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The effect of the probiotic *Pediococcus acidilactici* on the gut microbiota ecology of rainbow trout (*Oncorhynchus mykiss* Walbaum) analysed using DGGE

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Abstract

The microbiota of the gastrointestinal tract heavily influences the health, growth and survival of fish. Probiotics have proved effective in improving fish productivity in aquaculture. Research suggests that probiotics, supplemented in the feed, may elicit these benefits by altering the ecology of the gastrointestinal microbiota. The probiotic *Pediococcus acidilactici* has been successfully used for terrestrial animals and humans but its use in aquatic organisms has been less researched. In the present study, rainbow trout *Oncorhynchus mykiss* (Walbaum) were fed a diet that contained *Ped. Acidilactici,* or a control diet. Analysis of the posterior digesta bacteria, using a non-culture dependant technique, Denaturing Gradient Gel Electrophoresis (DGGE), showed that *Ped. acidilactici* had some effects on the ecology of the microbiota but this was not statistically significant compared to controls. This study suggests that *Ped. acidilactici* must be fed at a dose above the manufacturer's recommended dose, of 10^6 CFU g^{-1} of feed, for more than four weeks for significant changes in the microbiota to occur.

Key words: Rainbow trout, *Pediococcus acidilactici,* Probiotic action, microbiota, bacteria, ecology, DGGE

Introduction

The role of the gut microbiota in health, growth and survival of fish has been recognised as extremely important. Manipulation of the gut microbiota is key to improving efficiency and survival of animals in aquaculture. Probiotics have recently been the object of much research as they can have many beneficial effects such as improving the host's resistance to disease, health status, growth performance, feed utilization, stress response or general vigour (Merrifield *et al*. 2010). Probiotics are defined as a dead, live or component of a microbe that will provide one or many benefits when administered to a host or its environment. They are added to diets to help maintain a stable, beneficial gut microbial population (Merrifield *et al.* 2009a) and alter the ecology of the microbiota by antagonistic and/or synergistic interactions.

The gut microbiota has an essential role for food utilization and disease resistance. The gastrointestinal tract of a fish contains acids, bile, salts and enzymes that create a hostile environment for many pathogens (Gómez, & Balcázar 2008). It also contains a variety of microbes that provide additional protection against pathogens. Lactic acid bacteria (LAB) in particular are recognised as beneficial and have been demonstrated as part of the indigenous microbiota of fish (Gilberg *et al.* 1995; Gómez, & Balcázar 2008; Liu *et al.* 2008).

The microbiota of the gastrointestinal tract can be grouped into allochthonous and autochthonous microbes (Ringø & Berbeck 1999). Allochthonous microbes are transient and remain in the lumen of the gut and digesta contents, but are unable to colonise the gut. Autochthonous microbes are able to colonize and are permanently resident. In this study the allochthonous bacteria will be analysed.

A variety of microbes make up the gastrointestinal microbiota of any organism including, yeasts, protozoa, viruses, bacteriophages and bacteria. Several studies have demonstrated that bacteria are the most abundant and the bulk of these bacteria in fish are gram-negative (Gatasoupe 1999). In rainbow trout, particular types of bacteria have been isolated, dominated by the gamma subclass *Proteobacteria* that includes *Citrobacter*, *Aeromonas* and *Pseudomonas;* also present were bacteria from the genus *Carnobacterium* and the beta subclass of *Proteobacteria* (Spanggaard *et al*. 2000). These communities of microbes in the gut are highly variable and dependent on the environment (Spanggaard *et al*. 2000; Liu *et al.* 2008). They are affected by dietary manipulations, however it is unlikely that under intensive rearing conditions a stable microbial community can be achieved (Vershuere *et al.* 2000). Complex interrelations have evolved between aquatic organisms and their indigenous microflora; including their pathogens (Olafsen 2001).

