2009

Evaluation of selected probiotics and bovine lactoferrin as feed supplements for rainbow trout (Oncorhynchus mykiss Walbaum) for applications in aquaculture

Merrifield, Daniel Lee

Evaluation of selected probiotics and bovine lactoferrin as feed supplements for rainbow trout (*Oncorhynchus mykiss* Walbaum) for applications in aquaculture

A thesis submitted in fulfilment of the

requirement for the degree of

DOCTOR OF PHILOSOPHY

The University of Plymouth

School of Biological Sciences

Faculty of Science

By Daniel Lee Merrifield

Feb 2009

Copyright Statement

This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognise that the copyright rests with the author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.

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.

The study was funded by BASF AG (Ludwigshafen, Germany) and all investigations reported in this thesis conform to the UK 1986 Animal Scientific Procedures Act under the specific Project Licence (PPL #30/2135). This was also in compliance with the University of Plymouth Ethical Review Committee.

Word count: 47 811

Signed.....D. L. Merrifield.....

Date.....11/02/09.....

Contents

List of abbreviations

2B	BioPlus 2B diet
AD	Anterior digesta
AM	Anterior mucosa
AU	Arbitrary units
BLAST	Basic local alignment search tool
CFU	Colony forming units
DAPI	4',6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
EDTA	Ethylenediaminetetraacetic acid
Ef	*Enterococcus faecium* diet
EM	Electron microscopy
FCR	Feed conversion ratio
FM	Fishmeal
FOS	Fructo-oligosaccharides
GOS	Galacto-oligosaccharides
K-facter	Condition factor
LAB	Lactic acid bacteria
Lf	Lactoferrin
LyoLo	Lyophilised low level *P. acidilactici*
LyoHi	Lyophilised high level *P. acidilactici*
MOS	Mannan-oligosaccharides
MRS	de Man, Rogosa and Sharpe
MS222	Tricane methyl sulphonate
NRC	National Research Council
S&B	Slanetz and Bartley medium
PA	*Pediococcus acidilactici* diet
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PD	Posterior digesta
PER	Protein efficiency ratio
PM	Posterior mucosa
RISA	Ribosomal intergenic spacer analysis
RNA	Ribonucleic acid
SBM	Soybean meal
SEM	Scanning electron microscopy
SCFA	Short-chain fatty acid
SGR	Specific growth rate
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TSA	Tryptone soy agar
TSB	Tryptone soy broth
VC	Viable count
VegLo	Vegetative low level *P. acidilactici*
VegHi	Vegetative high level *P. acidilactici*

Commonly discussed bacterial genera abbreviations

A. *Aeromonas*
B. *Bacillus*
C. *Carnobacterium*
E. *Enterococcus*
Es. *Escherichia*
L. *Lactobacillus*
La. *Lactococcus*
Leu. *Leuconostoc*
P. *Pediococcus*
Ps. *Pseudomonas*
Psy. *Psychrobacter*
V. *Vibrio* (including *Listonella* in the case of *Listonella anguillarum*, which will be referred to as *V. anguillarum* within this document)

List of tables

List of figures

List of plates

Acknowledgements

I would like to thank all the individuals who have helped and supported me during the completion of these investigations.

Firstly, I would like to thank Prof. Simon Davies, my director of studies, for giving a young enthusiastic student the opportunity and continued guidance to undertake this study. Dr Graham Bradley, my second supervisor, for his academic as well as technical support.

I am especially indebted to Dr Colin Munn for his guidance and supervision during my undergraduate degree which eventually led me into postgraduate research.

I am also indebted to Dr Remi Baker, my industrial supervisor during his time at BASF, for providing sponsorship, advice and generosity at the pub.

I would like to thank BASF AG (Ludwigshafen, Germany) for funding the project. I also thank Lallemand Inc. for the provision of Bactocell® and Aller Aqua for their generous supply of diets used throughout the studies.

I am of course thankful to the Plymouth Ponies, my friends and colleagues at the University of Plymouth: Dr James The Trifle Reeves, Arkadios Sonny Dimitroglou, Dr Jerome Ja Rome Laporte, Sebastian Jock Rider, Dr Stuart Maudling, Matthew Owen, Mark Rawling, Paul Waines and Siddiq El-Ajnaf, for helping maintain my sanity (in part at least) during the many highs and lows which comprise a PhD.

I also extend my thanks to the technical staff that have always been willing to help: Glenn Harper, Mike Hocking, Robert Serwata, Kelly Serwata, Liz Folland, Nikki Blatchford, Rick Preston, Ben Eynon, Michele Kiernan and Sarah Jamieson.

I would like to thank all my aunts, uncles, grandparents and cousins for conscientiously fostering my interest and enthusiasm to learn throughout my childhood. A special acknowledgement is reserved for my parents and my brother for their unconditional love and support (especially financial!) throughout my student years.

This thesis is dedicated to the memory of Alec Salaun, who helped spark my interest in the natural sciences.

Evaluation of selected probiotics and bovine lactoferrin as feed supplements for rainbow trout (*Oncorhynchus mykiss* Walbaum) for applications in aquaculture

Abstract

A series of investigations were carried out to assess the intestinal microbiota of rainbow trout and the potential applications of probiotics and bovine lactoferrin (Lf).

Farm and aquarium reared rainbow trout were examined with specific emphasis on the autochthonous microbial communities. Culture-based, culture-independent and electron microscopical investigations revealed mixed, complex microbial communities in all intestinal regions. DGGE based analysis revealed unique species present either only as allochthonous populations or autochthonous populations. 16S rRNA sequence analysis allowed species level identification of a range of isolates, many of which have not been identified from the rainbow trout digestive tract previously.

Two further investigations were carried out to assess the potential of using commercial probiotics and bovine Lf on growth, feed utilisation, health and intestinal colonisation of rainbow trout. Standard commercial diets were supplemented with *B. subtilis*, *B. licheniformis* and *Enterococcus faecium* either singularly or synergistically. When comparing the findings of the joint study it can be concluded that the application of probiotics with rainbow trout, and likely other finfish species, is highly complicated. Full intestinal replacement of indigenous microbiota is not likely to be a good idea when using *E. faecium*; the results indicate that a synergistic relationship with the indigenous microbiota is likely to be involved in providing host benefits. *Bacillus* probiotics only appeared to be effective at high intestinal levels indicating that a synergistic relationship with the indigenous microbiota may not be as important. The joint study also indicates that it is not always possible to reproduce probiotic benefits even when using the same probionts, the same fish species and similar rearing conditions. Thus, the physiological status of the fish and the indigenous microbiota are likely to play an important role in the outcome of probiotic administration.

A subsequent trial was conducted to evaluate *Pediococcus acidilactici* as a probiotic for rainbow trout. The experiment was conducted to supplement the diet with either vegetative cells or lyophilised powder (as commercially provided). Despite successful intestinal colonisation, irrelevant of supplementation form, few significant benefits were observed. SEM of the posterior mucosa revealed a localised colonisation pattern of *P. acidilactici* between the mucosal folds similar to the observed indigenous microbiota from the farmed fish. This revelation led to a further trial to investigate the nature of probiotic colonisation through the gastro-intestinal tract using electron microscopy. The study confirmed the high colonisation of *P. acidilactici* on the epithelium of the anterior intestine and posterior intestine. However, it was not possible to observe such colonisation with *Bacillus* spp. or *E. faecium*; despite culture-based results to the contrary. It is likely that the true mucosal colonisation may sometimes be confused with colonisation of the mucus layer as opposed to actual attachment to the epithelium itself. Therefore, it is crucial to utilise electron microscopy in order to confirm epithelial colonisation.

The nature of both the indigenous microbiota and the application of probiotics appears to be more complicated than previously thought and continued research is clearly warranted.

Chapter 1. Introduction

Aquaculture

The aquaculture industry represents one of the fastest growing food production sectors worldwide. Over the last four decades the aquaculture industry has been expanding at an average of 8.8% per annum, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production (FAO 2006). Rainbow trout (*Oncorhynchus mykiss* Walbaum) are a highly resilient, fast growing species which have become economically important worldwide, particularly in Europe. Total European production of rainbow trout exceeded over 272 706 tons in 2005, second only to Atlantic salmon (*Salmo salar*) (FAO FISHSTAT 2008). Despite such high levels of production, rainbow trout aquaculture has faced many challenges, some of which continue to be problematic.

Early salmonid farming under intensive conditions was continually set back by large scale mortalities and heavy economic losses, which even threatened the survival of the industry. It should not have come as a surprise, as the very economics that profitable aquaculture practices demand, are the very same factors that cause stress and increase exposure to disease. Elevated stress may increase susceptibility to disease and enhance transmission of pathogens (Hunter *et al.* 1980). Such contributory factors include rearing at high population densities, handling, confinement and improper husbandry. Good husbandry is unfortunately not enough to prevent many diseases. Disease brought about from exotic infections may well be prevented if good husbandry is practiced, but many infections are caused by indigenous potential pathogens which are opportunistic when fish become susceptible (Austin & Austin 1999; Dalsgaard & Madsen 2000).

Among the main culprits that contributed towards such heavy economic losses are bacterial diseases such as vibriosis and furunculosis. Vibriosis is caused by several *Vibrio*

spp., including *V. anguillarum*, *V. ordalii*, *V. alginolyticus*; *V. anguillarum* was the first to be identified and is generally regarded as the most prolific vibriosis causing agent (Schiewe 1981). *A. salmonicida* is the aetiological agent of furunculosis which is perhaps one of the most economically damaging agents of salmonid farming. These species are often indigenous to fish and one of the routes of infection is thought to be the gastro-intestinal tract (Austin & Austin 1999; Dalsgaard & Madsen 2000; Ringø *et al.* 2007). In fact the gastro-intestinal tract is thought to be a potential route of entry for many of the most destructive fish pathogens and it is suggested that the indigenous gut microbiota plays a role as a defensive barrier (Ringø & Birkbeck 1999; Holben *et al.* 2002; Birkbeck & Ringø 2005; Ringø *et al.* 2007; Salinas *et al.* 2008).

Another key issue regarding the sustainability of rainbow trout farming, as well as the aquaculture industry as a whole is the demand it places on wild fish stocks. This increasing demand fuels the never ending increase in fishmeal prices. Aquafeeds are one of the most costly outlays for the fish farmer; with the current EU ban on antibiotic growth promoters (Regulation 1831/2003/EC) assessment of novel alternatives is a high priority. Any alternative feed additive/supplement that could potentially stimulate growth and health performance would be of great economic importance. The intestinal microbiota of fish is likely to be involved in both health and nutrition of the host.

Intestinal microbiota of fish

In contrast to the intestinal microbiota of endotherms, which is often dominated by Gram-positive bacteria, most notably lactic acid bacteria [LAB] (Tucker & Taylor-Pickard 2004), Gram-negative bacteria generally seem to dominate the intestinal tract of fish (Cahill 1990). The composition of fish intestinal populations also appears to be more transient and variable than that of endothermic animals. Fish intestinal microbiota is

dependent on: the developmental stage, water chemistry, temperature, salinity, stress, dietary components and the microbiota of the rearing environment (Cahill 1990; Ringø *et al.* 1995; Ringø & Gatesoupe 1998; Ringø & Birkbeck 1999; Gatesoupe 1999; Ringø *et al.* 1999). Many studies have been undertaken to profile the microbiota of both marine and freshwater fish and the several papers review the findings (Trust *et al.* 1979; Cahill 1990; Ringø *et al.* 1995; Ringø & Gatesoupe 1999; Ringø & Birbeck 1999). Initial beliefs were that fish microbiota were either aerobic or facultatively anaerobic, however, obligate anaerobes have since been identified in many species including: carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), tilapia (*Oreochromis niloticus*), Ayu (*Plecoglossus altivelis*), Japanese eel (*Anguilla japonica*), Channel catfish (*Ictalurus punctatus*), oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*), southern flounder (*Paralichthys lethostigma*) and rainbow trout (Trust *et al.* 1979, Sakata *et al.* 1980; Sugita *et al.* 1985; Sugita *et al.* 1988; Cahill 1990; Sugita *et al.* 1997; Ramirez & Dixon 2003; Saha *et al.* 2006).

Despite a large number of investigations, the significance of the gut microbiota is not fully understood with regards to host health and nutrition. As well as providing a defensive barrier, the gut microbiota produces a range of digestive enzymes and vitamins which likely contribute towards host digestive function (Rimmer & Weibe 1987; Moriarty 1990; Sugita *et al.* 1996; Sugita *et al.* 1997; Sugita *et al.* 1998; Ringø & Birkbeck 1999; Holben *et al.* 2002; Ramirez & Dixon 2003; Birkbeck & Ringø 2005; Chabrillon *et al.* 2006; Ringø *et al.* 2007; Goldschmidt-Clermont *et al.* 2008; Salinas *et al.* 2008). As a result, much work has focused on modifying and stabilising indigenous gut populations with novel feed additives such as probiotics and prebiotics. In order to explore the full potential of probiotics/prebiotics we must first have a clear, comprehensive understanding of the indigenous gut microbial populations.

Indigenous microbiota of salmonids

A wide range of bacterial genera have been identified within the intestinal tract of salmonids, these include: *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Agrobacterium*, *Bacillus*, *Brevibacterium*, *Carnobacterium*, *Clostridium*, Cornyeforms, Enterobacteriaceae, *Enterobacter*, *Flavobacterium/cytophaga*, *Kurthia*, *Lactobacillus*, *Leuconostoc*, *Microbacterium*, *Micrococcus*, *Photobacterium*, *Pseudomonas*, *Staphylococcus*, *Streptomyces* *Vibrio* and *Xanthomonas* (Trust & Sparrow 1975; Yoshimizu *et al.* 1976; Ringø 1993; Ringø & Strom 1994; Ringø *et al.* 1995; Ringø *et al.* 1997; Ringø *et al.* 2000).

Typically *Aeromonas, Pseudomonas,* Enterobacteriaceae and *Lactobacillus* are among the dominant genera isolated from the digestive tract of Arctic charr, *Salvelinus alpinus* (Ringø *et al.* 1993; Ringø *et al.* 1997; Ringø & Strom 1994; Ringø & Olsen 1999). Similarly, Chum salmon (*Oncorhynchus keta*) also have high populations of *Pseudomonas* and *Aeromonas* along with high numbers of *Bacillus* spp. (Trust 1975). *Pseudomonas* and *Aeromonas* were also found to be the dominant genera in the Masu salmon (*Oncorhynchus masou*) digestive tract, but high levels of *Achromobacter* (now differentiated as either *Moraxella* or *Acinetobacter*) were also found (Yoshimizu *et al.* 1980). Ringø *et al.* (2000) identified culture-based isolates from the intestinal mucosa of Atlantic salmon (*Salmo salar*). Approximately 30% of the isolates were identified as LAB, predominantly *Carnobacterium* spp. (~29%), *Streptococcus* spp. and *Lactobacillus* spp. (~1%). The remaining microbial population was composed of *Brevibacterium*, *Microbacterium*, *Micrococcus*, *Staphylococcus*, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Cytophaga/Flexibacter*, *Moraxella*, *Photobacterium*, *Pseudomonas* and *Xanthomonas* spp.. More recently, identification to species level of isolates from several salmonid species has been possible using 16S rRNA sequence analysis and BLAST.

Carnobacterium piscicola, *Microbacterium oxydans*, *Microbacterium luteolum*, *Staphylococcus equorum*, *Acinetobacter lwoffi*, *Psychrobacter faecalis* and *Psychrobacter glacincola* have been isolated from the hindgut of Atlantic salmon fed a fishmeal based diet (Ringø *et al.* 2006a). Recently, Hovda *et al.* (2007) concluded that *Lactobacillus* spp., *Lactococcus* spp., *Bacillus* spp., *Acinetobacter* spp., *Pseudomonas* spp. and *Vibrio* spp were the dominant organisms isolated from Atlantic salmon but *Photobacterium phosphoreum* and *Psy. glacincola* were also isolated. This study was particularly insightful as the authors demonstrated that culture-based and culture-independent analysis of gut microbiota provided contradictory results. In a study evaluating the effects of dietary protein, Ringø *et al.* (2008) isolated *Gelidibacter salicanalis*, *Pseudoalteromonas elyakovii*, *Psychrobacter* spp. (*Psy. aquimaris*, *Psy. cibarius*, *Psy. fozii*, *Psy. maritimus*, *Psy. okhotskensis*, *Psy. psychrophilus*), *Arthrobacter bergeri*, *Arthrobacter psychrolactophilus*, *Arthrobacter rhombi*, *B. pumilus*, *B. subtilis*, *Exiguobacterium* spp., *Microbacterium oxydans*, *Planococcus maritimus*, *Sporosarcina ginsengisoli* and *C. inhibens* from the intestine of Atlantic salmon. However, the dominant species isolated was *Psy. cibarius*.

A. media, *A. salmonicida*, *A. sobria*, *Buttiauxella agrestis*, *C. maltaromaticum*, *Hafnia alvei*, *Moellerella wisconsensis*, *Serratia grimesii*, *Y. kristensenii and Y. mollaretii* have been isolated from the digestive tract of river trout, *Salmo trutta fario* L. (Skrodenyte-Arbaciauskiene *et al.* 2006).

The dominant intestinal microbiota of farmed trout has been demonstrated to be culturable (Spanggaard *et al.* 2000; Huber *et al.* 2004). Spanggaard *et al.* (2000) determined that that on average, 50% agreement was found between culture-based analysis using TSA and direct counts. Huber *et al.* (2004) also reported that, in most cases, culture-based analysis

accounted for between 50 - 90% of the rainbow trout gut microbiota. These values must be considered as conservative estimates, as direct counts (using DAPI) provide data for total counts not viable counts. These studies indicate that culture-based analysis is a useful, quantitative tool, for investigating trout gut microbiota. Investigations have focused on the characterisation of rainbow trout gut microbiota have demonstrated that in general the dominant bacterial population appear to belong to the β and γ subclass of Proteobacteria, namely the Enterobacteriaceae, Pseudomonadaceae and Vibrionaceae, but many Gram-positive bacilli have also been identified (Trust & Sparrow 1974; Trust *et al.* 1979; Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000; Spanggaard *et al.* 2001; Huber *et al.* 2004; Heikkinen *et al.* 2006; Pond *et al.* 2006; Kim *et al.* 2007). Using 16S rRNA analysis *Duganella zoogloeides*, *Buttiauxella noackiae*, *Hafnia alvei*, *Enterobacter intermedius*, *Es. coli*, *Pantoea agglomerans*, *Plesiomonas shigelloides*, *A. salmonicida*, *A. media*, *A. sobria*, *A. veronii*, *C. divergens*, *C. maltaromaticum*, *C. piscicola*, *Microbacterium phyllosphaerae*, *Micrococcus luteus*, *Kocuria kristinae* and *Sporocytophaga cauliformis* have been isolated from the rainbow trout digestive tract (Huber *et al.* 2004; Pond *et al.* 2006; Kim *et al.* 2007).

The majority of these investigations of the gut microbiota of salmonids have utilised culture-based methods which does not fully reflect the gut microbiota, even in species with high culturability such as rainbow trout. The incorporation of culture-independent techniques, such as DGGE, restriction fragment length polymorphism (RFLP) and PCR length heterogeneity analysis (LH-PCR) have been incorporated into recent studies (Heikkinen *et al.* 2006; Pond *et al.* 2006; Hovda *et al.* 2007; Kim *et al.* 2007). The importance of such techniques was demonstrated by Pond *et al.* (2006), Kim *et al.* (2007) and Hovda *et al.* (2007) where the dominant microbiota determined by culture-dependent

techniques were different from those determined by culture-independent methods. For example, both Pond *et al.* (2006) and Kim *et al.* (2007) determined from culture-independent methods that *Clostridium* spp. were among the dominant microbiota of rainbow trout but this was not the case with culture-dependent methods. Culture-independent methods should be incorporated in future studies.

Probiotics

It was suggested more than a quarter of a century ago that bacteria could be useful as biological activators of nutrient regeneration and controllers of fish disease (Yasuda & Taga 1980). However, it is only really over the last decade that sustained research has focused on biological control. The definition of a probiotic differs greatly depending on the source, but a good specific definition is "...a live microbial feed supplement which beneficially effects the host animal by improving its microbial balance" (Fuller 1989). This is very important, as the term is often defined differently. Microbes that are antagonistic to pathogens, but are not found to be present, either transiently or residentially, in the gastro-intestinal tract, are termed biocontrol agents (Maeda *et al.* 1997). Microbes that improve water quality by the breakdown of waste or pollutants are termed bioremediation agents (Moriarty 1997). Whilst most people now refer to all of these microbial applications as probiotic treatments it is important to distinguish the differences between them. Given the nature of fish farming and the different administration methods applied in previous fish studies an aquaculture based probiotic definition has been suggested as "an entire or component(s) of a micro-organism that is beneficial to the health of the host" (Irianto & Austin 2002a). Perhaps the most appropriate definition was proposed by Vershuere *et al.* (2000): "A probiotic is defined as 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". Furthermore, Spanggaard *et al.* (2001) proposed certain favourable characteristics for potential probionts:

- it must not be pathogenic to the host
- it must colonise the intestinal tract
- the organism should exhibit antagonism to a potential pathogen[a]
- it should stimulate the host immune system[a]
- it should be indigenous to the host and environment in which it is to be used[b]

[a] I do not believe these points are essential, as long as other benefits are achieved (e.g. aiding nutrition or improving gut histology). Also, it is possible that protection against a specific pathogen may occur indirectly via microbial shifts rather than immuno-stimulation.

[b] Providing the probiont is capable of successful survival and metabolism in the host intestinal tract and is not pathogenic I believe it is not strictly necessary for the probiont to be indigenous to the host.

The application of probiotics in aquaculture has led to a reduction in the use of antimicrobial compounds, an increase in growth performance and/or improved appetite (Irianto & Austin 2002a). The majority of probiotic species proposed as probionts for aquatic species are either lactic acid bacteria (LAB) or *Bacillus* spp.; however, yeast based probiotics are also stimulating much interest (Gatesoupe 2002; Tovar *et al.* 2002; Aubin *et al.* 2005; Gatesoupe 2007).

Lactic acid bacterial applications

LAB are Gram-positive, often non-motile, often catalase negative, oxidase negative, non-sporing bacteria that produce lactic acid as a product of fermentative metabolism (Ringø & Gatesoupe 1998). LAB have been identified as indigenous components of the intestinal microbiota of a range of fish species; including Atlantic salmon (Ringø *et al.* 2000; Ringø *et al.* 2006a); Atlantic cod (Ringø *et al.* 2006c); Arctic charr (Ringø *et al.* 2006b); European eel (*Anguilla anguilla*, Esteve & Garay 1991); carps (Sugita *et al.* 1985; Sugita *et al.* 1997; Cai *et al.* 1999: Hagi *et al.* 2004); goldfish (Sugita *et al.* 1988): catfish (Hagi *et al.* 2004); yellowtail (*Seriola quinqueradiata*, Takemaru & Kusuda 1988ab) and river trout (Skrodenyte-Arbaciauskiene *et al.* 2006). For reviews see Ringø & Gatesoupe (1998) and Gatesoupe (2008a). LAB have also been isolated from the digestive tract of rainbow trout (Wallbanks *et al.* 1990; Starliper *et al.* 1992; Spanggaard *et al.* 2000; Spanggaard *et al.* 2001; Huber *et al.* 2004; Heikkinen *et al.* 2006; Pond *et al.* 2006; Kim *et al.* 2007) but are not normally found to be among the dominant populations. LAB have been the focus of extensive research for probiotic applications because they are able to tolerate low pH, bile and pancreatic enzymes (Cai *et al.* 1999; Ouwehand *et al.* 2002). LAB can also produce of a range of antimicrobial proteins and peptides (Twomey *et al.* 2002, Ringø *et al.* 2005).

Most commonly used LAB in aquaculture studies include *Lactobacillus* spp., *Lactococcus* spp., *Enterococcus* spp. and *Carnobacterium* spp. (refer to Table 1.1).

***Bacillus* spp. applications**

Bacillus spp. are Gram-positive endospore forming rods. They are either aerobic or facultative anaerobes that are typically ubiquitous in nature. Several studies have isolated *Bacillus* spp. from the digestive tract of salmonids (Ringø *et al.* 2006b; Hovda *et al.* 2007;

Ringø *et al.* 2008) including rainbow trout (Austin & Al-Zahrani 1988; Heikkinen *et al.* 2006; Kim *et al.* 2007). *Bacillus* spp. have many favourable properties for probiotic applications. Firstly the fact that they are spore formers helps to avoid some of the constraints regarding issues of shelf life. They are generally regarded as safe, they produce antibiotic substances and a range of vitamins and digestive enzymes (Moriarty 1996; Rosvitz *et al.* 1998; Martens *et al.* 2002; Martirani *et al.* 2002; Urdaci *et al.* 2004; Azokpota *et al.* 2006).

Probiotic administration of *Bacillus* spp. in aquaculture has demonstrated a range of benefits, including enhanced brood stock performance, improved growth performance, heightened disease resistance and immunostimulation (refer to Table 1.1). Amongst the most commonly used species include *B. subtilis* and *B. licheniformis*, which are registered for use as dietary supplementation within the EU (Council Directive 70/524/EEC). Despite the increasing body of data regarding finfish species there has perhaps been a greater focus applied with *Bacillus* based probiotics in crustacean species (Rengpipat *et al.* 1998; Skjermo & Vadstein 1999; Rengpipat *et al.* 2000; Vaseeharan & Ramasamy 2003).

Probiotic applications with rainbow trout

The potential for probiotic control of disease, immunostimulation and improved growth has been demonstrated in rainbow trout (refer to Table 1.2).

Table 1.1. Selected LAB and *Bacillus* spp. probiotic investigations.

Probiotic (Host fish species)	**Observations**	**Administration**	**Supplement level** (duration)	**Suggested mode of action**	**References**
Lyophilized *C. divergens* (Atlantic cod)	Decrease of fry mortality after *V. anguillarum* challenge	Addition to diet	2×10^9 CFU g^{-1} (21 days)	-	Gildberg *et al.* 1997
Lyophilized *C. divergens* (Atlantic cod)	Short term decrease of fry mortality after *V. anguillarum* challenge	Addition to diet	10^8 CFU g^{-1} (14 days)	Antagonism	Gildberg & Mikkelsen 1998
Dead *A. hydrophila*, *V. fluvialis*, *Carnobacterium* spp. and an unidentified Gram-positive coccus (Goldfish)	Reduced mortalities when challenged with *A. salmonicida*. Increased number of erythrocytes, macrophages, leucocytes. Enhanced phagocytic activity	Addition to diet	2×10^7 CFU g^{-1} (20 days)	Stimulation of cellular immunity factors	Irianto & Austin 2003
La. lactis (Turbot)	Significantly increased macrophage CL response and the serum NO concentration	Force fed 100 µL daily	10^6 cells mL^{-1} (7 days)	-	Villamil *et al.* 2002
E. faecium (Sheat fish)	Increased growth. Also reduced incidence of *E. coli*, *S. aureus* and *Clostridium* spp. within the intestine	Addition to diet	1×10^8CFU g^{-1} (58 days)	-	Bogut *et al.* 2000
L. delbrueckii and *B. subtilis* (Gilthead sea bream)	Increased phagocytic and cytotoxic activity	Addition to diet	10^7 CFU g^{-1} (21 days)	Immuno-stimulation	Salinas *et al.* 2005

Continued on the following page

Table 1.1 – *continued*

B. subtilis, *L. acidophilus*, *Clostridium butyricum*, *Saccharomyces cerevisiae* (Nile tilapia)	Enhanced non-specific immune parameters such as lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in improvement of resistance to *Edwardsiella tarda* infection	Addition to diet & rearing water (separate treatments)	1% addition to diet. Product contained 10^7 -10^8 CFU g^{-1}. 10 g in 100 mL added to tank every 5 days (30 days)	-	Taoka *et al.* 2006
Biogens®; commercial product containing *B. subtilis* (Nile tilapia)	Increased weight gain, SGR, PER, protein productive value and energy retention	Addition to diet	0.5 - 2.5% inclusion of diet. Product contained 6 x 10^7 CFU g^{-1} (120 days)	Antagonism, detoxifying harmful feed compounds, producing vitamins	El-Haroun *et al.* 2006
E. faecium and *B. toyoi* (European eel)	Reduction of mortality after exposure to *E.tarda*	Addition to diet	0.1% of diet (14 days)	Antagonism	Chang & Liu 2002
B. subtilis (Ornamental species*)	Improved weight gain, survival and FCR. Additionally increased carcass ash and protein content. Increased specific activity of protease and amylase in the digestive tract	Addition to diet	5 x 10^5 - 5 x 10^8 cells g^{-1} (90 days)	Immuno-stimulation and improved enzymatic activity	Ghosh *et al.* 2008

* *Poecilia reticulate*, *Poecilia sphenops*, *Xiphophorus helleri* and *Xiphophorus maculatus*

Table 1.2. Selected rainbow trout probiotic investigations.

Probiotic	Observations	Method of administration	Supplement level (Duration)	Suggested mode of action	References
A. hydrophila, *V. fluvialis*, *Carnobacterium* spp. and an unidentified Gram + cocci	Reduced mortalities when challenged with *Aeromonas salmonicida*. Increased HK macrophages and enhanced phagocytic activity	Addition to diet	10^6 - 10^8 CFU g^{-1} (varied from 7 to 140 days)	Stimulation of cell mediated immunological response	Irianto & Austin 2002b
Carnobacterium strain K1	Cells remained metabolically active in the gastro-intestinal tract for several days after feeding ceased	Addition to diet	~ 4 x 10^7CFU g^{-1} (6 days)	-	Jöborn *et al.* 1997
B. subtilis and *B. licheniformis* (BioPlus 2B®)	Reduced mortalities when challenged with *Y. ruckeri*	Addition to diet	4 x 10^4 spores g^{-1} (42 days)	Stimulation of cell mediated immunological response	Raida *et al.* 2003
L. rhamnosus	Reduced mortalities when challenged with *A. salmonicida*	Addition to diet	10^9 and 10^{12} CFU g^{-1} (51 days)	-	Nikoskelainen *et al.* 2001
L. rhamnosus	Enhanced respiratory burst activity of blood cells, serum-mediated killing of *Escherichia coli*, serum immunoglobulin levels	Addition to diet	7.9 x 10^4 to 9.7 x 10^{10} CFU g^{-1} (14 days)	Immuno-stimulation	Nikoskelainen *et al.* 2003
L. rhamnosus	Enhanced serum lysozyme and complement. Elevated phagocytic activity of head kidney leucocytes	Addition to diet	10^9 and 10^{11} CFU g^{-1} (30 days)	Immuno-regulatory role	Panigrahi *et al.* 2004
L. rhamnosus	Viable forms induced better phagocytic activity and complement activity compared to that of the non-viable	Addition to the diet. (Heat-killed, live and freeze-dried)	~ 10^{11} CFU g^{-1} (30 days)	Viability is a factor when enhancing immune responses	Panigrahi *et al.* 2005

Continued on the following page

Table 1.2 - *continued*

Pseudomonas spp.	Improved survival of rainbow trout infected with vibriosis	Addition to rearing water	10^7 cells mL^{-1} (Added to the tank during infection)	Antagonism	Spanggaard *et al.* 2001
La. lactis, *L. sakei* and *Leu. mesenteroides**	Elevated serum alternative complement activity, lysozyme activity and immunoglobulin levels	Addition to the diet	10^6 CFU g^{-1} (14 days)	Stimulate of the humoral immune response	Balcázar *et al.* 2007a*
La. lactis, *L. sakei* and *Leu. mesenteroides*	Enhanced phagocytic activity of head kidney leukocytes, superoxide anion production and serum alternative complement activity. Reduced mortalities when challenged with *A. salmonicida*	Addition to the diet	10^6 CFU g^{-1} (14 days)	Elevated humoral and cellular immune response	Balcázar *et al.* 2007b
C. maltaromaticum and *C. divergens*	Protection against challenge with *A. salmonicida* and *Y. ruckeri*. Increased phagocytic activity of the head kidney macrophages, increases in respiratory burst, enhanced serum and mucosal lysozyme activity. Cultures persisted in the gut for up to 3 weeks after administration ceased	Addition to diet	$>10^7$ cells g^{-1} (14 days)	Immunostimulation	Kim & Austin 2006
B. subtilis and *B. licheniformis* (BioPlus 2B®)	Improved survival, FCR, SGR, K-factor, PER and carcass composition of fry	Addition to diet	10^8 & 10^9 CFU g^{-1} (63 days)	Stimulated digestive development and enzymatic activity	Bagheri *et al.* 2008

* Brown trout (*Salmo trutta*)

Probiotic modes of action

The specific mode of action which results in the observed benefits to the host is often difficult to elucidate conclusively due to the wide range of possibilities and the complicated synergistic multi-factorial relationships between possible modes. Some studies have made suggestions, but have not conclusively investigated the full range of possibilities. Suggested mechanisms include: production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, enhancement of the immune response, source of macro and/or micronutrients and enzymatic contribution to digestion (Vershuere *et al*. 2000).

Inhibitory compounds

The antimicrobial properties of probionts have been concluded to be as a result of the production of a combination of extracellular products, which include: lactic acid, hydrogen peroxide, carbon dioxide, siderophores, antibiotic peptides/proteins, organic acids, ammonia and diacetyl (Neilands 1981; Baldry 1983; Williams & Vickers 1986; Lewus *et al*. 1991; Vandenbergh 1993; Midolo *et al*. 1995; Severina *et al*. 1998; Ringø & Gatesoupe 1998; Naidu *et al*. 1999; Alakomi *et al*. 2000; Ringø *et al*. 2005; Vázquez *et al*. 2005; Beaulieu *et al*. 2006; Anastasiadou *et al*. 2008).

LAB proteins and peptides with antimicrobial properties can be subcategorised as large proteins (> 20kDa) with enzymatic activity inducing cell lysis (e.g. lysins) or bacteriocins (antimicrobial peptides) which induce cell lysis or prevention of cell wall synthesis via non-enzymatic mechanisms (Twomey *et al*. 2002). To date, many bacteriocins or bacteriocins-like products have been identified. For example, nisin is a bacteriocin-like substance that is produced by *La. lactis*; it is likely to have contributed towards the reduction of *V. anguillarum* growth (combined with the production of lactic acid, organic

acids, hydrogen peroxide & carbon dioxide) observed by Villamil *et al.* (2002). Undiluted extracellular products from *La. lactis* were shown to significantly reduce the growth of *V. anguillarum*. In addition to this *La. lactis* was able to modulate the turbot (*Scophthalmus maximus*) immune system both *in vitro* and *in vivo*. Vázquez *et al.* (2005) demonstrated that bacteriocins and organic acid solutions produced from cultured probiotics (*Lactobacillus* spp, *La. lactis*, *Leu. mesenteroides* and *P. acidilactici*) displayed antagonistic properties against a range of fish pathogens *in vitro*. Panigrahi *et al.* (2007) tested probiotics for antagonism against a variety of pathogens and found that *B. subtilis* exhibited activity against a wide range of pathogens including *V. anguillarum*, *A. salmonicida*, *A. sobria*, *A. hydrophila* and *E. coli*. Similarly, *E. faecium* showed strong antagonistic properties against *A. salmonicida*. A wide range of other probiotics have shown antagonistic properties to some of the most destructive fish pathogens, including *A. salmonicida*, *V. anguillarum*, *V. salmonicida*, *E. tarda* and *Y. ruckeri* (for a summary see Gatesoupe 2008b).

Siderophores can also be described as antimicrobial substances, but considering the mode of action, are perhaps better described in terms regarding competitive exclusion.

Competition

Competition for available resources within the gastro-intestinal tract is likely to antagonize potential pathogens by reducing the number of possible niches available. One of the most well studied resources, in regards to competitive antagonism, is iron, which is essential for growth as well as being an important virulence factor (Singer *et al.* 1991; Litwin & Calderwood 1993). Siderphores are extracellular products, produced by many bacteria, which display an exceptional ferric ion affinity. They can dissolve precipitated iron,

making it available for microbial growth; the significance of siderophores lie in their ability to scavenge essential iron (Verschuere *et al.* 2000). Strains of siderophore-producing *Ps. fluorescens* have been successfully applied as a biological control agent. Gram *et al.* (1999) demonstrated that *V. anguillarum* could be inhibited by *Ps. fluorescens* in co-culture experiments (under iron-limited conditions). The effect was tested *in vivo* by bathing rainbow trout in a probiotic suspension and challenging with *V. anguillarum*. Short term bathing, long term bathing and a combination of both led to a reduction of mortalities. The accumulated mortality of infected fish not treated reached 50% after 9 days; however, this figure was reduced to 44% and 35%, for long term and short term bathing respectively. By combining both treatments accumulated mortality was reduced to 32%. Another such example was demonstrated by Gatesoupe (1997); a siderophore producing *Vibrio* spp. fed to turbot larvae improved growth and resistance against *V. splendidus*.

The same principle is expected to hold true with regards to competition for organic substrates that can be used as a source of carbon or energy. However, to the authors knowledge no studies have focused on this and as a result data is scarce; but it cannot be ruled at as a possible contributory factor in the majority of investigations.

Competition for mucosal adhesion sites is another possible mode of action. Despite this being a widely accepted possibility, very little work has been conducted to confirm this hypothesis. Many studies have been conducted regarding attachment competition with human probiotics. In 2001, Forestier *et al.* demonstrated that the addition of the *L. casei* to an intestinal cell line reduced the adhesion success of 3 pathogens. It was suggested that the presence of a probiont can restrict the access of a pathogen by blocking receptors with specific adhesion analogues, or by steric hindrance (Tuomola *et al.* 1999; Forestier *et al.* 2001). Very little data exists regarding investigations using probiotic attachment with fish.

However, Vine *et al.* (2004) demonstrated *in vitro* that interactions between pathogens and probiotics resulted in alterations of attachment success of both bacterial groups to intestinal mucus. Surprisingly, the addition of the probiotic first seemed to enhance pathogenic attachment, but pathogenic attachment was reduced if the probiont was added after the pathogen. Chabrillon *et al.* (2006) found that two probiotic candidates significantly reduced the adhesion of *V. anguillarum* to gilthead seabream (*Sparus aurata*) intestinal, gill and skin mucus under exclusion, competition and displacement conditions. Oral administration of one of the probiotic candidates prior to *V. anguillarum* challenge significantly reduced mortalities. These studies begin to shed some light on the complicated nature of the interactions of probiotics with pathogens and the resulting interactions with attachment sites. However, these examples could well be linked to the production of inhibitory compounds as opposed to direct competition for attachment sites.

Gastro-intestinal colonisation

This continual application of bacterial cells which are able to compete with the indigenous microbiota may result in colonisation of the gastro-intestinal tract and modulation of the indigenous microbiota. Indeed, modulation of the fish gut microbiota has been demonstrated with the probiotic application of *Bacillus* spp. (Chang & Liu 2002; Newaj-Fyzul *et al.* 2007; Bagheri *et al.* 2008; Ghosh *et al.* 2008), LAB (Gildberg *et al.* 1995; Jöborn *et al.* 1997; Robertson *et al.* 2000; Nikoskelainen *et al.* 2003; Panigrahi *et al.* 2004; Aubin *et al.* 2005; Panigrahi *et al.* 2005; Kim & Austin 2006; Balcázar *et al.* 2007ab) and yeast (Gatesoupe 2002; Aubin *et al.* 2005).

The continual application of probiotic cells has been demonstrated to lead to potentially resident probiotic colonisation of the intestinal epithelium (Gildberg *et al.* 1995; Gildberg *et al.* 1997; Gildberg & Mikkelsen 1998; Aubin *et al.* 2005), intestinal mucus (Kim &

Austin 2006; Newaj-Fyzul *et al.* 2007) and transient digesta based populations (Gildberg *et al.* 1997; Bogut *et al.* 2000; Kim & Austin 2006; Newaj-Fyzul *et al.* 2007; Bagerhi *et al.* 2008; Ghosh *et al.* 2008). However, it has also been shown that some probionts are able to persist within the digestive tract for several weeks after reverting to non-supplemented diets (Nikoskelainen *et al.* 2003; Kim & Austin 2006; Balcázar *et al.* 2007a). Kim & Austin (2006) demonstrated that *C. maltaromaticum* and *C. divergens* (originally isolated from trout) displayed exceptional qualities for the colonisation of the rainbow trout intestine. Dietary application for 14 days at > 10^7 CFU g^{-1} led to levels of > 90% of the total populations in the intestinal contents and > 99% of the mucus. PCR-DGGE profiles were utilised to monitor the persistence of the probionts within the rainbow trout intestine. After reverting to non-supplemented feeds the probionts were still detected within the intestine for at least 3 weeks. Similarly, Balcázar et al. (2007a) showed that *La. lactis* ssp. *lactis*, *L. sakei* and *Leu. mesenteroides* also displayed an ability to persist within the rainbow trout intestine after reverting to non-supplemented diets. Short term persistence was relatively high as levels remained at log 4-5 CFU g^{-1} and < log 3 CFU g^{-1} after 1 and 2 weeks, respectively. Nikoskelainen *et al.* (2003) observed recoverable levels of *L. rhamnosus* in the intestine closely related to dietary supplementation level (10^4 - 10^{10} CFU g^{-1}) during 2 weeks feeding to rainbow trout. However, this level dropped drastically after reverting to an un-supplemented diet; within one week levels dropped several log scales and after 2 weeks the probiont was not recovered from any treatments.

Probiotic colonisation is not restricted only to the intestine. For example, Panigrahi *et al.* (2004) fed rainbow trout *L. rhamnosus* for 30 days at 10^9 and 10^{11} CFU g^{-1}. *L. rhamnosus* was recovered at levels of 10^6 - 10^8 CFU g^{-1} and 10^5 - 10^9 CFU g^{-1} intestine and stomach, respectively. After 30 days, *L. rhamnosus* in fish fed the low level supplementation

reached > 40% and > 70% of the culturable microbiota of the stomach and intestine, respectively. Furthermore, *L. rhamnosus* levels of fish fed the high level supplementation reached > 60% and > 80% in the stomach and intestine, respectively. Gildberg & Mikkelsen (1998) demonstrated that *C. divergens* (originally isolated from fish) displayed better colonisation capabilities with regards to Atlantic cod fry pyloric ceaca than the intestine.

Probiotic colonisation can alter the indigenous intestinal microbiota composition as well as total populations levels (Bogut *et al.* 2000; Aubin *et al.* 2005; Bagerhi *et al.* 2008; Ghosh *et al.* 2008). For example, after feeding rainbow trout *P. acidilactici* or *Saccharomyces cerevisiae* var. *boulardii* for periods of up to 5 months, Aubin *et al.* (2005) enumerated adherent probiotic levels and used 16S rRNA sequence analysis to identify the indigenous autochthonous microbiota. Changes in the relative and absolute abundance of the indigenous bacteria were observed. For example, after a period of 20 days feeding on supplemented diets the presence of unique genera such as *Buttiauxella* and *Citrobacter* were found in the control fed fish and *Serratia* was found only in the *P. acidilactici* fed fish. Bagheri *et al.* (2008) fed rainbow trout fry *B. subtilis* and *B. licheniformis* at levels ranging from ca. 5×10^8 - 6×10^9 CFU g^{-1} diet for two months. This led to a significant increase of intestinal viable counts compared to the control, total bacterial counts were significantly different among treatments which suggests that colonisation was directly affected by dietary bacterial level. The percentage of probiotic *Bacillus* spp. recovered ranged from 65% to over 99% of the total viable counts. Changes in the levels of Enterobacteriaceae, Vibrionaceae, *Pseudomonas* spp. and other Gram-negative species were also observed. Bogut *et al.* (2000) found that the probiotic application of *E. faecium* to sheat fish (*Silurus glanis*) resulted in a clear alteration of intestinal microbiota.

Specifically a reduction of *Staphylococcus aureus* and Enterobacteriaceae (including *Es. coli*). Also a complete removal of *Clostridium* spp. was observed after 2 weeks feeding.

The current literature suggests that probiotic colonisation and modulation of indigenous microbiota is not restricted by fish maturation as investigations have demonstrated the effects of the probiotics on the gut microbiota of fish across a range life stages including: larval (Ringø 1999; Gatesoupe 2002; Planas *et al.* 2006), fry (Gildberg *et al.* 1995; Gildberg *et al.* 1997; Gildberg & Mikkelsen 1998; Robertson *et al.* 2000; Ghosh *et al.* 2008), fingerling (Jöborn *et al.* 1997; Robertson *et al.* 2000) and juvenile stages (Panigrahi *et al.* 2005; Carnevali *et al.* 2006).

