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Expression of immune-related genes in rainbow trout (*Oncorhynchus mykiss*) induced by probiotic bacteria during *Lactococcus garvieae* infection

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ABSTRACT

The aim of the present study was to investigate the effect of lactic acid bacteria (LAB) on the control of lactococcosis as well as to assess the impact of probiotics on the expression of immune-related genes in the head kidney and intestine of rainbow trout (*Oncorhynchus mykiss*). *Lactobacillus plantarum*, *Lactococcus lactis* and *Leuconostoc mesenteroides*, were administered orally at 10^6 CFU g^{-1} feed to fish for 36 days. Twenty-one days after the start of the feeding period, fish were challenged with *Lactococcus garvieae*. Only the fish fed the diet containing *Lb. plantarum* showed significantly ($P < 0.05$) improved protection against *L. garvieae* compared to the control. Subsequently, real-time PCR was employed to determine the mRNA levels of IL-1β, IL-8, IL-10 and TNF-α in the head kidney, and IL-8, Tlr5 and IgT in the intestine of the control and *Lb. plantarum* groups. IL-1β, IL-10 and TNF-α gene expression were significantly up-regulated by *Lb. plantarum*. Moreover, the mRNA levels of IL-10, IL-8 and IgT were significantly higher in the Lb. plantarum group after *L. garvieae* infection, suggesting that *Lb. plantarum* can stimulate the immune response of rainbow trout.

PCR-DGGE revealed no detectable levels of the probiotics or the pathogen present on the distal intestinal mucosa. These findings demonstrate that direct probiotic–host interactions with the intestine are not always necessary to induce host stimulatory responses which ultimately enhance disease resistance. Furthermore, as *L. garvieae* did not colonise the intestinal tract, and therefore likely did not infect via this route, the antagonistic properties of the probiotic candidate towards *L. garvieae* were likely of little influence in mediating the improved disease resistance which could be attributed to the elevated immunological response.

1. Introduction

Lactococcus garvieae is the causal agent of lactococcosis, a disease that causes severe economic losses in farmed marine and freshwater fish species, particularly during the summer months, given its association with high water temperatures [1]. The disease is characterised by a haemorrhagic septicaemia and meningoencephalitis in several species of marine and freshwater fish [1,2] and mammals [3]. Moreover, *L. garvieae* has also been isolated from humans in several cases, suggesting that this bacterium could be catalogued as a potential zoonotic agent [4].

In fish farming, bacterial disease outbreaks are typically treated with antibiotics; however, they are often ineffective and their indiscriminate and prophylactic use has led to an increase in antibiotic resistances [1]. Commercial vaccines are available for some pathogens [5], but vaccination cannot prevent disease development in immunologically immature fish. In the case of lactococcosis, immunity after vaccination gives a good level of protection, but it only lasts for a short period of time [6].

Over the last decade there has been a growing interest in the use of beneficial microorganisms to prevent or control pathogenic microorganisms as an alternative to traditional disease control treatments [7,8]. Probiotics have been defined as a viable microbial feed supplements which beneficially influence the health of the host [9], and they offer potential alternatives by providing benefits to the host primarily via the direct or indirect modulation of the gut microbiota. Suggested modes of action resulting from increased

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favourable bacteria in the gastrointestinal tract include the production of inhibitory compounds, competition with potential pathogens, inhibition of virulence gene expression, enhancing the immune response, improved gastric morphology and aiding digestive function [10–12].

Remarkable progress has been achieved in isolating and characterising cytokine genes from fish in recent years [13]. In particular, pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) and the anti-inflammatory cytokine IL-10 [13–15], these stimulatory and inhibitory molecules are commonly used immune-regulatory genes studied in rainbow trout (*Oncorhynchus mykiss*). These cytokines are thought to contribute to defence mechanisms of the host in response to bacterial colonization or invasion [16]. In contrast, the main function of IL-10 seems to be regulation of the inflammatory response, thereby minimizing damage to the host induced by an excessive response [17]. IgT, an immunoglobulin specialized in mucosal immunity which acts like a mucosal antibody [18] and toll-like receptor 5 (Tlr5) is involved in host survival before adaptive immunity [19].

