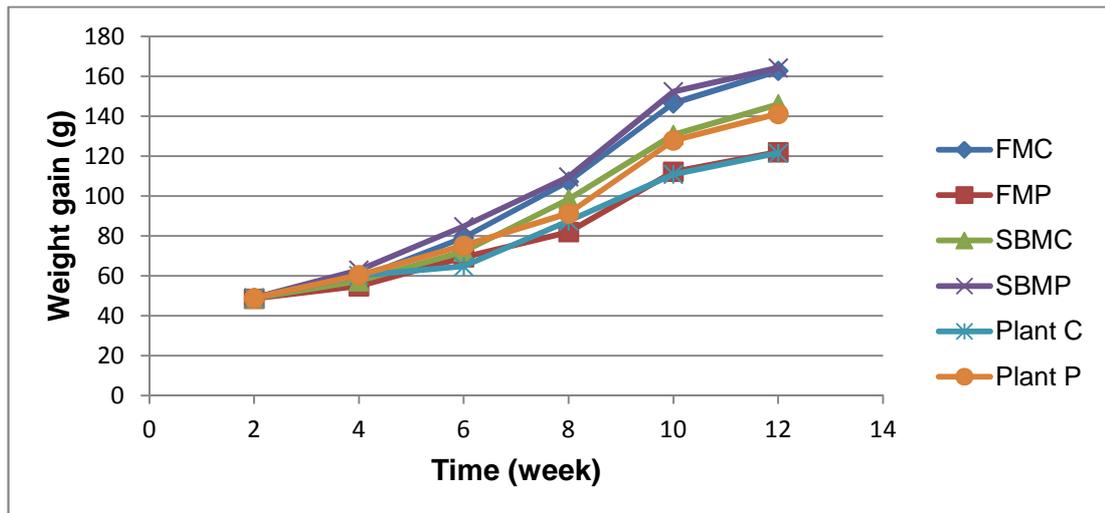


Figure 6.8 The mean weight gain of fish fed control and probiotic basal diets during the period of the trial. Sample codes, FMC- fishmeal control, FMP- fishmeal probiotic, SBMC- soybean meal control, SBMP- soybean meal probiotic, PlantMixC- PlantMix control and PlantMixP- Plantmix probiotic

6.5 Discussion

In the present study at both sampling points the intestinal LAB levels, were higher in the probiotic fed fish, compared to the control groups, in the SBM and PlantMix dietary groups. These LAB were identified as *P. acidilactici* in probiotic fed fish. LAB levels were increased by approximately 1 log value at week twelve compared to their levels at week five in the FMC fed fish and the PlantMix (control and probiotic) fed fish.

Vegetable meal, commonly used as a replacement for fish meal, could provide fermentable carbohydrates, such as oligosaccharides, which contain pentosans, cellulose and pectin. These substances may support the proliferation of different bacterial species in the intestinal tract. This may be the reason for the current results and explain why *P. acidilactici* levels were higher in the groups of rainbow trout fed vegetable diets. High levels of LAB were found in previous studies using this probiotic. For example, Ferguson *et*

al. (2010) also demonstrated that levels of *P. acidilactici* dominated in the probiotic group and these levels significantly higher compared with those groups of fish fed with a control diet of red Nile tilapia when fed *P. acidilactici* for a period of 32 days. In accordance with the present results, significant differences were found regarding the levels of *P. acidilactici* in the posterior digesta compared with the other intestinal tract regions at the end of the probiotic feeding period for rainbow trout (Chapter 4). Standen *et al.* (2013) found that the total cultivable levels in the digesta samples were not affected by the addition of *P. acidilactici*, while overall LAB levels were significantly higher in *P. acidilactici* fed tilapia. Low levels of LAB were detected only in sea bass larvae at 45 dph with low dosage of probiotic at 10^{-6} CFU g⁻¹, while high levels were detected at 40 dph with high dosage of probiotic at 10^{-7} CFU g⁻¹ (Lamari *et al.*, 2013). In addition, Skjermo and Vadstein (1999) reported that the colonization of probiotics in the gut of the host is likely to fluctuate depending on such factors including adhesion properties, attachment site, stress issues, dietary and rearing conditions.