Many of these studies have focused investigations of immune status and many have shown a resultant immunostimulating effect related to probiotic feeding.

Enhancement of the immune response

Nikoskelainen *et al.* (2003) demonstrated that rainbow trout immune parameters were enhanced by using LAB strains. Specifically, serum immunoglobulin levels were elevated along with serum mediated killing of *Es. coli*. Although Villamil *et al.* (2002) demonstrated a significant increase in macrophage chemiluminescent (CL) responses and an increase of nitric oxide (NO) production in turbot (*Scophthalmus maximus*) treated with *La. lactis*, the precise mechanism of the response was not determined. It was suggested that the enhancement of the CL response could be related to an improvement of disease resistance, as it may be of importance to phagocytic activity. NO has also been shown to be involved in the regulation of immune functions, as well as having antimicrobial properties against fish pathogens (Gunasegaran *et al.* 1993; Campos *et al.* 2000).

The effect of *L. rhamnosus* on rainbow trout serum lysozyme activity is variable with both no effect (Panigrahi *et al.* 2005) and improved activity having been reported (Panigrahi *et*

al. 2004). Rainbow trout treated with *C. divergens* demonstrated an increase in serum lysozyme activity whereas *C. maltaromaticum* treatment did not (Kim & Austin 2006). However, both species elevated lysozyme activity of gut mucus. Several other studies have demonstrated that serum lysozyme activity may be affected by using LAB probiotics in trout. Balcázar *et al.* (2007a) found a significant increase in lysozyme activity when administering *La. lactis* and *Leu. mesenteroides* to brown trout (*Salmo trutta*) but no difference was found using *L. sakei*. However, all 3 species failed to enhance serum lysozyme in rainbow trout (Balcázar *et al.* 2007b). An increase in lysozyme activity was also observed by Irianto & Austin (2002b) along with stimulation of rainbow trout cellular immunity including an elevated number of erythrocytes, macrophages and lymphocytes. The net result of probiotic feeding for up to 14 days was a reduction of mortalities after challenge with *A. salmonicida.*

Wang *et al.* (2008) found no improvement of Nile tilapia total serum protein, albumin, globulin and albumin/globulin ratio with the addition of *E. faecium* to aquaria water at 1 x 10^7 CFU mL^{-1} every 4 days for a total of 40 days. However, enhanced lysozyme activity, respiratory burst and complement component was found.

Improvement of gastro-intestinal structure

Maintaining a healthy gut microbiota is likely to beneficially affect the gut epithelial architecture. Reducing the number of potential pathogens present within the gastro-intestinal tract can prevent the reduction of intestinal surface area by reducing mucosal damage (Collet 2004). Indeed, many fish pathogens can disrupt the integrity of the intestinal epithelium (Austin & Austin 1993; Ringø *et al.* 2007).

Data regarding the effects of probiotics on the intestinal morphology of fish *in vivo* is scarce. However, the histological effect of probiotics on the gut epithelium of fish has

been assessed *in vitro* (Ringø *et al.* 2007; Salinas *et al.* 2008). Ringø *et al.* (2007) exposed the Atlantic salmon foregut to *C. divergens* as well as two pathogens: *A. salmonicida* and *V. anguillarum*. Light and electron microscopy demonstrated that pathogen induced damage to the Atlantic salmon foregut could not be prevented or reversed, but could be marginally reduced in some cases. Salinas *et al.* (2008) conducted a similar study; the Atlantic salmon foregut was assessed after exposure to a probiotic (*La. lactis*) and a pathogen (*A. salmonicida*). *A. salmonicida* caused disruption of enterocyte integrity when inoculated with foregut tissue that did not receive a *La. Lactis* pre-treatment. Confocal microscopy demonstrated that *La. Lactis* was able to colonise the foregut. Subsequently light and electron microscopy demonstrated that *La. Lactis* treatment provided protection against damage caused by *A. salmonicida*.

Despite the lack of *in vivo* literature regarding the effects of probiotic on fish gastro-intestinal morphology, studies using prebiotics, such as mannan oligosaccharides (MOS) have proven effective at enhancing the microvilli morphology of fish. Specifically, improved microvilli length has been observed in Cobia, *Rachycentron canadum* (Salze *et al.* 2008) and both improved microvilli length and density has been observed in rainbow trout (Dimitroglou *et al.* 2008) with 0.2% dietary MOS.

It is conceivable that benefits to gut histology, especially at the brush border level, could be a contributory factor to some of the improvements of feed utilisation and growth performance observed in probiotic studies discussed in the next section.

Nutritional role

It is generally accepted that the intestinal microbiota of mammals plays a key role in digestion (Bach-Knudsen *et al.* 1991; Titus & Ahearn 1992; Jensen & Jorgensen 1994; Stevens & Hume 1998), however, the role and importance of microbiota in lower

vertebrates remains largely unknown. Even so, many studies have demonstrated that fish intestinal bacteria can produce a range of enzymes which may contribute towards digestion (Kamei *et al.* 1985; Rimmer & Weibe 1987; Pollack & Montgomery 1994; Ringø *et al.* 1995; Sugita *et al.* 1997; Stevens & Hume 1998; Ramirez & Dixon 2003; Balcázar *et al.* 2006; Saha *et al.* 2006).

Bacteria indigenous to the gastro-intestinal tract of a variety of animals are reported to ferment carbohydrates into short-chain fatty acids (SCFAs; e.g. acetate, propionate and butyrate), which can both provide energy and enhance absorption of H_2O and Na (Stevens & Hume 1998). SCFAs are currently an area of high interest, as it is thought that microbial fermentation provides significant levels of nutrients to terrestrial host organisms in the form of SCFAs (Titus & Ahearn 1992). One of the most important energy substrates for intestinal microbial fermentation are non-digestible oligosaccharides (Bach-Knudsen *et al.* 1991; Jensen & Jorgensen 1994). The energy available, in the form of SCFAs, to the host animal after microbial fermentation is approximately 60% of that of the fermented substrate. Titus & Ahearn (1992) suggest that microbial fermentation could account for as much as 80% of maintenance energy requirements in ruminant herbivores, and up to 10% in carnivores and omnivores. Despite no such detailed reports specifically regarding symbiotic microbial fermentation in fish, it is highly likely that microbial fermentation could play a significant role in many fish species.

Possible examples of this include dietary application of oligosaccharides such as MOS, inulin and galacto-oligosaccharides (GOS). Staykov *et al.* (2007) found improved growth performance, feed utilization and survival of rainbow trout when feeding MOS at 0.2%. A significant increase of European sea bass (*Dicentrarchus labrax*) growth has also been observed with dietary supplementation of MOS at 0.2% and 0.4% for 67 days (Torrecillas *et al.* 2007). Perhaps more telling is a recent study demonstrating improved SBM

digestibility, particularly protein, by red drum (*Sciaenops ocellatus*) when fed MOS, inulin and GOS (Burr *et al.* 2008). Indeed, the indigenous microbiota of Siberian sturgeon (*Acipenser baerii*) has been documented to produce SCFAs from inulin and FOS (Mahious *et al.* 2006a). Many potential candidate probiotics, such as *Bacillus* spp. (Mahious *et al.* 2006b) and *Carnobacterium* spp. (Ringø & Holzapfel 2000), can utilise such oligosaccharides.

Candidate probiotics produce a wide range of extracellular enzymes and vitamins which are likely to contribute towards the host's digestive function (Moriarty 1996; Rosvitz *et al.*, 1998; Bairagi *et al.* 2002; Martens *et al.* 2002; Azokpota *et al.* 2006; Yanbo & Zirong 2006; Ziaei-Nejad *et al.* 2006). Indeed, stabilising or modulating the indigenous intestinal populations with probiotics population has proven to enhance the digestive function of several fish species (Bogut *et al.* 2000; El-Haroun *et al.* 2006; Yanbo & Zirong 2006; Bagheri *et al.* 2008; Wang *et al.* 2008). A study by Bagheri *et al.* (2008) demonstrated improved FCR, SGR, K-factor, PER and carcass composition of rainbow trout fry after dietary supplementation with *B. subtilis* and *B. licheniformis*. Similarly, improved weight gain, SGR, PER, protein productive value and energy retention of Nile tilapia has been observed with a commercial product containing *B. subtilis* (El-Haroun *et al.* 2006). Increased weight gain of sheat fish (Bogut *et al.* 2000) and Nile tilapia (Wang *et al.* 2008) has been observed after probiotic applications of *E. faecium*.

Thesis research objectives

The aim of the current research can be broken down into several objectives:

1. Investigate indigenous intestinal microbiota of farm reared rainbow trout
2. Investigate indigenous intestinal microbiota of aquarium reared rainbow trout
3. Investigate selected commercially available probiotics (LAB and *Bacillus* spp.) on rainbow trout growth performance, feed utilisation, intestinal colonisation, intestinal histology and selected health parameters
4. Investigate Lf on rainbow trout growth performance, feed utilisation, intestinal colonisation, intestinal histology and selected health parameters
5. Observe the effect of selected probiotics and Lf on rainbow trout growth performance, feed utilisation, intestinal colonisation, intestinal histology and selected health parameters after the administration of antibiotics
6. Investigate probiotic colonisation patterns

Chapter 2. General materials and methods

All experimental analyses were carried out with the following protocols unless otherwise stated. Chemicals, reagents and culture media were sourced from Fisher scientific Inc. (Hampton, NH, USA), Sigma-Aldrich corp. (St. Loius, MO, USA) or Oxoid Ltd unless otherwise indicated. All experimental work involving fish was carried out under the Home Office project licence #30/2135 and personal licence #30/7212.

Aquarium facilities

All feeding experiments were conducted at the University of Plymouth's Aquaculture and Fish Nutrition Research Aquarium. Unless otherwise indicated all trials were conducted in experimental system E (Plate 2.1). The system is a closed recirculation system with a total system volume of ~6200 L. Mechanical filtration is provided by an Aquasonic DF100 drum screen filter (Aquasonic Ltd, Wauchop, Australia; Plate 2). Biological filtration was provided by a submerged biological filter bed containing *Nitrosomonas* and *Nitrospira* spp.. The experimental holding unit comprised of 20 x 150 L fibreglass tanks, each provided with ~99% (the system received a constant flow of water at 1 L hr^{-1} to maintain volume) re-circulated aerated freshwater at a rate of ~750 L hr^{-1}. A 12 hr light, 12 hr dark photoperiod was maintained throughout the trial periods. Water changes (~900 L, approximately 15% of system volume) were conducted every 72 hr to minimise accumulation of background probiotic levels. The system pH, dissolved oxygen and temperature were monitored daily (Hach HQ 40d). The system was buffered with sodium bicarbonate ($NaHCO_3$) as required to maintain pH 6 - 8. The water temperature was maintained at 15 ± 1 °C with a thermostatic controlled chiller (Zodiac, France) and dissolved oxygen levels maintained > 80% saturation with additional aeration provided by

an air stone supplied by a low pressure side channel blower (Rietschle, UK). Nitrogenous waste was monitored on a weekly basis using a Hach Lange DR 2800 and cuvettes for ammonia (Lange LCK 304), nitrite (Lange LCK 341) and nitrate (Lange LCK 340). Acceptable levels were considered to be < 0.1 mg L^{-1}, < 1.0 mg L^{-1} and < 50 mg L^{-1} for ammonia (un-ionized), nitrite and nitrate, respectively. Levels were maintained with water changes when necessary.

Experimental fish

5 separate batches of fish were obtained to conduct the 5 experimental trials. Rainbow trout (*Oncorhynchus mykiss* Walbaum) of all female origin were obtained from Hatchlands Fisheries (Greyshoot Lane, Rattery, South Brent, UK). Fish were transported from the fish farm directly to the Aquaculture and Fish Nutrition Research Aquarium in a 1000 L tank supplied with pure oxygen (BOC, UK). Transportation time did not exceed approximately 30 min. Fish were allowed to recover from transport in darkness for 12 hr before light levels were slowly increased to full light intensity. Fish were fed commercial trout diets (Skretting) *ad libitum* until grading and random distribution into tanks to proceed with the experimental trials.

Feeding and weighing protocol

During the experimental trials fish were fed rations relative to % biomass day $^{-1}$, provided in equal rations at 0900 and 1700 hr. Daily feed was adjusted on an assumed FCR = 1 and was corrected every 2 weeks by batch weighing following 24 hr starvation.

Plate 2.1. System E, recirculation system, Aquaculture and Fish Nutrition Research Aquarium, The University of Plymouth (Above).

Plate 2.2. Aquasonic DF100 drum screen filter, providing mechanical filtration for experimental system E (left).

Growth and feed utilisation calculations

Growth performance and feed utilisation was assessed by mean final weight gain, SGR, FCR, PER and K-factor. Calculations were carried out as follows: SGR = 100((lnFW-lnIW)/T); FCR = WG/FI; PER = WG/PI; K = FW/(FL^3). Where FW = final weight, IW = initial weight, T = duration of feeding (days), WG = wet weight gain, FI = feed intake, PI = protein ingested and FL = final length (cm).

Chemical and proximate analysis

Fish carcass and diets were subjected to analysis for the determination of moisture, ash, protein, lipid and gross energy content. Fish were sampled at the beginning and end of the trial to determine carcass composition. Typically, samples were analysed in triplicate according to AOAC (1995) protocols.

Moisture

All samples were dried at 105 °C in a fan assisted Oven (Genlab ltd, UK) until a constant weight was achieved. Moisture content was calculated as: ((WW – DW)/WW)*100

Where WW is wet weight (g) and DW is dry weight (g).

Ash

Ash (total mineral or inorganic content) content was determined by adding a known weight of sample (~500 mg) to a pre-weighed crucible. The crucible was then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550°C for 12 hr. Ash was calculated as follows: ((SR - CW)/SW)*100

Where CW is crucible weight (g), SR is sample residue (including the crucible, g) and SW is the initial sample weight (g).

Lipid

Lipid content was determined using the Soxhlet extraction method. Approximately 3 g of sample material was placed into a cellulose thimble, lightly plugged with cotton wool and inserted into the condensers (rinsing position) of a SoxTec extraction system (Tecator Systems, Höganäs, Sweden; model 1043 and service unit 1046). Pre-weighed cups containing 40 mL of petroleum ether were clamped into the condensers and the extraction knobs were moved to the boiling position for 30 min. The extraction knobs are then moved to the rinsing position for 45 min. The cups were then transferred to a fume cupboard for 30 min before weighing. Lipid content is then determined as: (LW / SW) x 100

Where LW is lipid weight (determined from weight increase of cup, g) and SW is the initial sample weight (g).

Protein

Determination of crude protein was achieved by the Kjeldahl method to establish the total nitrogen content of samples. This amount is then multiplied by a factor 6.25 to calculate the crude protein content. Briefly, 100 mg of sample was transferred into a Kjeldahl digestion tube along with catalyst tablet (3 g $K_2 SO_4$, 105 mg $CuSO_4.5H_2O$ and 105 mg TiO_2; BDH Ltd UK) and 10 mL of concentrated H_2SO_4 (Sp.Gr. 1.84, BDH Ltd UK). Digestion was performed on a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) at 225 °C for 40 min and at 380 °C for 60 min. After the digestion stage the samples were distilled using Vapodest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany).

Crude protein is then determined as: (((ST - BT) x 0.10 x 14 x 6.25) / SW)*100

Where 0.1 is the molarity of the acid, 14 the relative atomic mass of nitrogen, 6.25 is the constant relationship between N and animal protein, ST is sample titre (mL), BT is blank titre (mL) and SW is the initial sample weight (mg).

Gross energy

Gross energy was determined using a Parr Adiabatic Bomb Calorimeter model 1356 (Parr Instrument Company, IL, USA).

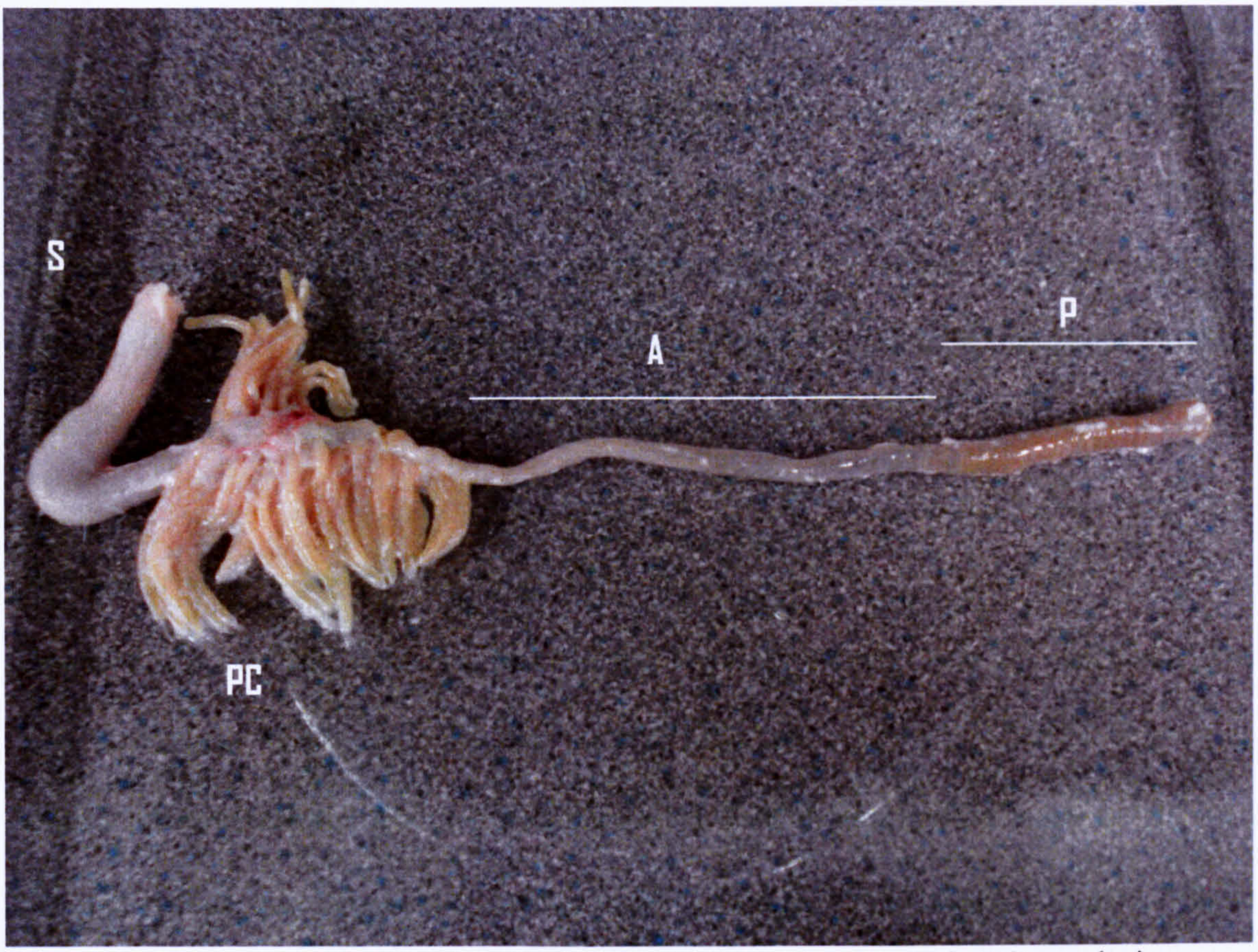


Plate 2.3. Rainbow trout gastro-intestinal tract. Regions: S = stomach, PC = pyloric ceaca, A = anterior intestine & P = posterior intestine.

Microbiological investigations

Fish dissection

Nitrile gloves, regularly wiped down with 99% ethanol, were used and aseptic technique maintained throughout the entire dissection process. After an external examination to ensure good health, fish were sampled as follows: the underside of the fish was washed with 70% ethanol and the peritoneal cavity was opened with a sterile scalpel blade. The entire gastro-intestinal tract was removed from the peritoneal cavity (refer to Plate 2.3). The anterior section was determined as the region between the distal most pyloric ceaca and the proximal border of the posterior section with the posterior section determined as the region between the increase in diameter of the gastrointestinal tract and the anus. After anterior and posterior regions were differentiated they were fixed using sterile clips in order to prevent the movement and mixing of intestinal contents within the intestinal tract. The intestine was divided into four sampling points, the anterior digesta (AD), posterior digesta (PD), anterior mucosa (AM) and the posterior mucosa (PM). After cutting at the proximal border between the regions (where clamped), digesta from the anterior section and posterior sections were removed separately. The anterior and posterior intestinal mucosal tissue was then excised and washed thoroughly 3 times with phosphate-buffered saline (PBS; pH 7.3) and homogenized in a macerator (MSE, London, UK).

Plating and colony counts

Typically, the resulting material from 3 fish was pooled into one sample. Feed was suspended in 10^{-1} dilution of PBS and homogenized in a stomacher (Seward laboratory, London, UK). Intestinal, feed and water samples were then serially diluted to 10^{-7} with PBS and 100 µL was spread onto appropriate duplicate agar plates and incubated. Viable counts were then performed using a Gallenkamp colony counter (Weiss-Gallenkamp,

Loughborough, UK). Aerobic heterotrophic counts were performed after 7 days aerobic incubation at 20 °C on duplicate TSA after Huber *et al.* (2004). TSA is a general purpose medium which supports a wide range of bacterial species and has shown high correlation between viable counts and the number of bacteria enumerated by direct counts (using DAPI) from rainbow trout (Spanggaard *et al.* 2000; Huber *et al.* 2004). Colony forming units (CFU) mL^{-1} or g^{-1} were determined for viable bacterial populations.

Phenotypic bacteriological identification

Approximately 25 colonies were randomly taken from all plates containing between 30-300 colonies and sub-cultured on TSA until pure cultures were achieved. All isolates were then tentatively placed into groups or genera, according to standard methods (Cowan & Steel 1993; Holt & Bergey 1994) based on colony morphology, cell morphology, Gram stain, production of catalase (3% H_2O_2), oxidase, glucose fermentation (glucose peptone water), motility (hanging drop) and endospore formation (observed from Gram stain).

Molecular microbial investigations

All molecular methodologies and protocols were carried out in a Labcaire PCR workstation (Labcaire Systems Ltd, Clevedon, UK).

16S rRNA sequence analysis of pure cultures

DNA extraction

A pure colony was suspended in 200 µL of distilled water and subjected to a 10 min boiling pre-treatment. DNA was extracted using a QIAamp® Stool Mini Kit (Qiagen), with slight changes to the manufacturer's instructions, as described in the Appendix 1.

Polymerase chain reaction (PCR)

PCR amplification of the V3 region of 16S rRNA genes was undertaken using the reverse primer P2 (5'-ATT ACC GCG GCT GCT GG-3') and the forward primer P3 with a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') after Muyzer *et al.* (1993). These primers correspond to position 341 to position 534 in the 16S rRNA of *Es. coli*, which produces a fragment of 233 bp. The following reagents were included in each PCR tube: 1 μL of primer P2 and 1 μL of P3 (50 pmol μL^{-1}; MWG-Biotech AG, Germany), 3 μL DNA template, 25 μL ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ and 21 μL PCR grade water. Giving a final concentration of 1.5 units Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs. Thermal cycling was conducted under the following conditions: 94 °C for 10 min, then 30 cycles at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min. Thermal cycling was conducted using either a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA) or a Techne TC-312 thermal cycler (MIDSCI, MO, USA).

Agarose gel electrophoresis

Before commencing downstream procedures DNA extracts and PCR products were run on agarose gels to assess quality. Unless otherwise indicated, all standard electrophoresis was conducted using 1.2% agarose (Cambrex Bio Sciences, ME, USA) containing ethidium bromide (EtBr, 0.5 mg mL^{-1}) run with 1 x Tris-borate-EDTA (TBE) buffer in a Pharmacia electrophoresis tank. Typically 5 μL of sample was loaded with 1 μL of loading buffer (Bioline). 5 μL of HyperLadder IV (Bioline) was used to assess the size of DNA products. Visualisation of agarose gels was achieved with a Bio-Rad universal hood II (Bio-Rad laboratories, Italy).

Sequence analysis

The PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions. DNA concentration was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop^{TM} 1000, DE, USA), diluted to 10 - 50 ng μL^{-1} with molecular grade water and sequenced by GATC laboratories (GATC-biotech laboratories, Germany). A BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was undertaken to retrieve the taxonomic groups for which the sequences showed the highest alignment identities.

Electron Microscopy

Scanning electron microscopy (SEM)

Fish were dissected as described on page 46. Samples for SEM were taken from 3 fish per tank unless otherwise indicated. Typically, intestinal samples from the anterior and posterior regions (ca. 2 mm^2) were excised and washed thoroughly in 1% S-carboxymethyl-L-cysteine for 30 sec in order to remove epithelial mucus. Samples were then fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (1:1 vol., pH 7.2, 3% NaCl). Fixative removal from samples was carried out by rinsing 3 times with 0.1 M sodium cacodylate buffer for 15 min. Dehydration was achieved by placing samples in graded ethanol solutions (30%, 50%, 70%, 90%) for at least 15 min each and then twice in 100%. After the dehydration process samples were critically point dried with ethanol as the intermediate fluid and CO_2 as the transition fluid (Emitech K850; Kent, UK). Dried samples are then mounted on aluminium stubs and gold coated using an Emitech K550 sputter coater (Kent, UK). Samples were then screened using a Jeol JSM 5600 LV electron microscope (Tokyo, Japan).

Transmission electron microscopy (TEM)

Fish were dissected as described in section 46. Samples for TEM were taken from 3 fish per tank unless otherwise indicated. Typically, intestinal samples from the anterior and posterior regions (ca. 1 mm^2) were excised and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1:1 vol., pH 7.2, 3% NaCl). Samples were rinsed with buffer twice for 15 min (minimum) in order to remove fixative. The samples were then secondary fixed for 1 hr in 1% osmium tetroxide (OsO_4). After, the samples were rinsed again with buffer (0.1 M sodium cacodylate) in order to remove the residual osmium. Samples were then dehydrated in graded ethanol solutions 30%, 50%, 70%, 90% (for 15 min) and then twice in 100% (for 15 min). Ethanol was then replaced with resin using an ethanol/resin solution at several graded concentrations: 30% resin (70% ethanol) for 24 hr, 50% resin (50% ethanol) for a minimum 5 hr, 70% resin (30% ethanol) for a minimum of 5 hr and finally in absolute resin for 24 hr. Tissues were placed in beem capsules in preparation for resin polymerisation (70 °C overnight). Blocks were trimmed; semi-thin sections (0.5 μm) were cut with a glass knife and stained with methylene blue for initial examination under light microscope. Ultrathin sections were cut with a diamond knife (~90 nm). Sections were placed in copper grids and stained with saturated uranyl acetate solution for 30 min, rinsed with distilled water and post stained with Reynolds lead citrate (Knight & Lewis 1977) for 30 min. Ultrathin sections were then screened with a JSM 1200 EX transmission electron microscope at 120kV (Jeol, Tokyo, Japan).

Sample screening and image analysis of microvilli

SEM images were taken at various magnifications for general structural observations and observations of bacterial communities. Images were taken at x 20 000 magnification and analysed using Image J 1.40 (http://rsb.info.nih.gov/ij/download.html) to assess microvilli

density. Images were converted to 8-bit and then the foreground (microvilli) was differentiated from the background (space between microvilli) by thresholding the image (as depicted in Plate 2.4). The ratio of foreground/background is then calculated to give a density value (as arbitrary units; AU). Images were analysed blind to prevent bias and typically 3 images per sample were analysed.

TEM images were analysed with Image J 1.40. TEM (Magnification x 20 000) in order to measure microvilli length after Hu *et al.* (2007). 10 well orientated individual microvilli were measured per image, with typically 3 images per sample.

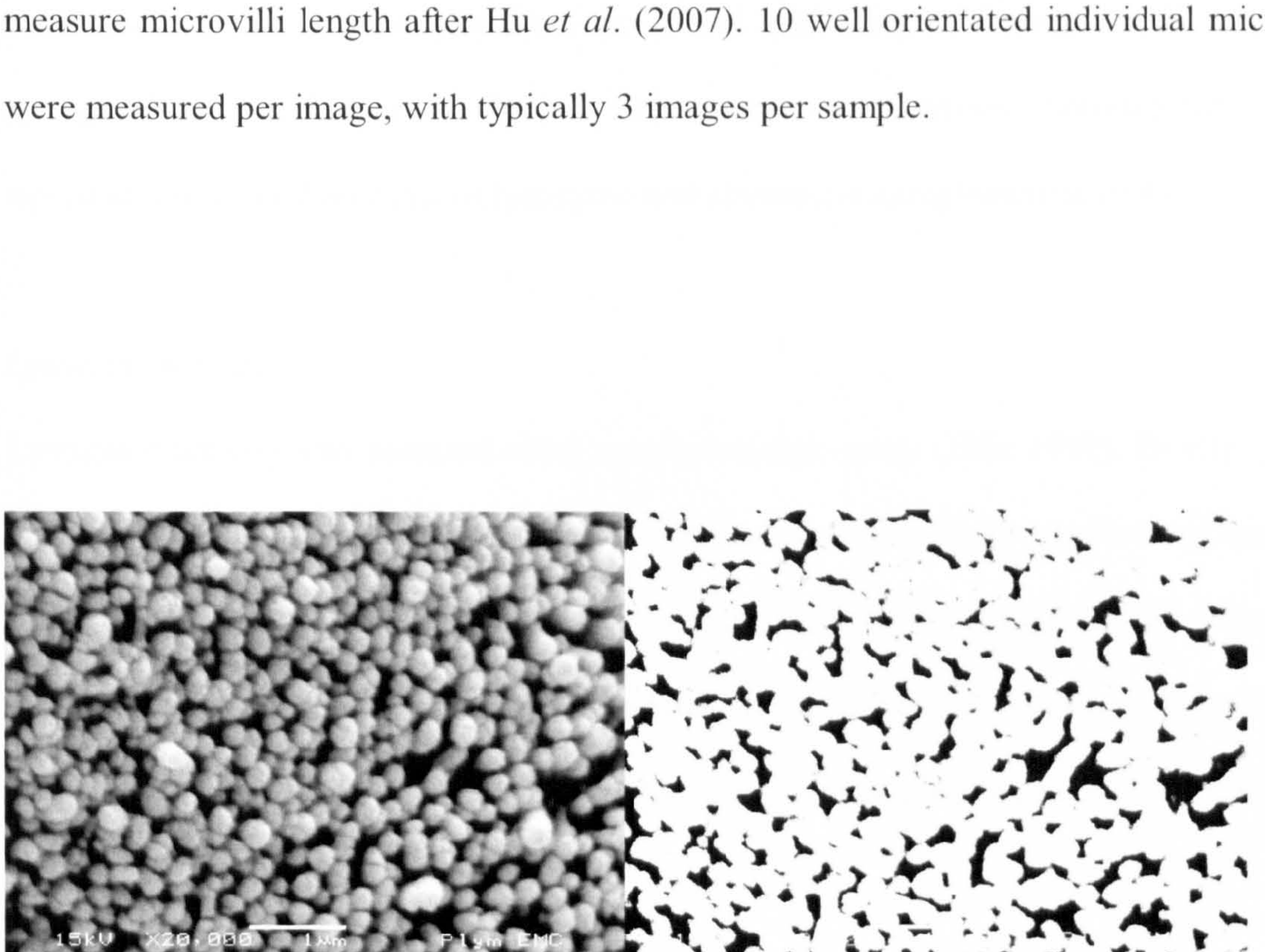


Plate 2.4. Example of threshold adjustments of SEM micrographs (rainbow trout anterior intestine) using Image J (V. 1.40) for the analysis of microvilli density. Left: sparse (top) and dense (bottom) microvilli. Right transformed images for analysis. Ratio of black to white is then calculated to give a density ratio (expressed as arbitrary units, AU).

Haematological and immunological parameters

Typically blood samples were taken from 3 fish per tank at the end of the growth trial. Fish were either A) euthanized by overdose of Tricaine methanesulfonate (MS222; Pharmaq, Fordingbridge, UK; 200 mg L^{-1} water for 15 min) followed by destruction of the brain, or B) sedated by transfer to an anaesthetic bath of 80 mg L^{-1} MS222 (Alpharma tech. bulletin). Fish were judged as suitably sedated for subsequent sampling by loss of equilibrium. Blood was sampled from the caudal vein using a 25 gauge needle and 1 mL syringe. Blood was left to clot for 3 hr before serum was removed. Serum samples were stored at -80 °C until analysis of lysozyme and alternative complement activity.

Lysozyme activity

Lysozyme activity was assessed using a turbidometric assay (Ellis 1990). Briefly, 50 µL of serum was added to 950 µL of the lysozyme sensitive Gram-positive *Micrococcus lysodeikticus*; at a concentration of 200 mg mL^{-1} in 0.05 M Na_2HPO_4 (pH 6.2). After mixing the reduction in turbidity was measured between 0.5 and 4.5 min at 530 nm at 22 °C. 1 unit of lysozyme activity was defined as a decrease in absorbance of 0.001 units min^{-1}.

Alternative complement activity

Alternative complement activity was assayed using rabbit red blood cells (RaRBC; TCS Biosciences, Claydon, UK) following the procedure of Yano (1992). Briefly, RaRBC were washed 3 times and adjusted to cell concentration of 2×10^8 mL^{-1} in 0.01M in EGTA-Mg-gelatin PBS buffer (pH 7.5). The 100% lysis value (OD 414 nm) was obtained by lysing 100 µL rabbit erythrocytes with 3.4 mL distilled water. Serum was subjected to an initial 1/20 dilution. Dilutions of serum were made using 0.01M in EGTA-Mg-gelatin PBS

buffer to give the following concentrations in a total volume of 250 µL: 5%, 4%, 3.2%, 2.5% and 2 % (v/v). 250 µL RaRBC was added and tubes and then incubated at 20 °C for 90 min with manual shaking. The volume of each tube was adjusted to 1 mL using 0.01M in EGTA-Mg-gelatin PBS buffer. Tubes were then centrifuged (1600 *g*) for 5 min and 200 µL of supernatant was transferred to a 96-well plate. The OD (414 nm) of the supernatant was measured in an Optimax microplate reader with Kinetic software SOFTmax® (Molecular Devices, USA) and % haemolysis was calculated. A lysis curve was obtained by plotting haemolysis against volume of serum. The volume of serum yielding 50% haemolysis was determined and used to calculate the complement activity of the sample (ACH50 value units mL^{-1}).

Haematocrit

In order to measure haematocrit fresh blood was drawn into microhaematocrit tubes by capillary rise and sealed with Cristaseal. Capillaries were centrifuged for 5 min (3600 *g*) in a microhaematocrit centrifuge. Haematocrit values were measured using a Hawksley reader as % packed cell volume (%PCV).

Leukocyte counts

In order to quantify total leukocyte counts 5 µL whole blood was smeared on to microscope slides. Slides were allowed to air dry and were then fixed in 95% methanol. Slides were stained with 6% Giemsa (BDH) for 20 min and mounted in DPX (BDH). Images were taken using a DCM130 digital camera (Brunel microscopes Ltd, Wiltshire, UK) using ScopePhoto (ScopeTek®, China) and a Medilux-12 light microscope (Kyowa). Counts were performed on digital images and levels were quantified as total number of leukocytes per 1000 blood cells.

Statistical analysis

Data transformations were carried out where necessary and unless otherwise indicated relevant statistical analysis was carried out using SPSS 15.0 (Chicago, IL, USA). Shapiro-Wilk test was conducted in order to test for normal distribution of data. Typically a One-Way ANOVA and *post hoc* LSD was used for the analysis of normally distributed data and Kruskal-Wallis and *post hoc* Bonferroni was used for analysis of non-normally distributed data. Significance was accepted at $P < 0.05$ level. Results are presented as mean ± standard deviation unless otherwise indicated.

Chapetr 3. The indigenous intestinal microbiota of rainbow trout

Chapter 3A. The indigenous intestinal microbiota of farm reared rainbow trout

Abstract

Bacterial communities from the intestinal tract of rainbow trout were investigated to assess transient and resident microbial communities using both culture-based and culture-independent techniques. Viable counts attached to the intestinal mucosa were in the range of log 4.77-5.38 CFU g^{-1} and log 6.67-6.79 CFU g^{-1} in the intestinal contents. *Pseudomonas* spp. and Enterobacteriacaea constituted nearly 80% of the allochthonous population but less than 60% of the autochthonous populations. This coincided with an elevated mucosal level of a group of Gram-positive rods from ~ 2% in the digesta to ~25-35% on the mucosa. This group was identified by 16S rRNA as *Arthrobacter aurescens* and *Janibacter* spp.. Analysis of DGGE banding patterns revealed complex communities in all intestinal regions. Similarity coefficients revealed that mucosal communities were ~70% similar to digesta communities yet due to the presence of bands found uniquely either in the digesta or on the mucosa the communities are distinctly different. Scanning electron microscopy confirmed mucosal bacterial populations and highlighted a possible localised colonisation between mucosal folds. The study highlights the complexity of resident microbial communities which have not been fully explored in previous rainbow trout studies; this is especially true with probiotics/prebiotic investigations.

Introduction

In order to fully analyse and understand the effects of probiotics on rainbow trout growth the indigenous gut microbiota must first be investigated. To achieve this, and develop relevant techniques, an investigation was carried out to characterise the microbial communities of different intestinal regions of farm reared rainbow trout. Previous investigations of the rainbow trout gut microbiota have given a broad solid foundation but are sometimes limited by the number of gut regions and the numbers of isolates tested (Trust & Sparrow 1974; Trust *et al.* 1979; Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000; Spanggaard *et al.* 2001; Huber *et al.* 2004; Heikkinen *et al.* 2006; Pond *et al.* 2006). Many of these previous studies have focused on the allochthonous microbiota as opposed to the autochthonous communities found associated with the intestinal lining. Early studies have suggested that the microbiota associated with the epithelial mucosa is considerably less than that in the digesta; 2 - 3 log units difference (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000). It is because of this that recent studies that explore the gut microbiota of rainbow trout are concerned with the transient populations found within the digesta. The microbiota associated with the gut epithelium is thought to play a role as a barrier against pathogenic infection (Ringø & Birkbeck 1999) and numerous papers have also demonstrated the influence of the adherent population on the integrity of the epithelial surface (for a review see Ringø *et al.* 2007). The key aim of the experimental investigation of this section is to give a comprehensive analysis of autochthonous and allochthonous intestinal microbial communities of the rainbow trout, as well as the bacteriology of the rearing environment.

Materials and methods

Fish

Rainbow trout were collected from a local fish farm (Rattery Devon, UK; page 41). A total of 12 fish were sampled in June and July 2005. Water temperatures ranged between 15 - 16 °C during this period. Fish were fed a commercial diet (Skretting elite; 42% protein, 20% lipid) and had a mean weight of 100.0 ± 6.7 g.

Dissection and sampling procedure

Fish were euthanized by a hard blow to the head, followed by destruction of the brain and placed on ice for transportation to the laboratory. Triplicate samples of water, originating from three different regions of the farm, along with triplicate samples of feed were taken. Time between termination and dissection did not exceed 2 hr. Fish were dissected (as described on page 46) to differentiate samples of the anterior intestinal mucosa (AM), anterior digesta (AD), posterior mucosa (PM) and posterior digesta (PD). The resulting material from 3 fish was pooled into 1 sample, thus, yielding a total of 4 samples.100 mg of sample material was then separately homogenized in 900 µL PBS (10^{-1}).

Bacterial identification

Culture-based methodologies

Plating and culture-based identification was carried out as explained on pages 46-47. Duplicate plates were also incubated anaerobically in a modular atmosphere controlled system (DW Scientific, Shipley, UK) to determine the number of obligate anaerobes. Anaerobic gas (10% H_2, 10% CO_2 and 80% N) was supplied by BOC (Surrey, UK). Isolates were picked and re-streaked on TSA for aerobic incubation to differentiate the strict anaerobes from the facultative anaerobes.

Sequence analysis

Several isolates were subjected to BLAST analysis following DNA extraction, PCR and sequence analysis as described on pages 47 - 49.

Culture-independent investigations

DNA extraction

Intestinal samples were incubated with lysozyme solution (50 mg mL^{-1} in TE buffer) for 30 min at 37 °C. DNA was then extracted from 2 pooled samples per gut region using a QIAamp® Stool Mini Kit (Qiagen) with minor modifications to the manufacturers' instructions (Appendix 1).

Ribosomal intergenic spacer analysis

PCR amplification of 16S and 23S intergenic spacer region was undertaken using the primers B1055 (5'-AAT GGC TGT CGT CAG CTC GT-3') and 23SOR (5'-TGC CAA GGC ATC CAC CGT-3') after Acinas *et al.* (1999). The following reagents were included in each PCR tube: 1 µL of primer B1055, 1 µL of primer 23SOR (100 pmol μL^{-1}; MWG-Biotech AG, Germany), 2 µL DNA template (25 ng μL^{-1}), 10 µL ReadyMix™ Taq PCR Reaction Mix with $MgCl_2$ and PCR grade water to a final volume of 20 µL. Thermal cycling was conducted using a Techne TC-312 thermal cycler, under the following conditions: 95 °C for 5 min, then 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min with a final extension step of 72 °C for 10 min. 5 µL of the PCR products were then run on a 2% agarose gel in 1 x TBE. For comparative purposes samples were run on several gels. Two gels containing EtBr (0.5 and 1.0 mg mL^{-1}) and one with GelRed (Biotium, 0.1 µL mL^{-1}). Gels were visualised with UV (Bio-Rad universal hood II).

Images were optimised for analyses by enhancing contrast and greyscale using Quantity one® version 4.6.3 analyses software (Bio-Rad laboratories, CA, USA).

Denaturing gradient gel electrophoresis (DGGE)

PCR amplification of the V3 16S rRNA genes was undertaken as described on page 48 with the following modifications:

- 5 μL DNA template
- Touchdown thermal cycling was conducted under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min. The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at 55 °C for the remaining cycles.

The DGGE was performed using a DGGE-2001 system (C.B.S. scientific, CA, USA; Plate 3.1). 15 μL of standardized PCR products (40 ng μL^{-1}) were run on 8% acrylamide gels (16 cm x 16 cm x 1 mm) with a denaturing gradient of 40 - 60% (where 100% denaturant is 7 M urea and 40% formamide). Gels were poured using a Bio-Rad gradient delivery system (model 475, Bio-Rad laboratories). All samples were run on the same gel to prevent issues of reproducibility and outside lanes were not used. The gel was run at 65 V for 16 hr at 60 °C in 1 x TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA). Preliminary staining using EtBr (up to 1 μg mL^{-1} for 30 min) was not sensitive enough as only a few bands were observed. Therefore, visualization of the DGGE bands thereafter was achieved by the more sensitive, optimized silver staining method of Benbouza *et al.*, (2006). Briefly, the gel was soaked in fixation buffer (dH_2O containing 10% ethanol and 0.5% acetic acid) for 5 min followed by 6 - 7 min in impregnation solution (1 L dH_2O containing 1.5 g $AgNO_3$, 1.5 mL 37% formaldehyde). The gel was then briefly washed in

dH_2O before transferring to development solution (1 L dH_2O containing 15 g NaOH, 2 mL 37% HCOH) for 3 - 5 min or until bands are clearly visible. The gel was then transferred to the fixation buffer to terminate development (2 min). The Gel was scanned in a Bio-Rad universal hood II (Bio-Rad laboratories, Italy) and optimized for analyses by enhancing contrast and greyscale.

Electron microscopy

Samples for SEM were also taken from 5 individual fish as described on page 49.

Statistical analysis

Statistical analysis of culture-based microbial populations was carried out as described on page 54. Due to the nature/sensitivity of the silver staining procedure, excessive staining of lanes may sometimes occur, therefore analysis of banding patterns from silver stained gels were conducted on a presence/absence basis instead of including band intensities. Jaccard similarity calculation was conducted from binary analysis of the presence/absence of bands present from the DGGE fingerprints in order to construct a similarity half matrix. Jaccard similarity was calculated as follows: $S = N_{AB}/(N_A + N_B - N_{AB})$, where N_{AB} is the number of common bands, N_A is the number of bands in lane A and N_B is the number of bands in lane B.