Understanding the gut microbiota and how it is affected by probiotics is key when developing probiotic treatments for use in aquaculture. Probiotics will likely improve the aquaculture industry. Aquaculture is designed to maximise production for profit. Any form of farming is a concentration of a species with an altered environment to protect and maximise yield. This may involve high

crowding of the same species, that causes disease to spread rapidly and stressful conditions that reduce immunity (Barton and Iwama, 1991). Chemotherapeutics have been relied upon but the widespread and unrestricted use of antibiotics in finfish aquaculture has created problems. Large amounts of non-biodegradable antibiotics have entered the marine environment and exerted selective pressure towards resistant bacteria and then these resistant determinants are transferred to human pathogens (Cabello 2006). Further the aquaculture products treated with antibiotics may contain residuals that can be toxic and/or cause allergies in humans (Cabello 2006). Because of these concerns antibiotics are no longer used as therapeutics or growth promoters in the EU (Regulation 1831/2003/EC). Probiotics are considered a much safer and environmentally friendly option to antibiotics (Gatasoupe 1999).

To date probiotics have been successful in laboratory studies and in the field. For example a number of probiotic species have been shown to increase survival of rainbow trout infected with *Aeromonas salmonicida*, that causes the common disease furunculosis (Irianto & Austin 2003 and 2002, Newaj-Fyzul *et al.* 2007, Robertson *et al.* 2000). A few examples of very successful studies on probiotic fed rainbow trout include: - Nikoskelainen *et al*. (2001b) where the probiotic *Lactobacillus rhamnosus* improved survival from infection with *Aeromonas salmonicida* to 81% compared with the control, Irianto & Austin, (2002) found a number of probiotics worked against *Aeromonas salmonicida* improving survival up to 94%, Brunt & Austin, (2005) showed that *Aeromonas sobria* GC2 could improve survival from *Lactococcus garvieae* to 90% and *Streptococcus iniae* to 82% and Newaj-Fyzul *et al*, (2007) found the commonly used probiotic *Bacillus subtilis* AB1 decreased mortality from *Aeromonas* infection by 95%. These studies are *in vivo* and are therefore more reliable in predicting the effects of probiotics compared to antagonism tests on agar.

In this study rainbow trout are used because they are industrially farmed around the globe and are therefore of significant economic importance (FAO, 2009). The probiotic, *Ped. acidilactici,* is investigated because so far studies have found promising results from its application (Harper *et al.* 2011) but they have been ambiguous for rainbow trout and finfish compared to studies on shrimp (Merrifield *et al.* 2011; Castex *et al.* 2008 and 2009). The use of the probiotic has been shown to increase survival significantly (by 12%) in rainbow trout (Ferguson *et al.* 2010). However other studies disagree and found little effect on survival of the rainbow trout (Merrifield *et al.* 2011; Aubin *et al.* 2005). Harper *et al.* 2011 suggested that *Ped. acidilactici* could potentially control *Vibrio anguillarum* infections by competition, elevating leukocyte levels and goblet cells in the epithelium of the gut and provide histological benefits to the anterior intestine, which is an important region for *V. anguillarum* colonisation. It has also been shown that *Ped. acidilactici* can reduce rainbow trout vertebral column compression syndrome observed by Aubin *et al.* (2005). Positive results for K-factor, leukocyte levels and colonization of intestinal mucosa may be the explanation (Merrifield *et al.* 2011). The probiotic was a much better option than the use of antibiotics

against the syndrome because it is not toxic to the fish and therefore reduces mortality. However, it took 5 months of feeding, compared to 10 days of the antibiotic, before *Ped*. *acidilactici* reduced incidences of the syndrome to a similar degree. (Aubin *et al.* 2005). The syndrome must be reduced in aquaculture because aesthetic quality is important in farmed fish (Merrifield *et al.* 2011). It remains to be seen if the probiotic *Ped. acidilactici* improves host nutrition, feeding and growth (Merrifield *et al.* 2011; Ferguson *et al.* 2010; Shelby *et al.* 2006). Studies have not found that the probiotic produces digestive enzymes and/or vitamins but this area is worth further research. To date the probiotic has not been shown to improve weight gain in rainbow trout (Aubin *et al.* 2005; Merrifield *et al.* 2011; Ferguson *et al.* 2010) but has had success when applied to larval Pollock and shrimp (Gatasoupe 2002; Castex *et al.* 2008, 2009).

Merrifield *et al.* 2010 emphasizes that investigation into the microbiota ecology within the gut after probiotic treatment is important to understand fish health and that there are few studies solely on the gut microbiota. The aim of the present study was to observe if the probiotic *Pediococcus acidilactici* has an effect on gut microbial ecology of the popularly farmed rainbow trout (*Oncorhynchus mykiss* (Walbaum)). This study has used Denaturing gradient gel electrophoresis (DGGE) analysis rather than traditional culture techniques, (Muyzer *et al.* 1993; Calhau *et al.* 2010), to compare the probiotic effect on bacteria in posterior digesta samples.