In the present study, the ACC levels were not significantly different in the SBM fed fish than the FM fed fish. SBM has been extensively studied as a replacement for FM, and in recent years studies have started to assess its role in the modulation of the microbiota of fish. The present findings are in agreement with the study of Heikkinen *et al.* (2006) who reported from culture-dependent techniques that the dietary inclusion of SBM (hexane extracted; included at a dietary level of 45%) did not affect the gut microbiota of rainbow trout compared to the control group fed a FM diet. Similar results were reported when rainbow trout were fed dietary SBM for 16 weeks; no

significant differences in the culturable bacteria were found between the SBM and FM groups in any of the intestinal regions (Merrifield *et al.*, 2009b). In contrast, the present results are not in agreement with the study of Bakke-McKellep *et al.* (2007) who investigated the microbiota of Atlantic salmon fed either FM as the sole protein source, a diet containing 25% de-hulled, extracted and toasted SBM instead of FM and extruded wheat, or a FM diet which partially replaced extruded wheat with 75 g inulin kg⁻¹. The authors demonstrated that the highest bacterial population level was found in SBM fed fish followed by the FM group. The reason for the different results in the above- mentioned studies are not fully understood, but the difference in feeding period, the different SBM inclusion level, the different SBM characteristics and the different culture conditions could all be reasons for the disparity (Merrifield *et al.*, 2009b).

The expression of immune related genes, including IL-8 and TNF- α and cell stress related genes HSP70 and casp-3, in the posterior intestine were investigated in order to evaluate if *P. acidilactici* had an effect on the localized intestinal immune system of rainbow trout. Cytokines are proteins which regulate the local and systemic immune response through the induction, enhancement or inhibition of a number of immune-related genes. TNF- α as pro-inflammatory cytokines are mainly produced by macrophages/monocytes and T-lymphocytes, which act to initiate the immune response by attracting lymphocytes and leucocytes to the site of the infection or by activating the secretion of other cytokines (Mulder *et al.*, 2007). Pro-inflammatory cytokines, including IL-8 and TNF- α , are commonly used as immune biomarker genes that are up-/down-regulated in fish (Laing *et al.*,

2002; Standen *et al.*, 2013) and the former has been reported to induce in the early stage of an immune response, attracting leucocytes, including neutrophils and T-lymphocytes, to the site of the infection (Kim and Austin, 2006a; Overturf and LaPatra, 2006; Gómez and Balcázar, 2008). Granulocyte cells and T cells have been reported to play important role in the SBM-induced enteritis processes in the posterior intestine (Bakke-McKellep *et al.*, 2007), which can result in increased levels of inflammatory cytokines.

In the present study, dietary *P. acidilactici* did not significantly influence TNF- α mRNA levels; this is in contrast with the results reported in previous investigations applied to other probiotic bacteria in rainbow trout (Kim and Austin, 2006a; Panigrahi *et al.*, 2007; Pérez-Sánchez *et al.*, 2011b), Nile tilapia (Pirarat *et al.*, 2011) and this probiotic in rainbow trout (Chapter 5) and Nile tilapia (Standen *et al.* 2013). Standen *et al.* (2013) reported that *P. acidilactici* on stimulating the innate immune response via expression of TNF- α in Nile tilapia; the results demonstrated that TNF- α mRNA levels were up-regulated in *P. acidilactici*-fed tilapia, which was also the case four rainbow trout in Chapter 5.

The available literature indicates that a number of probiotics can effectively modulate the expression of the IL-8 gene in fish (Pérez-Sánchez *et al.*, 2011b; Pirarat *et al.*, 2011), including *P. acidilactici* applications to rainbow trout, as was observed in Chapter 4. In contrast with these findings, IL-8 mRNA levels in the current study were not up-regulated in rainbow trout fed *P. acidilactici* supplemented diets.

The discrepancies between the effects observed on TNF- α and IL-8 in the present chapter and those reported elsewhere, might be due to the different dietary ingredients used, different probiotic doses or duration of probiotic feeding.

HSP70 is involved in programmed cell death and in defense against stress, which results in protein unfolding and protein aggregation; in this case the expression of HSP70 is increased to allow cells to prevent/reduce elevated concentrations of damaged proteins (Garrido *et al.*, 2006). Caspases belong to a family of cysteinyl aspartate-specific proteases which are coordinators of apoptosis. Active caspases induce apoptosis through several mechanisms, such as activation of DNases, promotion of mitochondrial cytochrome *c* release via Bcl-2 family proteins, and by degradation of structural and regulatory proteins within the cell (Elmore, 2007). Numerous caspases have been identified, but caspases 3 (5 and 7), are recognized as important biomarkers of apoptosis due to their role as executioner, or downstream, caspases, which are responsible for the destruction of the cell (Cols Vidal *et al.*, 2008).