Results

Culture-based analysis

Viable counts in the feed were log 5.87 CFU g^{-1}; dominant microbial groups were identified as Enterobacteriaceae, *Pseudomonas* spp., *Micrococcus* spp. and yeast. Viable counts in the rearing water were log 4.43 CFU mL^{-1}; dominant bacterial genera were Enterobacteriaceae, *Pseudomonas* spp. and other Gram-positive rods. The aerobic heterotrophic bacterial populations associated with the rainbow trout intestine is displayed in Table 3.1. The number of viable autochthonous microbes were less than the level of allochthonous microbes by approximately 1-1.5 log units. Mean viable counts were log 4.77 CFU g^{-1} in the anterior mucosa; log 5.38 CFU g^{-1} in the posterior mucosa; log 6.67 CFU g^{-1} in the anterior digesta; and log 6.79 CFU g^{-1} in the posterior digesta. The autochthonous level was significantly lower than the allochthonous level in the anterior intestine ($P = 0.014$) but not the posterior. *Staphylococcus* and *Acinetobacter* spp. were observed as minor allochthonous populations but were completely absent on the mucosa. Levels of *Pseudomonas* spp. were consistently found to be significantly higher in the digesta than the mucosa ($P < 0.05$). The percentage of allochthonous *Pseudomonas* spp. was 35.81% and 43.55% in the anterior and posterior intestine, respectively. However, autochthonous levels were only found at 16.80% and 18.58% in the anterior and posterior intestine, respectively. This coincided with a considerable increase in the number of unidentified Gram-positive rods. Dominant isolates from this group were identified by 16S rRNA as *Arthrobacter aurescens* (100 %) and *Janibacter* spp. HTCC2649 (99 %). Combined, they were found at levels lower than 2 % in the digesta but comprised 36.79% and 24.71% of the total microbial population identified on the anterior and posterior mucosa, respectively.

Table 3.1. Composition of rainbow trout microbiota isolated from different intestinal regions: AM, anterior mucosa; AD, anterior digesta; PM, posterior mucosa; PD, posterior digesta, F, feed; W, water. Microbial composition expressed as percentages.

	AM	AD	PM	PD	F	W
Viable count (CFU g^{-1})	4.77^{a}	6.67^{b}	5.38	6.79	5.87	4.43
Composition (%)						
Enterobacteriaceae	31.44	41.41	38.88	35.13	17.68	10.93
Pseudomonas spp.	16.80^{a}	35.81^{b}	18.58^{a}	43.55^{b}	27.26	59.71
Staphylococcus spp.	-	3.22	-	3.35	-	-
Micrococcus spp.	3.80	9.19	5.03	6.76	28.21	1.99
Acinetobacter spp.	-	0.49	-	2.89	0.46	2.48
Aeromonas spp.	7.80	7.98	12.80	7.35	4.17	8.95
Vibrio spp.	-	-	-	-	-	1.00
Bacillus spp.	1.89	0.49	-	-	3.47	3.99
Other Gram + rods*	36.79	1.41	24.71	0.98	6.28	10.95
Yeast	1.47	-	-	-	12.49	-
Total number of isolates	206	214	210	207	209	201

[ab] Different superscripts within the row and same intestinal region (i.e. anterior or posterior) denote a significant difference between mucosal (autochthonous) and digesta populations (allochthonous) ($P < 0.05$).

* Dominant isolates identified by 16S rRNA as *Arthrobacter aurescens* and *Janibacter* spp.

- not detected.

[z] CFU mL^{-1}

RISA of bacterial community profiles

PCR-RISA resulted in the presence of very few bands (Figure 3.1). Two distinct bands of size 600 - 700 bp were identified and were common to all samples. Several other faint bands were present in some samples. Feint

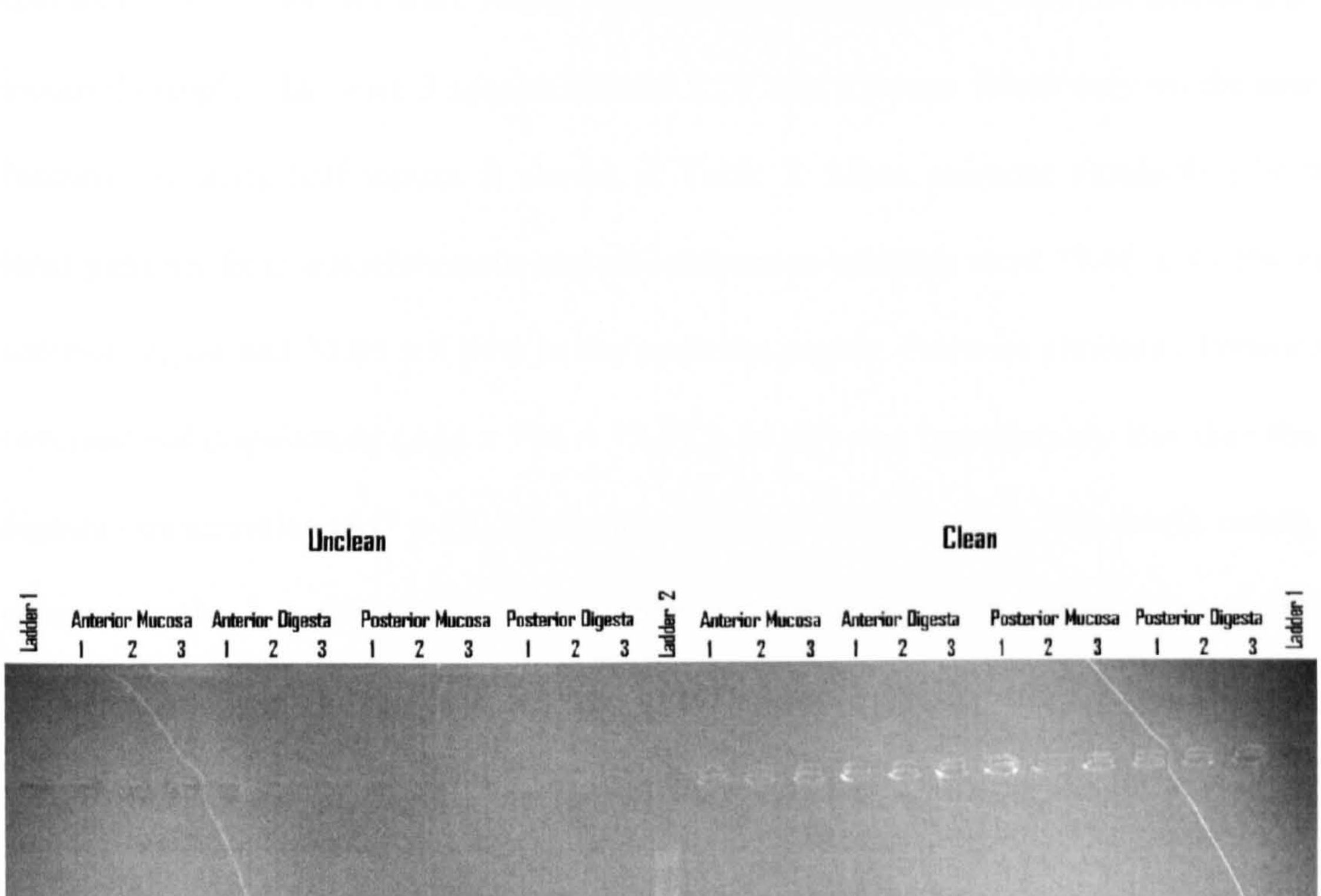


Figure 3.1. Negative image of clean and unclean PCR-RISA run on a 2% agarose gel. Ladder 1; 1000-100 bp ladder (HyperLadder IV, Bioline, 100 bp increments). Ladder 2; 2000-50 bp ladder (HyperLadder II, Bioline, 2000, 1800, 1600 1400, 1200, 1000, 800, 600, 500, 400, 300, 200, 100, 50 bp). Banding patterns represent fingerprints of the bacterial communities present in the intestinal tract of rainbow trout. Only 2 or 3 distinct bands present (ranging from ~500 - 700 bp).

DGGE analysis of bacterial community profiles

DGGE analysis revealed complex microbial communities present in both replicates from all intestinal samples (Figure 3.2). A total of 27 distinct bands (presumed species) were observed on the gel. The number of different species present in each of the samples ranged between 18 - 23; of these, 15 were common to all samples and all replicates. However, 2 species (bands C and K) were found present in the digesta that were not detected in any mucosal samples, likewise 3 species (bands V, Y and Z) were found only on the mucosa. Jaccard similarity half matrix is shown in Table 3. Mean pairwise similarities between band patterns from autochthonous and allochthonous samples were 73.46 ± 8.45% in the anterior region and 70.85 ± 8.84% in the posterior region. Pairwise similarity between the two mucosal populations (AM x PM = 79.34 ± 14.40) was considerably less than the two digesta communities (AD x PD similarity = 89.01 ± 5.38%). It is also worth noting that comparing the 2 replicated pooled samples for each region the posterior mucosal the pairwise similarity value was one of the lowest values recorded (PM1 x PM2 = 64.00%) as opposed to the other regions which were relatively similar (AM = 78.26 %, AD = 83.33 % and PD = 86.32 %).

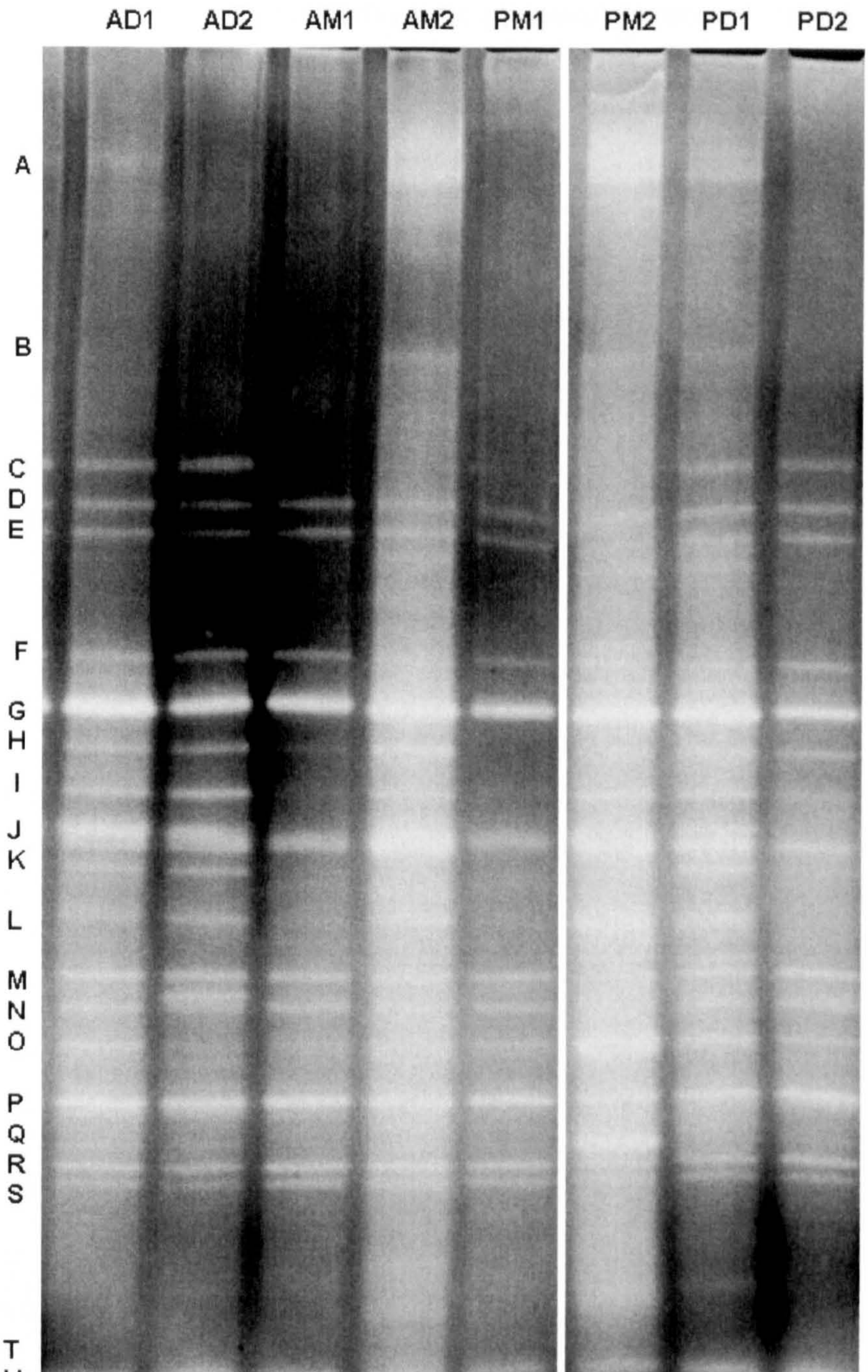


Figure 3.2. Negative image of a silver stained 40 - 60% DGGE gel, showing banding (A-Z2) patterns of PCR-amplified bacterial 16S rRNA fragments. Banding patterns represent fingerprints of the bacterial communities present on the anterior mucosa (AM), anterior digesta (AD), posterior mucosa (PM) and posterior digesta (PD) of rainbow trout. Each lane represents 2 samples pooled from 6 fish.

Table 3.2. Similarity half matrix between presence\absence of DGGE bands of rainbow trout microbial communities isolated from different intestinal regions (Jaccard calculation, values expressed as %). AD = anterior digesta; AM = anterior digesta; PD = posterior digesta; PM = posterior mucosa.

Region		AD		AM		PD		PM	
	Replicate	1	2	1	2	1	2	1	2
AD	1	100.00	83.33	64.00	84.00	87.50	82.61	61.54	80.00
	2		100.00	69.57	76.00	95.45	90.48	66.67	72.00
AM	1			100.00	78.26	60.00	68.18	94.74	60.00
	2				100.00	80.00	68.00	82.61	80.00
PD	1					100.00	86.32	64.00	83.33
	2						100.00	65.22	70.83
PM	1							100.00	64.00
	2								100.00

Table 3.3. Number of bacterial species present in samples from DGGE lanes and mean pairwise comparisons of similarity indices of the bacterial communities present in the rainbow trout intestine. Intestinal regions: anterior mucosa (AM), anterior digesta (AD), posterior mucosa (PM) and posterior digesta (PD).

Region	# Species
AD 1	23
AD 2	21
AM 1	18
AM 2	23
PM 1	19
PM 2	22
PD 1	22
PD 2	19
Pairwise comparisons (%)	
AD x AM	73.4 ± 8.6
PD x PM	70.9 ± 8.8
AD x PD	89.0 ± 5.4
AM x PM	79.3 ± 14.4

Electron microscopy

The scanning electron micrographs clearly demonstrate that bacterial populations are adhered to the mucosal lining (Figures 3.3 and 3.4) and can be considered as resident (autochthonous); thus, confirming the conventional and molecular microbial analysis. Mixed populations of rods and cocci can be seen on the surface of the mucosa. Sporadic colonisation by bacterial cells appeared throughout the anterior and posterior intestine; however, a noticeable localised effect was evident between the mucosal folds and at the bases of the villi (Figure 3.5).

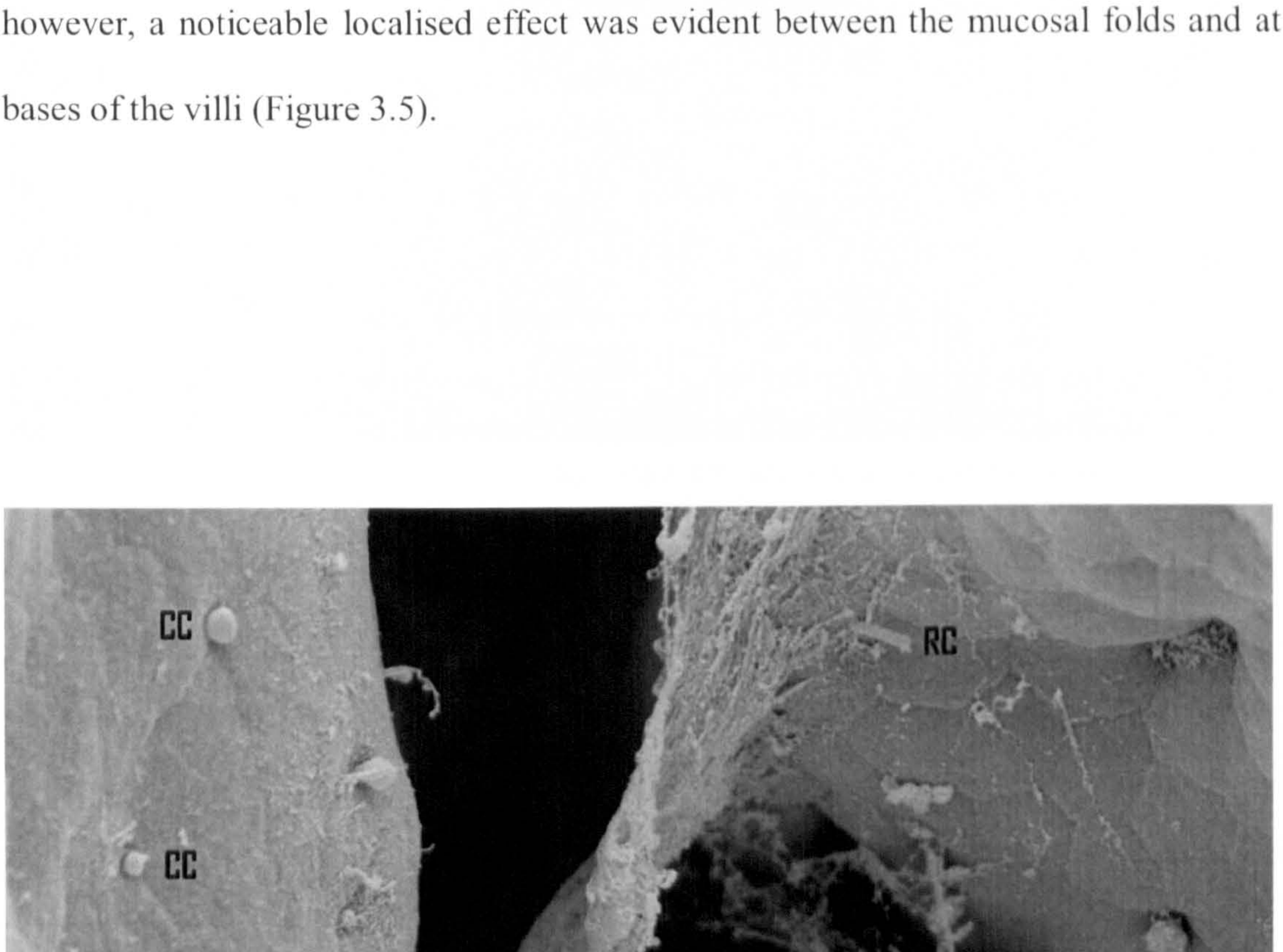


Figure 3.3. SEM micrograph of the anterior intestinal mucosa of trout. Mixed populations of rods (RC; rod cells in division) and cocci-like cells (CC) adhered to the mucosal surface. Scale bar = 10 µm.

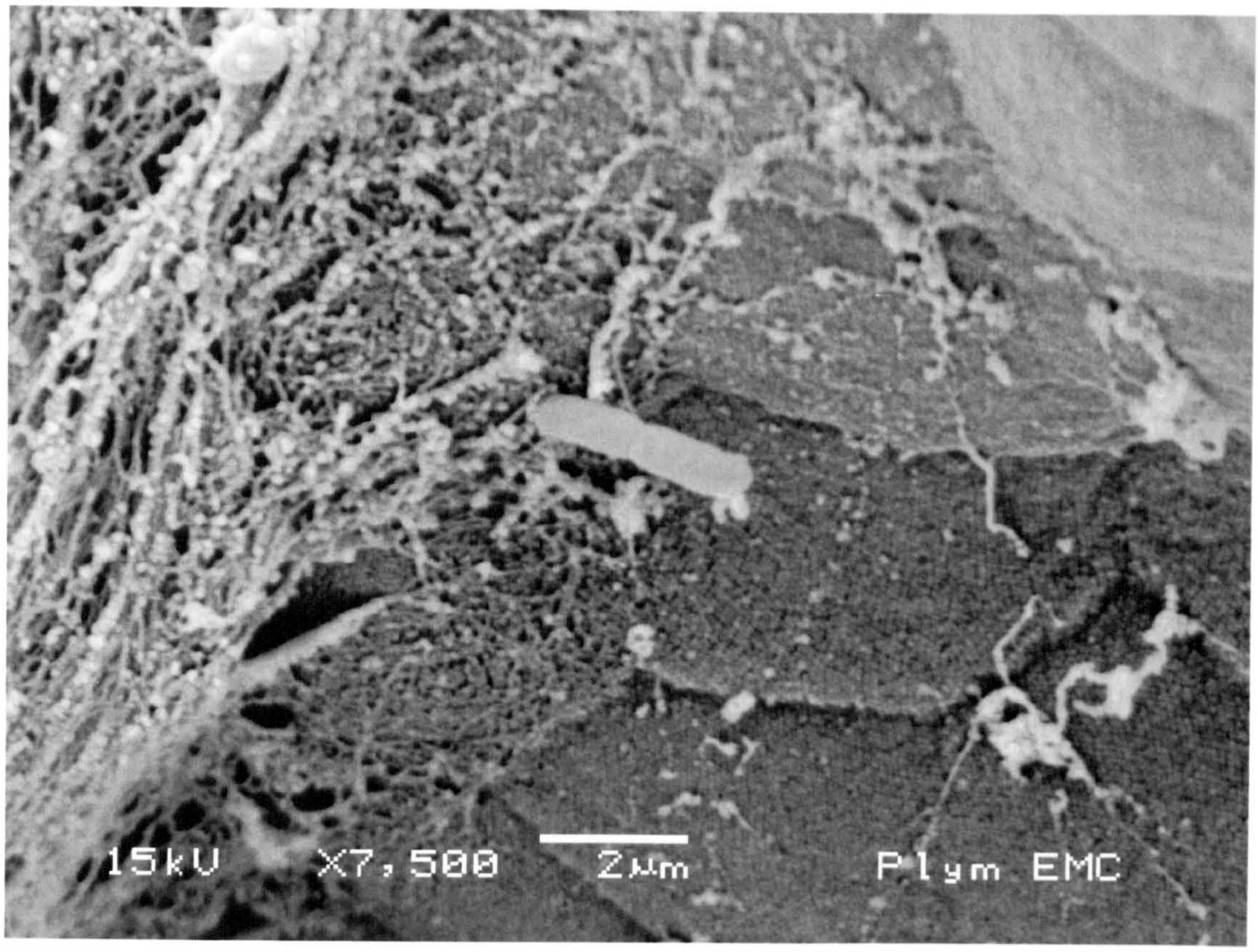


Figure 3.4. SEM micrograph of the anterior intestinal mucosa. Examples of close microvilli association of bacterial rod cells. Scale bar = 2 µm.

Figure 3.5. SEM micrograph of the posterior intestinal mucosa. Localised colonisation of bacterial cells between mucosal folds and at the base of the villi. Scale bar = 10 µm.

Discussion

The bacterial populations identified are in line with that of similar studies and confirms that the dominant groups associated with the gut of rainbow trout belong to the γ subclass of Proteobacteria, in particular *Pseudomonas* spp., *Aeromonas* spp. and Enterobacteriaceae (Austin & Al-Zahrani 1998; Spanggaard *et al.* 2000; Spanggaard *et al.* 2001; Huber *et al.* 2004; Heikkinen *et al.* 2006; Pond *et al.* 2006; Kim *et al.* 2007). Similarly, as reported in many of these papers, fluctuations of microbiota between samples and the 2 sampling visits were also observed. This is to be as expected as the bacterial populations found to be associated with fish are largely influenced by the fluctuating nature and bacteriology of their environments (Cahill 1990; Ringø *et al.* 1995; Ringø & Gatesoupe 1998; Ringø & Birkbeck 1999). It is clear from the present study that the most dominant groups associated with the feed and water are often reflected by their dominance within the gut regions. This is most evident with *Pseudomonas* spp. and Enterobacteriaceae. The number of viable microbes found in the digesta (10^5 - 10^7 CFU g^{-1}) is in agreement with earlier studies and it was also observed that the viable microbial communities associated with the intestinal mucosa are lower than that found passing through the lumen in the digesta. However, typically only a 1 – 2 log unit decrease in the viable microbial communities was observed on the mucosal surfaces, as opposed to 2 - 3 log units reported previously (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000). In the present study log viable counts in the intestine were in the range of 4.77 - 5.38 CFU g^{-1} on the mucosal surfaces and 6.67 - 6.79 CFU g^{-1} in the digesta. Comparably high levels of adherent gut mucosal populations have been identified in other fish species. Viable counts in the order of log 4.93 - 6.34 CFU g^{-1} have been found on the hindgut mucosa of Atlantic salmon (Ringø *et al.* 2006a) and counts in the range of log 4.55 - 5.68 CFU g^{-1} have been reported to be associated with the mucosa of Arctic charr (Ringø *et al.* 2006b). Similarly,

viable bacterial counts found adherent to the intestinal mucosa of Atlantic cod have been reported in the range of log 3.95 - 4.77 CFU g^{-1} in the mid intestine and log 4.73 - 5.50 CFU g^{-1} in the distal intestine (Ringø *et al.* 2006c). These values are only approximately one log unit lower than the values reported in the digesta; log 4.96 - 5.45 CFU g^{-1} in the mid intestine and log 5.35 - 5.56 CFU g^{-1} in the distal intestine. Kim *et al.* (2007) investigated rainbow trout microbial populations of the intestinal mucus and intestinal contents. Lower numbers of viable bacteria were found in the mucus (log 6.48 ± 6.66 CFU g^{-1}) than in the digesta (log 6.68 ± 6.81 CFU g^{-1}); however, these were not significantly different ($P > 0.05$). The microbial population found was similar, yet smaller in diversity, than the communities found in the digesta. This suggests that the mucus associated microbiota may be distinctly different from the transient population associated with the intestinal contents. The mucus bacterial population may itself be a population representing a different niche between the intestinal contents and the epithelial associated community. In the present study a higher level of the group identified as "other Gram-positive rods" were isolated from the mucosa than the digesta. These isolates were identified by 16S rRNA as closest nucleotide sequence matches to *Arthrobacter aurescens* and *Janibacter* spp. HTCC2649. *Arthrobacter* spp. have been identified from the rainbow trout digestive tract (Huber *et al.* 2004; Kim *et al.* 2007), however, to the author's knowledge *Arthrobacter aurescens* and *Janibacter* spp. HTCC2649 have not been isolated from the rainbow trout digestive tract previously. Both *Janibacter* and *Arthrobacter* are genera of the Actinomycetales order and are robust enough to survive environmental fluctuations and are thus able to survive gastric transit to be found in the rainbow trout intestine. *A. aurescens* are ubiquitous soil bacteria and were likely present in the soil and pond water at the trout farm. Arthrobacteria can degrade pesticides and other toxic compounds and could be important in terms of bioremedation (Megharaj, Avudainayagam, & Naidu 2003).

Janibacter spp. HTCC2649 is an aquatic bacterium originally isolated from the Sargasso Sea, Atlantic Ocean. The name *Janibacter* is derived from Janus, because the bacterium has a changing rod - cocci morphology during the growth cycle (Loubinoux, Rio, Mihaila, Foïs, Le Fleche, Grimont, Marie, & Bouvet 2005). Related Gram-positive rod genera, such as *Arthrobacter* also show a rod - cocci growth cycle and thus such species are difficult to identify with culture-based methods alone. These species may have been misidentified in previous studies which have not incorporated 16S rRNA analysis or they may not have been isolated previously due to the fact that these organisms were low in the digesta, the sampling region which has been the focal point of most previous investigations. Future studies focusing on the mucosa will reveal whether these species are commonly found as epithelial populations or whether this is an isolated case, confined to the present farm.

The results from the RISA based fingerprinting method in the present study were not very informative with regards to community analysis due to the very small number of bands. However, RISA has been used successfully to demonstrate differences of microbial communities associated with range environmental samples, including aquatic bacterial communities (Fisher & Triplett 1999; Yu & Mohn 2001). The ineffective nature of RISA in the present study may be due to that fact that the length of intergenic spacer region of the different species recovered were relatively similar. Despite this, differentiation of bands may have been more effective using Metaphor agarose gel or polyacrylamide gel electrophoresis (PAGE).

Statistical analysis of gel electrophoresis fingerprints is a useful tool for comparing complex microbial community profiles from different environmental samples and it can be utilised as a tool without the need for sequencing of bands (Eichner *et al.* 1999; Zhang & Fang 2000; Yang *et al.* 2003; Dilly *et al.* 2004). Although binary similarity calculations

are routinely used, these estimates are likely to overestimate similarities due to the non-weighted quantification of each band (Yu & Mohn 2001); thus, similarity coefficients presented in the current study should be considered as maximum estimates. The current study utilised banding pattern analysis to demonstrate the complexity of the microbiota found within the rainbow trout intestine, with specific emphasis on mucosal populations in a way that has not been conducted before. While gel electrophoresis techniques have been used previously in rainbow trout studies they have been used only as a means for excision and sequencing of bands (Spanggaard *et al.* 2000; Huber *et al.* 2004; Pond *et al.* 2006; Kim *et al.* 2007). The data from the present study show that the number of autochthonous species and allochthonous species within the intestine is not significantly different (~20 in both intestinal regions). However, when comparing the species from respective mucosal and digesta samples by banding similarity matrices, mean pairwise comparisons are only approximately 70%. Pairwise similarity between the two mucosal populations (~79%) was considerably less than when comparing the two digesta communities (89%). This suggests that the autochthonous microbial community may be more specific to different intestinal regions than the digesta populations, which appear to only alter slightly during intestinal passage. Further investigation is clearly required. DGGE based analysis appears to confirm the conventional culture-based findings and shows that autochthonous and allochthonous communities are distinctly different. Autochthonous communities in the posterior intestine appeared highly variable with one of the lowest pairwise values recorded when comparing the replicate fingerprints. Future studies should incorporate sequence analysis of DGGE bands isolated from intestinal epithelial samples in order to identify non-cultured species and further assess variability of autochthonous populations.

Electron microscopy is an important tool for investigating intestinal mucosal bacterial populations and in recent times the gut microbiota of several fish species have been

investigated with electron microscopy (Ringø *et al.* 2003; Ringø *et al.* 2007). Such species include Atlantic salmon (Ringø *et al.* 2006a), Arctic charr (Ringø *et al.* 2001; Ringø *et al.* 2006b) and Atlantic cod (Ringø *et al.* 2006c). However, the use of SEM as tool for the investigation of rainbow trout mucosal bacterial populations is not well documented. Lesel & Pointel (1979) used SEM to demonstrate bacterial colonisation of the rainbow trout mucosal epithelium; they found that it was only possible to observe bacteria associated with pyloric caeca and middle intestines, but not the posterior intestine. Austin & Al-Zahrani (1988) reported a lack of colonisation of the intestinal mucosa but demonstrated adherent bacteria on the mucosa of the stomach with the use of SEM. Contrary to these findings, the present study revealed sporadic colonisation of both the anterior and posterior intestinal mucosa; in addition to this, large levels of colonisation were evident between the folds of the mucosal surface. Presumably, residence is more easily established and sustained at the base of the villi and between the mucosal folds due to reduced exposure to shear (peristalsis) of the transient digesta as it passes through the lumen. SEM also demonstrated a very close association of bacterial cells with the epithelial mucosa which may play a role in host immunostimulation. SEM is an especially useful tool for demonstrating mucosal colonisation throughout the digestive tract of fish and should be incorporated in future investigations.

Chapter 3B. The indigenous intestinal microbiota of aquarium reared rainbow trout

Abstract

Rainbow trout were fed either a diet containing fishmeal (FM) as the crude protein source or a diet containing 50% replacement with soybean meal (SBM) for 16 weeks. An enteritis-like effect was observed in the SBM group; villi, enterocytes and microvilli were noticeably damaged compared to the FM group. The posterior gut microvilli of SBM fed fish were significantly shorter (1.19 ± 0.18 and 0.80 ± 0.15µm, respectively) and the anterior microvilli significantly less dense than the FM fed fish. Electron microscopy confirmed the presence of autochthonous bacterial populations associated with microvilli of both fish groups. Reduced density of microvilli consequently led to increased exposure of enterocyte tight junctions which combined with necrotic enterocytes is likely to diminish the protective barrier of the intestinal epithelium. No significant differences in total viable counts of culturable bacteria were found between the groups in any of the intestinal regions. A total of 1500 isolates were tentatively placed into groups or genera, according to standard methods and subsequent partial 16S rRNA sequencing revealed species that have not been identified from the rainbow trout intestine previously. Compared to the FM group, levels of *Psychrobacter* spp. and yeast were considerably higher in the SBM group; a reduction of *Aeromonas* spp. was also observed.

Introduction

It is highly likely that a more controlled aquarium system, with recirculation facilities, will reduce environmental variation and influences on the intestinal microbiota. This will likely result in a difference of the microbial populations found within the intestinal tract of aquarium reared rainbow trout compared to farmed or wild rainbow trout. As probiotic experiments are to be carried out under aquarium conditions it is also necessary to evaluate microbiota of aquarium reared trout. Due to current difficulties in the supply and price of fishmeal along with the knowledge that the gut microbiota is sensitive to changes of dietary components (Ringø *et al.* 1995; Ringø & Gatesoupe 1998; Ringø & Birkbeck 1999) it would be interesting to incorporate an alternative protein source into the present investigation. Plant feedstuffs are commonly utilised as key alternative protein sources due to competitive prices and relative availability (Gatlin *et al.* 2007). Soybean meal (SBM) is moderately rich in protein and is currently one of the major plant proteins included in salmonid diets (Storebakken *et al.* 2000). However, SBM has been demonstrated to induce histological and functional changes of the fish gastro-intestinal tract which include enteritis, increased susceptibility to bacterial infection, changes in absorptive cells, increased presence of inflammatory cells, shortening of villi/mucosal folds and irregular microvilli (Van den Ingh *et al.* 1991; Baeverfjord & Krogdahl 1996; Burrels *et al.* 1999; Krogdahl *et al.* 2000; Bakke-McKellep *et al.* 2000; Krogdahl *et al.* 2003; Heikkinen *et al.* 2006; Ringø *et al.* 2006c; Bakke-McKellep *et al* 2007a). While much effort has focused on evaluating the extent of SBM induced histological damage, the effect on the gut microbiota, which responds both directly and indirectly to dietary changes (Ringø *et al.* 1995; Ringø & Gatesoupe 1998; Ringø & Birkbeck 1999) is not so well documented. However, recent investigations have demonstrated SBM induced changes in gut microbiota of Atlantic cod (Ringø *et al.* 2006c) and Atlantic salmon (Bakke-McKellep *et*

al 2007b; Ringø *et al.* 2008). Moreover, a study by Heikkinen *et al.* (2006) begins to shed light onto the complicated dynamics of rainbow trout gut microbiota when fed dietary SBM. Changes in allochthonous bacterial populations were observed, however, autochthonous populations were not investigated.

Given the high numbers and complexity of the autochthonous bacterial communities identified in Chapter 3A, further research into the intestinal microbiota of aquarium reared rainbow trout and the effect of SBM is warranted. Therefore, the aim of the present study was to investigate the indigenous intestinal microbiota of aquarium reared rainbow trout. The study will also incorporate a SBM diet in order to further the research of Heikkinen *et al.* (2006) and provide a quantitative analysis of the effect of SBM on allochthonous as well as autochthonous gut microbial populations. Additionally, the ultrastructural morphology of the intestinal tract is assessed using electron microscopy.

Material and methods

Aquarium system

The feeding trial was conducted in system A at the Aquaculture and Fish Nutrition Research Aquarium (University of Plymouth; Plate 3.3). The system holds a total volume of ~5500 L. Mechanical filtration was provided by plastic scourers which trap large faecal waste; water is then pumped through a Lacron Hi Pressure sand filter (Lacron Limited, Kent, UK) to remove finer faecal solids. Biological filtration was provided by a submerged biological filter bed, composed of a porous clay filter medium containing *Nitrosomonas* and *Nitrospira* spp..

Experimental fish

Rainbow trout (25 - 35 g) in weight were obtained from a local farm (page 41). The fish were acclimated to the experimental system and fed a standard Skretting (2 - 3 mm) diet until reaching mean weight of ~40 g. All fish were graded and 25 fish were randomly distributed into 6 130 L, round edged (30°) square fiberglass tanks. Each tank was provided with ~99% re-circulated freshwater at a rate of 800 L hr^{-1}. Additional aeration was provided by an air stone supplied by a low pressure side channel blower (Rietschle, UK).

Plate 3.3. Recirculation system A, Aquaculture and Fish Nutrition Research Aquarium, The University of Plymouth.

Diet formulation and feeding

Experimental diets were formulated according to standard specifications to comply with known nutritional requirements for salmonid fish (NRC 1993). A standard diet was formulated with fishmeal (diet FM) as the protein source and a second diet was formulated with fishmeal and a SBM each providing 50% of dietary crude protein (diet SBM). The diets were prepared by a commercial feed manufacturer (Nutreco ARC, Norway) using a pilot extruder to produce low density 3 mm pellets with identical characteristics to commercial extruded trout diets. All nutrient analysis was undertaken according to AOAC (1995) methods. Diet formulation and chemical composition is shown in Table 3.4. Fish were fed 1.0 - 1.8% biomass day^{-1} for a period of 16 weeks. Water temperature was maintained at 15 ± 1 °C and dissolved oxygen levels maintained above 75% saturation. A 14 hr light/10 hr dark photoperiod was maintained throughout the entire trial duration.

Table 3.4. Formulation and nutrient analysis of experimental diets. All dietary ingredients provided by Nutreco ARC, Norway.

	Diet	
Ingredients (g kg^{-1})	FM	SBM
Fishmeal[1]	640	322
Marine fish oil	150	170
Soybean meal[2]	0	460
Whole wheat	199	37
Vitamins[3]	5	5
Minerals[4]	5	5
Yttrium oxide	1.0	1.0
Nutrient analysis		
Dry Matter	919	928
Protein	469	451
Lipid	218	220
Ash	102	83
NFE[5]	130	174
Gross energy[6] (MJ kg^{-1})	22.0	22.4

[1] LT-fishmeal, Norwegian herring meal
[2] HiPro soybean meal (48% protein), Central Soya Ltd
[3] Vitamin premix, formulated by Skretting to meet NRC (1993) requirements
[4] Mineral premix, formulated by Skretting to meet NRC (1993) requirements
[5] Nitrogen Free Extracts (NFE) = Dry Matter - (crude lipid + crude ash + crude protein)
[6] Gross Energy calculated using factors of 23.62, 39.5 and 17.56 kJ g^{-1} for protein, lipid and carbohydrate, respectively (NRC 1993)

Bacterial identification

Phenotypic characterisation

A total of 1500 isolates were tentatively placed into groups or genera, according to standard characterisation as described on page 47. Additionally, dominant colonies from each Gram-negative group/genera were identified to species level using Microbact™ 24E test kits (Oxoid Ltd, Plate 3.4).

Microbact™ 24E test kits

The Microbact™ 24E Gram-negative kit is used for the identification of aerobic and facultatively anaerobic Gram-negative bacteria (Enterobacteriaceae and miscellaneous Gram-negative bacteria). Organism identification is based on pH change and utilisation of up to 25 individual substrates. The kit was used as per manufacturer's instructions. Briefly, a loop full of fresh pure culture was suspended in 2.5 mL of saline and 100 µL aliquoted into each well. Results were read after 24 - 48 hr and assessed using Microbact™ 2000 Computer Aided Identification Package (V. 2.03; Oxoid Ltd). The kit allows for the identification of approximately 150 species from 40 genera (Appendix 2).

16S rRNA sequencing

25 of the dominant isolates were selected for 16S rRNA sequence analysis. DNA extraction, PCR, sequence analysis and BLAST search was carried out as described on pages 47 - 49. The majority of isolates were selected from Gram-positive genera and particular attention was paid to *Bacillus* spp..

Electron microscopy

Intestinal samples from 3 fish per tank were retained for histological examination by SEM and TEM. Sampling and processing procedures are described on pages 49 - 51. Microvilli length, microvilli density and microbial colonisation patterns were assessed.

Statistics

Data were transformed where necessary and an independent samples two-tailed T-test was carried out using SPSS 15.0 in order to evaluate the effect of SBM on intestinal histology and microbiota.

Results

Microbiology

Log total viable counts (TVC) of allochthonous and autochthonous microbiota from the anterior and posterior intestine of rainbow trout under different dietary regimes are shown in Tables 3.5 and 3.6, respectively. Mean log TVC were in the range 5.65 - 5.79 CFU g^{-1} on the anterior mucosa, 5.97 - 6.10 CFU g^{-1} on the posterior mucosa, 6.63 - 6.67 CFU g^{-1} in the anterior digesta and 6.90 - 6.93 CFU g^{-1} in the posterior digesta. In general the viable bacterial populations were found to be higher in the posterior intestine than the anterior intestine; however, these differences were only around 1 log unit in all cases, which was not significant ($P > 0.05$). No significant differences of viable populations between the dietary groups were found in any of the intestinal regions investigated. However, clear differences of microbial communities comprising the populations are evident (refer to Tables 3.5 and 3.6). Common groups identified belong to the γ subclass of Proteobacteria, in particular *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp. and Enterobacteriaceae. A clear reduction of the level *Aeromonas* spp. was isolated from the SBM fed fish. This was particularly true with allochthonous populations whereby no *Aeromonas* spp. were recovered from the SBM fed fish, however, *Aeromonas* spp. accounted for 37.3 % in the anterior intestine and 32.0 % in the posterior intestine of fish fed the FM based diet. Similarly, autochthonous *Aeromonas* spp. accounted for ~ 23 % of the total viable populations of the FM group but ≤4 % of the SBM group. Considerably higher numbers of yeast were observed in the SBM group. This was most evident regarding the allochthonous populations, with the relative abundance increasing from ~14% in the FM to ~50% in the SBM group. These isolates were presumptively identified as *Saccharomyces* spp. (smooth butyrous colony, oval/circular cell morphology, no pseudomycelium and positive glucose fermentation). According to Microbact™ 24E

identification the dominant *Aeromonas* spp. were *A. hydrophila* and *A. caviae*, *Vibrio* spp. were *V. alginolyticus*, *Pseudomonas* spp. were *P. stutzeri* and *P. putida* and members of the Enterobacteriaceae were *Enterobacter hormaechei* and *Citrobacter* spp. After 16s rRNA sequencing a GenBank BLAST search revealed the species with the closest phylogenetic relationship with the selected isolates. The 25 sequences identified corresponded to 7 genera. Dominant Enterobacteriaceae species were identified as *Enterobacter* spp. 638 and *Salmonella enterica.* The group of "Gram-negative cocci", which were only isolated from SBM fed fish, were identified as *Psychrobacter* spp. (species could not differentiated from *Psy.* spp. PRwf-1, *Psy. cryohalolentis* or *Psy. arcticus*). The group "other Gram-positive rods" were identified as *Arthrobacter aurescens, Janibacter* spp. and *Streptomyces coelicolor. Bacillus* spp. were identified as *Bacillus* spp. SG-1, *B. subtilis, B. licheniformis, B. cereus* and *B. pumilus.*

Table 3.5. Composition of rainbow trout culturable autochthonous intestinal microbiota from fish fed fishmeal (FM) as protein source and soybean meal (SBM) as 50% protein replacement. N=3, pooled from 9 fish.

	Anterior		Posterior	
	FM	SBM	FM	SBM
Viable population (CFU g^{-1})	5.65	5.79	5.97	6.10
Composition (%)				
Enterobacteria[1]	2.67	8.67	11.33	9.33
Pseudomonas spp.[2]	8.00	6.00	6.67	8.67
Staphylococcus spp.	-	0.67	-	-
Micrococcus spp.	-	12.67	-	2.67
Acinetobacter spp.	-	-	-	-
Aeromonas spp.[3]	23.33	2.00	22.67	4.00
Vibrio spp.[4]	8.00	-	7.33	1.33
Bacillus spp.[5]	16.00	0.67	2.67	13.33
Other Gram – cocci[6]	-	14.00	-	14.67
Other Gram – rods	-	0.67	-	-
Other Gram + rods[7]	33.33	15.34	32.00	26.67
Yeast	8.67	39.33	17.33	19.33
Total isolates	150	150	150	150

- not detected

[1] Dominant isolates identified by 16S rRNA as *Enterobacter* spp. 638 and *Salmonella enterica and* by Microbact™ 24E as *Enterobacter hormaechei* and *Citrobacter* spp.
[2] Dominant isolates identified by Microbact™ 24E as *P. stutzeri* and *P. putida*
[3] Dominant isolates identified by Microbact™ 24E as *A. hydrophila* and *A. caviae*
[4] Dominant isolates identified by Microbact™ 24E as *V. alginolyticus*
[5] Dominant isolates identified by 16S rRNA as *Bacillus.* spp. SG-1, *B. subtilis, B. licheniformis, B. cereus* and *B. pumilus*
[6] Dominant isolates identified by 16S rRNA as *Psychrobacter* spp.
[7] Dominant isolates identified by 16S rRNA as *Arthrobacter aurescens, Janibacter* spp. and *Streptomyces coelicolor*

Table 3.6. Composition of rainbow trout culturable allochthonous intestinal microbiota from fish fed fishmeal (FM) as protein source and soybean meal (SBM) as 50% protein replacement. N=3, pooled from 9 fish.