Methods

Experimental design*

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local commercial farm and the study was carried out in the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Before commencement of the experiment the fish where acclimatised for four weeks and fed a commercial diet (Sigma® 50, EWOS UK). Fish weighing 310 ± 9 g were randomly distributed in eight fibreglass tanks (80L capacity), at a density of 10 fish per tank. Four tanks were randomly assigned to the control diet and the other four tanks to the probiotic diet. Fish were hand-fed feed at 1% of biomass, twice daily, in equal rations at 9.00am and 5.00pm for a period of four weeks. Each tank was provided with 98% re-circulated-aerated freshwater at a rate of 100 L h^{-1}. A photoperiod of 12 hours light: 12 hours dark was controlled throughout the entire work period. During the trial, water temperature was maintained at 14 ˚C, pH was adjusted with $NaHCO_3$ to maintain pH 6.5 - 7.5 and dissolved oxygen was maintained above 90% saturation (dissolved oxygen and pH were measured daily using a HQ 40d multi HACH). Additionally, ammonia, nitrite and nitrate were measured weekly and maintained at 0.093 ± 0.029 mg L^{-1}, 0.002 ± 0.001 mg L^{-1} and 3.58 ± 3.58 mg L^{-1}, respectively.

**Probiotic and non-probiotic diets*

A *Ped. acidilactici* (CNCM MA 18/5 M) culture was made from 100 mg of lyophilised Bactocell® (Lallemand Inc., Montreal, Canada) in 50 ml of MRS (de Man, Rogosa and Sharp agar) broth that was incubated at 37 ˚C in a shaking water bath (Clifton UK) for 22 hours. The bacterial levels within the broth culture were determined using plate counts. After incubation, 1 ml aliquots from the culture were harvested by centrifugation (2.00 g for 5 min), in order to ensure the density of the *Ped. acidilactici* in the feed was 10^6 CFU (colony forming units) g^{-1}; this concentration was selected according to the manufacturers recommended dose. To produce the probiotic feed, pellets were washed once with PBS (phosphate buffered saline) and re-suspended in fish oil. Then using a Hobart mixer (Beater Co. Ltd Hobart House London UK) the probiotic/oil mixture was top-dressed onto the basal diet. The control diet had the same volume of fish oil added but without the probiotic. The basal diet used in this study (EWOS® Sigma 50) had a declared nutritional profile of crude protein 45 % and lipid 23%. The diets were air-dried at 25˚C for 24 hours and stored in plastic bags at 4˚C. New batches of diets were produced every two weeks to ensure that high levels of probiotics were maintained for the duration of the trial. To check the probiotic concentration in the experimental diet *Ped. acidilactici* colonies were counted on MRS plates using serial dilutions. The control diet was also checked for possible contamination by the probiotic strain. The survival of the probiotics, over the experimental period were determined by counts on MRS agar plates, where 1.0 g of feed was homogenised in 9.0 ml of PBS in a stomacher (Bag Mixer Interscience, France) and dilutions prepared to 10^{-7} CFU using PBS. Then, 100 µl of solution was spread over duplicate plates of MRS.

*Ali Abid PhD (University of Plymouth) provided the samples for analysis and therefore the treatment of the rainbow trout was conducted by him.

Molecular bacterial community (PCR-DGGE) analysis

At the end of the feeding trial, one fish per tank was sacrificed by immersion in a tank containing an overdose (200 mg l^{-1}) of tricaine methane sulfonate (MS222; Pharmaq, Fording bridge, UK), followed by a sharp blow to the cranium and destruction of the brain. Each fish was cleaned using 70% ethanol. Under sterile conditions, the intestine in its entirety was excised and divided into the anterior and the posterior regions. The posterior digesta was extracted aseptically from each fish by gentle squeezing of the removed intestine. Digesta from each fish was weighed into 200 mg ± 5 mg samples, more than one sample was taken were possible to enable further comparisons.