Contrary to the results presented in Chapter 4, the expression of the HSP70 gene was not affected by probiotic treatment in the present study. The present study demonstrated that intestinal HSP70 mRNA levels were however affected by the diets, with lower expression levels in the SBM and PlantMix fed fish than the FM fed fish. Previous studies have demonstrated that expression of these genes increase in response to different stresses, such as heat, irradiation and oxidative stress on the gut epithelium (Garrido *et al.*, 2006). Under normal conditions, the level of HSP70 mRNA expression

should be low in order to allow for constitutive cellular activities to proceed (Qian *et al.*, 2006). These authors reported that the up-regulation of the inducible HSP70, under various stress conditions, results in enhancement of the ability of stressed cells to cope with the increased misfolding of protein and inhibits apoptosis induced by a wide range of stimuli (Garrido *et al.*, 2006; Qian *et al.*, 2006). The reduced expression of HSP70 in the SBM and PlantMix fed fish, and the observation that the probiotic did not up-regulate HSP70 levels, is indicative that plant based diets and the probiotic did not have an irritant or stress- inducing effect.

It has previously been reported that salmonid distal enterocytes show elevated levels of casp-3 and subsequently elevated apoptosis during dietary SBM induced enteritis (Bakke-McKellep *et al.*, 2007). The down-regulation of casp-3 in the fish fed the probiotic supplemented diets, compared to the control diets at week 5 in the present chapter, is in agreement with the results reported in Chapter 4. This supports the hypothesis that the probiotic did not induce an enteritis effect even when used in diets containing high levels of ANFs. The large variation within the data set would indicate that more replicates were required in order for robust conclusions to be made.

Fish haematology profiles are reported to be useful tools for monitoring health (Ferguson *et al.*, 2010). In the present study at 12 weeks of probiotic feeding, leucocyte counts, Hb, PCV, MCV and MCHC were not affected by *P. acidilactici* treatment. These results are in accordance with the results presented in Chapters 4 and 5. Modulation of the immune system is regarded as one of the most important benefits of probiotics, which interact with

immune cells such as monocytes, macrophages and neutrophils to enhance nonspecific immune responses (Nayak, 2010a). Ringø *et al.* (2012) stated that lysozyme could be used as an indicator in fish to evaluate the immune system and disease resistance in response to internal and external factors. In the present study, serum lysozyme activity was significantly higher in the groups of rainbow trout fed FM and SBM diets supplemented with *P. acidilactici* compared to those groups fed control diets, which is in accordance with results presented in Chapters 4 and 5.

Serum lysozyme activity has been reported to be improved after *P. acidilactici* supplementation to the diet of red Nile tilapia (Ferguson *et al.*, 2010). Elevated levels of lysozyme activity could improve the defence of the host against bacterial diseases in fish fed probiotic supplemented diets (Nayak, 2010a). In the PlantMix feeding regime, lysozyme activity was not affected in the rainbow fed the *P. acidilactici* supplemented diet compared to the control group. In line with these findings, *P. acidilactici* failed to improve lysozyme activity in catfish (Shelby *et al.*, 2007) and tilapia (Standen *et al.*, 2013) fed practical diets (i.e. those with a diverse group of plant based ingredients).

Other studies, however, showed contradictions with regards to lysozyme activity in response to different probiotic bacteria. For example, lysozyme activity has been reported to be significantly higher in rainbow trout fed with *Lb. rhamnosus* (Panigrahi *et al.*, 2004) and *B. subtilis* (Merrifield *et al.*, 2010b). In addition, serum lysozyme activity has been significantly elevated in rainbow trout fed a diet supplemented with *Ca. divergens*, whereas