	Anterior		Posterior	
	FM	SBM	FM	SBM
Viable population (CFU g^{-1})	6.63	6.67	6.90	6.93
Composition (%)				
Enterobacteria[1]	5.33	3.33	4.67	5.33
Pseudomonas spp.[2]	4.00	5.33	4.67	2.00
Staphylococcus spp.	-	-	-	-
Micrococcus spp.	6.67	12.67	9.33	8.67
Acinetobacter spp.	-	0.67	2.00	-
Aeromonas spp.[3]	37.33	-	32.00	-
Vibrio spp.[4]	18.00	-	20.00	-
Bacillus spp.[5]	4.67	0.67	1.33	-
Other Gram – cocci[6]	-	5.33	-	4.67
Other Gram – rods	2.67	-	-	0.67
Other Gram + rods[7]	8.67	19.33	12.00	30.00
Yeast	12.67	52.67	14.00	48.67
Total isolates	150	150	150	150

- not detected

[1] Dominant isolates identified by 16S rRNA as *Enterobacter* spp. 638 and *Salmonella enterica and* by Microbact™ 24E as *Enterobacter hormaechei* and *Citrobacter* spp.
[2] Dominant isolates identified by Microbact™ 24E as *P. stutzeri* and *P. putida*
[3] Dominant isolates identified by Microbact™ 24E as *A. hydrophila* and *A. caviae*
[4] Dominant isolates identified by Microbact™ 24E as *V. alginolyticus*
[5] Dominant isolates identified by 16S rRNA as *Bacillus.* spp. SG-1, *B. subtilis, B. licheniformis, B. cereus* and *B. pumilus*
[6] Dominant isolates identified by 16S rRNA as *Psychrobacter* spp.
[7] Dominant isolates identified by 16S rRNA as *Arthrobacter aurescens, Janibacter* spp. and *Streptomyces coelicolor*

Histology

SEM and TEM confirmed the presence of bacterial - like populations of morphologically different rods and cocci associated with the epithelial mucosal layer (Figures 3.6 & 3.7). Populations of straight rod - like, curved rod - like and cocci - like bacteria can be seen associated with the microvilli (Figures 3.6 & 3.7). Electron microscopy also revealed enteritis-like symptoms in the fish fed the SBM diet. Damage to the mucosal epithelium was evident at the villi, enterocyte and microvilli level (Figures 3.8, 3.9 & 3.10). Microvilli morphology of apparently non-damaged enterocytes was significantly different compared to the control fish; microvilli appeared to be thicker, significantly shorter and less dense (Table 3.7, Figure 3.8, 3.9 and 3.10). Compared to the control fed fish, the density of microvilli in the anterior region of the intestine was significantly lower in the SBM fed fish (from 16.6 ± 4.0 AU in the FM group to 5.3 ± 0.7 AU in the SBM group). There was no significant difference in the anterior region. Figure 3.8b also reveals that the irregular and less densely packed microvilli on the enterocytes surface, lead to exposed tight junctions which appeared to be susceptible to bacterial infection/invasion (Figure 3.8c). Microvilli length from the posterior intestine was significantly longer in the FM group than the SBM (FM = 1.19 ± 0.18 μm, SBM = 0.80 ± 0.15 μm; $P = 0.001$), refer to Table 4 and Figure 5. However, there were no significant differences in the anterior region.

Table 3.7. Microvilli morphology of rainbow trout fed either a fishmeal based diet (FM) or soybean meal based diet (SBM). Values expressed as mean ± standard deviation.

Parameter	Intestinal region	FM	SBM
Microvilli density*	Anterior	16.6 ± 4.0[a]	5.3 ± 0.7[b]
	Posterior	11.7 ± 3.4	6.3 ± 0.9
Microvilli length (μm)	Anterior	1.65 ± 0.12	1.68 ± 0.47
	Posterior	1.19 ± 0.18[a]	0.80 ± 0.15[b]

*Arbitrary units (AU)

[ab] Values within the same row with different superscripts are significantly different ($P < 0.05$)

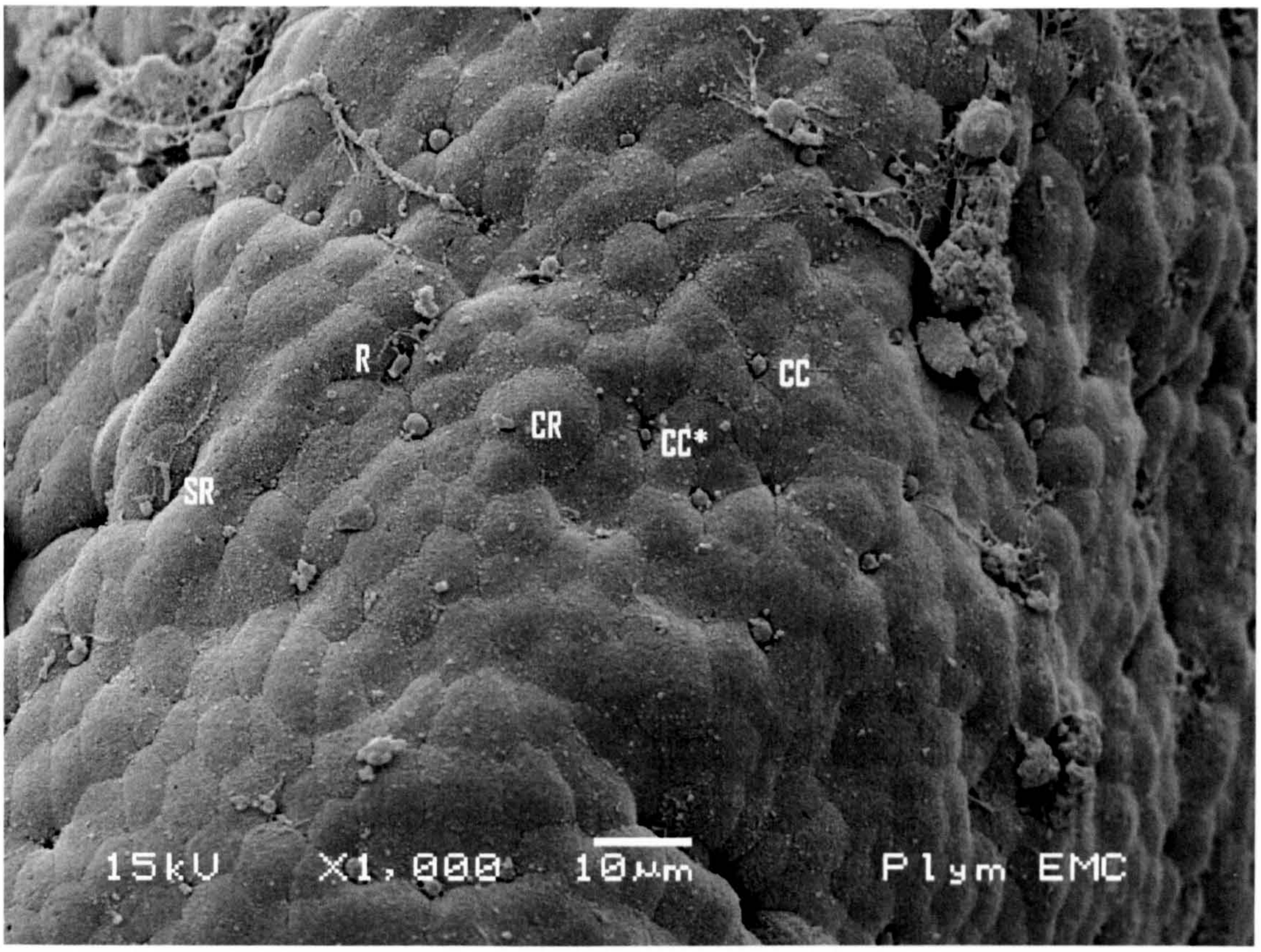


Figure 3.6. SEM micrograph of posterior intestine of FM fed rainbow trout. Mixed bacteria - like populations of rods (R; SR = straight rods; CR = curved rods) and cocci (C) are observed. Scale bar = 10 µm.

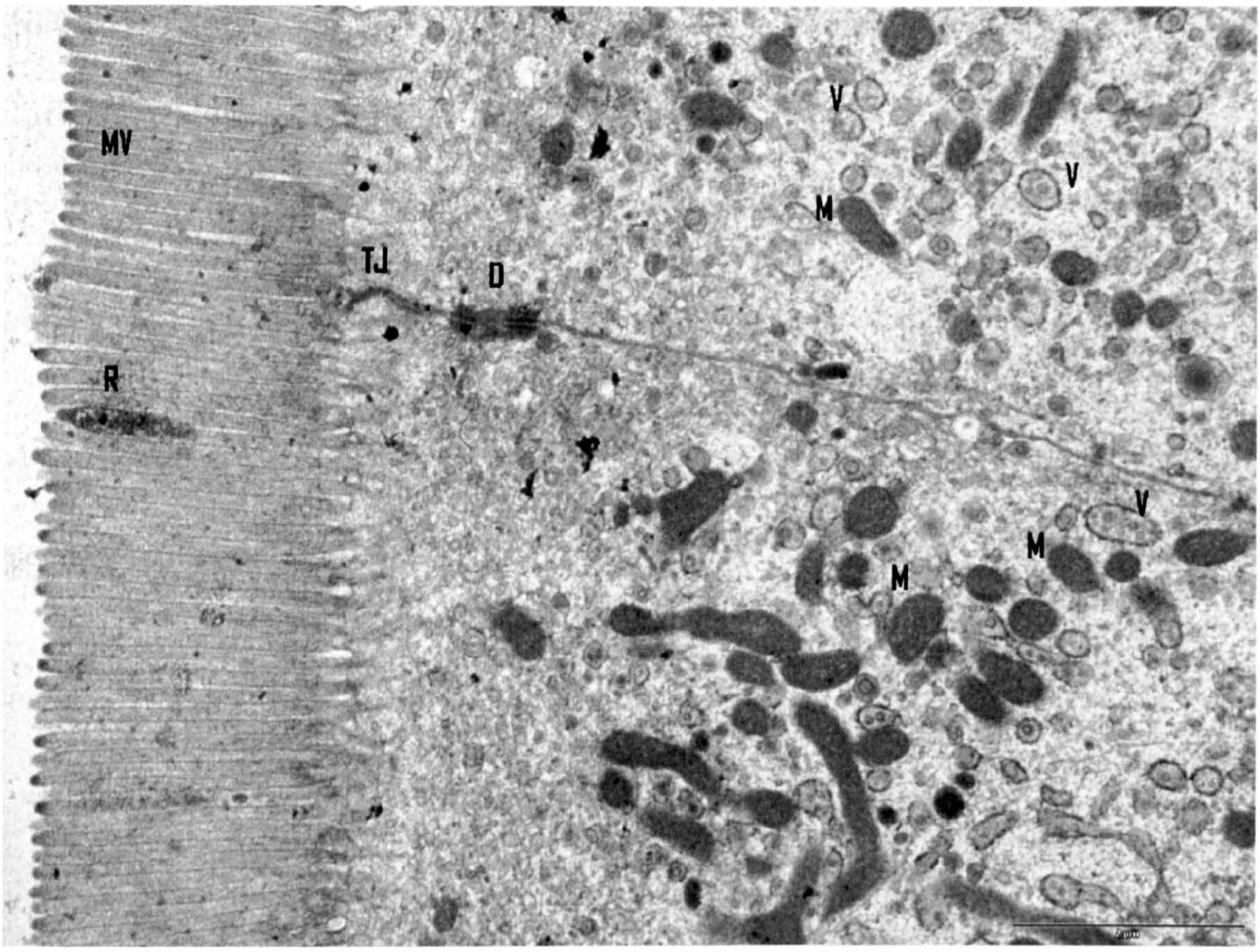


Figure 3.7. TEM micrograph of the anterior region FM fed rainbow trout showing a rod shaped bacterium (R) between the microvilli (MV). TJ = tight junction, D = desmosomes, M = mitochondria and V = vacuoles. Scale bar = 2 μm.

Figure 3.8. Comparative SEM micrographs of anterior intestine of rainbow trout. A) FM fed fish with healthy, regular shaped enterocytes (scale bar = 10 μm). B) SBM fed fish displaying irregular shaped enterocytes with irregular and deformed microvilli (scale bar = 10 μm). C) brush border of SBM fed fish showing exposed tight junctions and invading bacteria – like profile (B) (scale bar = 1 μm).

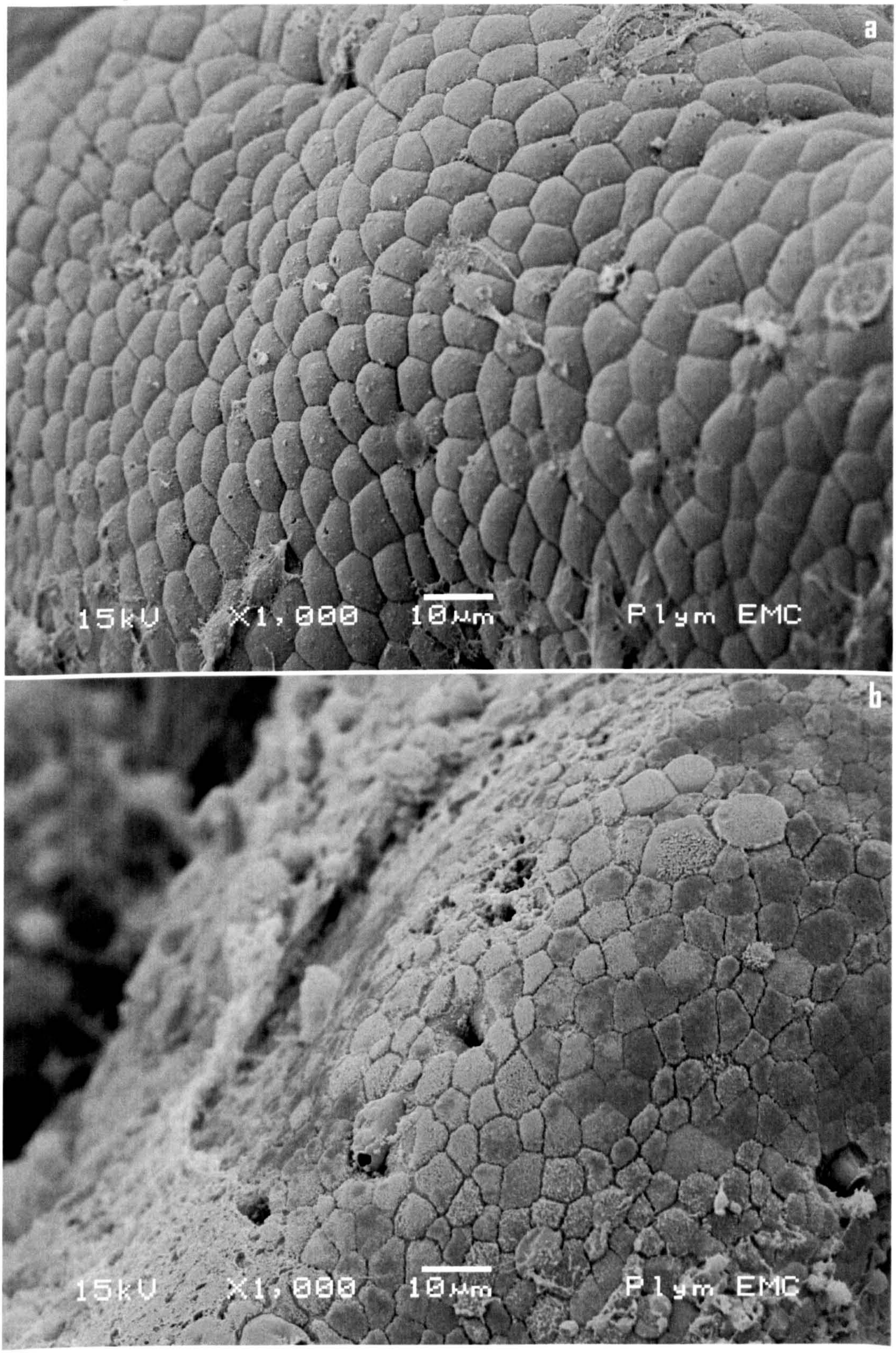
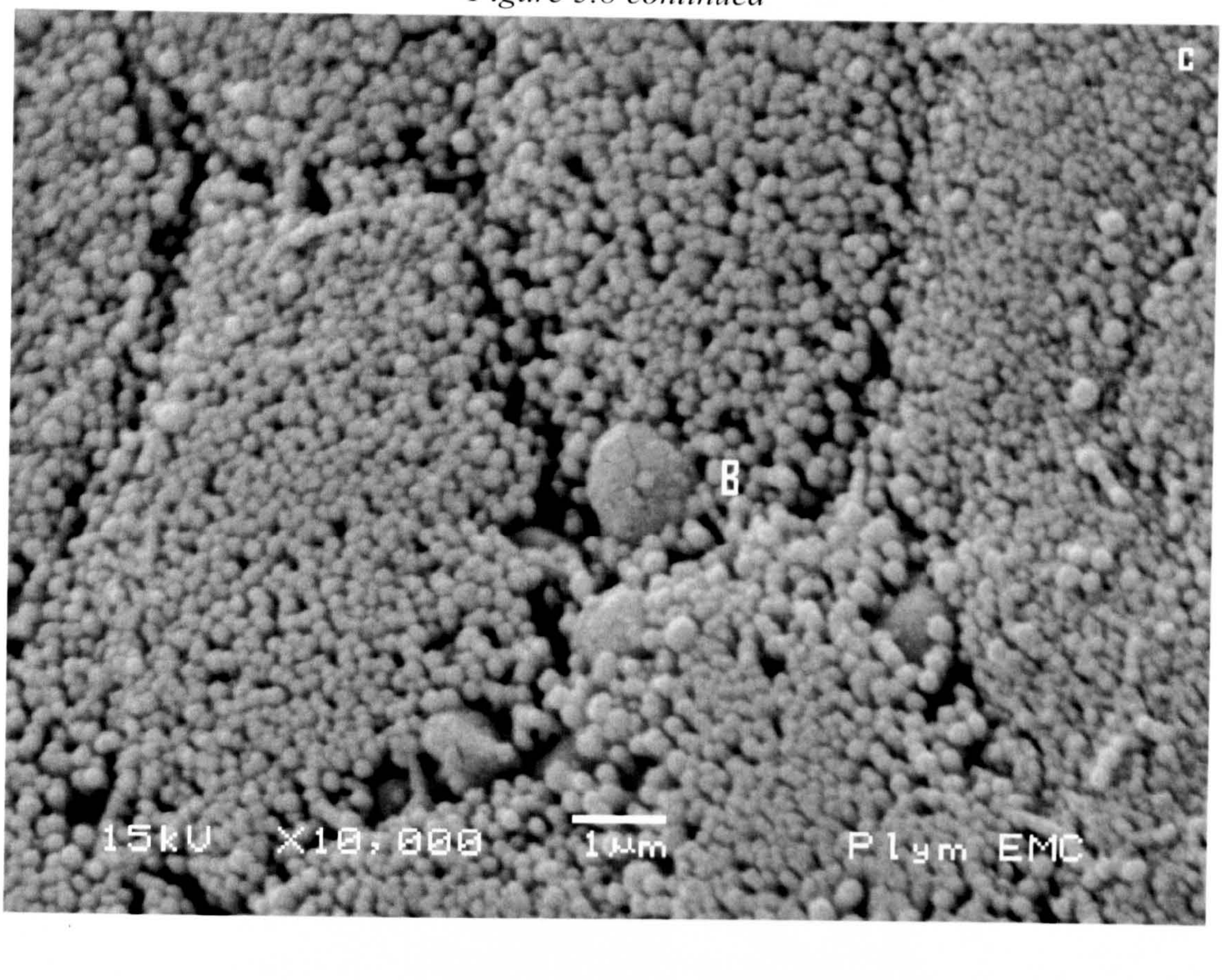


Figure 3.8 continued on the next page

Figure 3.8 continued



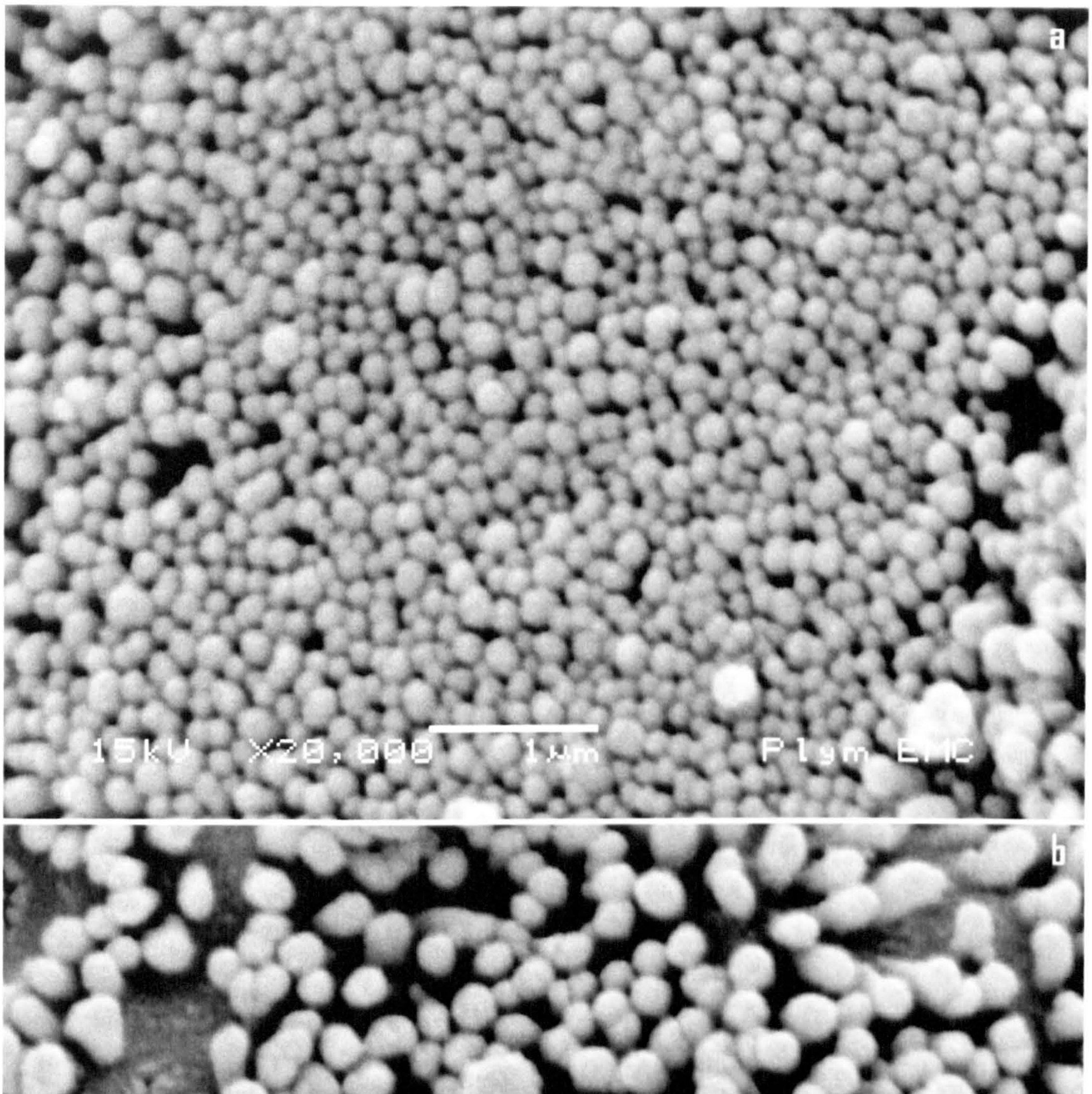


Figure 3.9. Comparative SEM micrographs of the anterior intestine of rainbow trout microvilli. A) FM fed fish, displaying dense, regular microvilli. B) SBM fed fish, displaying a more sparse and irregular arrangement of microvilli. Scale bar = 1 µm.

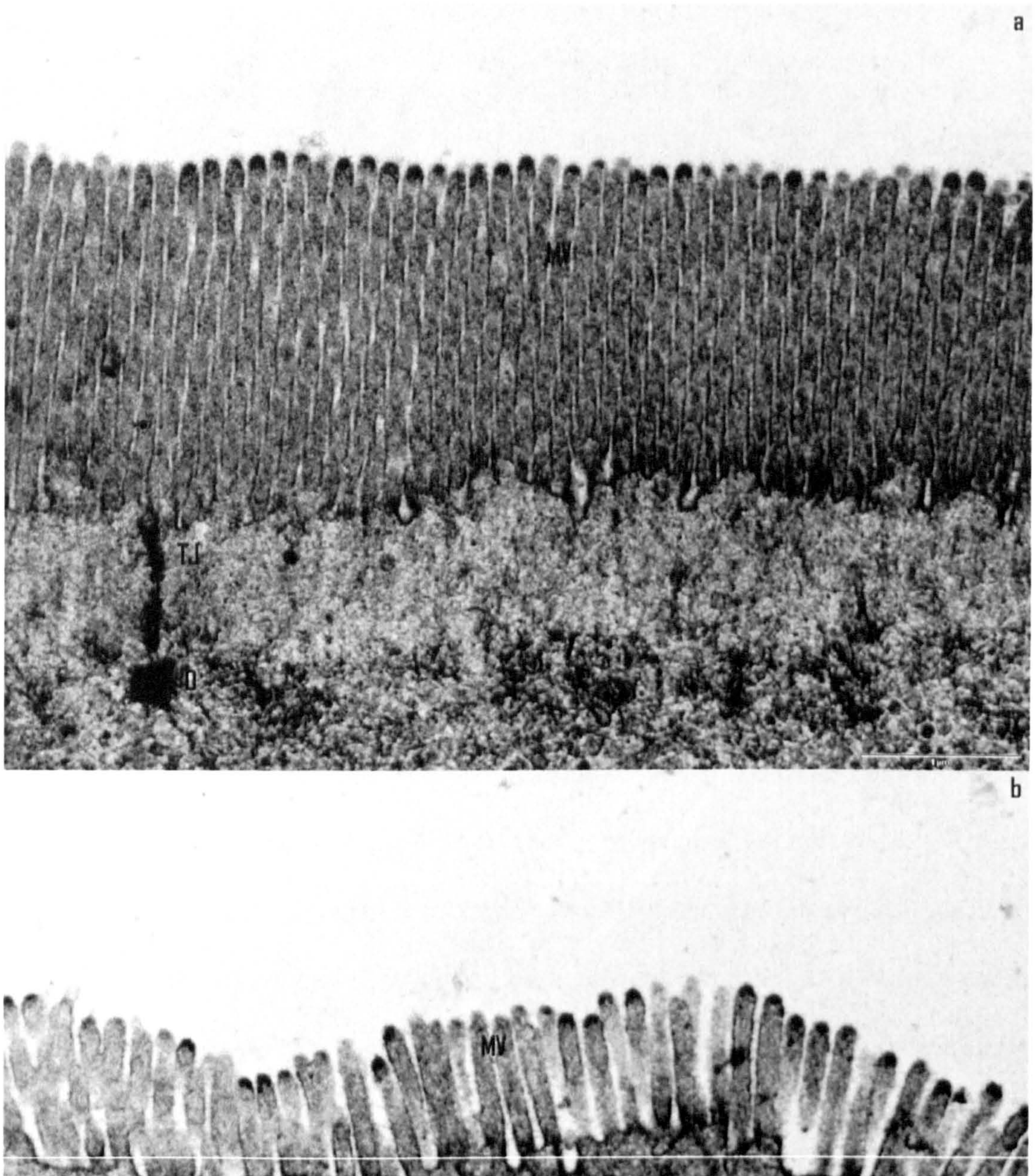


Figure 3.10. Comparative TEM micrographs of the posterior gut of rainbow trout. A) FM fed fish, with long, regular, healthy microvilli (MV) lining the surface of the enterocytes (E). B) SBM fed fish, with short, irregular microvilli. TJ = tight junction, D = desmosomes and M = mitochondria. Scale bar = 1 µm.

Discussion

Heterotrophic aerobic populations ranged between 10^5 and 10^7 CFU g^{-1}, which is within the range of values reported in previous rainbow trout investigations (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000, Heikkinen *et al.* 2006; Kim *et al.* 2007; Chapter 3A). SBM did not significantly alter viable microbial numbers in the intestinal tract. The results of the present study, along with those found by Ringø *et al.* (2008) suggest that quantitative changes of total viable populations of gut microbiota of salmonids may be less influenced by SBM in than other species, such as Atlantic cod (Ringø *et al.* 2006c) which often resulted in higher populations. However, contrary to this Heikkinen *et al.* (2006) and Bakke-McKellep *et al.* (2007b) observed changes in total microbial populations in rainbow trout and Atlantic salmon, respectively. Heikkinen *et al.* (2006) demonstrated that SBM fed rainbow trout displayed an initial increase of viable intestinal microbes but after 8 weeks feeding these levels dropped below that of the control fed fish. Bakke-McKellep *et al.* (2007b) observed significant increases of TVC of autochthonous populations in both the mid and distal intestine of SBM fed Atlantic salmon compared to the control group. Allochthonous populations in the distal intestine were also significantly higher in the SBM fed fish

However, despite no change in viable counts in the present study, changes of the microbiota populations comprising the microbial community were observed, confirming previous findings (Heikkinen *et al.* 2006; Ringø *et al.* 2006a; Bakke-McKellep *et al.* 2007b; Ringø *et al.* 2008). The inclusion of dietary SBM had a pronounced effect on *Aeromonas* levels isolated from the intestinal tract in the present study. The reason for the large reduction within the SBM fed fish is not clear but is comparable to the findings of Heikkinen *et al.* (2006). Heikkinen *et al.* (2006) identified *Aeromonas* spp. as approximately 19.6 % of intestinal isolates from FM fed rainbow trout yet *Aeromonas* spp.

where approximately half this value, 9.8 % of SBM isolates. However, these values should be viewed with caution as they are based on the identification of a total of only 94 isolates. Members of the *Aeromonas* genus, such as *Aeromonas salmonicida* are potentially pathogenic and are responsible for destructive diseases such as furunculosis (Austin & Austin 1993; Dalsgaard & Madsen 2000) and it has been suggested that the intestinal tract is a possible route of infection (Ringø *et al.* 2003; Birkbeck & Ringø 2005; Ringø *et al.* 2007). Taking this into consideration the findings of the current study are particularly interesting and worthy of further consideration when conducting future research focusing on the effect of SBM on the intestinal microbiota of fish.

Noticeably high levels of autochthonous and allochthonous *Saccharomyces* spp were identified in the SBM fed fish. Yeast have been isolated previously as part of the fish gut microbiota, including rainbow trout (Sakata *et al.* 1993; Andlid *et al.* 1995; Andlid *et al.* 1999; Gatesoupe *et al.* 2005a; Gatesoupe *et al.* 2005b; Waché *et al.* 2006; Gatesoupe 2007). Common strains from rainbow trout have been identified as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Candida* spp. and *Leucosporidium* spp.; natural proliferation of yeasts in the fish digestive tract can generally be considered as commensal populations in healthy fish reared under good conditions (Gatesoupe 2007). The reason for such a large increase in yeast populations in the present study is likely to be a direct result of fermentable carbohydrates provided by SBM. Oligosaccharides typically constitute about 4 - 5% of SBM by dry weight (Obendorf *et al.* 1998), of which stachyose (1.4 - 4.1%) and raffinose (0.1 - 0.9%) are the main components (Hymowitz *et al.* 1972). Raffinose and stachyose consists of fructose, glucose and galactose (Lan *et al.* 2007). Yeasts, including *Saccharomyces* spp. are able to ferment various sugars, including glucose and galactose (Doran & Bailey 1986; Lindén *et al.* 1992; Barnett 2003); hence, an increase in number of yeast may be a result of increased available sugars.

The 25 sequences identified by 16S rRNA sequencing corresponded to 7 genera. Closest matching alignments for the group identified as "other Gram-positives" were all Actinomycetes: *Arthrobacter aurescens, Janibacter* spp. and *Streptomyces coelicolor*. The former has been isolated from wastewater treatment plants, contaminated soil, river water, forest soil, and groundwater but all 3 species are probably ubiquitous soil bacteria and hardy enough to survive significant environmental changes and therefore are likely to be found in fish intestinal contents. Indeed, *Arthrobacter* spp. have previously been identified from the digestive tract of rainbow trout (Huber *et al*. 2004; kim *et al*. 2007; Chapter 3A). Dominant Enterobacteriaceae were identified as *Enterobacter* spp. 638 and *Salmonella enterica*. *Enterobacter* spp. 638 are endophytic plant bacteria and are ubiquitous in most plant species including soya beans (Roberts *et al*. 1999). *Salmonella enterica* are water borne organisms and may have been acquired through the ingestion of food or water. It is highly likely that these aerobic bacteria are likely to live in rich, organic intestinal contents.

The group categorised as "Gram-negative cocci" were identified as *Psychrobacter* spp. *Psychrobacter* spp. have been previously isolated from the intestinal tract of salmonids (Ringø *et al*. 2006b; Hovda *et al*. 2007; Bakke-McKellep *et al*. 2007b; Ringø *et al*. 2008) but interestingly were only isolated from the SBM fed trout in the present study. This is somewhat similar to the findings of Ringø *et al*. (2006a), Bakke-McKellep *et al*. (2007b) and Ringø *et al*. (2008). Ringø *et al*. (2006a) only isolated *Psychrobacter* spp. from 3 intestinal samples of Atlantic cod fed a FM based diet (out of 6 samples investigated); however, *Psychrobacter* spp. were isolated from all intestinal samples of fish fed either SBM or bioprocessed SBM rich diets. In particular *P. glacincola* was identified from virtually all regions (11 of 12) with levels ranging from log 2.57 – 4.79 CFU g^{-1}. Bakke-McKellep *et al*. (2007b) observed higher levels of *Psychrobacter* spp. in the intestinal

tract of Atlantic salmon fed SBM diets compared to the control. *Psychrobacter* isolates from the present study could not be differentiated from *Psychrobacter* spp. PRwf-1, *P. cryohalolentis* or *P. arcticus*; 16S rRNA sequences of the *Psychrobacter* isolates from the current study showed 100% similarity to submitted nucleotide sequences for the aforementioned species in the GenBank database. This highlights the fact that sometimes using short PCR amplicons (~200 bp in the current study) for sequence analysis may limit differentiation to genus level; using larger fragments produced with different primers may have led to identification to species level.

Due to recent interest in probiotic applications the present study paid particular attention to the identification of *Bacillus* species. *B. licheniformis* and *B. subtilis* are commonly used as probiotics in terrestrial studies due to their ability to produce antibiotic substances and a range of vitamins and digestive enzymes (Moriarty 1996; Rosvitz *et al.*, 1998; Martens *et al.* 2002; Yan *et al.* 2003; Azokpota *et al.* 2006). The advantage of using spore formers, such as *Bacillus* spp., for probiotic applications is the limitation of problems regarding shelf life and theses species are now commercially available with EU registration. Recently these species have proved effective as probiotics for improving rainbow trout disease resistance and immunostimulation (Raida *et al.* 2003; Newaj-Fyzul *et al.* 2007; Bagheri *et al.* 2008). *B. licheniformis* and *B. subtilis* were among the several *Bacillus* spp. identified in the present study, it is interesting to note that these species are indigenous to rainbow trout and adds further weight to their applications within rainbow trout aquaculture.

Many of the isolates identified have been isolated from environmental and fish intestinal samples previously, but it is interesting to note that data regarding isolation of the organisms identified in the present study from the epithelium of rainbow trout is either scarce or non-existent.

Electron microscopy of the gastro-intestinal epithelium showed an enteritis-like effect associated with the fish fed SBM. Microvilli morphology was noticeably different; fish fed SBM displayed missing, damaged, deformed, shorter and thicker microvilli. The net result of this was statistically significant shorter and less dense microvilli in fish fed the SBM diet. Electron microscopy is also an important tool for investigating autochthonous intestinal bacterial populations (Ringø *et al.* 2003; Ringø *et al.* 2007). SEM investigations of the rainbow trout autochthonous microbiota have reported limited colonisation (Lesel & Pointel 1979; Austin & Al-Zahrani 1988). Contrary to this, and confirming the findings of Chapter 3A, the present investigation demonstrates complex bacterial populations consisting of morphologically different rods and cocci in close association with the microvilli. However, contrary to the findings of Chapter 3A, no particular colonisation patterns were found in fish fed either diet butit was clear that SBM affected the level of exposure of intra-cellular tight junctions to bacterial cells. Tight junctions are complex protein structures between adjacent cells that help maintain the functional barrier. Enteric invasive pathogens have to overcome this obstacle; indeed, many bacterial pathogens, including *Es. coli*, *Yersinia* spp. and *Salmonella* spp. penetrate the junctional complex resulting in translocation (Sears 2000; Tafazoli *et al.* 2000; Köhler *et al.* 2007). Over exposure of tight junction complexes in combination with necrotic enterocytes and other SBM related damage severely compromises the epithelium as a defensive barrier mechanism. Increased susceptibility to gastro-intestinal pathogenic infection has been reported previously in SBM fed fish (Krogdahl *et al.* 2000); it is possible that tight junction exposure as observed in the current study may have been a contributory factor.

Chapter conclusion

The autochthonous mucosal community has previously been overlooked in trout intestinal microbial identification and probiotic/prebiotic studies. But the results from both the present investigations show that the microbiota found to be associated with the intestinal lining represent a complex microbial community. It is interesting to note that autochthonous populations were only found to be significantly lower than allochthonous populations in the anterior region of the farmed rainbow trout and not in aquarium reared rainbow trout. The reason for this is not clear but it may be due to the fact that the microbial populations associated with the rearing environments are different. Populations are likely to be more stable in the aquarium environment without the natural fluctuations of the more uncontrollable environment of a fish farm. The continual input of a more stable microbial population may allow for higher populations to colonise the intestinal epithelium. This autochthonous community appears to be relatively similar in many ways to that found in the digesta; however, they should be considered as distinctly different communities due to unique species found in the respective regions. This demonstrates, as to be expected, that some species are better adapted at colonisation of the rainbow trout intestinal lining. The importance of this potentially resident microbial community has not been fully explored in previous rainbow trout studies.

The results of Chapter 3B demonstrates that changing dietary protein sources has an effect on both autochthonous and allochthonous gut microbiota of rainbow trout; however, it is not clear which specific soy factors are responsible. An enteritis-like effect is clearly associated with the SBM fed fish, confirming previous studies. Damage at the microvilli level combined with increased exposure of tight junctions can severely reduce the effectiveness of the epithelium as a defensive barrier against potential invading pathogens. It would be interesting to combine the use of probiotics with a SBM rich diet in order to

assess the epithelial colonisation which could potentially help replenish the defensive barrier against pathogens or possibly influence the level of enteritis.

When considering the findings of the present chapter and those of Kim *et al.* (2007) it is clear that further investigation of the microbial communities in the mucus and on the mucosal surface is required. This is especially true when screening gut microbiota for potential probiotics, which for the most part, has taken place using transient, digesta, associated microbial populations. Further investigations on the microbiota associated with the pyloric ceaca.

Chapter 4. Comparison of commercial probiotics and lactoferrin on rainbow trout (*Oncorhynchus mykiss* Walbaum) with or without prior antibiotic treatment

Chapter 4A. Dietary supplementation of commercial probiotics and lactoferrin on rainbow trout (*Oncorhynchus mykiss* Walbaum)

Abstract

The effect of commercial probiotic strains (*B. subtilis*, *B. licheniformis* and *E. faecium* used singularly and synergistically) and bovine lactoferrin (Lf) on the growth performance, intestinal microbiota and health status of rainbow trout (*Oncorhynchus mykiss*) was assessed. High levels of probiotic species survived transit through the stomach and were observed in the posterior gastro-intestinal tract as transient digesta associated populations and potentially resident mucosal populations. Levels of *Bacillus* spp. reached over 35% of the microbial population on the mucosa and 60% in the digesta. Enterococci levels accounted for 45% of the mucosal population and 89% of the population in the digesta. A range of benefits, including significantly improved feed conversion ratio, lysozyme activity and elevated leukocyte levels were achieved after a 10 week nutrition trial. No change in proximal carcass composition was observed throughout the groups. The results of the current study demonstrate some potential role of *B. subtilis*, *B. licheniformis* and *E. faecium* to improve growth performance, gastrointestinal microbiota and health status of rainbow trout.

Introduction

The potential for probiotic LAB and *Bacillus* spp. for the control of disease, immunostimulation and improved growth has been demonstrated in trout (Nikoskelainen *et al.* 2001a; Spanggaard *et al.* 2001; Panigrahi *et al.* 2004; El-Haroun *et al.* 2006; Kim & Austin 2006; Balcázar *et al.* 2007a; Balcázar *et al.*2007b; Brunt *et al.* 2007; Bagheri *et al.* 2008; refer to Tables 1.1 and 1.2). Despite the increasing body of data regarding the health and disease benefits of these probionts, there is a lack of information concerning the effects of *E. faecium* and *Bacillus* spp. on growth, feed utilisation and gastro-intestinal microbiology of rainbow trout.

Lactoferrin (Lf) is an 80-kDa glycoprotein capable of binding 2 ferric ions, present in milk and, to a lesser extent, in exocrine fluids such as bile and tears (Lonnerdal & Iyer 1995). It consists of a single-chain polypeptide with two globular lobes. It is relatively resistant to proteolysis and is stable under acidic conditions, and therefore, most Lf can survive passage through the gastro-intestinal tract (Lönnerdal & Iyer 1995; Tomita *et al.* 2002). Lf has been used as a functional feed additive; it can protect against certain Gram-negative bacteria (Reiter *et al.* 1975; Arnold *et al.* 1977; Arnold *et al.* 1980; Stuart *et al.* 1984; Ellison & Giehl 1991). Lf has a range of immunostimulatory properties as well as the potential to modify gut microbiota. The application of dietary bovine Lf has shown a range of benefits in several fish species; including immunostimulation (Sakai *et al.* 1995; Kumari *et al.* 2003, Kumari & Sahoo 2006), enhanced disease resistance (Sakai *et al.* 1993; Kakuta & Kurokura 1995; Kumari *et al.* 2003) and suppressed stress response (Kakuta 1998; Yokoyama *et al.* 2005; Yokoyama *et al.* 2006).

The aim of this study was to observe the effect of *E. faecium*, *B. licheniformis/B. subtilis* and Lf on rainbow trout growth performance, feed utilisation, intestinal colonisation and related health parameters.

Materials and Methods

Diet preparation

B. licheniformis (DSM 5749) and *B. subtilis* (DSM 5750) were cultured by adding 1 g BioPlus 2B® (diet code 2B: Chr. Hansen A/S, Hørsholm, Denmark) to 50 mL TSB. After 24 hr incubation at 37 °C in a shaking water bath 1 mL aliquots were added to 50 mL fresh TSB and incubated for a further 24 hr. *E. faecium* (DSM 7134) was cultured by adding 1 g Bonvital® powder (diet code EF: Lactosan GmbH & Co. KG, Kapfenberg, Austria) to 50 mL TSB. After 24 hr incubation at 37 °C in a shaking water bath, 1 mL aliquots were added to 50 mL fresh TSB and incubated for a further 24 hr. After incubation, the probiotic cells were harvested by centrifugation (2150 *g* for 15 min) washed twice with PBS and re-suspended in fish oil.

Commercial feed (Aller 45/15; Aller Aqua, Christianfield, Denmark) was used as the basal diet. After heating (40 °C) for 2 hr the diet was top-dressed with fish oil containing either probiotic or PBS containing Lf (Fonterra NZMP, 90% purity) by slowly mixing in a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 - 10STDA mixer). All diets were top-dressed with same volumes of fish oil and PBS to produce isoenergetic, identical diets with the exception of the presence of probiotics/Lf. Experimental diet composition is shown in Table 4.1. New fresh diets were produced after 5 weeks to ensure that high levels of probiotics remained in the diets for the duration of the trial. Bacterial counts in the feed and the survival of the probiotics over the experimental period were determined by means of the total viable counts on TSA and S&B.