DNA extraction was performed using the QIAamp Stool Mini Kit (QIAGEN Manufacturers). The digesta samples where incubated for 30 minutes at 37°C with 0.507g of lysozyme and 10 ml of Tris EDTA. 800 µl of buffer ASL (provided in kit) was added and vortexed for 1 minute before the suspension was heated for 10 minutes at 90°C. After a further vortex and centrifuge for 2 minutes the inhibitor was removed from 750 µl of the supernatant by addition of half an Inhibitex tablet, then immediate vortex for 1 minute and standing for 1 minute. This was then centrifuged for 5 minutes and approximately 300 µl of

the supernatant was removed and centrifuged for 4 minutes. To remove the proteins, 250 μl of the supernatant was vortexed for 15 minutes with 15 μl Proteinase K and 250 μl buffer. After incubation at 70°C for 15 minutes, 250 μl of ethanol was added and the mixture was vortexed. Then 600 μl was applied to a QIAamp column and centrifuged for 1 minute. The column was then removed, 500 μl buffer AW1 (provided in kit) added, centrifuged for 2 minutes, removed again, 500 μl buffer AW2 (provided in kit) added, centrifuged for 5 minutes until the spin column was empty, removed again, 200 μl buffer AE (provided in kit) added, stood for 1 minute and centrifuged for 1 minute. The end product was the eluted DNA that was then amplified by PCR and run on a DGGE (after Muyzer *et al*. 1993).

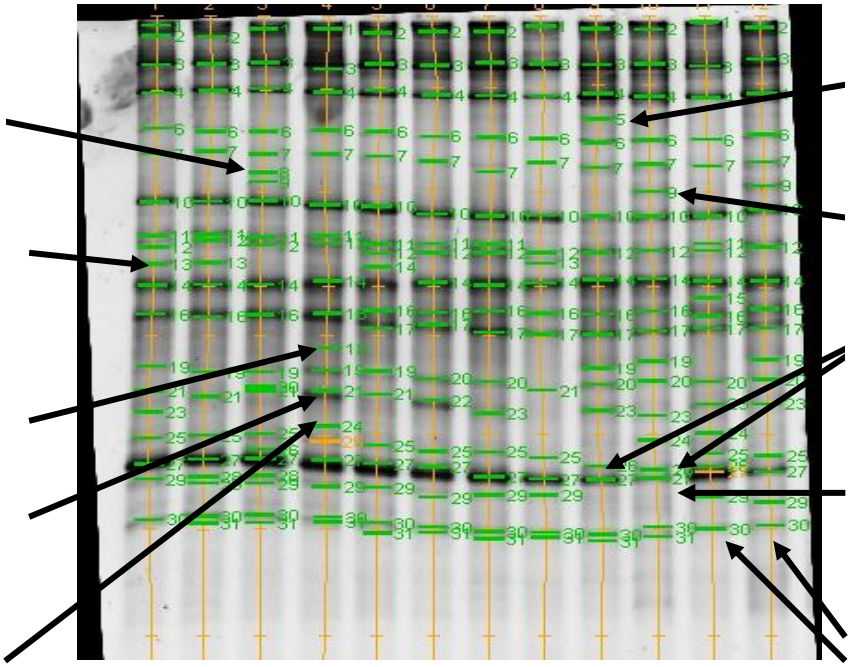
PCR amplification of the variable V3 region of 16S rRNA genes was carried out using 3 µl DNA template, 25 µl RedTaq™ PCR Reaction Mix (Sigma Aldrich, U.K.) 20 µl molecular biology-grade water, and 1 µl of the reverse primer P2 (5'-ATT ACC GCG GCT GCT GG-3') and 1 µl of the forward primer P3 (5'-CC TAC GGG AGG CAG CAG-3'), which had a GC clamp attached at the 5' end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') from Muyzer *et al.* (1993). Primers were synthesized by Eurofins MWG Biotech Ltd., Germany. PCR was carried out as described by Muyzer *et al.* (1993), using a GeneAmp PCR system 9700 and PCR products were run on 1.5% agarose gel to assess PCR success. DGGE was performed using a DGGE-2001 system (C.B.S. scientific, CA, USA). PCR products were run on 8% polyacrylamide gel (160 mm × 160 mm × 1 mm) containing a denaturing gradient of 40%-60% (where 100% denaturant is 7 M urea and 40% formamide). The gel was run at 65 volts for 17 hours at 60 °C in 1 × Tris-acetate-EDTA (TAE) buffer. The DGGE gel was stained for 20 minutes in 100 ml 1 × TAE buffer containing 10 µl of a 10000× stock solution of SYBR Gold nucleic acid gel stain (Molecular Probes, U.K.). Visualization was carried out in a BioRad 1387 universal hood II (BioRad laboratories, Italy).