lysozyme activity was not affected by a *Ca. maltaromaticum* supplemented diet (Kim and Austin, 2006b). Moreover, serum lysozyme activity in brown trout fed a diet supplemented with *La. lactis* sp. *lactis* or *Leu. mesenteroides* was significantly elevated compared to the control group after 3 weeks (Balcazar *et al.*, 2007). Lysozyme activity was found to be elevated in rainbow trout fed diets supplemented with *B. licheniformis* + *B. subtilis* or *E. faecium* (Merrifield *et al.*, 2010a), whilst *Lb. rhamnosus* induced no such differences compared to control fed fish (Panigrahi *et al.*, 2005b). Ridha and Azad (2012) reported that some immunological parameters, including serum lysozyme activity, were significantly higher in groups of Nile tilapia fed *Bacillus amyloliquefaciens* or *Lactobacillus* spp. individually supplemented diet for 99 days compared to the control group. The variation in the above results may possibly be due to such factors including species, exposure period, dosage of diet supplementation and the type of prebiotics and probiotics used (Cerezuela *et al.*, 2011), and feed composition (Standen *et al.*, 2013). Higher lysozyme activity in the current study could indicate an increase of the resistance of fish to diseases, since lysozyme has anticancer, antiviral, antibacterial and opsonization properties (Heo *et al.*, 2013).

Despite the aforementioned beneficial effects, the present study demonstrated that *P. acidilactici* supplementation diets did not significantly affect the growth performance. The literature regarding the effect of *P. acidilactici* supplementation on growth performance of aquatic species is inconclusive. Similar to the present study, previous investigations with rainbow trout (Aubin *et al.*, 2005; Merrifield *et al.*, 2011; Ramos *et al.*, 2013) and tilapia (Ferguson *et al.*, 2010; Standen *et al.*, 2013) showed that *P.*

acidilactici supplementation did not improve growth performance. Similarly, these findings are in agreement with the results obtained from 132 days scFOS plus *P. acidilactici* fed salmon, which demonstrated that weight gain, feed conversion ratio and survival rate were not affected (Chapter 5). Contrary to these studies, it has been reported that probiotic applications of *P. acidilactici* beneficially affected the growth performance of Pollack (*Pollachius pollachius*) larvae (Gatesoupe (2002), Oscar (*Astronauts ocellatus*) (Safari and Atash, 2013) and the green terror (*Andinoacara rivulatus*) (Neissi *et al.*, 2013). It is possible that the contradictory growth results in these investigations are attributable to the dosage of bacteria, bacterial strain, different fish species, diet ingredients and experimental circumstances. Whilst the precise reasons for these differences in observations are not clear, it may also be possible that there was limited potential for growth improvement in the present study, where the fish were reared under good (near optimal) conditions.

6.6 Conclusions

This study confirmed that dietary *P. acidilactici* can survive the rainbow trout transit through the GIT and reduced intestinal Casp-3 gene expression (at week 5). Further, serum lysozyme activity was enhanced in the probiotic fed fish which may contribute to enhanced resistance against disease. Despite these benefits, no detrimental effects of growth performance were observed. Further investigations should be conducted to ascertain the role of *P. acidilactici* as a stimulating agent which provokes the immune system to alleviate stress symptoms. Future work must consider the investigation of

histological changes of the GIT after diets feeding (inflammation symptoms)
using both SEM and LM.

Chapter 7

Chapter 7: General discussion and conclusions

Cultivation-based methods have been found to be insufficient to study the GIT microbiota of fish because of the high level of non-cultivable microbes present. Therefore, in the present body of research a combination of culture-dependent and culture-independent techniques including, clone libraries analysis, PCR-DGGE, qPCR, direct counts and FISH, were used to evaluate the potential impact of *P. acidilactici* on the intestinal microbial community of salmonids. In order to broaden our understanding regarding the role of microbiota, including probiotics microorganisms, in the host GIT many investigations have been conducted during last two decades.

In spite of the plethora of literature regarding the microbiota of salmonids, information on the microbial community of brown trout is very scarce. The aim of Chapter 3 therefore, which is the first report on the autochthonous gut microbiota of brown trout, was to identify and enumerate the bacterial composition of the GIT of brown trout using 16S rRNA gene analysis and PCR-DGGE. Culture-based assessment in Chapter 3 demonstrated that although posterior mucosa had a trend towards higher levels of the ACC and LAB, no significant differences between the regions were observed. The relative abundances of the species identified by 16S rRNA gene sequence analyses did not differ significantly between gut regions. Sequence analysis identified a range of phyla including Proteobacteria, Firmicutes, Bacteroidetes, with the most predominant bacteria from ACC being *C. freundii*. LAB populations on MRS media were exclusively identified as *Ca. maltaromaticum*. However, a different picture of the microbial community was provided by DGGE analysis where five OTUs (out of eleven total OTUs which