Table 4.1. Proximate composition and probiotic/Lf supplementation level of experimental diets (C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf = lactoferrin).

	Diets					
	C	2B	EF	2B+EF	Lf1	Lf2
Probiotic/Lf inclusion level	-	* 5.58×10^7	** 2.25×10^8	* 1.66×10^8, ** 1.86×10^8	***0.1	***0.2
Dry matter	912.6 ± 8.1	926.0 ± 6.5	919.5 ± 5.6	923.6 ± 6.7	911.1 ± 5.6	917.2 ± 14.4
Crude Protein	418.6 ± 0.7	425.4 ± 1.3	417.4 ± 9.4	425.4 ± 5.3	417.9 ± 1.9	423.7 ± 4.0
Lipid	210.9 ± 3.4	209.9 ± 3.0	211.5 ± 1.0	212.0 ± 1.4	209.5 ± 1.7	208.0 ± 1.8
Ash	59.7 ± 4.5	61.7 ± 4.3	60.2 ± 2.2	61.7 ± 4.2	62.7 ± 2.0	63.2 ± 1.5
Gross energy (MJ kg^{-1})	23.24 ± 0.10	23.37 ± 0.13	23.11 ± 0.11	23.01 ± 0.18	23.28 ± 0.16	23.19 ± 0.15

Values expressed as g kg^{-1}.

* CFU g^{-1} *B. subtilis/B. licheniformis*; ** CFU g^{-1} *E. faecium*; *** g kg^{-1} lactoferrin.

Experimental design

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local commercial farm (page 41) and acclimated for 4 weeks before beginning the trial. Each tank was randomly stocked with 20 fish (average ~70 g) and treatments were conducted in triplicate. Fish were fed 2.0% biomass day^{-1} for 10 weeks. Daily feed administration and weighing is described on pages 41. The water temperature was maintained at 15 ± 1 °C with oxygen levels maintained above 80% saturation. A 12 hr light/12 hr dark photoperiod was maintained throughout the entire trial duration.

Growth parameters and carcass analysis

Growth performance, feed utilisation and carcass analysis was assessed as described on pages 43 - 45.

Intestinal bacteriology

At the end of the growth trial 3 fish per tank were euthanized by overdose (200 mg L^{-1} water for 15 min) of MS222 (Pharmaq, Fordingbridge, UK) followed by destruction of the brain. After aseptic dissection, posterior intestinal digesta and mucosal material were sampled as described on page 46. The resulting material from 3 fish was pooled into 1 sample per tank. Samples were then serially diluted to 10^{-8} with PBS and 100 µL was spread onto duplicate agar plates. *E. faecium* levels were determined by CFU mL^{-1} or g^{-1} after 7 day aerobic incubation on selective medium (S&B at 37°C). *Bacillus* levels were determined by calculating the proportion of *Bacillus* colonies (presumptively identified by colony morphology and identified as Gram-positive endospore forming rods) from the total CFU (mL^{-1} or g^{-1}) after 7 day aerobic incubation on TSA at 20°C. 16S rRNA sequence analysis was used to confirm presumptive probiont identification (page 47 - 49).

Haematological and immunological parameters

Haematocrit, leukocyte levels, lysozyme and alternative complement activity was determined as described on pages 52 & 53.

Statistical analysis

Data transformations were carried out where necessary and relevant statistical analysis was carried out as described on page 54.

Results and Discussion

Growth performance and carcass composition

Growth rates by all treatments showed efficient utilisation of feed; fish biomass increased by nearly 300% in all groups with FCR < 0.98 and SGR > 2.01 (see Table 4.2). A significant improvement of FCR was found in EF (*E. faecium*; 0.93 ± 0.02) and 2B+EF (*B. subtilis*, *B. licheniformis* and *E. faecium*; 0.93 ± 0.02) fed fish compared to the control group (0.98 ± 0.02). Similarly, Bogut *et al.* (2000) demonstrated an increase in growth performance when feeding *E. faecium* supplemented diets to sheat fish. Although enhanced SGR was seen in the present experimental groups they were not significant. The PER of the experimental groups were not significantly different from the control, however, PER of EF fed group (1.90 ± 0.04) was significantly higher than the 2B group (1.74 ± 0.02). Despite our current findings, improved growth performance of fish fed *B. subtilis* probiotics has been demonstrated in previous studies (El-Haroun *et al.* 2006; Kumar *et al.* 2006; El Dakar *et al.* 2007). A recent study by Bagheri *et al.* (2008) also demonstrated that *B. subtilis* and *B. licheniformis* could improve growth performance of rainbow trout fry when supplemented at high dietary levels. El-Haroun *et al.* (2006) and Bagheri *et al.* (2008) also reported changes in carcass proximate composition; specifically an elevated level of protein and a reduction of crude lipid. However, proximate composition remained the same throughout all treatments in the present study (Table 4.3). Comparing the results of Bagheri *et al.* (2008) and the current findings it would appear that *B. subtilis* and *B. licheniformis* could be more effective at enhancing growth and carcass composition of fry rather than sub-adult rainbow trout. Application of Lf did not seem to affect growth performance of rainbow trout in the current study. Survival rates for all groups were in an acceptable range (> 95%).

Table 4.2. Growth performance of rainbow trout after 10 weeks feeding on experimental diets (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

	Treatment					
	C	2B	EF	2B+EF	Lf1	Lf2
Initial weight	69.38 ± 0.10	68.95 ± 0.56	68.98 ± 0.93	69.15 ± 0.36	69.5 ± 0.51	69.18 ± 0.49
Final weight	264.42 ± 12.00	264.14 ± 5.61	275.85 ± 5.87	278.85 ± 7.76	267.88 ± 11.09	271.40 ± 9.28
Weight gain (g)	195.04 ± 11.90	195.19 ± 5.37	206.87 ± 4.96	209.70 ± 7.87	198.38 ± 10.59	202.22 ± 9.76
Weight gain (%)	281.08 ± 16.77	283.09 ± 7.24	299.85 ± 3.27	303.28 ± 12.41	285.37 ± 13.24	292.37 ± 16.17
FCR	0.98 ± 0.02^{a}	0.98 ± 0.01^{a}	0.93 ± 0.02^{b}	0.93 ± 0.03^{b}	0.97 ± 0.04^{a}	0.95 ± 0.03^{a}
SGR	2.01 ± 0.16	2.05 ± 0.05	2.13 ± 0.01	2.15 ± 0.05	2.05 ± 0.10	2.10 ± 0.06
PER	1.80 ± 0.06^{a}	1.74 ± 0.02^{ab}	1.90 ± 0.04^{ac}	1.85 ± 0.08^{a}	1.80 ± 0.10^{a}	1.82 ± 0.08^{a}
Survival (%)	95.00 ± 8.66	98.33 ± 2.89	100.00 ± 0	100.00 ± 0	96.67 ± 5.77	100.00 ± 0

Values in the same row with the different superscripts are significantly different ($P < 0.05$)
FCR = feed conversion ratio; SGR = specific growth rate; PER = protein efficiency ratio

Table 4.3. Proximate composition of carcasses after 10 weeks feeding on experimental diets (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

		Treatment					
Carcass composition	Initial fish	C	2B	EF	2B+EF	Lf1	Lf2
Moisture	729.4 ± 14.5	687.3 ± 9.2	678.7 ± 8.2	677.1 ± 15.9	682.8 ± 5.5	685.7 ± 4.9	683.2 ± 4.5
Crude protein	146.9 ± 8.5	156.7 ± 7.4	162.6 ± 5.8	158.0 ± 05.5	155.7 ± 0.3	154.6 ± 4.4	159.2 ± 10.3
Crude lipid	55.3 ± 5.4	117.4 ± 21.7	117.6 ± 56.2	125.7 ± 47.1	123.3 ± 43.3	118.4 ± 41.6	122.6 ± 58.1
Ash	22.4 ± 2.5	18.1 ± 1.3	19.3 ± 1.6	19.2 ± 1.9	19.6 ± 1.1	18.8 ± 0.5	18.9 ± 0.2

Values expressed as g kg^{-1}

Microbial analysis

The level of *Bacillus* spp. probiotic remained relatively stable in the 2B and 2B+EF feeds, only decreasing by approximately 15% over a 5 week period (Figure 4.1). *E. faecium* probiotic levels in the EF and 2B+EF diets decreased by around 35 - 40% over the same time period. The number of viable and probiotic bacteria associated with the intestinal tract at the end of the feeding trial is displayed in Table 4.4 and Figure 4.2. Total viable counts within the digesta ranged from log 6 - 7 CFU g^{-1} which are within the range reported in previous rainbow trout studies (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000; Heikkinen *et al.* 2006; Kim *et al.* 2007). Viable microbial levels on the mucosa were several log scales lower, ranging from 2.5 - 4 CFU g^{-1}; similarly, mucosal populations corresponding to levels several log scales lower than the digesta have been reported (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000). *Bacillus* and enterococci detected in the control fish were found to comprise less than 10% of the total microbial populations. *Bacillus* isolates were not identified as *B. subtilis* or *B. lichenifromis* and remain as unidentified indigenous *Bacillus* spp.. However, *E. faecium* was identified as a minor component of the *Enterococcus* spp.. Indeed, *Bacillus* spp. and enterococci have been identified as minor components of the rainbow trout indigenous intestinal populations previously (Heikkinen *et al.* 2006; Kim *et al.* 2007; Dimitroglou *et al.* unpublished data; Chapters 3a & 3b).

Bacillus levels in the 2B group were found at levels of log 3.41 CFU g^{-1} on the intestinal mucosa and log 7.32 CFU g^{-1} in the digesta. This represents 36% of the microbial population on the mucosa and 62% in the digesta. Unlike isolates from the control group, *Bacillus* isolates from group 2B were identified as being composed entirely of the probiotic *B. subtilis* and *B. licheniformis*. These levels show that dietary application of

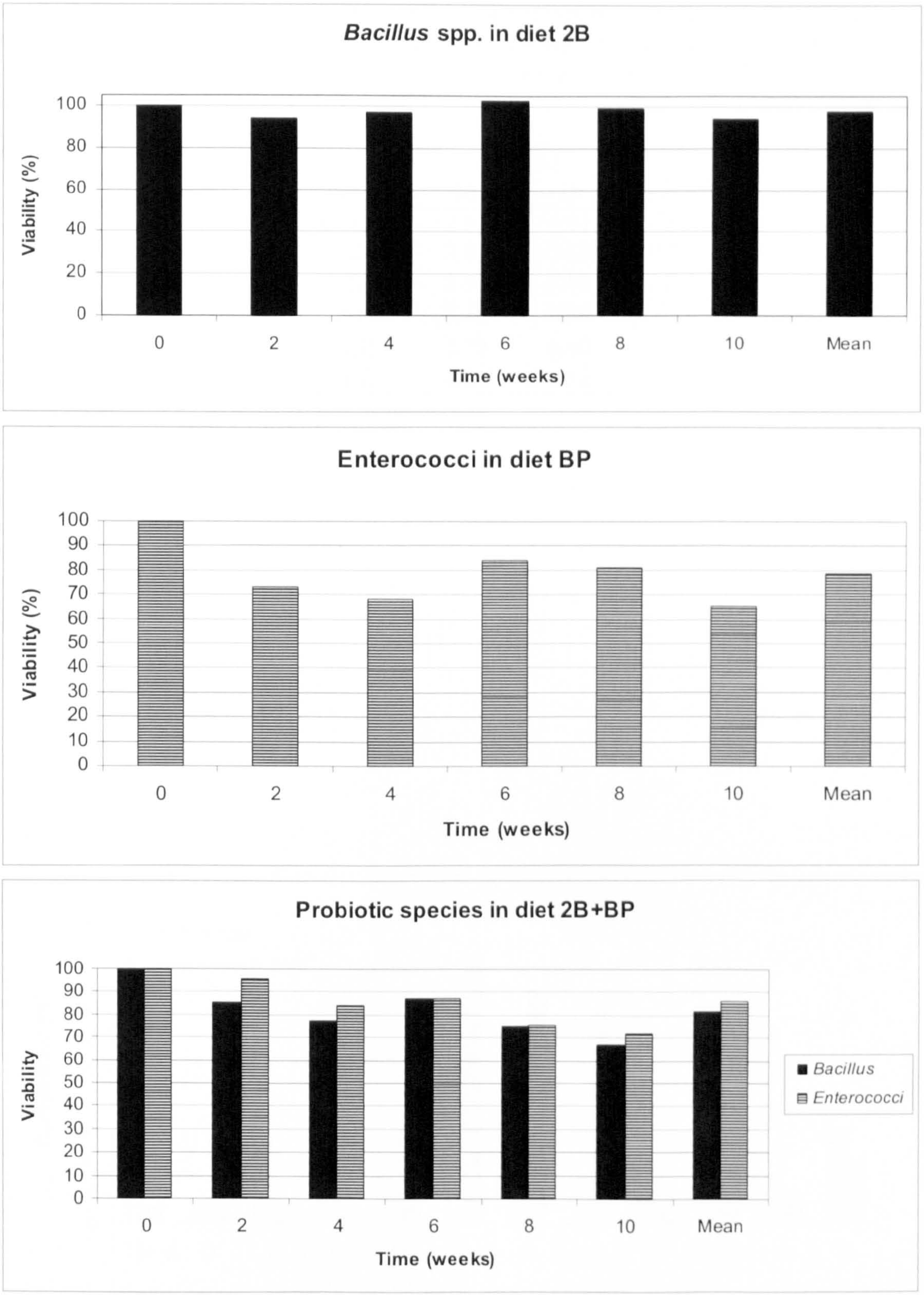


Figure 4.1. Probiotic viability of diets throughout the 10 week experimental period. New batch of diets produced at week 5.

Table 4.4. Log viable counts (CFU g^{-1}) from the rainbow trout intestinal tract. Intestinal regions: M = Mucosal lining and D = digesta. Dietary groups: 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

	Sample	
Group	M	D
Control	2.62	6.51
2B	3.85	7.53
EF	2.96	7.82
2B+EF	4.18	7.90
Lf1	2.78	6.60
Lf2	4.24	6.52

Figure 4.2. Intestinal microbial composition (%) of rainbow trout after 10 weeks feeding on the experimental diets. Intestinal regions: M = Mucosal lining and D = digesta. Dietary groups: 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

BioPlus 2B® can result in high numbers of *B. subtilis* and *B. licheniformis* surviving passage through the upper gastro-intestinal tract to the posterior intestine, where the potential for colonisation is demonstrated by the high levels associated with the mucosa. Newaj-Fyzul *et al.* (2007) also demonstrated that high levels of *B. subtilis* dominated (10^4 CFU g^{-1}) the gut and gut mucus of rainbow trout when fed the probiotic for a period of 14 days. Aside from continual introduction into the intestine via the feed high intestinal colonisation by *Bacillus* probiotics is likely to be due in part to antagonism against the indigenous microbiota. The antimicrobial properties of *B. licheniformis* and *B. subtilis* are likely due to a range of extracellular products but are perhaps most often attributed to the production of bacteriocins and of biosurfactants. Biosurfactants such as lichenysin are bacterial cyclic lipopeptides that have an antibiotic effect by reducing membrane surface tensions (Yakimov *et al.* 1998). Bacillocin and subtilisin are a types of bacteriocins isolated from *B. licheniformis* and *B. subtilis* respectively (Yakimov *et al* 1996; Martirani *et al.* 2002; Yan *et al.* 2003). Furthermore, certain *B. subtilis* strains are known to control the growth or inhibit the potentially pathogenic fish bacteria such as *Aeromonas* spp. (Newaj-Fyzul *et al.* 2007).

Levels of *Enterococcus* colonisation of the gastro-intestinal tract of EF treated fish were recorded at log 2.61 CFU g^{-1} on the mucosa and log 7.77 CFU g^{-1} in the digesta. These values accounted for 45% of the mucosa population and 89% of the bacterial population in the digesta (Figure 4.2). Enterococci isolates were successfully identified as *E. faecium*. Such high levels of *E. faecium* colonisation of the fish intestinal tract have been reported previously. Chang & Liu (2002) reported that dietary administration of *E. faecium* at levels of 10^6 - 10^8 CFU g^{-1} resulted in high intestinal recovery levels (73% of the intestinal population) when fed to European eel. Bogut *et al.* (2000) demonstrated that *E. faecium was* also able to modulate the intestinal microbiota of sheat fish. Most notably, reductions

of Enterobacteriaceae (including *Es. coli*), *Staphylococcus aureus* and *Clostridium* spp., which subsequently resulted in improved weight gain. *E. faecium* has also been shown to possess antimicrobial activities against *Aeromonas* spp. (Panigrahi *et al.* 2007), a genus commonly found to be indigenous in the rainbow trout gut (Spanggaard *et al.* 2000; Pond *et al.* 2006; Chapter 3). It has been demonstrated that *E. faecium* has good adhesive properties to both mammalian (Nikoskelainen *et al.* 2001b) and fish intestinal mucus (Marciňáková *et al.* 2004), which may help to explain the high levels found on the mucosa in the current study. Similarly, high levels of a range of LAB species have demonstrated the ability to persist in the intestine of brown trout (*Salmo trutta,* Balcázar *et al.* 2007b). *Carnobacterium* spp. have been shown to be even more successful at colonisation of the rainbow trout gut after 14 days feeding, with reported levels of > 90% of the total population in the digesta and > 99% in the gut mucus (Kim & Austin 2006). After oral administration with *L. rhamnosus,* high LAB counts were found in the stomach and gut of rainbow trout, representing up to 70% and 83% of the viable population in the stomach and intestine, respectively (Panigrahi *et al.* 2004).

Levels of *Enterococcus* and *Bacillus* spp. in the 2B+EF treated fish were also high; ranging from log 3 CFU g^{-1} on the mucosa to log 7 CFU g^{-1} in the digesta. However, the *Enterococcus* levels were approximately four-fold greater than *Bacillus* spp.. This suggests that *E. faecium* may be slightly more effective at colonising the gut of rainbow trout. Indeed, it has been suggested that *E. faecium* could be more suitable for rainbow trout than *B. subtilis* due to ambient temperature conditions (Panigrahi *et al.* 2007). Despite this, high levels of *Bacillus* spp. were still achieved when applied synergistically with *E. faecium*.

Relative to the control treatments, elevated levels of *Bacillus* spp. and enterococci were found in the Lf treated fish, ranging from 4 - 16% of the viable populations associated

with the mucosal epithelium and 15 - 31% in the digesta. The reason for higher levels colonising the gut of the Lf groups is not fully understood. LAB have been demonstrated to be able to utilise Lf bound iron in previous studies (e.g. *Bifidobacterium bifidum* species; Kim *et al.* 2002) and this may be a possible explanation for elevated levels of enterococci if iron is limited in the gut. However, this possibility does not explain why elevated levels of *Bacillus* spp. were observed. Lf does have bacteriostatic and antimicrobial properties (Wakabayashi *et al.* 2006) which may have caused a shift and/or reduction in the microbiota of Lf treated groups, resulting in a reduction of competition, or the opening of a niche for *Bacillus* spp. and enterococci (indigenous species and/or levels colonising from re-circulating rearing water). Lf is also known to de-stabilise the outer membrane of Gram-negative bacteria, which are dominant in the intestinal tract of rainbow trout, by releasing membrane lipopolysaccharides (Ellison *et al.* 1988). There is recent evidence to support such a theory; Kim *et al.* (2008) found that Lf has antibacterial and binding abilities against *Pseudomonas* spp., which have been shown to be amongst the dominant species of gut microbiota found in trout (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000; Huber *et al.* 2004; Kim *et al.* 2007; Chapters 3a & 3b).

The data clearly demonstrate that the probiotics are found at high levels within the posterior intestine, where the potential benefits to the host, such as aiding digestion, immunostimulation and providing a defensive barrier against pathogenic infection, may occur.

Haematological and immunological parameters.

Haematocrit levels (%PCV) remained the same throughout the probiotic treatments (Table 4.5). The results from this study confirm those of Raida *et al.* (2003), who also found that BioPlus 2B® had no effect on rainbow trout haematocrit levels. Lf did not affect

haematocrit levels in the present study and had no effect on carp haematocrit levels in a previous study either (Kakuta 1998). A significant increase in the total number of leukocytes was found in groups 2B+EF and Lf2 but no differences were found in the remaining treatments. Similarly, Raida *et al.* (2003) found no differences in lymphocyte levels when using BioPlus 2B®. Compared to the control group (471 ± 244 U mL^{-1}), elevated serum lysozyme activity was found in fish fed diets 2B (802 ± 469 U mL^{-1}) and 2B+EF (534 ± 256 U mL^{-1}), however, only 2B was found to be significantly different at the $P < 0.05$ level. There were no differences observed between the remaining treatments. Newaj-Fyzul *et al.* (2007) found that *B. subtilis* fed rainbow trout displayed an increase in serum and gut mucus lysozyme activity after 14 days dietary supplementation. Lysozyme activity of *B. subtilis* fed trout was approximately 2 - 3 times greater than the control group. This is somewhat similar to the observation in the present study; whereby 2B fed fish displayed nearly double the activity of the control fish. Previous studies have found that serum lysozyme may be affected when using LAB probiotics in trout. Balcázar *et al.* (2007a) found a significant increase in lysozyme activity when administering *La. lactis* and *Leu. mesenteroides* to brown trout but no difference was found using *L. sakei*. However, all 3 bacterial species failed to enhance serum lysozyme in rainbow trout (Balcázar *et al.* 2007b). The effect of the *L. rhamnosus* on rainbow trout serum lysozyme activity is variable with both no effect (Panigrahi *et al.* 2005) and improved activity having been reported (Panigrahi *et al.* 2004). Rainbow trout fed dietary *C. divergens* demonstrated an increase in serum lysozyme activity whereas *C. maltaromaticum* treatment did not (Kim & Austin 2006). However, both bacteria elevated lysozyme activity of intestinal mucus. This reported increase of intestinal mucus lysozyme activity may help to explain the observed changes of gut microbiota seen in the Lf fed fish. However, Lf supplementation did not directly affect serum lysozyme activity when

Table 4.5. Haematological and immunological parameters of rainbow trout after 10 weeks feeding on experimental diets (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

	Treatment					
	C	2B	EF	2B+EF	Lf1	Lf2
PCV (%)	29.61 ± 1.88	30.39 ± 2.22	28.33 ± 2.74	29.78 ± 2.48	30.83 ± 3.48	28.56 ± 2.88
Leukocyte count*	34 ± 11^{a}	47 ± 17^{a}	35 ± 12^{a}	54 ± 6^{b}	40 ± 2.5^{a}	58 ± 29^{b}
Lysozyme (U mL^{-1})	471 ± 244^{a}	802 ± 469^{b}	456 ± 230^{a}	534 ± 256^{a}	416 ± 149^{a}	451 ± 225^{a}
ACH50 (U mL^{-1})	343 ± 46	365 ± 31	407 ± 96	361 ± 65	383 ± 81	373 ± 45

Values in the same row with different superscripts are significantly different ($P < 0.05$).
*Number of leukocytes per 1000 blood cells.

supplemented at 100 or 200 mg kg $^{-1}$. Similarly, Lyrgen *et al.* (1999) found no effect on lysozyme activity in Atlantic salmon fed Lf at 135 - 140 mg kg^{-1} for 19 days. However, Lf has been demonstrated to elevate serum lysozyme activity in Asian catfish (*Clarias batrachus*, Kumari *et al.* 2003) and Japanese eel (Ren *et al.* 2007), when supplemented at levels between 50 - 500 mg kg^{-1}.

Alternative complement activity remained unaffected by any of the probiotic treatments in the current study (Table 4.5). Newaj-Fyzul *et al.* (2007) also found that *B. subtilis* fed rainbow trout displayed no increase in serum complement activity after 14 days dietary supplementation. On the contrary, Panigrahi *et al.* (2007) found significant improvements in complement activity when feeding *B. subtilis* and *E. faecium* to rainbow trout for 45 days. Increased complement activity has also been observed in a range of other LAB when administered to rainbow trout (Panigrahi *et al.* 2004; Panigrahi *et al.* 2005). Complement activity remained unaffected in Lf fed fish in the present study; similarly Lygren *et al.* (1999) found that 19 day dietary supplementation with Lf had no effect on the alternative complement activity of Atlantic salmon.

This study demonstrates that dietary *B. licheniformis*, *B. subtilis* and *E. faecium* can survive transit through the digestive tract and successfully colonise the intestine of rainbow trout as both transient and potentially resident populations. The current study gives a broad insight into the potential for probiotic and Lf applications with regards to rainbow trout growth performance, feed utilisation, health status and gastro-intestinal colonisation. A range of benefits, including significantly improved FCR, lysozyme activity, elevated leukocyte levels and high probiotic intestinal colonisation were observed after a 10 week nutrition trial. However, the full extent of benefits may not have been observed in the current investigation. The potential benefits may be greater expressed under poor rearing parameters, stress conditions or challenge trials.

Chapter 4B. Evaluation of commercial probiotics and lactoferrin on rainbow trout (*Oncorhynchus mykiss* Walbaum) following antibiotic administration.

Abstract

The effect of commercial probiotic strains (*B. subtilis*, *B. licheniformis* and *E. faecium*) and bovine lactoferrin (Lf) were assessed on rainbow trout (*Oncorhynchus mykiss*) previously treated with oxolinic acid. Parameters investigated include growth performance, feed utilisation, gastro-intestinal colonisation and health status. High levels of probiotic species survived transit through the stomach and were observed in the posterior gastro-intestinal tract as transient digesta associated populations and potentially resident mucosal populations. Levels of *Bacillus* spp. reached over 10^3 CFU g^{-1} on the mucosal epithelium and 10^7 CFU g^{-1} in the digesta. Enterococci levels reached 10^2 - 10^4 CFU g^{-1} on the mucosa and 10^7 CFU g^{-1} in the digesta. Compared to the control group, dietary Lf led to an increase of digesta associated viable populations (from 10^6 in the control to 10^7-10^8 CFU g^{-1} in the Lf groups). *Bacillus* fed fish displayed a significant improvement of FCR, SGR and PER. Fish fed *Bacillus* and *Bacillus+E. faecium* displayed elevated leukocyte levels. The results of the current study demonstrate the potential role of probiotics and Lf at stabilising/reinforcing the gastro-intestinal microbiota after antibiotic treatment. This may reinvigorate the intestinal defensive barrier mechanism and is likely to provide protection against secondary potential pathogens.

Introduction

Due to the current limitations and regulations on the use of antimicrobials in animal nutrition antibiotics are no longer used as therapeutics or growth promoters within the European Union (EU regulation 1831/2003/EC). However, they are still an important part of the arsenal of treatments for many salmonid bacterial diseases and are routinely used worldwide for treatment of bacterial infections. Such antimicrobial treatments are often very effective at reducing and preventing mortalities caused by the primary bacterial pathogen. However, the effect on the natural microbiota of the gastro-intestinal tract is not often considered. The period of time after antibiotic treatment is potentially dangerous; evidence suggests that indigenous gut microbiota may be altered in terms of total viable numbers and diversity of populations (Lesel *et al.* 1989; Bakke-McKellep *et al* 2007b). Any reduction of both diversity and quantity of microbes is likely to reduce the effective barrier mechanism normally provided by the indigenous microbiota; this may lead to a reduction of competition against secondary potential pathogens from the surrounding environment. Also, surviving bacterial species carrying genes for resistance may exchange genetic material conferring resistance to pathogens re-entering the gastro-intestinal tract (Moriarty 1990). Thus, leading to the spread of antibiotic resistance and a reduction of antibiotic efficacy against future disease outbreaks. This is a more dangerous scenario than in mammals due to the fact that the fish gut microbiota is largely transient and heavily influenced by the microbiota of the rearing environment, which itself readily supports and spreads bacterial pathogens. Some of the most common salmonid diseases, such as vibriosis, furunculosis and enteric red mouth, are caused by *Vibrio*, *Aeromonas* and *Yersinia* spp. (e.g. *V. anguillarum*, *V. ordalii*, *V. alginolyticus*, *A. salmonicida* and *Y. ruckeri*) which are often indigenous and thought to cause infection via the gastro-intestinal tract (Austin & Austin, 1993; Thune *et al.* 1993; Evelyn 1996).

The aim of the present study was to assess the effect of *B. licheniformis*, *B. subtilis*, *E. faecium* and bovine Lf on growth, health and gastro-intestinal microbiota of rainbow trout after antibiotic treatment.

Materials and methods

Diet preparation

Supplementation of basal diets with *B. licheniformis*, *B. subtilis*, *E. faecium* and bovine Lf is described on page 101. Dietary composition is displayed in Table 4.6.

Experimental design

Rainbow trout were obtained from a local commercial farm (page 41) and acclimated for 5 weeks before commencing the trial. During this period fish were subjected to antibiotic treatment: commercial trout diets (Skretting) were top dressed with oxolinic acid (Vetrepharm ltd, Fordingbridge, UK) according to manufacturer's instructions (20 mg kg^{-1}, fed daily over 10 day period). Oxolinic acid is commonly used for the treatment of Gram-negative bacterial pathogens. Post exposure to oxolinic acid, 25 fish (average ~45 g) were randomly distributed into experimental tanks. Fish were fed 2 - 2.5% biomass day^{-1} for 10 weeks, feeding and weighing protocol was conducted as described on page 41. The water temperature was maintained at 15 ± 1°C and dissolved oxygen maintained > 80% saturation. Water changes (approximately 25% of system volume, ~1500 L) were conducted every 72 hr to minimise accumulation of background probiotic levels.

Table 4.6. Proximate composition and probiotic/Lf supplementation level of experimental diets (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis*; *B. subtilis* + *E. faecium*; Lf = lactoferrin.

	Diets					
	C	2B	EF	2B+EF	Lf1	Lf2
Probiotic/Lf inclusion level	-	* 6.16×10^7	** 2.28×10^8	* 1.12×10^8, ** 1.69×10^8	*** 0.1	*** 0.2
Dry matter	918.8 ± 19.7	925.7 ± 19.8	921.0 ± 14.7	922.0 ± 14.2	912.5 ± 9.1	914.2 ± 5.1
Crude Protein	432.6 ± 6.4	429.9 ± 1.0	430.1 ± 6.7	427.4 ± 3.5	428.8 ± 7.3	430.2 ± 15.8
Lipid	194.6 ± 15.7	198.5 ± 4.9	196.7 ± 4.9	194.3 ± 2.2	202.6 ± 21.6	197.5 ± 34.7
Ash	61.3 ± 9.3	56.6 ± 4.3	56.9 ± 1.1	59.1 ± 1.4	59.4 ± 4.0	56.1 ± 3.3
Gross energy (MJ kg^{-1})	22.88 ± 0.08	21.90 ± 0.01	22.97 ± 0.00	22.98 ± 0.15	22.68 ± 0.15	22.19 ± 0.02

Values expressed as g kg^{-1}.

* CFU g^{-1} *B. subtilis/B. licheniformis*; ** CFU g^{-1} *E. faecium*; *** g kg^{-1} lactoferrin.

Growth parameters and carcass analysis

Growth performance, feed utilisation and carcass analysis was assessed as described on pages 43 - 45.

Intestinal bacteriology

Analysis of probiotic colonisation was conducted as described on page 103. 16S rRNA sequence analysis was used to confirm probiont identification (page 47 - 49).

Haematological and immunological parameters

Haematocrit, leukocyte levels and lysozyme activity was determined as described on pages 52 & 53.

Statistical analysis

Data transformations were carried out where necessary and relevant statistical analysis was carried out as described on page 54.

Results

Growth performance and carcass composition

Growth performance, FCR, SGR and PER are displayed in Table 4.7. High growth performance was observed; fish biomass increased by over 300% in all groups with FCR < 0.92 and SGR > 2.11. Compared to the control group, FCR and SGR of group 2B (*B. licheniformis*, *B. subtilis*) were significantly improved ($P < 0.05$). FCR reduced from 0.93 ± 0.03 in the control group to 0.85 ± 0.04 in the 2B group and SGR improved from 2.13 ± 0.03% to 2.22 ± 0.05%. Compared to the control group (1.93 ± 0.09) and the Lf1 group (1.94 ± 0.01) the fish fed diet 2B displayed a significantly increased PER (2.13 ± 0.12). No significant differences with the other groups were observed. Carcass analysis showed no significant differences (Table 4.8). Survival rates for all groups were acceptable (> 93%).

Table 4.7. Growth performance of rainbow trout after 10 weeks feeding on experimental diets after antibiotic treatment (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

	Treatment					
	C	2B	EF	2B+EF	Lf1	Lf2
Initial weight (g)	45.03 ± 0.95	45.00 ± 0.92	44.51 ± 1.43	44.17 ± 1.42	44.45 ± 0.29	44.20 ± 1.85
Final weight (g)	195.90 ± 9.53	202.02 ± 12.08	189.24 ± 12.01	189.53 ± 4.33	187.75 ± 5.27	187.50 ± 7.79
Weight gain (g)	150.88 ± 10.00	157.02 ± 11.36	144.73 ± 10.71	145.36 ± 2.93	143.30 ± 4.35	143.30 ± 5.49
Weight gain (%)	335.35 ± 26.42	348.76 ± 20.49	324.16 ± 15.47	329.16 ± 4.34	322.38 ± 10.61	324.21 ± 1.68
FCR	0.93 ± 0.03^{a}	0.85 ± 0.04^{b}	0.89 ± 0.04^{ab}	0.90 ± 0.02^{ab}	0.90 ± 0.05^{ab}	0.91 ± 0.01^{ab}
SGR (% day^{-1})	2.13 ± 0.02^{a}	2.22 ± 0.05^{b}	2.16 ± 0.07	2.16 ± 0.07	2.11 ± 0.18	2.14 ± 0.04
PER	1.93 ± 0.09^{a}	2.13 ± 0.12^{b}	2.00 ± 0.11^{ab}	2.00 ± 0.06^{ab}	1.99 ± 0.13^{ab}	1.94 ± 0.01^{a}
Survival (%)	93.33 ± 6.36	96.00 ± 4.17	97.33 ± 4.81	96.00 ± 4.17	94.67 ± 9.62	96.00 ± 0.00

Values in the same row with the different superscripts are significantly different ($P < 0.05$).
FCR = feed conversion ratio; SGR = specific growth rate; PER = protein efficiency ratio

Table 4.8. Proximate composition of carcasses after 10 weeks feeding on experimental diets, after antibiotic treatment (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

		Treatment					
Carcass composition (%)	Initial fish	C	2B	EF	2B+EF	Lf1	Lf2
Moisture	739.5 ± 4.3	680.3 ± 15.1	678.1 ± 6.6	685.3 ± 5.6	682.8 ± 5.5	680.0 ± 9.1	685.8 ± 9.9
Crude protein	153.6 ± 3.9	144.1 ± 5.5	143.5 ± 6.6	146.2 ± 4.6	149.0 ± 2.0	147.0 ± 10.9	143.9 ± 9.2
Crude lipid	56.0 ± 2.6	105.1 ± 20.0	107.5 ± 14.7	102.8 ± 33.8	94.6 ± 9.0	100.2 ± 27.5	95.9 ± 28.0
Ash	21.6 ± 1.4	19.3 ± 0.8	18.9 ± 2.2	18.0 ± 1.1	18.2 ± 1.7	18.9 ± 1.4	20.1 ± 1.7

Values expressed as g kg^{-1}.

Intestinal microbial analysis

Microbial analysis of the posterior intestine of rainbow trout is presented in Table 4.9 and Figure 4.3. Viable bacterial counts associated with the intestinal mucosa were log 2 - 4 CFU g^{-1} and log 6 - 8 CFU g^{-1} in the digesta. *Bacillus* and enterococci detected in the control fish were found to comprise less than 12% of the total microbial populations. *Bacillus* isolates were not identified as *B. subtilis* or *B. lichenifromis* and remain as unidentified indigenous *Bacillus* spp.. However, *E. faecium* was identified as a minor component of the *Enterococcus* spp.. *Bacillus* levels accounted for 75% (log 3.74 CFU g^{-1}) of the mucosal populations and 82% (log 7.41 CFU g^{-1}) of the digesta populations in the 2B fed group. 16S rRNA sequence analysis confirmed that these *Bacillus* populations were composed entirely of *B. subtilis* and *B. licheniformis*. *E. faecium* levels in the EF fed fish accounted for 72% (log 2.84 CFU g^{-1}) the epithelial mucosal populations and 90% (log 7.78 CFU g^{-1}) of the digesta populations. Total probiotic levels in the 2B+EF treated fish accounted for nearly 100% in both the mucosa and digesta. However, *E. faecium* dominated with around 70% colonisation compared to 30% *Bacillus* colonisation. Total viable populations in the digesta increased from log 6.57 CFU g^{-1} in the control group to log 7.66 CFU g^{-1} ($P = 0.047$) in the Lf1 group and log 8.21 CFU g^{-1} ($P < 0.001$) in Lf2 group. No significant increases in populations were found on the mucosa. *Bacillus* and enterococci levels in the Lf groups remained less than 16% in the digesta and 12% on the mucosa.

Table 4.9. Log viable counts (CFU g^{-1}) from the rainbow trout intestinal tract. Intestinal regions: M = Mucosal lining and D = digesta. Dietary groups: 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

	Sample	
Group	M	D
Control	2.68	6.57
2B	3.85	7.49
EF	2.98	7.83
2B+EF	4.23	7.91
Lf1	2.83	7.66
Lf2	4.50	8.21

Figure 4.3. Intestinal microbial composition (%) of rainbow trout after 10 weeks feeding on the experimental diets. Intestinal regions: M = Mucosal lining and D = digesta. Dietary groups: 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

Haematological and immunological parameters

Heamatological and immunological results are displayed in Table 4.10. Haematocrit levels (%PCV) remained the same throughout the probiotic treatments, at approximately 30%. Total number of leukocytes were significantly elevated in the 2B and 2B+EF groups, from 39 ± 7 in the control to 52 ± 16 and 51 ± 14 in 2B and 2B+EF, respectively. Compared to the control (523 ± 44 U mL^{-1}), an elevation of serum lysozyme activity was found in groups 2B (658 ± 154 U mL^{-1}), EF (618 ± 168 U mL^{-1}), Lf1 (633 ± 150 U mL^{-1}) and Lf2 (639 ± 159 U mL^{-1}). However, these were not significant ($P > 0.05$).

Table 4.10. Haematological and immunological parameters of rainbow trout after 10 weeks feeding on experimental diets after antibiotic treatment (mean ± standard deviation). C = control; 2B = *B. licheniformis, B. subtilis*; EF = *E. faecium*; 2B+EF = *B. licheniformis, B. subtilis* + *E. faecium*; Lf1 = lactoferrin 0.1 g kg^{-1}; Lf2 = lactoferrin 0.2 g kg^{-1}.

	Treatment					
	C	2B	EF	2B+EF	Lf1	Lf2
PCV (%)	30.44 ± 3.32	31.00 ± 2.50	29.44 ± 3.47	30.33 ± 3.54	31.33 ± 3.57	31.44 ± 3.78
Leukocyte count*	39 ± 7^{a}	52 ± 16^{b}	45 ± 9^{a}	51 ± 14^{b}	44 ± 8^{a}	45 ± 6^{a}
Lysozyme (U mL^{-1})	523 ± 44	658 ± 154	618 ± 168	556 ± 321	633 ± 150	639 ± 159

Values in the same row with different superscripts are significantly different ($P < 0.05$).
*Number of leukocytes per 1000 blood cells.

Discussion

A significant improvement of FCR, SGR and PER was found in the *Bacillus* fed fish (group 2B) in the present study. Similarly, Bagheri *et al.* (2008) found significant improvements of FCR, SGR and PER in rainbow trout fry with the administration of dietary *B. licheniformis* and *B. subtilis* at 10^9 cells g^{-1}. However, Bagheri *et al.* (2008) found that no significant benefits were achieved until the level of *Bacillus* spp. accounted for over 85% of the intestinal microbiota. Dietary *Bacillus* levels of 10^8 CFU g^{-1} (leading to ~65% intestinal colonisation) showed no improvements in growth or feed utilisation. Similarly, when using *B. licheniformis* and *B. subtilis* in Chapter 4A (which resulted in ~60% colonisation of the digestive tract) no improvements of growth performance or feed utilisation were observed. These studies indicate that a high level of *B. subtilis* and *B. licheniformis* colonisation within the intestinal tract is required to influence rainbow trout growth performance (i.e. > ~80%). The precise mechanism for enhanced growth performance is not clear, however, *Bacillus* spp., including *B. subtilis* and *B. licheniformis* produce a wide range of extracellular enzymes (e.g. esterase, protease & amylase; Moriarty 1996; Azokpota *et al.* 2006) and vitamins (e.g. vitamin K and B-12; Rosvitz *et al.* 1998; Martens *et al.* 2002). Compared to fish enzymes, bacterial enzymes often have a broader pH range; which has been suggested to prolong digestion time for the host (Ochoa & Olmos 2006). *Bacillus* spp. are known to produce an array of antibiotic and antimicrobial compounds, some of which are known to be effective against *Vibrio* spp. (Moriarty 1990; Moriarty 1998). *Vibrio* spp. are indigenous to the rainbow trout gut and are often potentially pathogenic, any reduction of potential pathogens within the gut may also have been a contributory factor to the improved feed utilisation seen in the group 2B.

This may help to explain why high levels of *B. subtilis* and *B. licheniformis* within the gut can aid digestive function. Improved growth performance of fish fed *B. subtilis* probiotics has also been demonstrated in Nile tilapia (El-Haroun *et al.* 2006), rohu (*Labeo rohita,* Kumar *et al.* 2006) and spinefoot rabbitfish, (*Siganus rivulatus*, El-Dakar *et al.* 2007). Compared to the control group, no significant differences of growth performance was observed in the present study. Contradictory to the present findings, the findings from Chapter 4A demonstrated that dietary *E. faecium* significantly improved FCR. Bogut *et al.* (2000) also observed enhanced growth performance when using *E. faecium* probiotics (with sheat fish). The reason for a lack of improvement of growth performance and feed utilisation in the current study is unclear, it may be that the level of gastro-intestinal colonisation in the present study was too high and a possible synergistic effect with the indigenous gut microbiota in the previous investigations was negated. Also, it is possible that the oxolinic acid pre-treatment may have removed bacterial species that were potentially important in a synergistic role with *E. faecium*.

Bacillus and enterococci levels dominated both the mucosal and digesta microbial populations in the respective treatments (> 70% in all cases). However, when administered synergistically to group 2B+EF, probiotic colonisation reached > 99%. Of this population, enterococci levels dominated the gastro-intestinal tract, more than doubling *Bacillus* levels (a ratio of ~70%/30%, respectively). The level enterococci in the diet was slightly higher than *Bacillus* levels (a ratio of ~60%/40%, respectively over the duration of the trial); it seems that *E. faecium* may have a competitive advantage over *B. licheniformis* and *B. subtilis* in the rainbow trout digestive tract. These results are somewhat similar to those found in Chapter 4A and appear to confirm the suggestion of Panigrahi *et al.* (2007) that *E. faecium* may be a more suitable probiotic to the specific conditions of the rainbow trout gut than *B. subtilis*. The lack of improvements regarding growth and feed utilisation in the

group 2B+EF may again be explained by the fact that the level of gastro-intestinal colonisation was too high and any possible synergistic effect with the indigenous gut microbiota was negated.

Background levels of *Bacillus* and *Enterococcus* spp. were found in the rearing water; *Bacillus* spp. (log 3.86 CFU mL^{-1}) accounted for less than 14% of the total bacterial population and enterococci (log 3.61 CFU mL^{-1}) accounted for less than 8%. This small background build up indicates that large levels of background probiotics may be problematic and cause cross contamination between treatments in re-circulating systems if not controlled effectively (i.e. by UV filtration or frequent water changes). This did not affect the current study as levels of *Bacillus* and *Enterococcus* spp. only accounted for less than 12% and 9% (respectively) in the control group. *Bacillus* and *Enterococcus* isolates from the control were not identified as the probionts and are thus assumed to be indigenous gut populations (Heikkinen *et al.* 2006; Kim *et al.* 2007; Arkadios conference; Chapters 3a & 3b). Thus, water changes of approximately 25% system volume every 72 hr is deemed sufficient in the present study. Viable counts after oxolinic acid administration at the beginning of trial resulting in microbiota viability within the digesta < log 4 CFU g^{-1}; at the end of the trial counts ranged between log 5 - 7 CFU g^{-1}, which is within the range of gut microbial levels in previous rainbow trout studies (Austin & Al-Zahrani 1988; Spanggaard *et al.* 2000, Heikkinen *et al.* 2006; Kim *et al.* 2007; Chapter 3).