Statistical Analysis

The DGGE fingerprint bands were labelled manually using Quantity-One Analysis Software (Bio-Rad lab, CA, USA). The gel did not set correctly causing the bands to distort but manual labelling accounted for this (Figure 1). The intensities of the bands were measured as an indicator of abundance. A *p*-value of < 0.05 was used to indicate a significant difference. A resemblance matrix was created using Primer 6 (V6.1.13) software and a dendrogram plotted to visualise how similar the samples were relative to each other. An analysis of similarity (ANOSIM) was performed comparing replicates both within and between groups. A Shannon diversity index and a Margelef richness index were calculated in Primer 6. Two-dimensional multidimensional scaling (MDS) analysis was used to represent the relative similarities between three replicates in each treatment. Further analysis of variance, a one-way ANOVA, was performed using Minitab V6 and a two-way t-test was also used to compare sample differences.

Results

The DGGE banding pattern is shown in figure 1. Each band represents a bacteria species being present in the sample and the abundance of this bacterium is related to the intensity. The raw data showed no obvious signs of differences between the samples, but on close examination of the main bands present some differences were revealed. Pointed out by arrows on figure 1 are example differences between the samples with bands indicated in green.



Code	C1	C1	P3	P1	C2	P1	P3	C1	P2	C2	C3	P1
Lane	1	2	3	4	5	6	7	8	9	10	11	12

Figure 1: Labelled PCR-DGGE fingerprints showing the bacteria species present in samples from the posterior digesta of control and probiotic fed rainbow trout: Each lane is labelled with a number and a code to the sample used. C stands for control fish and P stands for probiotic fed fish. Samples with codes of the same letter and number have been taken from the same fish. The individual band labels are arbitrary numbers, bands with same number are assumed to be of the same species. Arrows indicate example differences between the samples that are explained either side of the figure.

There is no distinct clustering or significant differences between any of the samples indicated by the dendrogram (Figure 2) that represents the similarities between the presence of species and their abundance. The differences between samples from the same fish are not significant, indicating no contamination was introduced during the extraction process, however not all samples from the same fish are over 90% similar to each other. Three replicates from the control and probiotic groups were chosen at random.

Results from lane 6 and 8 (Figure 1) were avoided however due to pipetting problems during the loading of the DGGE gel that may have reduced the intensity of the bands. An MDS similarity plot of these six samples is given in figure 3. There is no clustering of the data points to suggest a relationship between any of the samples. The stress value is close to 0 and therefore the data is well represented.

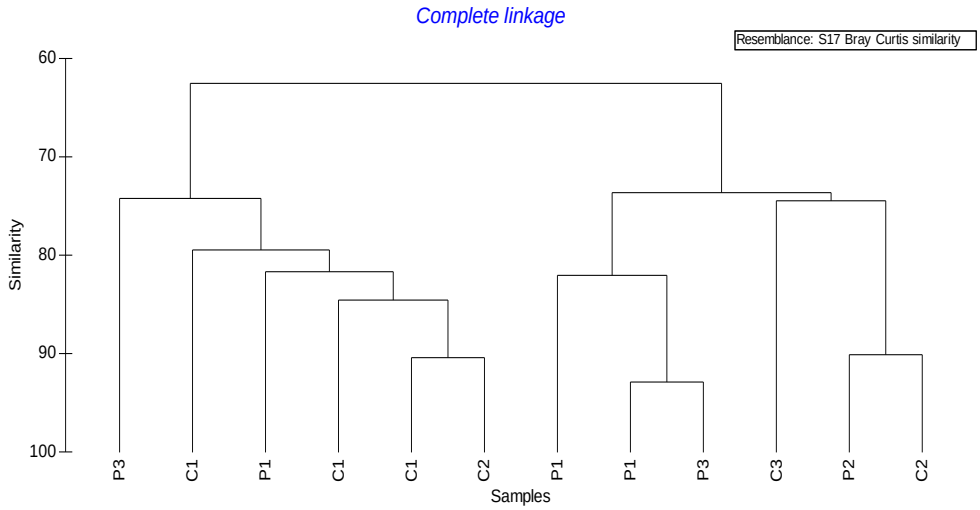


Figure 2: Dendrogram of the resemblance and similarity of bacteria presence and abundance between all samples taken from control and probiotic fed fish: C stands for control fish and P stands for probiotic fed fish. Samples with the same code have been taken from the same fish.