sequenced) were identified as members of the LAB group, including *Streptococcus* sp., *La. lactis* subsp. *lactis* and *W. cibaria*, but no carnobacterial bands were detected. Further, *C. freundii* was not detected from the sequenced OTUs. The fact that these dominant culturable species' were not detected as part of the dominant total bacteria from DGGE analyses clearly highlights the need to utilise multiple techniques to investigate the gut microbiota of fish. The reasons for the discrepancy between approaches might be related to inherent flaws in the respective methods. For example, in DGGE, each band may not represent one bacterial strain and may in fact be composed of several different strains with the same/similar denaturing properties which migrate to the same point in the gel and this can hinder sequencing of bands/OTUs. In addition, the culture-based approaches favour certain bacteria, which are well adapted to utilizing the nutrient sources in the media and grow quickly under the specific cultivation conditions; this can bias the results. Highlighting this point, different genera were identified in when using 16S rRNA gene sequencing of cultivated isolates and when using sequencing of DGGE bands, in Chapter 3. Data obtained from DGGE showed trends towards progressively higher diversity, richness and the number of OTUs from the pyloric caeca to the posterior intestine. Conversely, the culturable bacterial levels progressively decreased from the pyloric caeca to the posterior mucosa.

These findings provide an important basis for our understanding of the GI microbiota of brown trout; however, further investigation of the allochthonous community of brown trout is required. The use of a 16S rRNA gene clone library analysis and/or NGS in future work is recommended to provide

information on the abundance of the populations comprising the microbial community. More quantitative molecular methods such as direct counts (i.e. with nucleic acid staining), FISH and qPCR would also be valuable.

In the latter chapters, the emphasis moved on to the investigation of the microbial communities of other salmonid species and to elucidating the effects of different dietary ingredients and feed additives on these microbial communities. Specifically, these investigations focused on feed additives such as probiotics and prebiotics as well as important dietary protein sources used in aquafeeds to replace fishmeal.

Chapter 4 assessed the impact of a probiotic (*Pediococcus acidilactici*) supplemented diet on rainbow trout gut microbiota and the subsequent impact on host health status. Culture-based methods revealed high ($> \log 3.5$ CFU g^{-1}) levels of *P. acidilactici* present in the digesta and mucosa of the probiotic fed fish. Interestingly, clone library analysis revealed that *P. acidilactici* represented $\leq 1\%$ of the total (cultivable and non-cultivable) bacterial populations. Despite this low presence, the constructed clone libraries demonstrated that *P. acidilactici* could still influence the microbial community composition. The probiotic feeding reduced the abundance of certain bacteria, such as *Aeromonas* spp., *Acinetobacter* spp. and *Staphylococcus pasteurii* in some intestinal regions. Similarly, the DGGE analysis also revealed that the probiotic could modulate the total community structure and composition despite a relatively low level presence. This interesting observation requires more attention, and NGS would allow for a

more accurate estimation of the probiotic abundance relative to the total community composition.

The probiotic driven microbial modulations in the GIT led to host improvements in terms of the elevated intestinal goblet cell levels and modulated gene expression profiles, in the probiotic group. Real time PCR results demonstrated that IL-1 β , IL-8 and IgT mRNA levels were up-regulated in the *P. acidilactici* fed fish in the posterior intestine at both sampling points compared to the control group. In the anterior intestine, IL-8 was up-regulated at both time points while IL-1 β and IgT were up-regulated at week four only. IL-1 β , IL-8 and TNF- α are pro-inflammatory cytokines which regulate the activity of immune cells in response to colonization or invasion of bacteria and/or their cell surface components (Mulder *et al.*, 2007). IL-10 was down-regulated in the anterior and posterior of the intestine at week four. IL-10 suppresses inflammatory responses and prevents tissue damage by regulating T-lymphocytes and natural killer cells and down-regulating free radical and TNF- α production. The concomitant elevation of pro-inflammatory cytokines and the decrease in the anti-inflammatory IL-10 is suggestive of an elevated inflammatory status. It is hypothesised that this inflammatory status is not excessive to the extent that it is detrimental to the host given the observations of normal and healthy mucosal appearance from histological analysis. In addition, PCNA, HSP70 and casp-3 mRNA levels were down-regulated in the probiotic group compared to the control group in all samples at two and four weeks; HSP70, casp-3 and PCNA contribute to cyto-protective effects, apoptosis or programmed cell death and cellular repair. The down-regulation may be indicative of a low level of stress at the cellular

level and better epithelial integrity in the probiotic group. It is likely therefore that the induced pro-inflammatory response, with the elevations of goblet cells, and the increased expression of IgT mRNA could improve intestinal immunity and may aid in defending against pathogenic insult at the mucosal level. Further studies are required to validate this hypothesis.