The results clearly show that high levels of probiotics survived transit through the gastro-intestinal tract and colonised the posterior intestine, confirming the findings from Chapter 4A. Therefore it is possible that benefits such as aiding digestive function, enhancing the microbial defensive barrier mechanism and immunostimulation may arise. Such high levels of gastro-intestinal colonisation, both within the digesta, and especially those associated with the mucosal epithelium, increases competition and is likely to play an

important protective role. However, it is evident that total replacement of indigenous populations with probiotics may not be desirable with regards to improving growth performance. A probiotic presence within the digestive tract may alter the complex microbial communities and cause a shift to a more functional microbial population, rather than merely direct benefits provided by the probiont itself.

Total viable populations in the digesta of Lf groups increased significantly, from log 6 CFU g^{-1} in the control group to log 7 CFU g^{-1} and log 8 CFU g^{-1} in Lf1 and Lf2 groups, respectively ($P < 0.05$). This was not evident from the study conducted in Chapter 4A. The reason for this may be that levels within the intestine when not administered with antibiotics may be already at/or near saturation. Lf can have both a direct independent effect and synergistic effect with host defensive mechanisms (e.g. lysozyme) which results in bacteriostatic or bactericidal effects on a range of bacterial pathogens. Such pathogens include: *Listeria* spp., *Vibrio* spp., *Salmonella* spp., *Pseudomonas* spp., *Burkholderia* spp., *Staphylococcus* spp. and *Es. coli* (Ellison *et al.* 1988; Ellison & Giehl, 1991; Leitch & Willcox 1999; Farnaud & Evans 2003; Branen & Davidson 2004; Berlutti, *et al.* 2005; Jenssen & Hancock 2009; Leon-Sicairos *et al.* 2009). Lf releases membrane lipopolysaccharides thus bacterial cells are more susceptible to lysis from secondary antimicrobial compounds (Kim *et al.* 2008). Lf also chelates iron, which is an important virulence factor for many bacterial pathogens (Singer *et al.* 1991; Litwin & Calderwood 1993), thus, making it unavailable to certain potential pathogens (Reiter *et al.* 1975; Arnold *et al.* 1977; Arnold *et al.* 1980; Stuart *et al.* 1984; Ellison & Giehl 1991). However, unlike some pathogens certain LAB are able to utilise Lf-bound iron (Kim *et al.* 2002). Although the microbial composition in the Lf treated groups in the present study were not characterised it can be assumed that the increase in the viable microbial populations is a positive outcome. Lf treated fish remained healthy throughout the trial

duration, with mortalities of less than 5%. Thus, it can be assumed that the increased numbers of bacteria are not likely to be pathogenic; this increase (which at the very least can be considered as a commensal population) increases competition and reduces niches for secondary potential pathogens.

Haematocrit levels (%PCV) remained the same throughout the probiotic treatments, confirming the findings of Raida *et al.* (2003) and those of Chapter 4A, which also showed that BioPlus 2B® had no effect on rainbow trout haematocrit. Lf did not affect haematocrit levels in the current study, confirming the findings of Chapter 4A. A significant elevation of leukocytes was found in fish fed diets 2B and 2B+EF, confirming the findings of Chapter 4A. Similarly, Kumar *et al.* (2008) found that *B. subtilis* caused significant differences of rohu leukocytes; specifically with regards to granulocytes. However, Raida *et al.* (2003) found no differences in rainbow trout lymphocyte levels when using *B. licheniformis* and *B. subtilis*. Compared to the control fish, elevated serum lysozyme activity of between 20 - 25% were found in groups 2B, EF, Lf1 and Lf2 in the present study, however, these were not significant at the $P < 0.05$ level. Contrary to these findings; probiotics, including *Bacillus* spp., *E. faecium* and LAB, have often led to significantly enhanced trout serum lysozyme activity (Newaj-Fyzul *et al.* 2007; Balcázar *et al.* 2007a; Chapter 4A). It may have been the case in the current long term study that the probiotics/Lf exposure may have resulted in an initial increase in activity followed by a gradual reduction to near control levels. Several studies have demonstrated the variable effects of probiotics on serum lysozyme activity (Panigrahi *et al.* 2004; Panigrahi *et al.* 2005; Balcázar *et al.* 2007a; Balcázar *et al.* 2007b). The effect of Lf on serum lysozyme activity of fish also appears to be variable. Studies by Kumari *et al.* (2003) and Ren *et al.* (2007) demonstrated enhanced activity; however, other studies have shown no significant improvements (Lyrgen *et al.* 1999; Chapter 4A).

General chapter conclusions

In conclusion, variability of results, even when using the same probiotic with the same fish species (i.e. the findings of Panigrahi *et al.* 2004 and Panigrahi *et al.* 2005; Chapter 4A and 4B) suggests that the physiological status of the host and rearing factors play a large role in determining the outcome of probiotic and Lf supplementation. Probiotics/Lf (and likely prebiotics) will not always show a benefit, even when attempting to reproduce studies using the same probiont with the same fish species; the host animal's current status at any given time may not always require, nor be able to yield enhanced immunological status or growth performance. Thought should be given as to the timing of these dietary functional supplements as the nature of circumstances dictate. It is also clear that there is a fine line between supplementing the indigenous commensal populations and direct removal/replacement of indigenous populations. Probiotic colonisation of the gastro-intestinal tract reaching near 100% of the total microbiota does not appear desirable. Further work needs to be undertaken to determine appropriate levels; such levels are likely to vary depending on the probiont species, host fish species, host physiological status and the rearing conditions. It is clear from the present investigation that probiotic and Lf supplementation after antimicrobial treatment increases the number of viable bacteria within the digestive tract and associated epithelial mucosa. This creates competition for potential pathogens re-entering the digestive tract and stabilises/reinforces the gut microbial defensive barrier after antibiotic treatment. Subsequently, enhanced growth, feed utilisation and health status may be achieved. It would be interesting to apply this strategy with challenge studies following antibiotic treatment and probiotic/Lf feeding.

Continued work should be conducted to further explore the potential of these products and to determine optimal inclusion levels. Further work should also be conducted using

electron microscopy to investigate the extent of the epithelial mucosal colonisation throughout the digestive tract and examine any possible colonisation patterns.

Chapter 5. Comparison of vegetative and lyophilised *Pediococcus acidilactici* as a probiotic for rainbow trout (*Oncorhynchus mykiss* Walbaum).

Abtsract

A study was conducted to assess the probiotic effect of different dietary forms of *P. acidilactici* on rainbow trout (*Oncorhynchus mykiss* Walbaum). Growth performance, feed utilisation, intestinal colonisation and basic health status were investigated after a 10 week feeding trial. Fish were fed either vegetative (Veg) or lyophilised (Lyo) cells incorporated into a basal diet at either 10^7 (Lo) or 10^8 (Hi) CFU g^{-1}. *P. acidilactici* successfully colonised the digestive tract (as both epithelium associated and transient populations) in all probiotic groups. Scanning electron microscopy confirmed the presence of localised colonisation of coccoid cells (presumptively identified as *P. acidilactici*) between intestinal folds of the probiotic fed fish. Compared to the control group, no significant improvements in growth performance, feed utilisation or carcass composition were observed in the probiotic fed fish ($P > 0.05$). However, a significant reduction of condition factor (K) was evident in fish fed the lyophilised diets. Increased leukocyte levels and lysozyme activity were observed in fish fed the probiotic diets, however only leukocyte levels were significant at the $P < 0.05$ level. The study demonstrates some potential for the application of *P. acidilactici* with rainbow trout but further research is required.

Introduction

Although much probiotic interest has focused on LAB, most notably *Lactobacillus* spp., *Carnobacterium* spp. and *Lactococcus* spp. (refer to Chapter 1) there remains a distinct lack of literature regarding *P. acidilactici*. A range of bacteriocins (pediocins) have been identified from *P. acidilactici* strains (Bhunia *et al*. 1990; Schved *et al*. 1993; Huang *et al*. 1996; Anastasiadou *et al*. 2008). The production of these pediocins and organic acids (such as lactic and acetic acid) results in antagonistic properties against a range of Gram-positive and Gram-negative bacteria, most notably towards *Vibrio* strains (Cintas *et al*. 1995; Villamil *et al*. 2002; Vázquez *et al*. 2005; Beaulieu *et al*. 2006; Castex *et al*. 2008). Some studies have begun to provide valuable information of the probiotic potential of *P. acidilactici* for finfish (Gatesoupe 2002; Aubin *et al*. 2005; Shelby *et al*. 2007). Due to the variability of results obtained so far and many unanswered questions, the probiotic application of *P. acidilactici* in finfish aquaculture remains unclear. It has been suggested that further work should be carried out to investigate the effect *P. acidilactici* on rainbow trout, with specific emphasis on gastro-intestinal microbiology (Aubin *et al*. 2005).

It is also interesting to note that most probiotic studies do not seem to consider the importance of the mode of probiotic administration to the diet. Some studies have compared live and dead cells (Irianto *et al*. 2003; Panigrahi *et al*. 2005; Taoka *et al*. 2006) but it is often not considered important when using live cells whether the probiont is added as vegetative cells or added in a lyophilised powdered form (as supplied by the manufacturer). It is of course more practical to add the probiotic to a basal diet mixture as a dietary ingredient rather than culturing cells and top dressing pellets. Thus, commercial probiotics are supplied in a lyophilised form. Both administration methods have been used previously but there is very little data regarding a comparison of the two live supplementation methods. The aim of the present investigation is to assess the effect of

vegetative and lyophilised *P. acidilactici* on growth performance, feed utilisation, intestinal colonisation and selected health parameters of rainbow trout.

Materials and methods

Diet preparation

P. acidilactici (CNCM MA 18/5 M) cultures were prepared by culturing Bactocell® (Lallemand Inc, Montréal Canada) in MRS broth for 24 hr and 48 hr at 37 °C for diets VegLo and VegHi respectively. After incubation the cells were harvested by centrifugation (2150 *g* for 15 min), washed twice with PBS and re-suspended in fish oil.

A commercial feed (Aller 45/15; Aller Aqua, Christianfield, Denmark) was taken as the basal diet and milled in order to produce a fine powder. Warm water was added to the milled basal mixture in a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 - 10STDA) before cold press extrusion (PTM P6 extruder, Plymouth, UK) to produce 2 mm pellets. This was used as the basal diets for the control and vegetative (Veg) groups. The Veg diets were then topped dressed with fish oil containing the probiont at the two desired levels (see Table 5.1). In order to produce the lyophilised (Lyo) diets the desired volumes of Bactocell® powder was diluted in 50 mL PBS and added dropwise into the basal mixture prior to cold press extruding after Shelby *et al.* (2007). All diets were top dressed with the same volume of oil in order to produce identical diets with the exception of the probiont. New batches of feed were produced every 4 weeks after Shelby *et al.* (2007). Diets produced 10^7 and 10^8 CFU g^{-1} *P. acidilactici* for the low and high level diets respectively. Diets were stored at 4 °C.

Table 5.1. Proximate composition (mean ± standard deviation) and *P. acidilactici* supplementation level (log CFU g^{-1}) of experimental diets. C = control; VegLo = vegetative low; VegHi = vegetative high; LyoLo = lyophilised low; LyoHi = lyophilised high.

	Diets				
	C	VegLo	VegHi	LyoLo	LyoHi
Probiotic inclusion (log CFU g^{-1})	-	7.46	8.11	7.57	8.25
Dry matter (g kg^{-1})	938.4 ± 1.4	937.3 ± 6.5	937.8 ± 3.7	941.8 ± 5.5	940.7 ± 4.0
Crude Protein (g kg^{-1})	398.1 ± 20.6	399.6 ± 16.8	402.9 ± 8.1	405.0 ± 10.6	400.4 ± 10.7
Lipid (g kg^{-1})	243.5 ± 18.6	235.8 ± 17.1	241.4 ± 22.3	234.3 ± 8.0	240.8 ± 5.3
Ash (g kg^{-1})	50.7 ± 2.1	52.5 ± 11.6	50.9 ± 2.9	59.3 ± 1.0	57.5 ± 8.7
Gross energy (MJ kg^{-1})	23.50 ± 0.11	23.44 ± 0.13	23.59 ± 0.18	23.59 ± 0.08	23.56 ± 0.06

Experimental design

Rainbow trout were obtained from a local commercial farm (page 41) and acclimated for 6 weeks before beginning the trial. Each tank was randomly stocked with 25 fish (average ~ 9 g) and treatments were conducted in triplicate. Fish were fed between 2 - 2.5% biomass day^{-1}; feeding and weighing protocol is described on page 41. Water temperature was maintained at 15 ± 1 °C and dissolved oxygen was maintained at > 85% saturation. ~20% water changes (~1200 L) were conducted every 72 hr to prevent background system build up of probiotic levels.

Growth parameters and carcass analysis

Growth performance, feed utilisation, K-factor and carcass analysis was assessed as described on pages 43 - 45.

Intestinal bacteriology

At the end of the growth trial 3 fish per tank were euthanized by overdose of MS222 followed by destruction of the brain. After aseptic dissection samples were taken from the posterior intestine (as described on page 46). The resulting material from 3 fish per tank was pooled into one sample per intestinal region. Samples were then serially diluted to 10^{-8} with PBS and 100 µL was spread onto duplicate TSA and MRS agar. Plate counts were performed after 7 days aerobic incubation at 20 °C for TSA and 5 days at 45 °C for MRS (Aubin *et al.* 2005). CFU mL^{-1} or g^{-1}) were determined for viable bacterial populations. 16S rRNA sequence analysis was used to confirm identification of presumptive *P. acidilactici* isolates (Gram-positive tetrad cocci isolated from MRS) as described on pages 47 - 49.

Haematological and immunological parameters

Blood was sampled from 3 fish per tank after anaesthetisation as described on page 52. Haematocrit, leukocyte levels and lysozyme activity were determined as described on pages 52 & 53.

Electron microscopy

Samples for SEM were taken from 5 separate fish per treatment as described on page 49 - 51.

Statistical analysis

Data transformations were carried out where necessary and relevant statistical analysis was carried out as described on page 54.

Results

Growth performance and carcass composition

Growth performance, feed utilisation and K-factor are displayed in Table 5.2. Fish biomass increased by over 300% in all groups with FCR < 1.02 and SGR > 2.02. Compared to the control group a marginal improvement of FCR (from 1.02 to 1.01 - 0.99) and a considerable improvement of SGR was observed in the probiotic groups (from 2.02% to 2.08 - 2.15%); however, these were not significant at the $P < 0.05$ level. A non-significant improvement of PER was also seen in the Lyo fed groups, from 1.77 ± 0.19 in the control to 1.84 ± 0.07 in LyoLo and 1.83 ± 0.14 in LyoHi. K-factor were significantly improved in LyoLo (1.38 ± 0.01, $P = 0.050$) and LyoHi (1.37 ± 0.08, $P = 0.041$) groups compared to the control (1.45 ± 0.05). No significant differences with the other groups were observed. Carcass analysis showed no significant differences between dietary groups (Table 5.3). Survival rates for all groups were acceptable (> 95%).

Table 5.2. Growth performance of rainbow trout after 10 weeks feeding on experimental diets (mean ± standard deviation). C = control; VegLo = vegetative low; VegHi = vegetative high; LyoLo = lyophilised low; LyoHi = lyophilised high.

Measured parameter	C	VegLo	VegLo	LyoLo	LyoHi
Survival (%)	95.45 ± 4.55	98.48 ± 2.62	96.97 ± 5.25	98.48 ± 2.62	100.00 ± 00
Initial mean weight (g)	9.32 ± 0.04	9.32 ± 0.11	9.28 ± 0.07	9.25 ± 0.08	9.23 ± 0.05
Final mean weight (g)	37.97 ± 4.23	38.17 ± 1.05	38.19 ± 0.89	39.54 ± 1.20	38.14 ± 2.59
Weight gain (g)	28.65 ± 4.19	28.85 ± 0.95	28.91 ± 0.95	30.29 ± 1.23	28.91 ± 2.56
Weight gain (%)	307.30 ± 43.79	309.53 ± 6.94	311.58 ± 12.35	327.36 ± 14.68	313.27 ± 26.69
FCR	1.02 ± 0.08	1.01 ± 0.02	1.01 ± 0.03	0.99 ± 0.03	1.00 ± 0.06
SGR (% day^{-1})	2.02 ± 0.16	2.08 ± 0.01	2.08 ± 0.08	2.15 ±0.04	2.12 ± 0.10
PER	1.77 ± 0.19	1.77 ± 0.05	1.76 ± 0.06	1.84 ± 0.07	1.83 ± 0.14
K-factor (%)	1.45 ± 0.05^{a}	1.40 ± 0.00^{ab}	1.39 ± 0.05^{ab}	1.38 ± 0.01^{b}	1.37 ± 0.08^{b}

[ab] Values within the same row with different superscripts are significantly different ($P < 0.05$).
FCR = feed conversion ratio; SGR = specific growth rate; PER = protein efficiency ratio

Table 5.3. Proximate composition of carcasses after 10 weeks feeding on experimental diets (mean ± standard deviation). C = control; VegLo = vegetative low; VegHi = vegetative high; LyoLo = lyophilised low; LyoHi = lyophilised high.

	Initial	C	VegLo	VegLo	LyoLo	LyoHi
Moisture (%)	72.65 ± 1.59	70.36 ± 0.80	70.71 ± 0.65	70.74 ± 0.54	70.22 ± 0.22	69.61 ± 0.31
Crude protein (%)	15.35 ± 0.48	14.62 ± 0.28	14.53 ± 0.20	14.32 ± 0.47	14.86 ± 0.37	14.53 ± 0.37
Lipid (%)	5.53 ± 0.54	10.61 ± 1.05	9.97 ± 1.02	10.12 ± 0.21	10.78 ± 1.08	11.27 ± 1.02
Ash (%)	2.24 ± 0.25	2.14 ± 0.09	2.11 ± 0.03	2.12 ± 0.09	2.02 ± 0.09	2.12 ± 0.13

Microbial analysis

Indigenous and probiotic levels within the intestinal tract of rainbow trout fed the experimental diets are presented in Table 5.4. TVC of bacteria associated with the mucosa ranged from log 3.60 CFU g^{-1} in the control fish to log 3.75 - 4.63 CFU g^{-1} in the probiotic fed fish. TVC of allochthonous bacteria (transient) ranged from log 8.14 CFU g^{-1} in the control group to log 7.42 - 8.64 CFU g^{-1} in the probiotic groups. Mucosal based *P. acidilactici* levels ranged from log 3.20 in the Lyo groups to 3.41 CFU g^{-1} in the Veg groups. Digesta based levels were considerably higher, within the range of log 5.64 in the Lyo groups to 5.93 CFU g^{-1} in the Veg groups. *P. acidilactici* identification was confirmed with 16S rRNA sequences analysis. *P. acidilactici* levels were statistically too low to confidently determine (i.e. total LAB levels < 20 CFU at the lowest dilution) in the control fed fish and rearing water.

Scanning electron microscopy

SEM revealed large numbers of coccoid cells morphologically corresponding to *P. acidilactici* (clusters of spherical cells in the order of ~1 - 2 μm in diameter) in the posterior intestine of the probiotic fed groups; these populations were not present in any of the control fed fish. A colonisation pattern was observed and noticeable build up of cells were present on the brush border between the mucosal folds and at the base of the villi (Figure 5.1). Close association of cells with the brush border was observed (Figure 5.1, inset).

Table 5.4. Viable and probiotic counts from the gastro-intestinal tract of rainbow trout. Values expressed as mean log CFU g^{-1} for the intestinal mucosa and digesta. C = control; VegLo = vegetative low; VegHi = vegetative high; LyoLo = lyophilised low; LyoHi = lyophilised high.

Intestinal sample	C	VegLo	VegHi	LyoLo	LyoHi
		Total viable counts (log CFU g^{-1})			
Mucosa	3.60	4.63	3.75	4.12	4.03
Digesta	8.14	7.58	8.03	8.64	7.42
		P. acidilactici (log CFU g^{-1})			
Mucosa	TFTC*	3.37	3.41	3.20	3.25
Digesta	TFTC*	5.82	5.93	5.64	5.66

* TFTC – too few to count (statistically too low for reliable estimation).

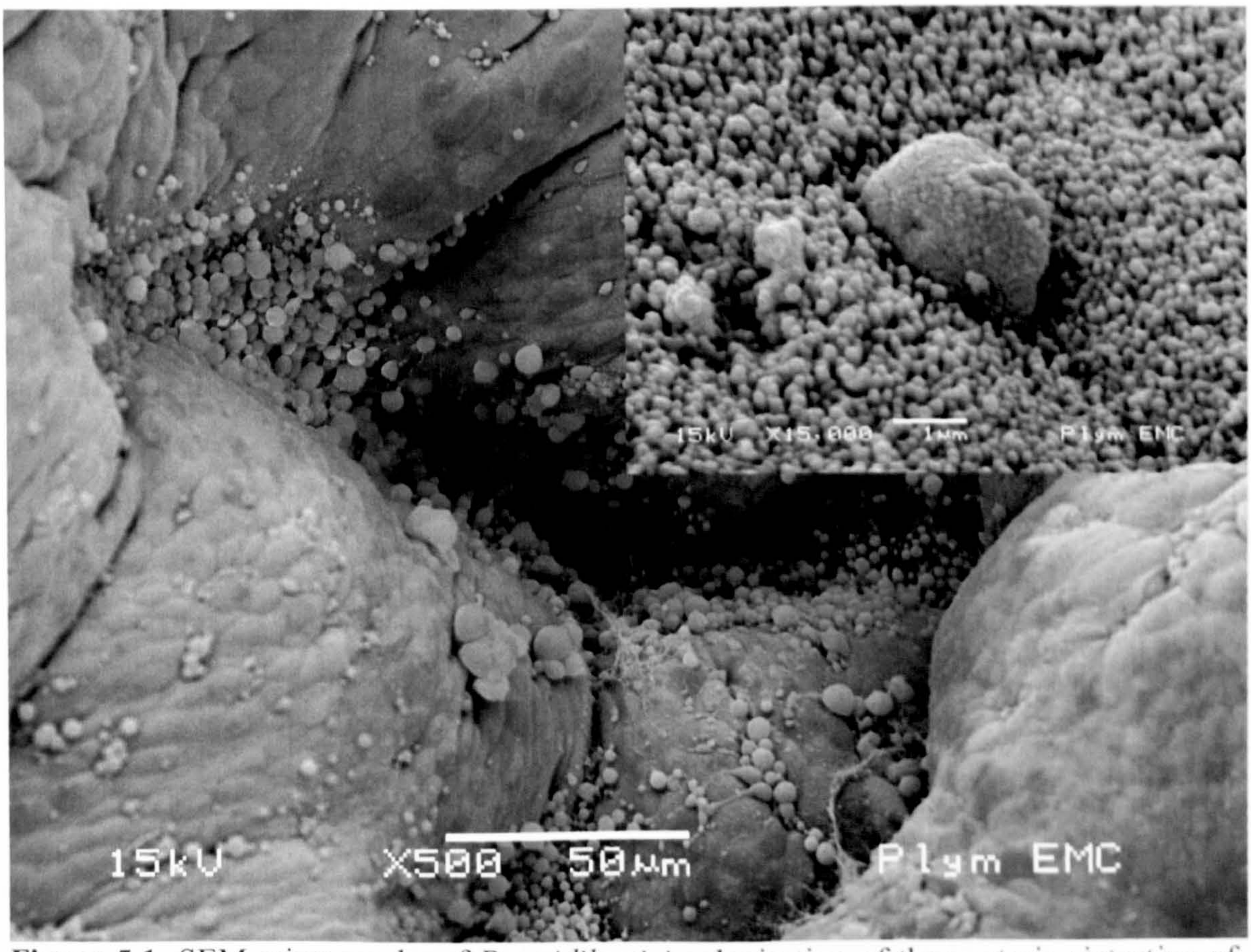


Figure 5.1. SEM micrographs of *P. acidilactici* colonisation of the posterior intestine of trout fed probiotic diets. Localised colonisation is evident between mucosal folds and at the base of the microvilli. Inset: possible initial stages of endocytosis of *P. acidilactici*. Scale bar = 50 μm (Inset scale bar = 1 μm).

Haematological and immunological parameters

Haematological and immunological results are displayed in Table 5.5. Haematocrit levels remained unaffected by the probiotic treatment, PCV of ~29% was observed in all groups. The total number of leukocytes was significantly higher in the VegLo group (76 ± 15) than all other groups, including the control (52 ± 17; $P = 0.009$). The remaining dietary groups were not affected. A considerable yet non significant elevation of serum lysozyme activity was found in groups VegLo (432 ± 136 U mL^{-1}) and VegHi (483 ± 202 U mL^{-1}) compared to the control (328 ± 105 U mL^{-1}). The Lyo groups were not affected.

Table 5.5. Haematological and immunological parameters of rainbow trout after 10 weeks feeding on experimental diets (mean ± standard deviation). C = control; VegLo = vegetative low; VegHi = vegetative high; LyoLo = lyophilised low; LyoHi = lyophilised high.

	Treatment				
	C	VegLo	VegHi	LyoLo	LyoHi
PCV (%)	29.6 ± 2.5	29.4 ± 3.1	28.0 ± 3.1	30.4 ± 4.3	28.6 ± 2.9
Leukocyte count*	52 ± 17[a]	76 ± 15[b]	55 ± 18[a]	55 ± 33[a]	52 ± 17[a]
Lysozyme (U mL^{-1})	328 ± 105	432 ± 136	483 ± 202	314 ± 75	319 ± 156

*Number of leukocytes per 1000 blood cells.

Discussion

Although improved growth performance, feed utilisation and lower mortalities were consistently found in the probiotic fed fish there were no significant differences relative to the control group. Among the largest improvements, especially in the Lyo fed groups, was SGR and mean weight gain (%). SGR increased from 2.02% in the control group to 2.15% in the LyoLo group and 2.12% in the LyoHi group. Mean final weight gain increased from 307% in the control group to 327% in the LyoLo group. Compared to the control group, a small, non significant, elevation of PER was observed in the Lyo fed fish (from 1.77 to 1.83 - 1.84). K-factor was significantly improved in both Lyo groups. A reduction of K-factor may increase aesthetic quality of salmonids to consumers; a more streamlined appearance is an important quality. Current trout farming practices are based around high energy, high lipid diets which often result in the production of fish of lesser quality than their wild counterparts. Whilst economically successful, these practices are not always acceptable to the consumer. Several studies have compared the use of live and dead probiotic cells for aquaculture applications and benefits have been observed with both supplementation forms (Irianto *et al.* 2003; Panigrahi *et al.* 2005; Taoka *et al.* 2006). However, with the exception of a study by Panigrahi *et al.* (2005), there remains a distinct lack of information regarding comparisons between live-sprayed (vegetative) and freeze-dried (lyophilised) dietary supplementation of probiotics. Panigrahi *et al.* (2005) concluded that both viable (live-sprayed and freeze-dried) applications of *L. rhamnosus* induced elevated immunological responses compared to heat killed cells, particularly with regards to phagocytic and complement activity. However, contradictory to the results of the present study Panigrahi *et al.* (2005) did not observe differences of immunological status between fish fed live-sprayed or freeze-dried supplemented diets.

Similar to the present study, Aubin *et al.* (2005) also found that probiotic application of *P. acidilactici* failed to significantly improve weight gain of rainbow trout after 5 months supplemented feeding. However, Gatesoupe (2002) observed improved weight gain of larval pollock (*Pollachius pollachius*) when fed Artemia enriched with *P. acidilactici*. Successful colonisation of the digestive tract by the *P. acidilactici* was achieved in all of the probiotic fed groups. Levels were consistently higher in the Veg groups; but it is interesting to note that the colonisation levels were not significantly different between the application forms. However, the lyophilised cells may not necessarily be as metabolically active within the digestive tract as the cells recovered from the digestive tract of the fish fed the vegetative diets. Digesta based levels were considerably higher than the mucosal based populations. SEM showed the presence of large numbers of bacteria on the mucosal epithelium; these cells were presumptively identified as *P. acidilactici* on the basis of size and morphology and that these cells were not found to be present in the control group.

Electron microscopy is an invaluable tool for the investigation of mucosal associated bacterial populations which has been incorporated into several studies in recent years (Ringø *et al.* 2006abc). However, it remains under utilised in regards to probiotic studies and to the authors knowledge very little data exists regarding electron microscopy with probiotic trials. In the present study a localised presence was evident between the folds of the mucosal surface. This appeared to be somewhat similar to the localised colonisation observed in farm reared trout (observed in Chapter 3A). Close association of the bacterial cells with the epithelial brush border was also evident and is likely to play a role in host immunostimulation. The present study provides further evidence of the use of SEM as a tool for demonstrating mucosal colonisation patterns throughout the digestive tract and should be incorporated in future probiotic investigations. The observations from the present study are contrary to previous rainbow trout findings (Aubin *et al.* 2005) where it

was suggested that *P. acidilactici* demonstrated little noticeable mucosal association of trout when fed dietary levels of 1.5×10^6 CFU g^{-1}. However, it should be noted that Aubin *et al.* (2005) sampled after a 20 hr starvation period which may not provide an accurate representation of intestinal levels. Comparing the results of the present study to those found by Aubin *et al.* (2005) it would appear that in order to achieve adherent mucosal populations either high dietary levels are required or that continual supplementation is necessary to prevent the removal of resident cells (due to continual mucus and brush border turnover) during starvation or reverting to non-supplemented feeds.

A limited potential for elevating immune status was demonstrated in the present study. However, a significant increase in leukocyte numbers were observed in the fish fed the VegLo diet. Also a considerable improvement of lysozyme activity was observed in both vegetative probiotic fed groups (~30% higher than the control group); however, this was not significant at the $P < 0.05$. Similar to these findings, previous studies also appear to indicate a limited potential for *P. acidilactici* with regards to immunostimulation (Bhunia *et al.* 1990; Villamil *et al.* 2002; Shelby *et al.* 2007). For example, Shelby *et al.* (2007) found no significant differences in any of the immune parameters measured (serum protein, immunoglobulin, alternative complement or lysozyme activity) when feeding lyophilised *P. acidilactici* to catfish. Immunological improvements, albeit limited, from the current study were found only with the vegetative diets. It is possible that vegetative *P. acidilactici* may yield a more positive immunological improvement than the lyophilised-based diets. Further work should be conducted specifically focusing on immunological and haematological parameters to reveal the full potential of *P. acidilactici* on the rainbow trout health status. However, *P. acidilactici* has proved effective at reducing vertebral column compression syndrome in rainbow trout after feeding on supplemented diets for 5 months (Aubin *et al.* 2005). Despite the mixed responses of probiotic application of *P.*

acidilactici in finfish studies the potential for application in recent shrimp studies seems promising (Chim *et al.* 2005; Castex *et al.* 2006; Chim *et al.* 2007; Castex *et al.* 2008).

Conclusion

Although virtually all parameters measured were improved in the probiotic groups very few improvements were significant ($P > 0.05$). It can be concluded that either supplementation levels in the present study were not appropriate or that *P. acidilactici* is not particularly effective at enhancing growth and health parameters of rainbow trout reared under good conditions. Benefits may present themselves under poor environmental conditions or situations conducive of stress. In general, improvements (however small) in health parameters were found with the vegetative fed probiotic and improvements in growth and feed utilisation were found with lyophilised fed probiotic. Intestinal colonisation seemed successful (particularly in the Veg diet groups) in terms of both transient and adherent mucosal populations. Considering the antimicrobial and antagonistic properties of *P. acidilactici* and the effective colonisation of the epithelial mucosa, there may be a potential role in providing protection against intestinal pathogenic infections. It would be interesting to conduct challenge studies in order to evaluate this possibility as there is no information addressing this issue currently available. From the little information currently available regarding *P. acidilactici* application in finfish aquaculture it would appear that either: (a) dosage rates and/or form of supplementation are not yet optimised, (b) the effect could be highly species specific, (c) *P. acidilactici* may be more effective at the larval stage, and/or (d) could be effective as a protective agent against enteric pathogenic challenge due to successful intestinal colonisation. Continued research is clearly warranted to evaluate these hypotheses; special attention should be focused on expanding research to other important aquaculture species, short term immunological investigations and challenge studies.

Chapter 6. Assessment of probiotic colonisation of the rainbow trout gastro-intestinal tract: an electron microscopical study

Abstract

Probiotic (*B. subtilis*, *B. licheniformis*, *E. faecium and P. acidilactici*) colonisation of the rainbow trout (*Oncorhynchus mykiss*) gastro-intestinal tract was assessed using culture-based techniques and electron microscopy. After 5 weeks feeding on the experimental diets colonisation of the stomach, pyloric ceaca, anterior and posterior intestine was investigated. Culture-based assessment suggested that all the probionts were able to colonise all intestinal regions (the pyloric caeca was not assessed). Contrary to this, scanning electron microscopy did not show cells morphologically corresponding to *Bacillus* or *E faecium* adherent to the gastro-intestinal epithelial mucosa in the respective probiotic groups. However, coccoid cells morphologically similar *to P. acidilactici* were observed in close association with the brush border of *P. acidilactici* fed fish. Subsequently, microvilli length in the anterior intestine was significantly improved ($P = 0.004$) in the *P. acidilactici* fed fish (2.03 ± 0.37 µm) compared to the control fed fish (1.48 ± 0.51 µm). However, microvilli density was not affected by dietary probiotics in any group.

Introduction

Recent studies have demonstrated high levels of probiotic colonisation of the intestinal mucosa (Kim & Austin 2006; Newaj-Fyzul *et al.* 2007; Chapters 4 and 5), however, no investigation with EM has been carried out to confirm epithelial attachment or investigate colonisation patterns. The influence of fish intestinal microbiota on the gut morphology is evident (Ringø *et al.* 2007). Recent prebiotic studies have demonstrated improved fish intestinal histology at the brush border/microvilli level, presumably by beneficial improvements of the microbial balance (Salze *et al.* 2007; Dimitroglou *et al.* 2008); however, little information is currently available regarding probiotic effects to host intestinal morphology. Salinas *et al.* (2008) demonstrated *in vitro* that the probiotic *L. delbrueckii* could improve Atlantic salmon intestinal enterocyte condition and prevent subsequent damage by *Aeromonas salmonicida* subsp. *salmonicida*.

Given the high level of probiotic mucosal colonisation observed in Chapters 4 and 5, together with the distinct lack of *in vivo* literature regarding probiotic gastric colonisation patterns and resultant effects on gut ultrahistology, the aim of the present study was to assess potential colonisation patterns and the effects on microvilli morphology.

Materials and methods

Diet preparation

Vegetative cultures of *B. licheniformis*+*B. subtilis* (Diet 2B), *E. faecium* (Diet EF) and *P. acidilactici* (Diet PA) were top dressed as described on pages 101 and 136.

Experimental design

Rainbow trout were sourced from a local farm (page 41). The feeding experiment was conducted using 35 trout (average ~100 g) per group in 150 L fibreglass tanks. Fish were

fed 2.0% biomass day^{-1} for 5 weeks. The water temperature was maintained at 15 ± 1 °C with oxygen levels maintained above 80% saturation. A 12 hr light/12 hr dark photoperiod was maintained throughout the entire trial duration. The mean fish weightof sampled fish at the end of the 5 week feeding phase was 189.9 ± 9.4 g for the control group, 191.6 ± 15.1 g for the 2B group, 188.6 ± 10.6 g for the EF group and 200.8 ± 10.0 g for the PA group.

Gastro-intestinal microbiology

Intestinal colonisation of *B. licheniformis*, *B. subtilis*, *E. faecium* and *P. acidilactici* was assessed as described on pages 103 and 138. Samples were also taken from the pyloric caeca and stomach as described for the intestine. 16S rRNA sequence analysis was used to confirm probiont identification (page 47 - 49).

Electron microscopy

Additionally, intestinal samples from 5 fish per experimental group were retained for histological examination of probiotic colonisation and microvilli density by SEM as described on pages 49-51. TEM examination of probiotic colonisation and microvilli length of 5 fish from the control group and *P. acidilactici* group as described on pages 50-51.

Statistical analysis

Data transformations were carried out where necessary and relevant statistical analysis was carried out as described on page 54.

Results

Gastro-intestinal microbiology

Culture-based results show high probiotic populations were present along the gastro-intestinal tract (Table 6.1). Culture-based assessment of mucosal populations showed that all probiotics were found present within all gastro-intestinal regions examined. Probionts were in the range of log 3 - 4 in all gastro-intestinal regions. 16S rRNA analysis confirmed the identification of the presumptive probiotics.

SEM analysis of probiotic populations throughout the digestive tract is shown in Table 6.2. Sporadic colonisation of indigenous bacterial cells, presumably originating from the digesta populations, was identified in all fish groups. However, large populations of epithelium associated *P. acidilactici* (presumptively identified as clusters of spherical cells in the order of ~1 - 2 μm in diameter not present in the other groups) were consistently observed in both intestinal regions of all 5 fish fed the *P. acidilactici* diet (Figures 6.1 & 6.2). Such populations were also evident in the pyloric ceaca of 1 fish but no populations resembling *P. acidilactici* were observed in the stomach. Aside from sporadic indigenous bacteria, no noticeable colonisation of cells morphologically corresponding to *Bacillus* or Enterococci were observed in any gastro-intestinal region of the remaining groups.

Table 6.1. Culture-based assessment of mucosal colonisation by probiotics. Values expressed as log CFU g^{-1}. Diet codes: C = control; PA = *P. acidilactici*; 2B = *B. subtilis/B. licheniformis*; EF = *E. faecium*. Gastro-intestinal regions: S = stomach; AM = anterior mucosa; PM = posterior mucosa.

	Gastro-intestinal region			
	S	AM	PM	Mean
C*	3.99	4.87	5.02	4.63
PA	3.58	3.55	4.04	3.72
2B	4.98	4.69	4.56	4.74
EF	3.29	3.12	3.22	3.21

* Viable population

Microvilli morphology

EM analysis of intestinal microvilli morphology is shown in Table 6.2. There were no significant differences of microvilli density observed between any of the treatments (microvilli density ranged between 13.7 - 18.1 AU in the AM and 13.0 - 17.1 AU in the PM). However, compared to the control (1.48 ± 0.51 μm), *P. acidilactici* fed fish (2.03 ± 0.37 μm) displayed significantly longer microvilli in the anterior intestine ($P = 0.004$; Figure 6.3). This improvement was not observed in the posterior intestine.

Table 6.2. Rainbow trout microvilli morphology after probiotic feeding for 5 weeks. Diet codes: C = control; PA = *P. acidilactici*; 2B = *B. subtilis/B. licheniformis*; EF = *E. faecium*. Intestinal regions: AM = anterior mucosa; PM = posterior mucosa.

		Group			
Parameter	Intestinal region	C	PA	2B	EF
Microvilli density*	AM	15.4 ± 4.5	13.8 ± 3.2	13.7 ± 2.0	18.1 ± 12.0
	PM	15.2 ± 2.3	14.7 ± 1.6	13.0 ± 3.9	17.1 ± 5.2
Microvillus length (μm)	AM	1.48 ± 0.51[a]	2.03 ± 0.37[b]	-	-
	PM	1.99 ± 0.45	1.78 ± 0.20	-	-

* Arbitrary units (AU)

[Ab] Values with different superscripts denotes a significant difference ($P < 0.05$)

- not assessed

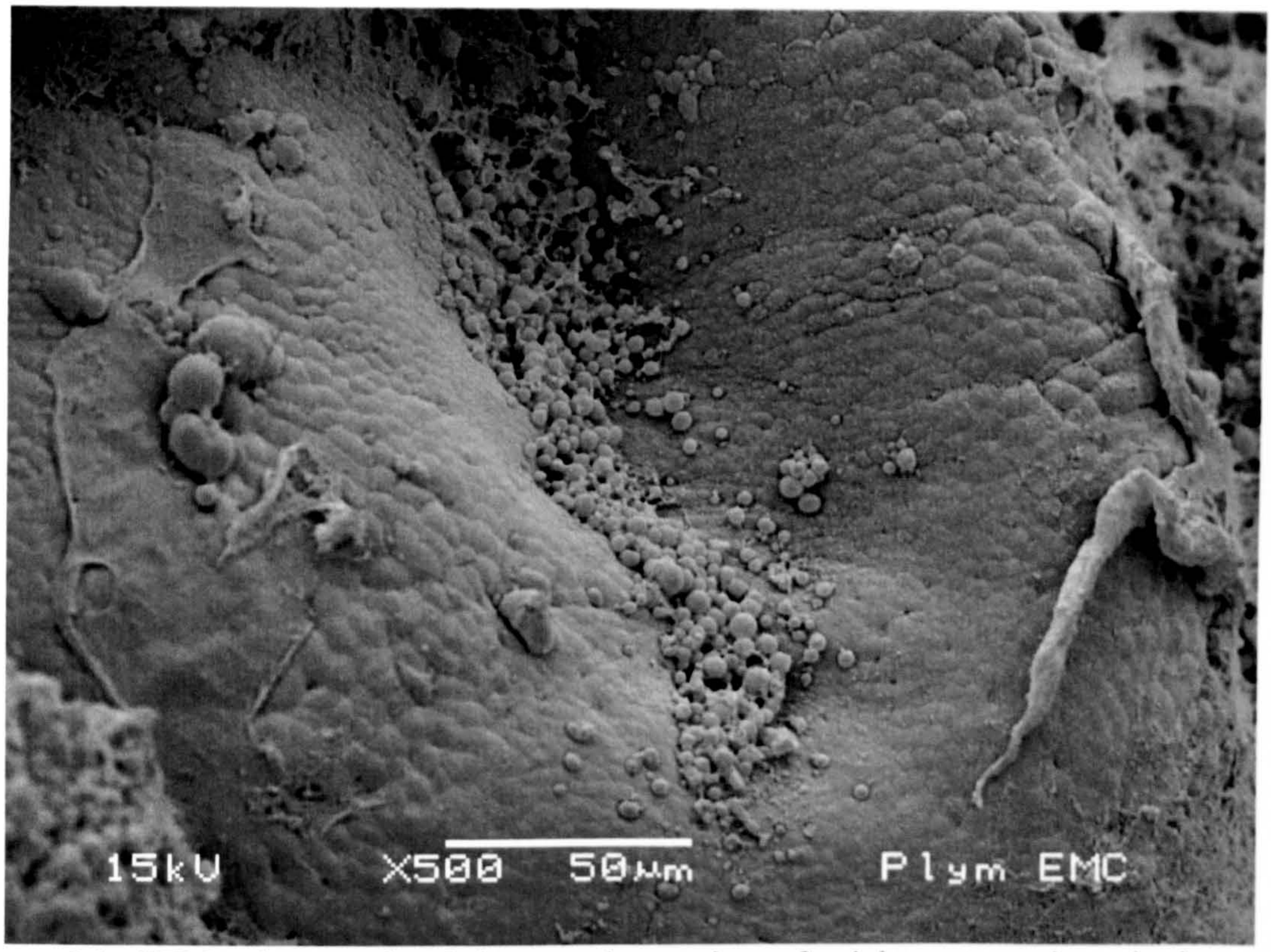


Figure 6.1. SEM micrograph of the anterior region of rainbow trout intestinal mucosa showing a localised colonisation of cocci cells (presumed *P. acidilactici* due to cell size, morphology and absence of observed cells in the other dietary treatments). Scale bar = 50 µm.