Figure 3: Non-metric multidimensional scaling analysis plot of the Bray Curtis relative similarity of the bacteria community in samples. Shown are three replicates from control (empty squares) and three from probiotic fed fish (solid black squares).

Statistical analysis is summarized in table 1. There is no difference between the average numbers of bands, representing bacteria species, between control and probiotic fed fish (table 1). The calculated species richness and diversity index show that the probiotic treatment has reduced both the richness and diversity of the microbiota in the posterior digesta of the rainbow trout, compared to the control (table 1). However, a two-way t-test shows that these are in fact insignificant (p >0.05). SIMPER analysis indicates a slight reduction in the similarity of the replicates from the probiotic fed fish. This is not significant as the p-value from an ANOSIM is greater than 0.05 and the groups are only 24.23% dissimilar (table 1).

Table 1: Statistics summary of the microbial community detected in the posterior digesta of fish fed a probiotic diet and controls, n = 3 per group.

Treatment	**(1) N**	**(2) Shannon Diversity**	**(3) Margalef Richness**	**(4) SIMPER similarity**
Control	18.67 ± 0.58	2.93 ± 0.03	1.59 ± 0.048	75.17
Probiotic	19.67 ± 2.08	2.10 ± 0.10	1.67 ± 0.17	70.60
	R-value	**P-value**	**Dissimilarity**	
(5) Pair wise comparison	-0.30	0.80	24.23	

Values expressed as means ± the standard deviation. (1) N is the number of bands in each lane of the DGGE gel. (2) Diversity is the Shannon diversity index of each sample [H= $\sum$ Pi. ln(Pi)] (3) Richness is the Margalef species richness index of each sample [M= (S-1) / ln(N)]. (4) SIMPER, similarity percentage within group replicates. (5) ANOSIM, Analysis of similarities between groups.

Discussion

The results of the present study show that the probiotic *Pediococcus acidilactici* has little effect on the bacteria in the posterior digester of rainbow trout (*Oncorhynchus mykiss* (Walbaum). The MDS plot shows no clear clustering of the replicate sample results to suggest the probiotic having any effect on the bacterial population, compared to controls. This result is not expected as previous studies have found that *Ped. acidilactici* survives transit through the gut and can colonize parts of the gut in rainbow trout (Harper *et al.* 2011; Merrifield *et al.* 2011). Therefore high levels of the probiotic in the posterior digesta could be expected. Other studies that have looked at the gut microbial composition find that a probiotic species added is in greater abundance than the other microbes present. For example Merrifield *et al.* 2009 a and b found that the percentage of an added probiotic would increase up to around 80% in the posterior digesta during feeding and this was generally more than in the mucosa but less than the amount fed. In this study it is not known what bacterial species the bands represent due to problems with the sequencing process. A valuable improvement would to repeat this study but include the banding pattern of *Ped. acidilactici.*

There is some indication that *Ped. acidilactici* has altered the ecology of the microbiota in the posterior digesta of the rainbow trout in terms of reducing the species richness and diversity (table 1 and figure 1). *Ped. acidilactici* may be present in higher numbers and displace other microbes completely or reduce their abundance, which would cause the bacterial individuals to be spread less evenly across the species encountered in the samples. However a two-way t-test gave a $p > 0.05$ for both statistics, confirms no statistical difference. There is large variation in the gut microbiota between fish from the same conditions (Spanggard *et al.* 2000), and therefore future work will need to use more replicates that may pronounce the effects on the species richness and diversity with less error. In addition Ferguson *et al.* 2010 found that replicates from the *Ped. acidilactici* fed group were significantly more similar to each other than replicates from the initial and control groups. The present study is in contradiction. The SIMPER analysis suggests slightly less similarity of the bacterial communities between replicate samples from the probiotic treatment (70.60%) compared to the control treatment (75.17%), however this is not significant ($p > 0.05$).