At the systemic level, probiotic supplementation elevated serum lysozyme activity which may also provide benefits against infections. Further investigations are required and should study selected humoral and cellular immune markers such as complement activity, phagocytosis activity, total Ig, acute-phase proteins (APPs) and disease challenge models.

After the cessation of probiotic feeding, the probiotic population was able to persist for at least 24h. These findings are not in agreement with study of Ferguson *et al.* (2010) using the same probiotic in red Nile tilapia, which reveal recovery for at least 17 days. The contradictions in the persistence duration of the probiotic within the intestine of these species are likely to be reflective of the different conditions in the GIT. The GIT of these species differ considerably with respect to the hosts selective pressures such as differences in host immunity, gastric juices (bile, pH, digestive enzymes), feed components (which are the probiotic substrates), temperature (i.e. rearing temperature), salinity (freshwater and marine fish) and the differing indigenous microbiomes in which the probiont has to compete to establish a presence. Perhaps the most obvious influencing factor is the rearing temperature (15 °C in trout versus ca. 25 -26 °C in tilapia).

Chapter 5 assessed the gut microbiota of Atlantic salmon, using a variety of approaches, and then investigated the effect of *P. acidilactici* and scFOS (as a potential synbiotic) on the microbial communities. The effects of potential microbial modulation were assessed in the context of host health and growth performance. Unfortunately, due to reduced feeding in the last week at the feed trial unit at the end of the study (day 132), reliable data was constrained to the first sampling point (day 63). It was observed that the simultaneous application of *P. acidilactici* and scFOS reduced total bacterial cell numbers in all intestinal regions (except the anterior digesta) compared to the control group at day 63. PCR-DGGE demonstrated that the bacterial richness, diversity and the numbers of OTUs were significantly higher in the anterior digesta of the synbiotic fed fish compared to the control group.

IELs were found to be significantly higher in the synbiotic group compared to the control group in the posterior intestine at both sampling point. These findings are in accordance with some previous studies which have reported elevated IELs in the intestine of *P. acidilactici* fed fish (Harper *et al.*, 2011; Standen *et al.*, 2013) and are contrary to some studies which have shown no effect on intestinal IELs (Chapter 4; Ferguson *et al.*, 2010). The reasons for these contradictory results are not clear, but are likely to be due to the different probiotic doses used, the different rearing conditions and the different dietary ingredients; all of these factors may influence the indigenous gut microbiota which will have a considerable effect on the efficacy of probiotics and prebiotics.

A significant up-regulation in the levels of immune genes (IL-1 β , TNF- α , IL-8, TLR3 and MX-1) in the intestinal regions (at both sampling points) was confirmed by RT-PCR in fish fed the synbiotic diet. These results were consistent with the findings reported in Chapter 4 for the same regions of rainbow trout intestine and again, may indicate an improved intestinal mucosal immune status. These observations correlate with the elevated IELs levels observed in the intestine since the inflammatory cytokines initiate inflammatory signals, regulate the phagocytes cells (either the resident or arriving groups) to destroy the invading pathogen and to initiate the specific immune response by regulation of antigen presenting cells (APCs) migration (Wang and Secombes, 2013). In keeping with Chapter 4, the histological evaluation of the GIT of salmon at the mid- and end sampling points of the trial period revealed that the intestinal tissue was in good condition and conformed to that of the control group. Indeed, the mucosal fold (villi) length was significantly increased in the synbiotic group of the anterior mucosa compared to the control group at the mid sampling point. Further studies are required to identify the mechanisms behind this host benefit, but FOS and other prebiotics can be fermented by bacteria, such as lactobacilli and clostridia and results in the production of short chain fatty acids (SCFAs; e.g. acetate, propionate, butyrate), that can be absorbed by fish gut epithelial cells (Mountfort *et al.*, 2002) and might play an important role in increasing the villus height (Pelicano *et al.*, 2005).