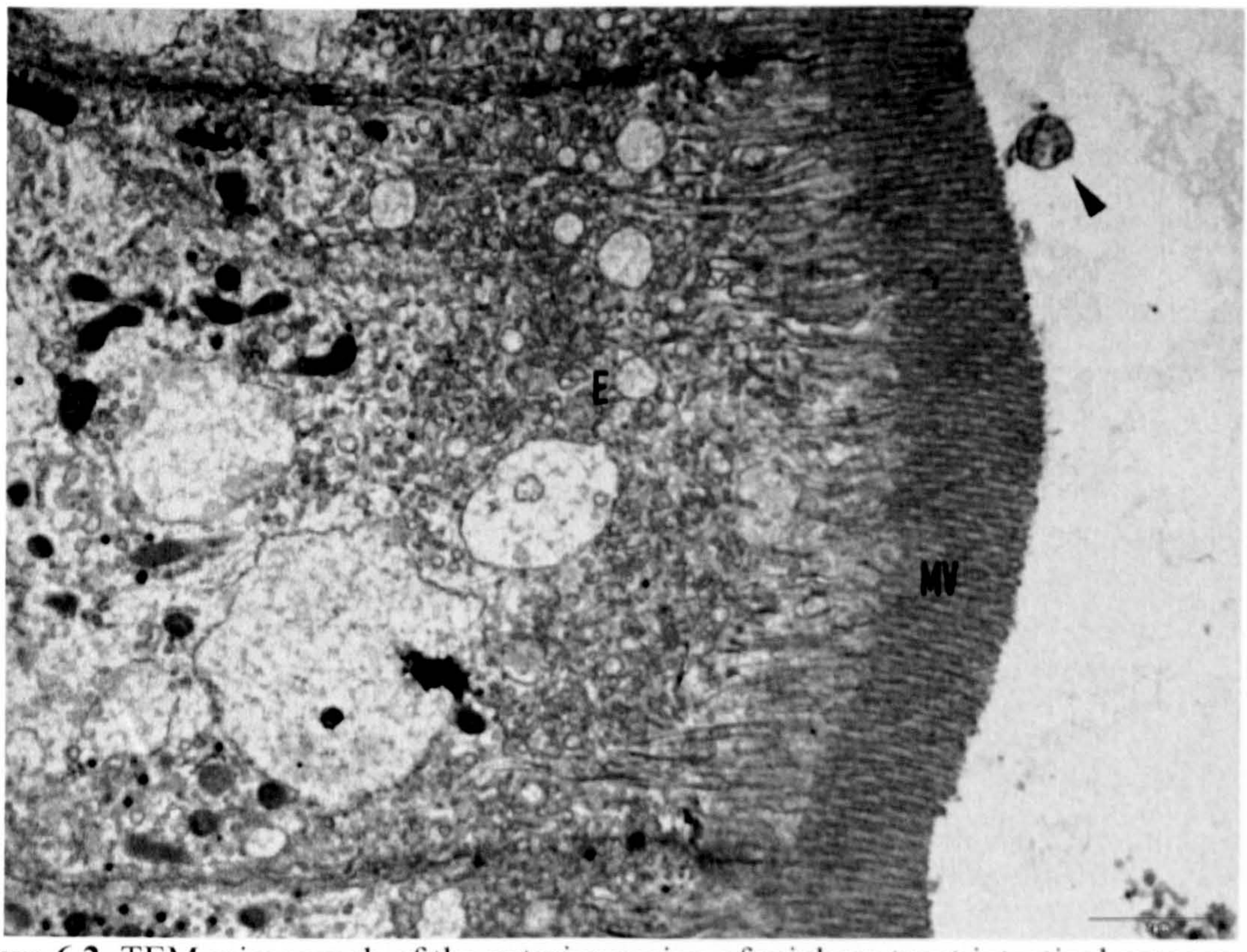


Figure 6.2. TEM micrograph of the anterior region of rainbow trout intestinal mucosa showing bacterial-like cell (arrow; presumed *P. acidilactici*). E = enterocyte, MV = microvilli.. Scale bar = 2 µm.

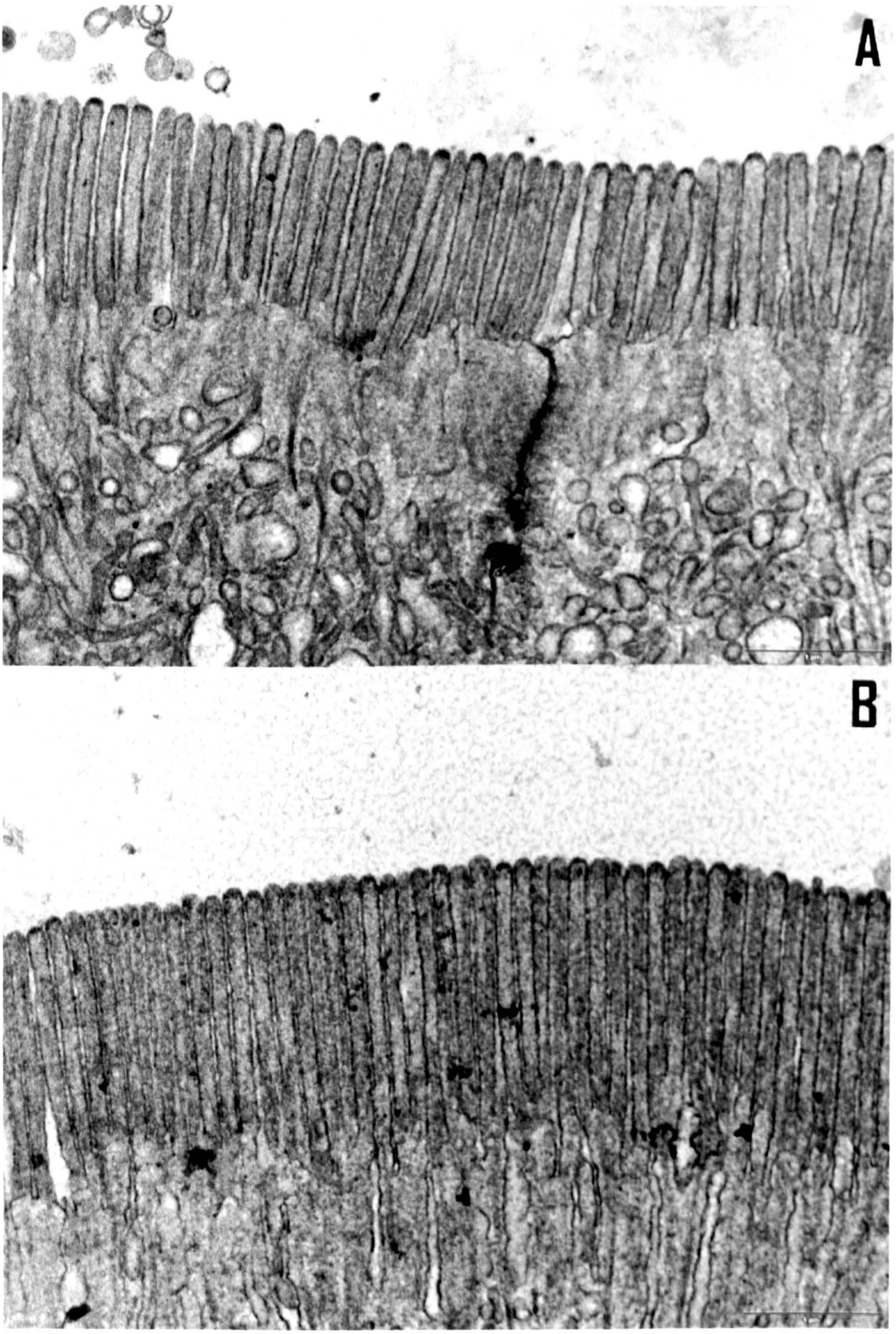


Figure 6.3. Comparative TEM micrographs of the anterior intestine of control fed fish (a) and *P. acidilactici* fed fish (b). Although microvilli appear healthy in both groups they are significantly longer in the *P. acidilactici* group ($P = 0.004$). Scale bar = 1 μm.

Discussion

Despite culture-based confirmation of probiotic colonisation of the mucosa (confirming previous studies such as Kim & Austin 2006; Newaj-Fyzul *et al.* 2007; Chapters 4 and 5), SEM and TEM revealed a distinct lack of probiotic colonisation of the gastro-intestinal tract of the fish fed *E. faecium* and *Bacillus* spp.. This would appear to suggest that either a) these probiotics form a loose association with the mucosa and become disassociated when washed or b) true epithelial colonisation is not achieved. The former option is hard to imagine as the association would have to be relatively strong/stable in order to withstand mucus turnover, sloughing of surrounding enterocytes and peristalsis of transient digesta. The latter option seems more likely. It would appear that instead of colonising the epithelial surface, *E. faecium*, *B. subtilis* and *B. licheniformis* are able to colonise the mucus layer and presumably proliferate at a rate high enough to sustain a considerable population (with continual dietary supplementation at least). With the current sampling methods it is very difficult to differentiate between a purely mucus associated population and an epithelium associated population. It is very easy to misidentify the nature this colonisation and EM must be incorporated in order to confirm true epithelial colonisation.

Confirming the findings of Chapter 5, EM in the present study shows true epithelial colonisation by *P. acidilactici*. *P. acidilactici* was frequently observed in both the anterior and posterior intestine, occasionally in the pyloric ceaca but not in the stomach. Again the mucosal folds either appear to provide protection against shear of digesta or a favourable site for initial colonisation. Epithelial attachment, bacterial-host cross-talk and translocation is an extremely complex process; many factors and mechanisms have been identified and a full review will not be presented here (for a more comprehensive discussion see Lu & Walker 2001; Sansonetti 2006; Ringø *et al.* 2007). Briefly,

colonisation of the epithelial surface requires intimate contact between the bacterial cell and the epithelium. In order to achieve this, attraction by van der Waal's forces closes the distance to a point where electrostatic interactions allow a close association to form in regions of low ionic strength (Ringø *et al.* 2007). Hydrophobic interactions or receptor binding may then occur. Bacteria express surface adhesins which bind carbohydrates linked to glycoproteins or glycolipids present on the epithelial cells of the brush border (Lu & Walker 2001). The epithelial cells are often actively involved in the process of internalisation, including internalisation of pathogenic bacteria. Indeed, some Gram-negative organisms produce specialized protein secretions (termed type III) which can alter host cellular functions resulting in the bacterium dictating the interaction process (Blocker *et al.* 2003), essentially the bacterial cells are actively co-opting the host cell into the infection process. Interactions can result in an extracellular attachment or endocytosis with or without cellular rearrangement. Two distinct internalisation mechanisms have been identified in mammals: "zippering" and "trigger" process (Sansonetti 2006) but such mechanisms are not well documented in fish.

From the results it is clear there is a distinct difference between the nature of mucosal colonisation between the probionts investigated. However, it should be noted that colonisation of the mucus layer as opposed to the epithelium is still a positive outcome as such populations will likely enhance the protective barrier function provided by the mucus layer, which potential pathogens have to overcome, or possibly colonise/populate in order to attach or come into contact with the host epithelium. Mucus associated populations of probiotics are also likely to release bacteriocins and other antimicrobial compounds in the mucus, furthering protective function. Extracellular production of digestive enzymes will also likely aid digestive function as nutrients come into contact with the mucus. Probiotic cells (viable or dead) may also exert an immunostimulatory role when colonising the

intestinal mucus. Indeed, Kim & Austin (2006) saw an increase in intestinal mucus lysozyme activity, likely due to changes in the intestinal microbiota (and/or mucus colonisation by *C. maltaromaticum* and *C. divergens*). Newaj-Fyzul *et al.* (2007) also reported an increase in intestinal mucus lysozyme activity and demonstrated successful colonisation of the rainbow trout intestinal mucus by *B. subtilis*. Whether true epithelial colonisation was achieved was not confirmed, however, colonisation of the intestinal contents and mucus layer resulted in host immunostimulation. Specifically significant improvements of leukocyte levels, phagocytic levels, bactericidal activity, respiratory burst activity, serum lysozyme activity, total antiprotease activity and total proteins was observed.

Establishing a probiotic population within the intestinal mucus is highly likely to strengthen the protective function of the intestinal barrier. Several probiotics have been demonstrated to attach to and grow well in fish intestinal mucus (Olsson *et al.* 1992; Jöborn *et al.* 1997; Vine *et al.* 2004) and utilisation of such probionts could provide competition and antagonism against pathogens. Indeed, Newaj-Fyzul *et al.* (2007) observed a significant improvement of rainbow trout survival after challenge with *Aeromonas* spp. ABE1. Several pathogens, particularly *V. anguillarum*, are well documented to adhere to and grow well in fish intestinal mucus (Olsson *et al.* 1992; Garcia *et al.* 1997; O'Toole *et al.* 1999; Ormonde *et al.* 2000). It would be interesting to test the probiotics that have been demonstrated to colonise intestinal mucus *in vivo*, such as those investigated by: Kim & Austin (2006); Newaj-Fyzul *et al.* (2007) and the probionts used in this thesis *in vivo* against *V. anguillarum* challenge.

Intestinal microvilli density has shown morphological changes in response to alterations of intestinal microbiota in previous studies (Dimitroglou *et al.* 2008) yet none of the of the probiotics assessed were able to significantly alter microvilli density in the rainbow trout

anterior or posterior intestine. Despite this, *P. acidilactici* fed trout produced significantly longer microvilli in the anterior intestine than the control fed fish. Similarly, prebiotics such as MOS have led to increased microvilli length in trout (Dimitroglou *et al.* 2008) and cobia (Salze *et al.* 2008). It can easily be suggested that a more beneficial microbial balance with a reduced number of potential pathogens or toxin producing organisms within the digestive tract will lead to improved gut development and morphology. The precise mechanism by which this is achieved is not clear and requires further investigation to fully elucidate. Likewise it is not clear why this improvement was observed only in the anterior intestine and not the posterior intestine. Further investigation with *B. subtilis, B. licheniformis* and *E. faecium* should be assessed in order to evaluate whether a probiotic mucus population is enough to achieve these benefits or whether true epithelial colonisation is required. If this observation is not restricted to *P. acidilactici* and is a common occurrence with other probiotic candidates then it cannot be ruled out that improved gut morphology, at least at the brush border level, may have contributed to improved feed utilisation/growth performance observed in previous studies (Bogut *et al.* 2000; El-Haroun *et al.* 2006; Bagheri *et al.* 2008; Wang *et al.* 2008; Chapters 4 and 5). Indeed, rainbow trout fed *P. acidilactici* resulted in a marginal improvement of FCR and considerable improvements in SGR and PER in Chapter 5.

Chapter conclusions

The present study highlights the importance of incorporating EM in probiotic studies and suggests that microbes apparently isolated from epithelial mucosa in previous investigations may be (at least in part) a mucus associated population which is not fully removed when washed prior to culture-based extraction. The mucus population may represent an intermediate population between the communities found in the digesta and on the epithelium. Future studies should address this possibility. The current study further indicates that *P. acidilactici* may be a useful probiotic to prevent or reduce gastro-intestinal infections and future challenge studies should be conducted. The study demonstrates the potential of probiotics to enhance intestinal ultra-structure. Future studies should incorporate light microscopy to assess any possible alterations at the villi level.

Chapter 7. General discussion and conclusions

Both culture-based and culture-independent techniques have demonstrated that rainbow trout mucosal communities are more complex and often higher in number than previously thought. Communities also appear to differ throughout the intestinal regions investigated. In contrast to previous investigations (Lesel & Pointel 1979; Austin & Al-Zahrani 1988) the current work using electron microscopy demonstrated a close association of bacterial cells with the brush border throughout the entire digestive tract. Most noticeably, a localised colonisation pattern was often observed between the villi and mucosal folds. The use of electron microscopy in future fish epithelial studies is highly recommended for visualisation of autochthonous populations. The intestinal microbiota of trout was sensitive to dietary SBM confirming the findings of Heikkinen *et al.* (2006); however, no changes of total viable culturable populations were observed. Despite this, communities comprising both the viable allochthonous and autochthonous population were altered in composition. Perhaps most notably, a large increase of yeast, presumptively identified as *Saccharomyces* spp., and *Psychrobacter* spp. were found associated with the intestinal tract of SBM fed rainbow trout. 16S rRNA analysis identified a range of species, most of which have not been identified from the rainbow trout digestive tract previously. Both *B. subtilis* and *B. licheniformis* were indigenous members of the rainbow trout intestinal microbiota of the fish, further supporting the application of these species as rainbow trout probiotics by fulfilling one of the requirements as suggested by Spanggaard *et al.* (2001; refer to page 23). Confirmation of enteritis-like damage was observed in rainbow trout fed SBM rich diets for 16 weeks. Microvilli morphology was clearly altered leading to a reduction of both microvilli density and length. Tight junctions were also exposed to epithelium associated bacteria and it appeared that some bacteria-like cells may have used

exposed tight junctionsas a vector for infection. It is concluded that the provision of 50% dietary protein by SBM compromises the epithelial barrier mechanism in salmonids. Future work must consider the indigenous autochthonous communities of rainbow trout when assessing microbial communities or scanning for probiotics. Improved SBM digestibility has been demonstrated with prebiotics (Burr *et al.* 2008), seemingly due to a shift to a more favourable microbial population. Probiotic supplementation of SBM rich diets could also result in such benefits, particularly if the probiont is selected from the indigenous microbiota of omnivorous fish species, such as carp or tilapia.

Culture-based assessment in Chapter 4 demonstrated that *E. faecium*, *B. subtilis* and *B. licheniformis* were highly effective at colonisation of the rainbow trout digestive tract. Chapter 4A demonstrated that *E. faecium* supplemented diets used singularly or combined with *Bacillus* probiotics significantly improved FCR of rainbow trout. PER of *E. faecium* fed fish was significantly higher than the *Bacillus* probiotic fed fish. Yet no other improvements of growth or feed utilisation were found. Fish fed diets with *B. subtilis, B. licheniformis* and *E. faecium* synergistically induced significant elevations of leukocyte levels. High level Lf (200 mg kg^{-1}) also resulted in elevated leukocyte levels. Rainbow trout fed *B. subtilis* and *B. licheniformis* significantly improved lysozyme activity but lysozyme was not affected in the remaining groups. No significant differences were found in haematocrit levels or complement activity.

Contradictory to the findings of Chapter 4A, the effects of feeding probiotics after antibiotic supplementation in Chapter 4B resulted in significant improvements of FCR, SGR and PER with the *Bacillus* fed fish but no improvements with *E. faecium* fed fish. These findings suggest that high intestinal levels of *B. subtilis* and *B. licheniformis* (~80 - 90% of the intestinal microbiota) are required to exert benefits to the host. This appears to confirm the findings of Bagheri *et al.* (2008) with regards feeding *B. subtilis* and *B.*

licheniformis to rainbow trout fry. The lack of response with *E. faecium* after antibiotic treatment of trout (which yielded > 70% intestinal colonisation as opposed to > 45% in non antibiotic treated fish) suggests an important synergistic relationship between *E. faecium* and the indigenous microbiota that seems to be negated with the use of antibiotics. Most probiotic studies to date, including those conducted within this thesis; do not consider the probiotic effects on the indigenous microbiota. Some studies do not even assess the intestinal probiotic colonisation; those that do normally only assess viable allochthonous counts and transient probiotic counts. Perhaps future studies may wish to investigate the overall shifts in the microbiota as opposed to enumeration of only the probionts in the digesta. Had this approach been undertaken in the present studies the synergistic relationship between *E. faecium* and the indigenous microbes may have been more clearly understood. Although elevated lysozyme activity was detected in the probiotic fed fish these improvements were not significant compared to the control group. *Bacillus* fed trout displayed significantly elevated leukocyte levels compared to the control. It is concluded that under the current circumstances when using Lf at levels of 100 & 200 mg kg^{-1} there are no significant effects on growth performance or feed utilisation. The use of the probiotics and Lf in the present investigations resulted in no significant differences of carcass composition at the end of the experimental trials. The chapter suggests that there may be a role for incorporating probiotic feeding strategies after the use of antibiotics, most notably with *B. subtilis/B. licheniformis*. Comparative results from the two investigations indicate that probiotic results are not reproducible even when using the same fish species under extremely similar circumstances. Lf continually demonstrated no benefits in growth or feed utilisation, but it did appear that leukocyte levels may be elevated under certain circumstances. Perhaps greater benefits of Lf may be seen under different circumstances, such as after short term supplementation or during periods of

stress. However, it must be concluded that the application of Lf was somewhat limited for rainbow trout under the conditions of the present studies.

Chapter 5 explored the potential of using *P. acidilactici* in a vegetative and lyophilised form. After 10 weeks feeding *P. acidilactici* successfully colonised the intestinal tract as both allochthonous and autochthonous populations. Mucosal colonisation was confirmed by SEM, which showed a localised presence between mucosal folds and villi somewhat similar to the findings of the indigenous microbiota of farm reared trout (Chapter 3A). These results seem contradictory to those observed by Aubin *et al.* (2005) where intestinal colonisation of rainbow trout intestinal epithelium did not appear particularly successful. Successful intestinal colonisation of the probiotic fed fish in the present study resulted in improved performance in virtually all parameters measured; however, very few improvements were significant at the $P < 0.05$ level. Significant improvements of K-factor was seen in the lyophilised *P. acidilactici* fed fish and elevated leukocyte levels were observed in the trout fed the vegetative *P. acidilactici* diet. From the little information currently available regarding the potential for *P. acidilactici* application in finfish aquaculture it would appear that either a) dosage rates and/or form of supplementation are not yet optimised, b) the effect could be highly species specific, c) that *P. acidilactici* is more likely to be effective at the larval stage or d) due to reported antagonistic properties and successful intestinal colonisation may be more effective as a protective agent pathogenic challenge. Continued research is clearly warranted. Further studies with both rainbow trout and other important aquaculture species may help to elucidate the extent of use of *P. acidilactici* for aquaculture applications.

Probiotic colonisation patterns were explored using electron microscopy in Chapter 6, which is a technique not utilised in previous fish probiotic studies. Culture-based assessment demonstrated successful mucosal colonisation throughout the digestive tract

by *B. subtilis*, *B. licheniformis*, *E. faecium* and *P. acidilactici*. However, electron microscopy revealed clear differences in epithelial colonisation by the various probionts evaluated. *P. acidilactici* seemed particularly well adapted, successfully colonising the anterior and posterior intestine along with the pyloric ceaca to a minor extent. However, *Bacillus* spp. and *E. faecium* cells were not observed in any regions of the digestive tract. The results would suggest that some probionts, such as *B. subtilis*, *B. licheniformis* and *E. faecium*, appear to colonise the mucus layer as opposed to the intestinal epithelium directly. None of the probionts successfully colonised the stomach epithelium; this was probably due to the specific conditions and a limited residence time in the stomach. The findings would suggest that previous studies investigating autochthonous populations may sometimes confuse true epithelial colonisation with mucus associated populations. Microvilli morphology has not been investigated in probiotic studies to date but the present investigation suggests that probiotics have the potential to enhance intestinal ultra-structure. Feeding rainbow trout diets supplemented with *P. acidilactici* resulted in improved microvilli length in the anterior intestine but microvilli density was not significantly altered in any of the intestinal regions with any of the probiotics tested. If such improvements are not restricted to just *P. acidilactici*, improved microvilli morphology could have been a contributory factor in previous studies where improved feed utilisation and growth performance have been observed (Bogut *et al.* 2000; El-Haroun *et al.* 2006; Bagheri *et al.* 2008; Wang *et al.* 2008; Chapters 4 and 5). Future studies should include electron microscopy and gastro-intestinal histological investigations in order to assess this possibility.

This body of research suggests that there is a potential role for probiotic applications in rainbow trout aquaculture. However, to exert the full benefits further research is necessary and special consideration is required with regards to probiotic treatment strategies.

Probiotic supplementation leads to improved competition within the digestive tract and so should be thought of as an insurance policy as opposed to merely assessing direct host benefits. By increasing competition within the intestinal tract we can reduce the potential of pathogen establishment/colonisation. This possibility requires further attention, and challenge studies induced by pathogenic bathing or feeding contaminated diets should be conducted. The results also indicate a potential role for the application of probiotics after antibiotic treatment in order to replenish the gut microbiota as a defensive barrier. Results are not reproducible and fish physiological status may not always require the potential benefits provided by probiotic supplementation. Perhaps probiotics are best utilised during, or after times of acute and chronic stress; further work must be conducted in order to evaluate this possibility. Continued research is required to elucidate correct dosage forms and dietary levels in commercial aquafeeds. Studies should also investigate histological changes of the gastro-intestinal tract after probiotic applications as the present work begins to shed light on this area.

Despite the growing body of knowledge accumulated over the last decade regarding probiotic applications for finfish species I believe that our knowledge still remains somewhat limited; in order to assess the applicability of the probionts used within these investigations, as well as other commonly used probiotics, research must be applied at the fish farm level. With different feeding strategies, husbandry practices and a more variable rearing environment the outcomes are likely to differ. Research conducted within laboratory facilities is a good indicator of benefits at the industrial farm rearing scale but future work is required to validate results. Indeed the effects of probiotics may vary during summer temperatures as well as when fish reach market and adult size. The smoltification process in salmonids for example is likely to have a major impact on the efficacy of the probiotics and currently little research has addressed this topic. Similarly cost benefit

analysis should also be included in studies where improvements of growth, health performance and disease resistance have been observed. With the exception of El-Haroun *et al.* (2006) very few several studies have considered cost benefit and further analysis should be conducted from farm level research trials. It is also clear that there is a distinct lack culture-independent probiotic colonisation investigations. Culture-dependent techniques are often sufficient to track probiotic colonisation levels but studies such as Kim & Austin (2006) clearly show the importance of utilising culture-independent techniques. Further probiotic studies should include techniques such as DGGE where applicable and also assess colonisation patterns using electron microscopy.

Our knowledge of probiotic applications within aquaculture continues to expand for several key species but data for many important aquaculture fish species is relatively scarce. Sustained research must focus on rectifying this problem; species where our knowledge is particularly lacking include: cobia, red drum (*Sciaenops ocellatus*), milkfish (*Chanos chanos*), snakehead (*Channa striatus*), sheat fish, mullet (*Mugil* spp.), amberjack (*Seriola dumerili*), tunas (*Thunnus* spp.), whitefish (*Coregonus* spp.), sturgeon (*Acipenser* spp.), barramundi (*Lates calcarifer*), tench (*Tinca tinca*), sea trout, wolffish and Arctic charr.

The combined application of both probiotics and prebiotics, termed synbiotics, is an area that also requires further attention. Whilst many synbiotic studies have been conducted with terrestrial animals synbiotic studies with fish are not well documented.

There remains a great deal that we have yet to learn but the outlook for use of probiotics within the aquaculture industry looks promising and we must continue to pursue this interesting area.

1. QIAamp Stool Mini Kit DNA extraction

Optimised from manufacturers instructions for extraction of a) mixed communities from digesta/mucosal sample or b) pure isolate from a single culture

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

Lysis

1. **a) Incubate 200 mg of digesta/mucosa sample with 500 µL of lysozyme (50 mg mL^{-1} in TE) for 30 min at 37°C.**

   b) Add a loop full of pure culture to 200 µL molecular grade water and boil for 10 min.
2. **Add 700 µL of buffer ASL and vortex for 30 sec.**
3. **Heat for 5 min at 90 °C.**
4. **Vortex for 15 sec and centrifuge for 1 min**

Inhibitor removal

5. **Place 700 µL of the supernatant into an Eppendorf and add half an Inhibitex tablet. Vortex for 30 sec or until suspended. Stand for 1 min.**
6. **Centrifuge for 4min and place 215 µL of the supernatant into a new tube.**
7. **Centrifuge for 4min.**

Protein removal

8. **Place 15 µl of Proteinase K into a fresh tube and add place 200 µL of the supernatant.**
9. **Add 200 µL of buffer AL and vortex for 15 sec.**
10. **Incubate at 70 °C for 10min.**
11. **Add 200 µL of ethanol and vortex.**

Cleanup

12. **Apply the entire sample to a QIAamp column and centrifuge for 1 min.**
13. **Place column into a new collection tube and add 500 µL buffer AW1. Centrifuge for 1 min.**
14. **Place column into a new collection tube and add 500 µL buffer AW2. Centrifuge for 3 min.**
15. **Place column into a new collection tube and add 200 µL Buffer AE. Stand for 3 min and centrifuge for 1 min.**

2. Database of Gram-negative organisms identified by Microbact™ 24E test kits
Identification based upon:
Lysine decarboxylase, gelatin liquefacien, ornithine decarboxylase, malonate inhibition, hydrogen sulphide, inositol fermentation, glucose fermentation, sorbitol fermentation, mannitol fermentation, rhamnose fermentation, xylose fermentation, sucrose fermentation, ONPG, lactose fermentation, indole, arabinose fermentation, urease, adonitol fermentation, Voges Prokauer, raffinose fermentation, citrate utilization, salicin fermentation, tryptophan deamnase and arginine dihydrolase.

Microbact™ 24E database of oxidase negative Gram-negative organisms.

***Acinetobacter* spp.**
A. baumannii
A. lwoffii
A. haemolyticus
***Budvicia* spp.**
B. aquatica
***Buttiauxella* spp.**
B. agrestis
***Cedecea* spp.**
C. davisae
C. lapagei
C. neteri
Cedecea sp 3
Cedecea sp 5
***Citrobacter* spp.**
C. freundii
C. diversus
C. amalonaticus
C. amalonaticus biogp 1
C. farmeri
C. youngae
C. braakii
C. werkmanii
C. sedlakii
Citrobacter sp 9
Citrobacter sp 10
Citrobacter sp 11
***Edwardsiella* spp.**
E. tarda
E. tarda biogp 1
E. hoshinae
E. ictaluri
***Enterobacter* spp.**
E. aerogenes
E. cloacae
E. agglomerans
E. gergoviae
E. sakazakii
E. taylorae
E. amnigenus biogp 1
***Hafnia* spp.**
H. alvei
H. alvei biogp 1
***Klebsiella* spp.**
K. pneumoniae
K. oxytoca
K. ornithinolytica
K. planticola
K. ozaenae
K. rhinoscleromatis
K. terrigena
Klebsiella gp 47
K. ascorbata
K. cryocrescens
***Leclercia* spp.**
L. adecarboxylata
***Leminorella* spp.**
L. grimontii
L. richardii
***Moellerella* spp.**
M. wisconsensis
***Morganella* spp.**
M. morganii
M. morganii ssp. *morganii*
M. morganii biogp 1
M. morganii ssp. Siboni 1
***Obesumbacterium* spp.**
O. proteus biogp 2
***Pragia* spp.**
P. fontium
***Proteus* spp.**
P. mirabilis
P. vulgaris
P. penneri
P. myxofaciens
***Providencia* spp.**
P. rettgeri
P. stuartii
P. alcalifaciens
P. rustigianii
***Serratia* spp.**
S. marcescens
S. marcescens biogp 1
S. liquefaciens
S. rubidaea
S. odorifera biogp 1
S. odorifera biogp 2
S. plymuthica
S. ficaria
S. entomophila
S. fonticola
***Shigella* spp.**
Shigella serogp.AB&C
S. sonnei
Trabulsiella spp.
T. guamensis
***Xenorhabdus* spp.**
X. luminescens(25C)
X. luminescens gp 5
X. nematophilis (25C)
***Xanthomonas* spp.**
X. (S.)maltophilia
***Yersinia* spp.**
Y. enterocolitica
Y. frederiksenii
Y. intermedia
Y. kristensenii
Y. rohdei
Y. aldovae
Y. bercovieri
Y. mollaretii
Y. pestis
Y. pseudotuberculosis
Y. ruckeri
***Yokenella* spp.**
Y. regensburgei
Enteric Gp17
Enteric Gp41
Enteric Gp45
Enteric Gp58

E. amnigenus biogp 2
E. asburiae
E. hormaechei
E. intermedium
E. cancerogenus
E. dissolvens
E. nimipressuralis
***Escherichia* spp.**
E. coli
E. coli-inactive
E. fergusonii
E. hermannii
E. vulneris
E. blattae
***Ewingella* spp.**
E. americana
P. heimbachae
***Rahnella* spp.**
R. aquatilis
***Salmonella* spp.**
Salmonella subsp.1
S. typhi
S. cholerae-suis
S. paratyphi A
S. gallinarum
S. pullorum
Salmonella subsp.2
S. arizonae subsp.3A
Salmonella subsp.3B
Salmonella subsp.4
Salmonella subsp.5
Salmonella subsp.6
Enteric Gp59
Enteric Gp60
Enteric Gp63
Enteric Gp64
Enteric Gp68
Enteric Gp69

Microbact™ 24E database of oxidase positive Gram-negative organisms

***Pseudomonas* spp.**
P. aeruginosa
P. fluorescens-25
P. fluorescens-35
P. putida
P. stutzeri
P. diminuta
***Burkholderia* spp.**
B. cepacia
B. pseudomallei
***Shewanella* spp.**
S. putrefaciens
***Alcaligenes* spp.**
A. faecalis type 11
A. faecalis
A. xylosoxidans ssp. *xylos* (*Achromobacter xylosoxidans*)
***Flavobacterium* spp.**
F. meningosepticum (*Chryseobacterium meningosepticum*)
F. odoratum (*Myroides odoratus*)
F. breve (*Empedobacter brevis*)
F. indologenes (*Chryseobacterium indologenes*)
***Moraxella* spp.**
***Plesiomonas* spp.**
P. shigelloides
***Aeromonas* spp.**
A. hydrophila
A. veronii bio *sobria*
A. veronii bio *veronii*
A. caviae
***Weeksella* spp.**
W. virosa
W. zoohelcum
***Pasteurella* spp.**
P. multocida
P. haemolytica
***Actinobacillus* spp.**
***Vibrio* spp.**
V. fluvialis
V. furnissii
V. mimicus
V. vulnificus
V. hollisae
V. cholerae
V. parahaemolyticus
V. alginolyticus

References

Acinas, S. G., Antón, J. & Rodríguez-Valera, F. (1999) Diversity of free-living and attached bacteria in offshore Western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl. Environ. Microb.* **65**, 514-522

Alakomi, H. L., Skytta, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000) Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl. Environ. Microbiol.* **66**, 2001-2005.

Alpharma animal health Ltd. Technical Bulletin 5/2001, revised 9/01

Anastasiadou, S., Papagianni, M., Filiousis, F., Ambrosiadis, I., & Koidis P. (2008) Pediocin SA-1, an antimicrobial peptide from *Pediococcus acidilactici* NRRL B5627: production conditions, purification and characterization. *Bioresource Technol.* **99**, 5384-5390.

Andlid, T., Vázquez-Juárez, R. & Gustafsson, L. (1995) Yeast colonizing the intestine of rainbow trout (*Salmo gairdneri*) and turbot (*Scophtalmus maximus*). *Microbial. Ecol.* **30**, 321-334.

Andlid, T., Vázquez-Juárez, R. & Gustafsson, L. (1998). Yeasts isolated from the intestine of rainbow trout adhere to and grow in intestinal mucus. *Mol. Mar. Biol. Biotechnol.* **7**, 115-126.

Andlid, T., Blomberg, L., Gustafsson, L. & Blomberg, A. (1999) Characterization of Saccharomyces cerevisiae CBS 7764 isolated from rainbow trout intestine. *Syst. Appl. Microbiol.* **22**, 145-155.

AOAC (1995) Official Methods of Analysis of Association of Official Analytical Chemists (16th ed.). Association of Analytical Chemists, Arlington, VA, USA.

Arnold, R. R., Cole, M. F. & McGhee, J. R. (1977) A bactericidal effect for human lactoferrin. *Science.* **197**, 263-265.

Arnold, R. R., Brewer, M. & Gauthier, J. J. (1980) Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. *Infect. Immun.* **28**, 893-898.

Aubin, J., Gatesoupe, F.-J., Labbe, L. & Lebrun, L. (2005) Trial of probiotics to prevent the vertebral column compression syndrome in rainbow trout (*Oncorhynchus mykiss* Walbaum) *Aquacult. Res.***36**, 758-767.

Austin, B. & Al -Zahrani, A. M. J. (1988) The effect of antimicrobial compounds on the gastro – intestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. *J. Fish Biol.* **33**, 1-14.

Austin, B. & Austin D. A. (1993) Bacterial Fish Pathogens. Ellis Horwood Limited, England.

Austin, B. & Austin D. A. (1999). Bacterial Fish Pathogens. Springer-Verlag, London Ltd, England.

Azokpota. P., Hounhouigan, D. J., Nago, M. C. & Jakobsen, M. (2006) Esterase and protease activities of *Bacillus* spp. from afitin, iru and sonru; three African locust bean (*Parkia biglobosa*) condiments from Benin. *Afr. J. Biotech.* **5**, 265-272.

Bach Knudsen, K. E., Jensen, B. B., Andersen, J. O., & Hansen, I. (1991) Gastrointestinal implications in pigs of wheat and oat fractions. 2 Microbial activity in the gastrointestinal tract. *Br. J. Nutr.* **65**, 233-248.

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

Bagheri, T., Hedayati, S. A., Yavari, V., Alizade, M. & Farzanfar, A. (2008) Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet Supplemented with probiotic during the two months of first feeding. *Turk. J. Fish. Aquat. Sci.* **8**, 43-48.

Bairagi, A., Sarkar Ghosh, K., Sen, S. K. & Ray, A. K. (2002) Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquacult. Int.* **10**, 109-121.

Bakke-McKellep, A. M., Press, C. M. L., Baeverfjord, G., Krogdahl, Å. & Landsverk, T. (2000) Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. *J. Fish Dis.* **23**, 115-127.

Bakke-McKellep, A. M., Koppang, E. O., Gunnes, G., Sanden, M., Hemre, G.-I., Landsverk, T. & Krogdahl, Å. (2007a) Histological, digestive, metabolic, hormonal and some immune factor responses in Atlantic salmon, *Salmo salar* L., fed genetically modified soybeans. *J. Fish Dis.* **30**, 65-79

Bakke-McKellep, A. M., Penn, M. H., Salas, P. M., Refstie, S., Sperstad, S., Landsverk, T., Ringø, E. & Krogdahl, Å (2007b) Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). *Br. J. Nutr.* **97**, 699-713

Balcázar, J. L., de Blas, I., Ruiz-Zazuela, I., Cunningham, D., Vandrell, D. & Muzquiz, J. L. (2006) The role of probiotics in aquaculture. *Vet. Microbiol.* **114**, 173-186.

Balcázar, J L., de Blas, I., Ruiz-Zazuela, I., Calvo, A. C., Márquez, I., Gironés, O. & Muzquiz, J. L. (2007a) Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *Brit. J. Nutr.*, **97**, 522-52.

Balcázar, J L., de Blas, I., Ruiz-Zazuela, I., Vandrell, D., Gironés, O. & Muzquiz, J. L. (2007b) Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunol. Med. Microbiol.* **51**, 185-193.

Baldry, M. G. C. (1983) The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. *J. Appl. Bacteriol.* **54**, 417-423.

Barnett, J. A. (2003) Beginnings of microbiology and biochemistry: the contribution of yeast research. *Microbiology*. **149**, 557-567.

Beaulieu, L., Aomari, H., Groleau, D. & Subirade, M. (2006) An improved and simplified method for the large-scale purification of pediocin PA-I produced by *Pediococcus acidilactici*. *Biotechnol. Appl. Biochem.* **43**, 77-84.

Berlutti, F., Morea, C., Battistoni, A., Sarli, S., Cipriani, P., Ammendolia, M. G. & Valenti, P. (2005) Iron availability influences aggregation, biofilm, adhesion and invasion of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, *Int. J. Immunopathol. Pharmacol.* **18**, 661-670.

Benbouza, H., Jacquemin, J. L., Baudoin, J. P. & Mergeai, G. (2006) Optimization of a reliable, fast, cheap and sensitive silver staining method to detect ssr markers in polyacrylamide gels. *Biotechnol. Agron. Soc. Environ.* **10**, 77-81.

Bhunia, A. K., Johnson, M. C., Ray, B. & Belden, E. L. (1990) Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. *J. Appl. Bacteriol.* **69**, 211-215.

Birkbeck, T. H. & Ringø, E. (2005) Pathogenesis and the gastrointestinal tract of growing fish. In: Holzapfel W, Naughton P (eds) Microbial ecology in growing animals. Elsevier, Edinburgh, 208-234.

Blocker, A., Komoriya, K., & Aizawa (2003) Type III secretion systems and bacterial flagella: Insights into their function from structural similarities. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 3027-3030.

Bogut, I., Milakovic, Z., Brkic, S., Novoselic, D. & Bukvic, Z. (2000) Effects of *Enterococcus faecium* on the growth rate and intestinal microflora in sheat fish (*Silurus glanis*). *Vet. Med. - Czech.* **45**, 107-109.

Burr, G., Gatlin III, D. & Ricke, S. (2005) Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. *J. World Aquac. Soc.* **36**, 425-436.

Burr, G., Hume, M., Niell, W. H. & Gatlin III, D. (2008) Effects of prebiotics on nutrient digestibility of a soybean-meal-based diet by red drum *Sciaenops ocellatus* (Linnaeus). *Aquacult. Res.* **39**, 1680-1686.

Burrels, C., Williams, P. D., Southgate, P. J. & Crampton, V. O. (1999) Immunological, physiological and pathological responses of rainbow trout (*Oncorhynchus mykiss*) to increasing dietary concentrations of soybean proteins. *Vet. Immunol. Immunopathol.* **72**, 277-288.

Branen, J. K. & Davidson, M. P. (2004) Enhancement of nisin, lysozyme, and monolaurin antimicrobial activities by ethylenediaminetetraacetic acid and lactoferrin. *Int. J. Food Microbiol.* **90**, 63-74.

Brett J. R., & Groves T. D. D. (1979) Physiological energetics. In: Fish Physiology (Hoar W. S., Randall D. J. & Brett J. R. eds). Vol. 8: 280-351.

Brunt, J., Newaj-Fyzul, A. & Austin, B. (2007) The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* **30**, 573-579.

Cahill, M. M. (1990) Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41.

Cai, Y., Suyanandana, P., Saman, P. & Benno, Y. (1999) Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. *J. Gen. Appl. Microbiol.* **45**, 177–184

Carnevali, O., de Vivo, L., Sulpizio, R., Gioacchini, G., Olivotto, I., Silvi, S. & Cresci A. (2006) Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture*. **258**, 430-438.

Castex, M., Chim, L., Wabete, N., Lemaire, P. & Usache, V. (2006) Feeding evaluation of probiotic bacteria *Pediococcus acidilactici* (Bactocell®) in sub adult shrimp *Litopenaeus stylirostris*: microbial, nutritional and zootechnical aspects. WAS Annual Meeting, 9-13 May 2006. Florence, Italy.

Castex, M., Chim, L., Pham, D., Lemaire, P., Wabete, N., Nicolas, J.-L., Schmidely, P. & Mariojouls, C. (2008) Probiotic *P. acidilactici* application in shrimp *Litopenaeus stylirostris* culture subject to vibriosis in New Caledonia. *Aquaculture.* **275**, 182-193.

Chang, C.-I. & Liu, W-Y (2002) An evaluation of two probiotic bacterial strains,

Enterococcus faecium SF68 and *Bacillus toyoi*, for reducing edwardsiellosis in cultured European eel, *Anguilla anguilla* L. *J. Fish Dis.* **25**, 311-315.

Chim, L., Maisonneuve, V., Lemaire, P., Wabete, N. & Usache, V. (2005) Dietary probiotic *Pediococcus acidilactici* MA 18/5 (Bactocell®) study to a juvenile marine shrimp *Litopenaeus stylirostris* reared in tanks and in pond. book of abstracts. WAS annual meeting. 9-13 May 2005. Bali, Indonesia.

Chim, L., Castex, M., Wabete, N., Lemaire, P., Pham, D. & Brun, P. (2007) Development of an original tool for shrimp culture studies using floating cages in earthen pond. First trial carried out to evaluate lactic acid probiotic (Bactocell®) in shrimp *Litopeneus stylirostris* reared in commercial farm subject to vibriosis. Book of Abstract Asian Pacific Aquaculture, 5-8 August 2007. Hanoi, Vietnam.

Cintas, L. M., Rodriguez, J. M., Fernandez, M. F., Sletten, K., Nes, I. F., Hernandez, P. E. & Holo, H. (1995) Isolation and characterization of pediocin L50, a new bacteriocin from *Pediococcus acidilactici* with a broad inhibitory spectrum. *Appl. Environ. Microbiol.* **61**, 2643-2648.

Collet, S. R. (2004) Controlling gastrointestinal disease to improve absorptive membrane integrity and optimize the digestion efficiency. In Interfacing immunity, gut health and performance (Tucker, L. A. & Taylor-Pickard, J. A. eds) Nottingham Press. 77-91.

Council Directive 70/524/EEC, 2004. List of the authorized additives in feedingstuffs published in application of Article 9t (b) of Council Directive 70/524/EEC concerning additives in feedingstuffs. Off. J. Eur. Union, C50-C144.

Cowan, S. T. & Steel, J. (1993) In: *Cowan & Steel's Manual for the Identification of Medical Bacteria* (3rd Edition ed.), Cambridge University Press, Cambridge.

Dalsgaard, I., & Madsen, L. (2000). Bacterial pathogens in rainbow trout, *Oncorhynchus mykiss* (Walbaum), reared at Danish freshwater farms. *J. Fish Dis.* **23**, 199-209.

Dilly, O., Bloem, J., Vos, A. & Munch, J. C. (2004) Bacterial diversity in agricultural soils during litter decomposition. *Appl. Environ. Microb.* **70**, 468-474.

Dimitroglou, A., Davies, S. & Sweetman, J. (2008) The effect of dietary mannan oligosaccharides on the intestinal histology of rainbow trout (*Oncorhynchus mykiss*). Abstracts *Comp. Biochem. Physiol. A.* **150**, S56-63.