The limited effect of the probiotic in this study may be due to either the dosage in the feed or the duration of the feeding. In other probiotic studies the dose and the durations of the feeding can alter results significantly. Studies such as Nikoskelainen *et al*. (2001a and 2003), Brunt & Austin, (2005), Newaj-Fyzul *et al*. (2007), Bagheri *et al*. (2008) and Merrifield *et al*. (2011) all found varying results when probiotics were fed at different dosages (colony forming units per gram of feed, CFU/g feed) to rainbow trout, with one specific dosage often giving best results. The duration of feeding also has an effect on results. Nikoskelainen *et al*. 2001a fed a *Lactobacillus rhamnosus sp.* probiotic at 10^9 CFU/g feed for 51 days to rainbow trout and got more significant results than Panigrahi *et al*. (2004) who fed the fish similarly for 30 days and Panigrahi *et al*. (2007) for 45 days. However different parameters were measured and therefore comparison as to which gave most benefit is difficult. In the present study the dose of the probiotic and the duration of feeding are lower to other successful studies. Merrifield *et al*. (2011) found that the optimum dose of *Ped. acidilactici* was 10^8 CFU/g feed and fed for 10 weeks. The study by Aubin *et al*. (2005) had successful results using about 10^6 CFU/g feed of *Ped. acidilactici* but best results came from feeding for 5 months. Ferguson *et al*. (2010) found that *Ped. acidilactici* fed at 10^7 CFU/g feed for 32 days had a significant effect on the gut microbial communities but this was in Tilapia, as was the study by Shelby *et al.* (2006), which may react differently to rainbow trout.

The gut microbiota could be altered by a probiotic, such as *Ped. acidilactici* by a number of modes of action (Merrifield *et al.* 2010). Probiotic modes of action in fish include: production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing, improvement of water quality, enhancement of the immune response, source of macro and/or micronutrients and enzymatic contribution to digestion (Sugita *et al.* 1996, 1997; 1998; Vershuere *et al.* 2000; Olafsen, 2001; Vine, 2004; Defoirdt *et al.*

2004; Gatesoupe 2008; Gòmez & Balcàzar, 2008; Ringø, 2008; Tinh *et al.* 2008 – from Merrifield *et al.* 2010). *Ped. acidilactici* has been shown to compete for adherence sites (Merrifield *et al.* 2010; Harper *et al.* 2011) and therefore displace pathogens. The ability to adhere and persist in the gastrointestinal tract would allow smaller doses of probiotics to be used and reduce the need for continual supplementation. It should be noted however that it is improbable that a single exogenous addition of a probiotic to an established microbial community will result in long-term dominant colonization (Vershuere *et al.* 2000). Another way in which *Ped. acidilactici* alters the ecology of the gut microbiota is to successfully compete for nutrients .The study by Nikoskelainen *et al*. (2001b) found that this was generally true for the LAB tested in that study. For example some lactic acid probiotics produce siderophores that competitively absorb iron, which is a limited nutrient and virulence factor to certain pathogens (Neilands 1995; Vershuere *et al*. 2000).

In addition a range of bacteriocins has been identified from *Ped. acidilactici* strains (Bhunia *et al*. 1990; Schved *et al.* 1993; Huang *et al*. 1996; Halami *et al*. 1999; Anastasiadou *et al*. 2008) and it is the bacteriocins that seem to have the most effect on other microbes (Gilberg *et al*. 1995). Anastasiadou *et al.* (2008) investigated the production of bacteriocins and pediocins from *Pediococcus spp.* and found that *Ped. acidilactici* produced the most resilient forms making them suitable for industrial production to be injected directly without the need to have viable cells. *Ped. acidilactici* produces organic acids (de Arauz *et al*. 2009), lowering the pH levels which inhibits growth of some pathogenic bacteria (Dicks and Botes 2010). Other inhibitory substances produced by some probiotics such as *Bacillus sp.*, are enzymes or molecules that disrupt quorum sensing between pathogenic bacteria, which will reduce their virulence, (Defoirdt *et al.* 2004). The lack of literature on this subject, however, means further studies are necessary to detect if *Ped. acidilactici* is producing such chemicals.