At the systemic level, synbiotic fed salmon displayed significantly elevated serum lysozyme activity compared to the control group. This result was consistent with the findings reported in Chapter 4. Despite the elevated

immunological status the final weight, FCR, SGR and TGC were not significantly affected by synbiotic diet in this chapter. Unfortunately, given the logistical difficulties it was not possible to conduct treatments with the individual use of the probiotic and prebiotic. Future studies should address this issue and elucidate which feed additive (probiotic or prebiotic), or both, were the driving force behind the host benefits. It has been reported that factors including the fish species, rearing conditions, feeding duration as well as the types of prebiotics and probiotics used can significantly affect the efficacy of synbiotics (Cerezuela *et al.*, 2011). NGS of the GIT microbiome and the quantification of SCFAs in the salmon GIT should be investigated utilized where possible in future studies in order to give a clear picture for the microbial community abundance and activity in Atlantic salmon.

Chapter 6 investigated the potential role of *P. acidilactici* inclusion, into different dietary formulations, on the health status of rainbow trout. The ACC were similar to the findings at the mid sampling point in Chapter 5. At both sampling points, with exception of fish fed FM diet at week twelve, LAB levels were higher in fish groups fed plant based diets supplemented with *P. acidilactici* compared to the respective control diets. LAB from the probiotic fed fish were confirmed as *P. acidilactici* by 16S rRNA gene analysis. In agreement with findings reported in Chapters 4 and 5, serum lysozyme activity was significantly higher in fish fed containing *P. acidilactici* (i.e. in FM and SBM diets), compared to the non-probiotic controls. Previous studies have reported that red Nile tilapia serum lysozyme activity has been improved after *P. acidilactici* supplementation (Ferguson *et al.*, 2010), whilst on the contrary, Standen *et al.* (2013) reported no effect on *P. acidilactici*

application on red Nile tilapia lysozyme activity and Shelby *et al.* (2007) reported no effect on Channel catfish serum lysozyme activity.

The variation in the above results might possibly be due to such factors including fish species, exposure period, dosage of probiotic supplementation and feed composition. Higher lysozyme activity in the current research could indicate an increase the resistance of salmonids to diseases, since lysozyme has anticancer, antiviral, antibacterial and opsonization properties (Heo *et al.*, 2013). Further studies with both rainbow trout and other important aquaculture species may help to elucidate the effect of *P. acidilactici* on fish immunological and haematological profiles. Contrary to the results of Chapter 4, HSP70, TNF- α and IL-8 mRNA levels were not affected by *P. acidilactici* in Chapter 6. In line with Chapter 4, casp-3 levels were reduced (at week 5). One consideration that must be acknowledged when drawing conclusions from these observations is the limited number of replicates available, due to limited fish availability.

The growth performance and feed utilisation were not affected by probiotic provision and these findings are in agreement with those of Atlantic salmon fed a combination of *P. acidilactici* and scFOS in Chapters 5. The literature regarding the effect of *P. acidilactici* supplementation on growth performance of aquatic species is inconclusive. Similar to the present study, previous investigations with rainbow trout (Aubin *et al.*, 2005; Merrifield *et al.*, 2011; Ramos *et al.*, 2013) and tilapia (Ferguson *et al.*, 2010; Standen *et al.*, 2013) showed that *P. acidilactici* supplementation did not improve growth performance. However, *P. acidilactici* has beneficially affected the growth

performance of Pollack larvae (Gatesoupe, 2002), green terror (Neissi *et al.*, 2013) and Oscar (Safari and Atash, 2013). The differences in probiotic dosage, feeding period, feeding administration, temperature, fish species and the gut microbiota could be reasons for the differences reported with respect to the effect of *P. acidilactici* on fish growth performance (Soccol *et al.*, 2010). An additional factor that could be considered is that there may be limited potential for growth improvement when the fish are reared under good (near optimal) and stable conditions such as those in laboratory scale aquaria. It has been hypothesised that there might be more scope for improving these parameters under poor rearing conditions or on farm scale trials (Merrifield *et al.*, 2010c). However, further research is needed to investigate the effects and potential benefits of these bacteria on the growth of the aquatic animals. Future work must consider the investigation of histological changes of the GIT when using challenging diets (i.e. high levels of plant proteins and ANFs), and probiotic applications, using both SEM and LM. In addition, the influence of the dietary formulation (i.e. the ingredients used and their ratios) on the efficacy of probiotics, in terms of their viability and activities is highly recommended in order to develop appropriate diets for use as a delivery mechanisms for probiotics.