Dimitroglou A., Merrifield D. L., Moate, R., Davies, S. J., Spring, P., Sweetman J. & G. Bradley (unpublished data). Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Anim. Sci.* Under review.

Doran, P. M. & Bailey, J. E. (1986) Effects of immobilization on growth, fermentation properties, and macromolecular composition of *Saccharomyces cerevisiae* attached to gelatin. *Biotechnol. Bioeng.* **28**, 73-87.

Eichner, C. A., Erb, R. W., Timmis K. N., & Wagner-Dobler, I. (1999). Thermal gradient gel electrophoresis analysis of bioprotection from pollutant shocks in the activated sludge microbial community. *Appl. Environ. Microb.* **65**, 102-109.

El-Dakar, A. Y., Shalaby, S. M. & Saoud, I. P. (2007) Assessing the use of a dietary probiotic/prebiotic as an enhancer of spinefoot rabbitfish *Siganus rivulatus* survival and growth. *Aquacult. Nutr.* **13**, 407-412.

El-Haroun, E. R., Goda, A. M. A.-S. & Kabir Chowdhury, M. A. (2006) Effect of dietary probiotic Biogens supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). *Aquacult. Res.* **37**, 1473-1480.

Ellis, A. E. (1990) Lysozyme assays. In: *Techniques in Fish Immunology* (Stolen, J. S., Fletcher, T. C., Anderson, D. P., Robersen, B. S. & Van Muiswinkel, W. B. Eds.), pp. 101-104. SOS Publications. Fair Haven, New Jersey, USA.

Ellison, R. T., Giehl, T. J. & Laforce, F. (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infect. Immunol.* **56**, 2774- 2781.

Ellison, R. T. & Giehl, T. G. (1991) Killing of Gram negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest.* **83**, 1080-1091.

Esteve, C. & Garay, E (1991) Heterotrophic flora associated with European eel *Anguilla anguilla* reared in freshwater. *Nip. Sui. Gak.* **57**, 1369-1375.

EU Regulation (EC) no. 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition.

Evelyn, T. P. T. (1996) Infection and disease. *Fish Physiol.* **15**, 339-366.

FAO (2006) World review of fisheries and aquaculture. In: The state of world fisheries and aquaculture (SOFIA). http://www.fao.org/docrep/009/A0699e/A0699e00.htm. Food and Agriculture Organization of the United Nations, Rome.

FAO (2008) FAO FISHSTAT PLUS 2008 Database. Food and Agriculture Organization of the United Nations, Rome.

Farnaud, S. & Evans, R. W. (2003) Lactoferrin - a multifunctional protein with antimicrobial properties. *Mol. Immunol.* **40**, 395-405.

Fisher, M. M. & Triplett, E. W. (1999) Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microb.* **65**, 4630-4636.

Fuller, R. (1989) Probiotics in man and animals. *J. Appl. Bacteriol.* **66**, 365-378.

Garcia, T., Otto, K., Kjelleberg, S., & Nelson, D. R. (1997) Growth of *Vibrio anguillarum* in salmon intestinal mucus. *Appl. Environ. Microb.* **63**, 10434-1039.

Gatesoupe, F.J. (1997) Sidephore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. *Aquat. Living Resour.* **10**, 239-246.

Gatesoupe, F.-J. (2002) Probiotic and formaldehyde treatments of *Artemia nauplii* as food for larval pollack, *pollachius pollachius*. *Aquaculture*. **212**, 347-360.

Gatesoupe, F. J., Zambonino Infante, J. L., Cahu, C. & Quazuguel, P. (1997) Early weaning of sea bass larvae, *Dicentrarchus labrax*: the effect on microbiota, with particular attention to iron supply and exoenzymes. *Aquaculture*. **158**, 117-127.

Gatesoupe, F.-J. (1999) The use of probiotics in aquaculture. *Aquaculture*. **180**, 147-165.

Gatesoupe, F.-J. (2007) Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health and development. *Aquaculture*. **267**, 20-30.

Gatesoupe F.-J. (2008a) Updating the Importance of Lactic Acid Bacteria in Fish Farming: Natural Occurrence and Probiotic Treatments. *J. Mol. Microbiol. Biotechnol.* 14, 107-114.

Gatesoupe, F.-J. (2008b) Different methods to reduce antibiotic use in farmed fish. In: Improving farmed fish quality and safety (Lie, Ø. Ed.) Woodhead Publishing Ltd, Cambridge, UK. 199-223.

Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E. J., Stone, D., Wilson, R. & Wurtele, E. (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquacult. Rese.* 38, 551-579.

Ghosh, S., Sinha, A. & Sahu, C. (2007) Effect of probiotic on reproductive performance in female livebearing ornamental fish. *Aquacult. Res.* 38, 518-526.

Ghosh, S., Sinha, A. & Sahu, C. (2008) Dietary probiotic supplementation in growth and health of live-bearing ornamental fishes. *Aquac. Nutr.* 14, 289-299.

Gibson, G. R., & Roberfroid, M. B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nut.* 125, 1401-1412.

Gildberg, A., Johansen, A. & Jarl Bøgwald, J. (1995) Growth and survival of Atlantic salmon *(Salmo salar)* fry given diets supplemented with fish protein hydrolysate and lactic acid bacteria during a challenge trial with *Aeromonas salmonicida. Aquaculture.* 138, 23-34.

Gildberg, A., Mikkelsen, H., Sandaker, E. & Ringø, E. (1997) Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod *(Gadus morhua). Hydrobiologia.* 352, 279-285.

Gildberg, A. & Mikkelsen, H. (1998) Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulating peptides during a challenge trial with *Vibrio anguillarum. Aquaculture.* 167, 103-113.

Goldschmidt-Clermont, E., Wahli, T., Frey, J. & Burr, S. E. (2008) Identification of bacteria from the normal flora of perch, *Perca fluviatilis* L., and evaluation of their inhibitory potential towards *Aeromonas* species. *J. Fish Dis.* 31, 353-359.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I. & Nielsen, T. F. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. *Appl. Environ. Microbiol.* 65, 969-973.

Hagi, T., Tanka, D., Iwamura, Y., & Hoshino, T. (2004) Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture* 234, 335-346.

Heikkinen, J., Vielma, J., Kemiläinen, O., Tiirola, M., Eskelinen, P., Kiuru, T., Navia-Paldanius, D., & von Wright, A. (2006) Effects of soybean meal based diet on growth performance, gut histopathology and intestinal microbiota of juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 261, 259-268.

Holben, W. E., Williams, P., Saarinen, M., Särkilahti, L. K. & Apajalahti, J. H. A. (2002) Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microbial Ecology* 44, 175-185.

Holt, J. G. & Bergey, D. H. (Eds.) (1994) Bergey's manual of determinative bacteriology. (9th edition) Williams and Wilkins, London.

Hovda, M. B., Lunestad, B. T., Fontanillas, R. & Rosnes J. T. (2007) Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar L.*). *Aquaculture*. **272**, 581-588.

Hu, C. H., Y. Xu, M. S. Xia, L. Xiong, and Z. R. Xu. 2007. Effects of Cu^{2+} exchanged montmorillonite on growth performance, microbial ecology and intestinal morphology of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*. **270**, 200-206.

Huang, J., Lacroix, C., Daba, H. & Simard, R. E. (1996) Pediocin 5 production and plasmid stability during continuous free and immobilized cell cultures of *Pediococcus acidilactici* UL5. *J. Appl. Bacteriol.* **80**, 635–644

Huber, I., Spanggaard, B., Appel, K.F., Rossen, L., Nielsen, T. & Gram, L. (2004) Phylogenetic analysis and *in situ* identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). *J. Appl. Microbiol.* **96**, 117-132.

Hunter, V. A., Knittel, M. D. & Fryer, J. L. (1980) Stress-induced transmission of *Yersinia ruckeri* infection from carriers to recipient steelhead trout *Salmo gairdneri* Richardson. *J. Fish Dis.* **3**, 467-472.

Hymowitz, T., Collins, F. I., Panczer, J. & Walker, W. M., (1972) Relationhsip between the content of oil, protein, and sugar in soybean seed. *Agron J.* **64**, 613-616.

Irianto, A. & Austin, B. (2002a) Probiotics in aquaculture. *J. Fish Dis.* **25**, 633-642.

Irianto, A. & Austin, B. (2002b) Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum) *J. Fish Dis.* **25**, 333-342.

Irianto, A. & Austin, B. (2003) Use of dead probiotic cells to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* **26**, 59-62.

Jensen, B. B. & Jørgensen, B. B. (1994) Effect of dietary fibre on microbial activity and microbial gas production in various regions of the gastriointestinal tract of pigs. *Appl. Environ. Microbiol.* **60**, 1987-1904.

Jenssen, H. & Hancock, R. E. W. (2009) Antimicrobial properties of lactoferrin. *Biochimie*. **91**, 19-29.

Jöborn, A., Olsson, J. C., Westerdahl, A., Conway, P. L. & Kjelleberg, S. (1997) Colonization in the fishintestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. *J. Fish Dis.* **20**, 383-392.

Kakuta, I. (1996a) Effects of orally administrated bovine lactoferrin on growth and blood properties of goldfish. *Suisanzoshoku*. **44**, 419-426.

Kakuta, I. (1996b) Protective effect of orally administrated bovine lactoferrin against experimental infection of goldfish *Carassius auratus* with *Ichthyophthirius multifiliis*. *Suisanzoshoku*. **44**, 427-432.

Kakuta, I. (1998) Reduction of stress response in carp, *Cyprinus carpio* L., held under deteriorating environmental conditions, by oral administration of bovine lactoferrin. *J. Fish Dis.* **21**, 161-167.

Kakuta, I. & Kurokura, H. (1995) Defensive effect of orally administrated bovine lactoferrin against *Cryptocaryon irritans* infection of red sea bream. *Fish Pathol.* **30**, 289-290.

Kamilya, D., Ghosh, D., Bandyopadhyay, S., Mal, B. C., & Maiti, T. K. (2006) *In vitro* effects of bovine lactoferrin, mushroom glucan and *Abrus* agglutinin on Indian major carp, catla (*Catla catla*) head kidney leukocytes. *Aquaculture* **253**, 130-139.

Kamilya, D., Maiti, T. K., Joardar, S. N. & Mal, B. C. (2006) Adjuvant effect of mushroom glucan and bovine lactoferrin upon *Aeromonas hydrophila* vaccination in catla, *Catla catla* (Hamilton). *J. Fish Dis.* **29**, 331-337.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J. & Gibson, L. (2008) Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture.* **274**, 1-14.

Kim, W., Tanaka, T., Kumura, K. & Shimazaki, K. (2002) Lactoferrin-binding proteins in *Bifidobacterium bifidum*. *Biochem. Cell Biol.* **80**, 91-94.

Kim, D.-H. & Austin, B. (2006) Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. *Fish Shellfish Immunol.* **21**, 513-524.

Kim, D.-H., Brunt, J. & Austin, B. (2007) Microbial diversity of intestinal contents and mucus in rainbow trout (*Oncorhynchus mykiss*). *J. Appl. Microbiol.* **102**, 1654-1664.

Kim, W.-S., Rahman, M. M. & Shimazaki, K.-I. (2008) Antibacterial activity and binding ability of bovine lactoferrin against *Pseudomonas* spp.. *J. Food Safety*. **28**, 23-33.

Klaenhammer, T. R. (1988) Bacteriocins of lactic acid bacteria. *Biochemie*. **70**, 337-379.

Köhler, H., Sakaguchi, T., Hurley, B .P., Kase, B. J., Reinecker, H.-C. & McCormick' B. A. (2007). *Salmonella enterica* serovar Typhimurium regulates intercellular junction proteins and facilitates transepithelial neutrophil and bacterial passage. *Am. J Physiol. Gastrointest. Liver Physiol*. **29**, 178-187.

Krogdahl, Å., Bakke-McKellep, A. M., Røed, K. H. & Baeverfjord, G. (2000) Feeding Atlantic salmon *Salmo salar* L. soybean products: effects on disease resistance (furunculosis), and lysozyme and IgM levels in the intestinal mucosa. *Aquac. Nutr.* **6**, 77-84.

Krogdahl, Å., Bakke-McKellep, A. M. & Baeverfjord, G. (2003) Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquac. Nutr.* **9**, 361-371.

Kumari, J. & Sahoo, P. K. (2006) Non-specific immune response of healthy and immunocompromised Asian catfish (*Clarias batrachus*) to several immunostimulants. *Aquaculture*. **255**, 133-141.

Kumari, J., Swain, T. & Sahoo, P. K. (2003) Dietary bovine lactoferrin induces changes in immunity level and disease resistance in Asian catfish *Clarias batrachus*. *Vet. Immunol. Immunopathol*. **94**, 1-9.

Kumar, R. Mukherjee, S. C., Prasad, K. P. & Pal, A. K. (2006) Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.) *Aquacult. Res.* **37**, 1215-1221.

Kumar, R., a, Mukherjee, S. C., Ranjan, R., & Nayak, S. K. (2008) Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. *Fish Shellfish Immunol.* **24**, 168-172.

Lan, Y., Williams, B. A., Verstegen, M. W. A., Patterson, R. & Tamminga, S. (2007) Soy oligosaccharides in vitro fermentation characteristics and its effect on caecal microorganisms of young broiler chickens. *Anim. Feed Sci. Tech.* **133**, 286-297.

Leitch, E. C. & Willcox, M. D. (1999) Lactoferrin increases the susceptibility of *S. epidermidis* biofilms to lysozyme and vancomycin. *Curr. Eye Res.* **19**, 12-19.

León-Sicairos, N., Canizalez-Román, A., de la Garza, M., Reyes-Lopez, M., Zazueta-Beltrán, J., Nazmi, K., Gómez-Gil, B. & Bolscher, J. G. (2009) Bactericidal effect of lactoferrin and lactoferrin chimera against halophilic *Vibrio parahaemolyticus*. *Biochimie* **91**, 133-140.

Lesel, R. & Pointel, J.-G. (1979) Implantation de la flore bactere´enne dans le tube digestif de la truite arc-en-ciel. Etude au microscope e´lectronique a` balayage. *Ann. Zool. Ecol. Anim.* **11**, 327-335.

Lesel, R., de la Noüe, J. & Choubert, G. (1989) Fecal bacterial flora of rainbow trout under antibiotic treatment: effect of the number of pyloric caeca and the lipid content of food (De Pauw, N., Jaspers, E., Ackerfors, H. & Wilkins, N. Eds). In: Aquaculture – A Biotechnology in Progress. Vol. 2. pp. 897-903

Lewis, P. R. & Knight, D. P. (1977) Staining methods for sectioned material (Glauert, A. M. ed.). North-Holland publishing company. Elsevier/North-Holland Biomedical press pages 43-44.

Lewus, C. B., Kaiser, A., & Montville, T. J. (1991) Inhibition of food-borne pathogens by bacteriocins from lactic acid bacteria isolated from meat. *Microbiology*. **57**, 1683-1688.

Lindén, T., Peetre, J. & Hahn-Hägerdal, B. (1992) Isolation and characterization of acetic acid-tolerant galactose- fermenting strains of *Saccharomyces cerevisiae* from a spent sulfite liquor fermentation plant. *Am. Soc. Microbiol.* **58**, 1661-1669.

Litwin, C. M. & Calderwood, S. B. (1993) Role of iron in regulation of virulence genes. *Clin. Microbiol. Rev.* **6**, 137-149.

Lönnerdal B. & Iyer S. (1995) Lactoferrin: molecular structure and biological function. *Annu. Rev. Nutr.* **15**, 93-110.

Lu, L. & Walker, A. (2002) Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. *Am. J. Clin. Nutr.* **73**, 1124-1130.

Lygren, B., Sveier, H., Hjeltnes, B. & Waagbø, R. (1999) Examination of the immunomodulatory properties and the effect on disease resistance of dietary bovine lactoferrin and vitamin C fed to Atlantic salmon (*Salmo salar*) for a short-term period. *Fish Shellfish Immunol.* **9**, 95-107.

Mahious, A. S., Van Loo, J. & Ollevier, F. (2006a) Impact of the prebiotics, inulin and oligofructose on microbial fermentation in the spiral valve of Siberian sturgeon *Acipenser baerii*. WAS Annual Meeting, 9-13 May 2006. Florence, Italy.

Mahious, A. S., Gatesoupe, F.-J., Hervi, M., Metailler, R. & Ollevier, F. (2006b) Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). *Aquac. Int.* **14**, 219-229.

Marciňáková, M., Simonová, M. & Lauková (2004) Probiotic properties of *Enterococcus faecium* EF9296 strain isolated from silage. *Acta. Vet. Brno.* **73**, 513-519.

Martens, J. H., Barg, H., Warren, M. J. & Jahn, D. (2002) Microbial production of vitamin B-12. *Applied Microbiol. Biot.* **58**, 275-285.

Martirani, L., Varcamonti, M., Naclerio, G. & De Felice, D. (2002) Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of ***Bacillus licheniformis***. *Microb. Cell Fact.* **1**,1

Midolo, P. D., Lambert, J. R., Hull, R., Luo, F., & Grayson, M. L (1995) In vitro inhibition of Helicobacter pylori NCTC 11637 by organic acids and lactic acid bacteria. *J. Appl Bacteriol.* **79**, 475-479.

Mishra, C., & Lambert, J. (1996) Production of anti-microbial substances by probiotics. *Asia Pacific J. Clin. Nutr.* **5**, 20-24.

Moriarty, D. J. W. (1990) Disease Control in Shrimp Aquaculture with Probiotic Bacteria. Proceedings of the 8th International Symposium on Microbial Ecology. Bell C. R., Brylinsky M. & Johnson-Green P. (eds). Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Moriarty, D. J. W. (1990) Interactions of microorganisms and aquatic animals, particularly the nutritional role of the gut flora. In: Lesel, R. Microbiology in Poecilotherms. Elsevier, Amsterdam 217-222.

Moriarty, D. J. W. (1996) Probiotics and bioremediation in aquaculture. *Asian Shrimp News.* **26**, 3.

Moriarty, D. J. W. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture.* **164**, 351-358.

Murray, R. (2004) The prokaryotes: an evolving electronic resource for the microbial community. Springer Verlag, New York.

Muyzer, G., de Waal, E. C. & Uitierlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695-700

Naidu, A. S., Bidlack, W. R., & Clemens, R. A. (1999) Probiotic spectra of lactic acid bacteria. *Crit. Rev. Food Sci.* **39**, 13-126.

National Research Council (NRC), 1993. Nutrient Requirements of Fish, National Academy Press, Washington. DC, USA.

Neilands, J. B. (1981) Iron absorption and transport in microorganisms. *Ann. Rev. Nutr.* **1**, 27-46.

Newaj-Fyzul, A., Adesiyunz, A. A., Mutani, A., Ramsudhag, Brunt, J. & Austin, B. (2007) *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum) *J. Appl. Microbiol.* **103**, 1699-1706.

Nikoskelainen, S., Ouwehand, A., Salminen, S. & Bylund, G. (2001a) Protection of rainbow trout *Oncorhynchus mykiss* from furunculosis by *Lactobacillus rhamnosus*. *Aquaculture.* **198**, 229-236.

Nikoskelainen, S., Salminen, S., Bylund, G. & Ouwehand, A. (2001b) Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. *Appl. Environ. Microb.* **67**, 2430-2435.

Nikoskelainen, S., Ouwehand, A. C., Bylund, G., Salminen, S., & Lilius, E. M. (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish Shellfish Immunol.* **15**, 443-52

Obendorf, R., Horbowicz, M., Dickerman, A. M., Brenac, P. & Smith, M. E. (1998) Soluble oligosaccharides and galactosyl in maturing soybean seeds. *Crop Sci.* **38**, 78-84.

Ochoa, S. J. L. & Olmos, S. J. (2006) The functional property of *Bacillus* for shrimp feeds. *Food Microbiol.* **23**, 519-525.

Olsson, J. C., Westerdahl, A., Conway, P. L. & Kjelleberg, S. (1992) Intestinal colonization potential of turbot (*Scophthalmus maximus*)- and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Appl. Environ. Microbiol.* **58**, 551-556.

Ormonde, P., Hörstedt, P., O'Toole, R., & Milton, D. L. (2000). Role of motility in adherence to and invasion of a fish cell line by *Vibrio anguillarum*. *J. Bacteriol.* **182**, 2326-2328.

O'Toole, R., Lundberg, S., Fredriksson, S., Jansson, A., Nilsson, B., & Wolf-Watz, H. (1999) The chemotactic response of *Vibrio anguillarum* to fish intestinal mucus is mediated by a combination of multiple mucus components. *J.Bacteriol.* **181**, 4308-4317.

Ouwehand, A. C., Salminen, S. & Isolauri, E. (2002) Probiotics: an overview of beneficial effects. In Lactic acid bacteria: genetics, metabolism and applications (Siezen, R. J., Kok, J., Abee, T. & Schaafsma, G. ed.). FEMS. Kluwer Academic Publishers. **82**, 279-289.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S. & Sugita, H. (2004) Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. *Vet. Immunol. Immunopathol.* **102**, 379-388.

Panigrahi, A., Kiron, V., Puangkaew, J., Kobayashi, T., Satoh, S. & Sugita, H. (2005) The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture*. **243**, 241-254.

Panigrahi, A., Kiron, V., Satoh, S., Hiron, I., Kobayashi, T., Sugita, H., Puangkaew, J. & Aoki, T. (2007) Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. *Dev. Comp. Immunol.* **31**, 372-382.

Peypoux, F., Bonmatin, J. M. & Wallach J. (1999) Recent trends in the biochemistry of surfactin. *Appl. Microbiol. Biotechnol.* **51**, 553-563.

Picchietti, S., Mazzini, M., Taddei, A. R., Renna, R., Fausto, A. M., Mulero, V. Carnevali, O., Cresci, A., & Abelli, L. (2007) Effects of administration of probiotic strains on GALT of larval gilthead seabream: Immunohistochemical and ultrastructural studies. *Fish Shellfish Immunol.***22**, 57-67.

Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø. & Pintado, J. (2006) Probiotic effect *in vivo* of *Roseobacter* strain 27-4 against *Vibrio* (*Listonella*) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture*. **255**, 323-333.

Pond, M. J., Stone, D. M. & Alderman, D. J. (2006) Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **261**, 194-203.

Raida, M. K., Larsen, J. L., Nielsen, M. E. & Buchmann, K. (2003) Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). *J. Fish Dis.* **26**, 495-498.

Ramirez, R. F. & Dixon B. A. (2003) Enzyme production by obligate intestinal anaerobic bacteria isolated from oscars (*Astronotus ocellatus*), angelfish (*Pterophyllum scalare*) and southern flounder (*Paralichthys lethostigma*). *Aquaculture*. **227**, 417-426.

Reiter, B., Brock, J. H. & Steel, E. D. (1975) Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E. coli*. *Immunology*. **28**, 83-95.

Refstie, S., Landsverk, T., Ringø, E., Sundby, A., Shearer, K.D. & Kroghdahl, Å. (2006). Digestive responses of 1- and 2-year-old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal. *Aquaculture*. **261**, 269-284.

Ren, T., Koshio, S., Ishikawa, M., Yokoyama, S., Micheal, F. R., Uyan, O. & Tung, H. T. (2007) Influence of dietary vitamin C and bovine lactoferrin on blood chemistry and non-specific immune responses of Japanese eel, *Anguilla japonica*. *Aquaculture*. **267**, 31-37.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., & Menasveta, P. (1998) Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* **167**, 301-313.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., & Menasaveta, P. (2000). Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). *Aquaculture* **191**, 271-288.

Rimmer, D. W. & Wiebe, W. J. (1987) Fermentative microbial digestion in herbivorous fishes. *J. Fish Biol.* **31**, 229-236.

Ringø, E. (1993) Does dietary linoleic acid affect intestinal microflora in Arctic Charr, *Salvelinus alpinus*. *Aquacult. Fish. Manag.* **24**, 133-135.

Ringø, E. & Strom, E. (1994) Microflora of Arctic charr, *Salvelinus alpinus*. The gastro – intestinal microflora of free - living fish and the effect of diet and salinity on intestinal microflora. *Aquacult. Fish. Manag.* **25**, 623-629.

Ringø, E., Strøm, E. & Tabachek, J.-A. (1995) Intestinal microflora of salmonids: A review. *Aquacult. Res.* **26**, 773-789.

Ringø, E. & Gatesoupe F.-J. (1998) Lactic acid bacteria in fish: a review. *Aquaculture*. **160**, 177-203.

Ringø, E. (1999) Does Carnobacterium divergens isolated from Atlantic salmon, Salmo salar L., colonize the gut of early developing turbot, Scopthalamus maximus L., Larvae? *Aquacult. Res.* **30**, 229-232.

Ringø, R. & Birkbeck, T. H. (1999) Intestinal microflora of fish larvae and fry. *Aquacult. Res.* **30**, 73-93.

Ringø, E. & Olsen, R. E. (1999) The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr (*Salvelinus alpinus* L.). *J. Appl. Microbiol.* **86**, 22-28.

Ringø, E. & Holzapfel, W. H. (2000) Identification and characterization of carnobacteria associated with the gills of Atlantic salmon (*Salmo salar* L.). *Syst. Appl. Microbiol.* **23**, 523-527.

Ringø, E., Bendiksen, R., Wesmajervi, M. S., Olsen, R. E., Jansen, P. A. & Mikkelsen, H. (2000) Lactic acid bacteria associated with the digestive tract of Atlantic salmon (*Salmo salar* L.) *J. Appl. Microbiol.* **89**, 317-322

Ringø, E., Lødemel, J. B., Myklebust, R., Kaino, T., Mayhew, T. M. & Olsen, R. E. (2001) Epithelium-associated bacteria in the gastrointestinal tract of Arctic charr (*Salvelinus alpinus* L.). An electron microscopical study. *J. Appl. Microbiol.* **90**, 294-300.

Ringo, E., Lodemel, J. B., Myklebust, R., Jensen, L., Lund, V., Mayhew T. M. & Olsen, R. E. (2002) The effect of soybean,linseed and marine oils on aerobic gut microbiota of Arctic charr (*Salvelinus alpinus* L.) prior to and post challenge with *Aeromonas salmonicida* ssp. *Aquacult. Res.*33, 591-606.

Ringø, E., Olsen, R. E., Mayhew, T. M. & Myklebust, R. (2003) Electron microscopy of the intestinal microflora of fish. *Aquaculture.* 227, 395-415.

Ringø, E., Schillinger, U., & Holzapfel, W. (2005) Antibacterial abilities of lactic acid bacteria isolated fromaquatic animals and the use of lactic acid bacteria in aquaculture. In: Microbial Ecology in Growing Animals. Holzapfel, W. and Naughton, P. (Eds.) Biology of Growing Animals series. Pierzynowski, S.G. and Zabielski, R. (Eds.). 418–453. Elsevier, Edinburgh, UK.

Ringø, E., Sperstad, S., Myklebust, R., Mayhew, T. M., Mjelde, A., Melle, W. & Olsen, R. E. (2006a) The effect of dietary krill supplementation on epithelium-associated bacteria in the hindgut of Atlantic salmon (*Salmo salar* L.): a microbial and electron microscopial study. *Aquacult. Res.* 37, 1644-1653.

Ringø, E., Sperstad, S., Myklebust, R., Mayhew, T. M. & Olsen, R. E. (2006b) The effect dietary inulin on aerobic bacteria associated with the hindgut of Arctic charr (*Salvelinus alpinus* L.). *Aquacult. Res.* 37, 891-897.

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S. & Krogdahl, Å. (2006c) Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.). The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture* 261, 829-841.

Ringø, E., Myklebust, R., Mayhew, Kaino, T., Olsen, R.E., Mayhew, T.M., Myklebust, R., 2006d. Endocytosis of indigenous bacteria and cell damage caused by Vibrio anguillarum in the foregut and hindgut of spotted wolffish (*Anarhichas minor* Olafsen) fry: an electron microscopical study. *Aquacult. Res.* 37, 647-651.

Ringø, E., Myklebust, R., Mayhew, T. M. & and Olsen, R. E. (2007) Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. *Aquaculture.* 268, 251-264.

Ringø, E., Sperstad, S., Kraugerud, O. F. & Krogdahl, Å. (2008) Use of 16S rRNA gene sequencing analysis to characterize culturable intestinal bacteria in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. *Aquac.Res.* 39, 1087-1100.

Roberts, D. P., Dery P. D., Yucel, I., Buyer, J. S., Holtman, M.A. & Kobayashi, D.Y. (1999) Role of pfkA and general carbohydrate catabolism in seed colonization by *Enterobacter cloacae*. *Appl. Environ. Microbiol.* 65, 2513-2519.

Robertson, P. A. W., O'Dowd, C., Burrells, C., Williams, P., & Austin, B. (2000) Use of Carnobacterium sp as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture.* 185, 235-243.

Robleto, E. A., Borneman, J. & Triplett, E. W. (1998) Effects of bacterial antibiotic production on rhizosphere microbial communities from a culture-independent perspective. *Appl. Environ. Microb.* 64, 5020-5022.

Rosovitz, M. J., Voskuil, M. I. & Chambliss, G. H. (1998) *Bacillus*. In: Balows, A., Duerden, B.I. (Eds), Systematic Bacteriology. Arnold Press, London: 709-720.

Saha, S., Roy, R. N., Sen, S. K., & Ray, A. K. (2006) Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes). *Aquacult. Res.* 37, 380-388.

Sakai, M., Otubo, T., Atsuta, S. & Kobayashi, M. (1993) Enhancement of resistance to bacterial infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by oral administration of bovine lactoferrin. *J. Fish Dis.*, 16, 239-247

Sakai, M., Kobayashi, M. & Yoshida, T., (1995) Activation of rainbow trout, *Oncorhynchus mykiss*, phagocytic cells by administration of bovine lactoferrin. *Comp. Biochem. Physiol. B.* **110**, 755–759.

Sakata, T., Oshiro, T. & Teshima, S. I. (1993) Characteristics and fatty acid composition of yeast isolated from the intestines of rainbow trout *Oncorhynchus mykiss*. *Mem. Fac. Fish.* **42**, 51-59.

Salinas, I., Cuesta, A., Estaban, M. Á. & Meseguer, J. (2005) Dietary administration of *Lactobacillus delbrüeckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish Shellfish Immunol.* **19**, 67-77.

Salinas, I., Myklebust, R., Esteban, M. A., Olsen, R. E., Meseguer, J., & Ringø, E. (2008) *In vitro* studies of *Lactobacillus delbrueckii* subsp. *lactis* in Atlantic salmon (*Salmo salar* L.) foregut: Tissue responses and evidence of protection against *Aeromonas salmonicida* subsp. *salmonicida* epithelial damage. *Vet. Microbiol.* **128**, 167-177.

Salze, G., McLean, E., Schwarz, M. H. & Craig S. R. (2008) Dietary mannan oligosaccharide enhances salinity tolerance and gut development of larval cobia. *Aquaculture* **274**, 148-152.

Sansonetti, P. (2006) Host–pathogen interactions: the seduction of molecular cross talk. *Gut* .**50**, 2-8.

Schiewe, M. H., Trust, T. J., & Crosa, J. H. (1981) *Vibrio ordali* sp. Nov.: a causative agent of vibriosis in fish. *Curr. Microbiol.* **6**, 343-348.

Schved, F., Lalazar, Y., Henis, Y. & Juven, B. J. (1993) Purification, partial characterization and plasmid-linkage of pediocin SJ-1, a bacteriocins produced by *Pediococcus acidilactici*. *J. Appl. Bacteriol.* **74**, 67-77.

Sears, C. L. (2000) Molecular physiology and pathophysiology of tight junctions V. Assault of the tight junction by enteric pathogens. *Am. J Physiol. Gastrointest. Liver Physiol.* **279**, 1129-1134.

Severina, E., Severin, A., & Tomasz, A. (1998) Antibacterial efficacy of nisin against multidrug-resistant Gram-positive pathogens. *J. Antimicrob. Chemother.* **41**, 341-347.

Shelby, R. A., Lim, C., Yildirim-Aksoy, M. & Klesius, P. H. (2007) Effects of Probiotic Bacteria as Dietary Supplements on Growth and Disease Resistance in Young Channel Catfish, *Ictalurus punctatus* (Rafinesque). *J. Appl. Aqua.* 19, 81-91

Simon, O. (2004) Interaction of nutrition with intestinal microbial communities. In: Interfacing immunity, gut health and performance (Tucker, L. A. & Taylor-Pickard, J. A. ed.) Nottingham Press. 33-47

Singer, J. T., Schmidt, K. A. & Reno, P. W. (1991) Polypeptides p40, pOM2, and pAngR are required for iron uptake and for virulence of the marine fish pathogen *Vibrio anguillarum* 775. *J. Bacteriol.* **173**, 1347-1352.

Skrodenyte-Arbaciauskiene, V., Sruoga, A., & Butkauskas, D. (2006). Assessment of microbial diversity in the river trout *Salmo trutta fario* L. intestinal tract identified by partial 16S rRNA gene sequence analysis. *Fish. Sci.* 72, 597-602.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K. F. & Gram, L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. *Aquaculture.* 182, 1-15.

Spanggaard, B., Huber, I., Nielsen, J., Sick, E. B., Pipper, C. B., Martinussen, T., Slierendrecht, W. J. & Gram, L. (2001) The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environ. Microbiol.* 3, 755-765.

Starliper, C. E., Shotts, E. B. & Brown, J. (1992) Isolation of *Carnobacterium piscicola* and an unidentified Gram-positive bacillus from sexually mature and post-spawning rainbow trout *Oncorhynchus mykiss*. *Dis. Aquatic Org.* 13, 181-187.

Stevens, C. E. & Hume, I., D. (1998) Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiol. Rev.* 78, 393-427.

Storebakken, T., Refstie, S. & Ruyter, B. (2000) Soy products as fat and protein sources in fish feeds for intensive aquaculture. In: Drackley, J.K. (Ed.), Soy in Animal Nutrition. Federation of Animal Science Societies, IL, USA, pp. 127-170.

Stuart, J., Norrell, S. & Harrington J. P. (1984) Kinetic effect of human lactoferrin on the growth of *Escherichia coli* 0111. *Int. J. Biochem.* 16, 1043-1047.

Sugita, H., Tokuyama, K. & Deguchi, Y. (1985) The intestinal microflora of carp *Cyprinus carpio*, grass carp *Ctenopharyngodon idella* and tilapia *Sarotherodon niloticus*. *Bull. Jpn. Soc. Sci. Fish.* 51, 1325-1329.

Sugita, H., Tsunohara, M., Ohkoshi, T. & Deguchi, Y. (1988) The establishment of an intestinal microflora in developing goldfish *Carassius auratus* of culture ponds. *Microbiol. Ecol.* 15, 333-344.

Sugita, H., Shibuya, K., Shimooka, H. & Deguchi, Y. (1996) Antibacterial abilities of intestinal bacteria in freshwater cultured fish. *Aquaculture.* 145, 195-203.

Sugita, H., Kawasaki, J. & Deguchi, Y. (1997) Production of amylase by the intestinal microflora in cultured freshwater fish. FEMS *Lett. Appl. Microbiol.* 24, 105-108.

Sugita, H., Hirose, Y., Matsuo, N. & Deguchi, Y. (1998) Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. *Aquaculture.* 165, 269-280.

Tafazoli, F., Holmstrom, A., Forsberg, A. & Magnusson, K .E. (2000) Apically exposed, tight junction-associated *beta* 1-integrins allow binding and YopE-mediated perturbation of epithelial barriers by wild-type *Yersinia* bacteria. *Infect Immun.* 68, 5335-5343.

Takemaru, I. & Kusuda, R. (1988a) *In vitro* antibacterial activity of josamycin against *Streptococcus* sp. and various fish pathogenic bacteria. *Nippon Suisan Gakkaishi.* 54, 1527-1531.

Takemaru, I. & Kusuda, R. (1988b) Effect of josamycin administration on intestinal microflora of cultured yellowtail. *Nippon Suisan Gakkaishi.* 54, 837-840.

Taoka, Y., Maeda, H., Jo, J.-Y., Kim, S.-M., Park, S.-I., Yoshikawa, T. & Sakata, T. (2006) Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. *Fish. Sci.* 72, 755-766.

Taoka, Y., Maeda, H., Jo, J.-Y., Jeon, M.-J., Bai, S. C., Lee, W.-J., Yuge, K., & Koshio, S. (2006) Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fish. Sci.* **72**, 310-321.

Thune, R. L., Stanley, L.A. & Cooper, R. K. (1993) Pathogenesis of gram negative bacterial infections in warm water fish. *Annu. Rev. Fish Dis.* **3**, 37-68.

Titus, E., & Ahearn, G A. (1992) Vertebrate gastrointestinal fermentation - transport mechanisms for volatile fatty-acids. *Amer. J. Physiol.* **262**, 547-553.

Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S. & Hayasawa, H. (2002) Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochem. Cell Biol.* **80**, 109-112.

Tovar, D., Zambonino, J., Cahu, C., Gatesoupe, F. J., Vazquez-Juarez, R., & Lesel, R. (2002) Effect of live yeast incorporation in the compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* **204**, 113-123.

Tucker, L. A. & Taylor-Pickard, J. A. (ed.) (2004) In: Interfacing immunity, gut health and performance. Nottingham Press.

Trust, T. J. & Sparrow, R. A. H. (1974) The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Can. J. Microbiol.* **20**, 1219-1228.

Trust, T. J., Bull, L. M., Currie, B. R. & Buckley, J. T. (1979) Obligate anaerobic bacteria in the gastrointestinal microflora of the Grass carp (*Ctenopharyngodon idella*), Goldfish (*Carassius auratus*), and Rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Board Can.* **36**, 1174-1179.

Twomey, D., Ross, R. P., Ryan, M., Meaney, B. & Hill, C. (2002) Lantibiotics produced by lactic acid bacteria: structure, function and applications. In Lactic acid bacteria: genetics, metabolism and applications (Siezen, R. J., Kok, J., Abee, T. & Schaafsma, G. ed.). FEMS. Kluwer Academic Publishers. **82**, 165-185.

Urdaci, M. C., Bressollier, P. & Pinchuk, I. (2004). *Bacillus clausii* probiotic strains: antimicrobial and immunomodulatory activities. *J. Clin. Gastroenterol.* **38**, 86-90.

Van den Ingh, T. S. G. A. M., Krogdahl, Å., Olli, J. J., Hendriks, H. G. C. J. M. &

Koninkx, J. G. J. F. (1991) Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. *Aquaculture*. **94**, 297-305.

Vandenbergh, P. (1993) Lactic acid bacteria, their metabolic products and interference with microbial growth. *FEMS Microbiol. Rev.* **12**, 221-238.

Vaseeharan, B. R. & Ramasamy P. (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett. Appl. Microbiol.* **36**, 83-87.

Vazquez, J. A., Gonzalez, M. P., & Murado, M. A. (2005) Effects of lactic acid bacteria cultures on pathogenic microbiota from fish. *Aquaculture* **245**, 149-161.

Vázquez-Juárez, R., Andlid, T., Gustafsson, L. & Wadström, T. (1993) The expression of potential colonization factors of yeasts isolated from fish during different growth conditions. *Can. J. Microbiol.* **39**, 1135-1141.

Vázquez-Juárez, R., Andlid, T. & Gustafsson, L. (1997) Adhesion of yeast isolated from fish gut to crude intestinal mucus of rainbow trout, *Salmo gairdneri*. *Mol. Mar. Biol. Biotechnol.* **6**, 64-71.

Villamil, L., Tafalla, C., Figueras, A. & Novoa, B. (2002). Evaluation of Immunomodulatory Effects of Lactic Acid Bacteria in Turbot (*Scophthalmus maximus*). *Clin. Diagn. Lab. Immunol.* **9**, 1318-1323.

Vine, N. G., Leukes, W. D., Kaiser, H., Daya, S., Baxter, J. & Hecht, T. (2004) Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. *J. Fish Dis.* **27**, 319-325.

Waché, Y., Auffray, F., Gatesoupe, F.J., Zambonino, J., Gayet, V., Labbé, L. & Quentel, C. (2006) Cross effects of the strain of dietary *Saccharomyces cerevisiae* and rearing conditions on the onset of intestinal microbiota and digestive enzymes in rainbow trout, *Onchorhynchus mykiss*, fry. *Aquaculture*. **258**, 470-478.

Wakabayashi, H., Yamaauchi, K. & Takase, M. (2006) Review: Lactoferrin research, technology and applications. *Int. Dairy J.* **16**, 1241-1251.

Wallbanks, S., Martinez-Murcia, A. J., Fryer, J. L., Phillips, B. A. & Collins, M. D. (1990) 16S rRNA sequence determination for members of the genus *Carnobacterium* and related lactic acid bacteria and description of *Vagococcus salmoninarum* sp. nov. *Int. J. Syst. Bacteriol.* **40**, 224-230.

Wang, Y.-B., Tian, Z-Q., Yao, J.-T. & Li, W.-F. (2008) Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Aquaculture*. **277**, 203-207.

Welker, T. L., Lim C., Yildirim-Aksoy, M., & Klesius , P. H. (2007) Growth, immune function, and disease and stress resistance of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of bovine lactoferrin. *Aquaculture* **262**, 156-162.

Westerdahl, A., Olsson, J. C., Kjelleberg, S. & Conway, P. L. (1991) Isolation and characterization of turbot (*Scophtalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. *Appl. Environ. Microbiol.* **57**, 2223-2228.

Williams, S. T., & Vickers, J. C. (1986) The ecology of antibiotic production. *Microb. Ecol.* **12**, 43-52.

Yakimov, M. M., Fredrickson, H. L., & Timmis, K. N. (1996) Effect of heterogeneity of hydrophobic moieties on surface activity of lichenysin A, a lipopeptide biosurfactant from *Bacillus licheniformis* BAS50. *Biotechnol. Appl. Biochem.* **23**, 13-18.

Yakimov, M. M., Kroger, A., Slepak, T. N. S. C., Giuliano, L., Timmis, K. N. & Golyshin, P. N. (1998) A putative lichenysin A synthetase operon in *Bacillus licheniformis*: initial characterization. *Biochim. Biophys. Acta Gene Struct. Expr.* **1399**, 141-153.

Yan, L., Boyd, K. G., Adams, D. R., & Burgess, J. G. (2003) Biofilm-specific cross-species induction of antimicrobial compounds in Bacilli. *Appl. Environ. Microbiol.* **69**, 3719-3727.

Yanbo, W. & Zirong, X. (2006) Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Anim. Feed Sci. Tech.* **127**, 283-292.

Yang, Y. J., Dungan, R. S., Ibekwe, A. M., Valenzuela-Solano, C., Crohn, D. M. & Crowley, D. E. (2003) Effect of organic mulches on soil bacterial communities one year after application. *Biol. Fert. Soils.* **38**, 273-281.

Yano, T. (1992) Assay of hemolytic complement activity. In: Techniques in Fish Immunology (Stolen, J. S., Fletcher, T. C., Anderson, D. P., Hattari, S. L. & Rowley, A. F. Eds.), pp. 131-141. SOS publications, Fair Haven, New Jersey, USA.

Yokoyama, S., Koshioa, S., Takakurac, N., Oshidac, K., Ishikawab, M., Gallardo-Cigarroaa F. J. & Teshimaa, S. (2005) Dietary bovine lactoferrin enhances tolerance to high temperature stress in Japanese flounder *Paralichthys olivaceus*. *Aquaculture*. **249**, 367-373.

Yokoyama, S., Koshioa, S., Takakurac, N., Oshidac, K., Ishikawab, M., Gallardo-Cigarroaa F. J., Catacutan, M. R. & Teshimaa, S. (2006) Effect of dietary bovine lactoferrin on growth response, tolerance to air exposure and low salinity stress conditions in orange spotted grouper *Epinephelus coioides*. *Aquaculture*. **255**, 507-513.

Yoshimizu, M., Kimura, T. & Sakai, M. (1976) Studies on the intestinal microflora of salmonids. *Bull. Jap. Soc. Sci. Fish*, **42**, 1281-1290.

Yoshimizu *et al*. 1980

Yu, Z. & Mohn, W. W. (2001) Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic spacer analyses and 16S ribosomal DNA sequencing. *Appl. Environ. Microbiol.* **67**, 1565-1574

Zhang, T. & Fang, H. H. P. (2000) Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial communities. *Biotechnol. Lett.* **22**, 399-405.

Ziaei-Nejad, S., Rezaei, M. H., Takami, G. A., Lovett, D. L., Mirvaghefi, A. & Shakouri, M. (2006) The effect of *Bacillus* ssp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. *Aquaculture*. **252**, 516-524.