One suggestion is that *Ped. acidilactici* has beneficial effects to a host by stimulating the immune system (Sakai 1999), which would not alter the ecology of the gut microbiota as significantly (Liu *et al.* 2008). Lactic acid bacteria have been documented to combine direct antagonism with immunostimulation (Villamil *et al*. 2002; Nikoskelainen *et al*. 2003). However previous investigations have found a lack of improvements in immune responses to *Ped. acidilactici* (Bhunia *et al.* 1990; Villamil *et al*. 2002; Shelby *et al*. 2006). Ferguson *et al.* (2010) found that *Ped. acidilactici* had little effect on haematology and immunology parameters except for lower haematocrit levels, increased number of leucocytes and increased serum lysozyme activity. Merrifield *et al.* (2011) found similar results but the haematocrit levels and serum lysozyme activity remained unaffected.

There are limits to this investigation. Using just one technique to analyse such a diverse community as the gut microbiota of fish cannot produce a complete picture of a probiotics effect on the ecology. Spanggard *et al.* (2000) suggests that only 50% of the microbiota in rainbow trout is culturable therefore traditional culture techniques are inadequate. Culture independent techniques

have therefore been developed such as DGGE, which has proved very useful at analysing complex microbial communities (Muyzer *et al*. 1993). The types of microbes that are detected depend on the primers used and Calhau *et al*. (2010) advises a combined use of more than one primer set. In the present study, changes in bacteria have been investigated using two general primers. The gut microbiota is predominantly bacteria and therefore any significant effects are likely to be seen but there is a lack of literature addressing how important it is to recognise changes in the other microbiota present. Merrifield *et al.* 2011 found that the use of electron microscopy was an invaluable tool to physically see what microbes were where in the gut, but noted that this must be used in combination with identification techniques. Further study of *Ped. acidilactici's* effect on the gut microbiota, using these other techniques, are needed.

The DGGE method has limits. Manipulating the image of the gel altered the intensity of the bands and therefore results are relative to each other and not to other separate studies. Intense wide bands may have been many bands clustered together but counted as one abundant species. Sekiguchi *et al.* (2001) explains that a single DGGE band does not represent a single bacterial strain and bands that migrated to the same position in different lanes may consist of different bacteria. To test and resolve both problems, the band can be excised and run on a further, shorter denaturant gradient to provide high-resolution DGGE profiles of particular parts of the original profile (Muyzer *et al*. 1993; Gavin and Spratt 2005) but this is time consuming and expensive. There may be bias in the PCR process that will make abundance measurements unreliable. It has to be assumed that the general primers will target all the different species and the PCR process will work equally. It is also assumed that the DNA extraction is of equal success for every bacteria, but *Ped. acidilactici* is a coccus type bacterium that will be generally harder to break up and remove the DNA compared to other bacterium types such as rods.

Further research should include challenge studies, where the effects of the *Ped. acidilactici* on the gut microbiota ecology after challenge with known pathogens is investigated and survival rates recorded. It should also be considered how important the gastrointestinal tract route of infection is, as the level of protection from a probiotic will depend on this (Gilberg *et al.* 1995). Included in such a study should be a range of dosages to find the optimum effective dose and investigation of the immune response. In addition studies on the nutritional benefits of *Ped. acidilactici* are needed. The mode of action of *Ped. acidilactici* is still not clear, as is the case for many of the probiotics used today. Aubin *et al.* (2005) suggest that the probiotic must be tested in rainbow trout with other environmental conditions, where the dominant microbes may be different, because the autochthonous microbes interfere with the effect of probiotics. The probiotic *Ped. acidilactici* is worth further research as it is a good candidate for use in aquaculture based on the criteria proposed by Merrifield *et al.* (2010).

In summary the gastrointestinal tract of fish contains a diverse community of microbes that aid digestion and disease resistance. Adding certain microbial strains, called probiotics, can alter this community to increase benefits to the host. The probiotic *Ped. acidilactici* has been found to be antagonistic or synergistic to other microbes by competition for adherence sites and production of a range of bacteriocins. Although this investigation has its limits there was no contamination indicated and changes in the bacteria ecology have been shown. Compared to other studies that found significant benefits from *Ped. acidilactici* application, this study indicates a minimum threshold dosage and duration of treatment, required for significant changes in the gut bacterial ecology of rainbow trout, is 10^6 CFU g^{-1} of feed for four weeks.

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