Conclusion

This programme of research revealed complex GIT microbiomes present in three salmonids species. Further, it was demonstrated that *P. acidilactici* has the potential to populate the gut of salmonids and compete with other microbial components of the GIT, modulate the microbial components of the GIT and improve intestinal morphology and gene expression. In addition,

some whole system responses, such as serum lysozyme activity, may also be improved without impairing growth performance. However, these benefits are influenced by a number of factors (such as dietary formulation) and further analyses are required to improve our understanding of how *P. acidilactici* modulates the gut microbiota composition; this should be pursued with the application of NGS technologies. Further, it is important that future studies focus not only on microbial composition, but also the activity of the modulated community by assessing SCFAs levels and microbial gene expression.

The determination of the impacts of intestinal microbial modulation on the host localised immune status should be further evaluated by the investigation of the expression of genes involved in cytokine expression pathways (e.g. TLRs, adaptor proteins, transcription factors, and the final cytokines expressed). The application of proteomic techniques such as two dimensional gel electrophoresis (2DE) and mass spectrometry (MS) would allow for the direct detection and identification of proteins modulated by the probiotics. A deep understanding of the interaction of probiotic with various components of the host's immune system is required, and the mechanisms underlying their immunostimulatory effect needed further investigation. Finally disease resistance studies must investigate the mechanisms by which gut microbiota interacts with the mucosal intestine in health and disease cases. Further work is required to be optimising the dose, route, frequency, and the long- and short-term effects of *P. acidilactici* on the salmonids.

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Appendix 1: Protocol of DNA extraction

Alternative DNA extraction from complex mixed samples

- Wear gloves, use new/ filter tips
- Ensure awareness of relevant COSHH/ Risk regulations
- Work on ice where possible
- Labelling all tubes in advance will save time!
- Ensure isopropanol is in the -20 °C freezer

Lysis

1. Use up to 350 mg of sample and add 500 µl of lysozyme (fresh, 50mg/ml in TE). Incubate minimum 30 min at 37 °C.
2. Add 800 µl of Buffer ASL and vortex until thoroughly mixed.
3. Heat the suspension for 10 min at 90 °C.
4. Vortex for 5 secs and centrifuge for 1 min/ max speed (4800g).

Inhibitor removal

5. Place 800 µl of the supernatant into an 1.5 mL MTCs and add half an Inhibitex tablet. Vortex immediately until suspended. Stand for 1 min. (process tubes in pairs)
6. Centrifuge for 3 min and pipette all of the supernatant into a new tube. Retain the remaining sample for future extraction if required.
7. Centrifuge for 3 min.

Protein removal

8. Place 20 µl of Proteinase K into a fresh 1.5 mL MTC and place 400 µl of the supernatant into this tube.
9. Add 400µl of Buffer AL and vortex.
10. Incubate at 70°C for 60 min.

Phenol Chloroform clean-up

Wear goggles. Perform 11-12, 14 in fume hood.

11. Carefully pour the entire sample into a 15 ml falcon tube and add an equal volume of ice cold Tris-buffered phenol solution. Mix by hand and leave on ice for 10 minutes.

12. Add an equal volume of chloroform/ isoamyl alcohol (24:1) and mix.
13. Centrifuge 3000g 5 mins in D301.
14. Carefully pipette off the aqueous layer and place in new 1.5 ml Eppendorf tube. Discard the organic layer into appropriate waste container.

Precipitation

15. Add 400 μ l ice-cold isopropanol. Vortex and place in -20°C freezer for 10 min-overnight. (recovery may be enhanced by addition of 0.3/ 0.5M Na Acetate)
16. Centrifuge 14K for 30 minutes (at 4°C)
17. Carefully pipette off supernatant and discard
18. Slowly add 1000 μ l 70% molecular grade ethanol. Pipette up and down carefully, and discard.
19. Repeat (18)
20. Dry pellet for 5 minutes maximum, ideally under vacuum
21. Resuspend overnight at 4°C using 30 μ l of either MGW or 1/10 TE.
22. Check yield on Nanodrop/ Nanovue/ agarose gel before progressing.