Therefore, the aim of the present study was to determine the protective effects of three lactic acid bacteria against pathogenic *L. garvieae* and to assess the impact of probiotics on the expression of immune-related genes in the head kidney and intestine in rainbow trout.

2. Materials and methods

2.1. Bacterial strains

Lactobacillus plantarum subsp. *plantarum* CLFP 3, *Lactococcus lactis* subsp. *cremoris* CLFP 25 and *Leuconostoc mesenteroides* CLFP 68, isolated from rainbow trout and identified by 16S rRNA gene sequencing, were selected as potential probiotics [20]. These strains were chosen from a pool of 335 isolates obtained from distal intestinal mucosa, cutaneous mucus and gills of healthy rainbow trout, because of their positive *in vitro* characteristics, which include resistance to pH and bile, positive adhesion characteristics and antagonism against *L. garvieae* [20]. These strains were grown aerobically in the de Man, Rogosa and Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 22 °C. Stock cultures stored at −80 °C were prepared from overnight cultures to which 15% (vol/vol) glycerol (Scharlab, Barcelona, Spain) was added just prior to freezing.

2.2. Preparation of the feed

The three selected strains were grown in MRS broth in a shaking incubator at 22 °C overnight. After incubation, the cells were harvested by centrifugation (2000 × *g*), washed twice with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride [pH 7.2]), and re-suspended in the same buffer. The absorbance at 600 nm was adjusted to 0.25 ± 0.05 which corresponded to 10^7–10^8 CFU mL^{-1}. Dilution plating was used to verify the relationship between absorbance at 600 nm and CFU mL^{-1}.

Commercial feed (Skretting, Burgos, Spain; 42% protein and 24% lipid) was used as the basal diet for the supplementation with the three selected strains. In order to reach a final concentration 10^6 CFU g^{-1} feed, after [21,22], bacterial suspensions were slowly applied into the feed, mixing part by part in a drum mixer. The probiotic concentration in each feed was determined by plate counting on MRS agar.

2.3. Fish and experimental conditions

Rainbow trout were obtained from a commercial fish farm in the Autonomous Community of Aragon, Spain. The fish were fed a standard commercial feed at a rate of 1.5% of the biomass per day. The fish had not been vaccinated nor exposed to fish diseases and were deemed pathogen free by standard microbiological techniques and by a previously described PCR technique for the detection of *L. garvieae* [23], *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri* [24]. The fish were acclimated for 2 weeks to laboratory conditions in tanks before the start of the trial. After the acclimation period, the average weight of the fish was 26 g and the fish were divided into five 1000 L tanks (one for each treatment, control group and cohabitation fish), each containing 45 fish. All the fish were maintained in re-circulating aerated freshwater at 15 ± 1 °C with a 25% water change everyday and a 12 h dark/12 h light photoperiod. During 36 days of probiotic supplementation, the water temperature was increased progressively from 15 ± 1 °C to 19 ± 1 °C to induce the experimental infection.

The first two groups were fed unsupplemented feed during the entire trial period. The first group served as the control, while the second group served to keep fish that were used for experimental infection (cohabitation method). The third, fourth and fifth groups were fed diet containing different viable lactic acid bacteria during the entire trial period. The third group was fed a diet supplemented with 10^6 CFU g^{-1} *Leu. mesenteroides*, the fourth one supplemented with 10^6 CFU g^{-1} *L lactis* subsp *cremoris* and the last group a diet supplemented with 10^6 CFU g^{-1} *Lb. plantarum*. Fish in all groups were fed at 1.5% of biomass over two feeding periods daily.

2.4. Experimental infection

After 21 days of probiotic feeding, the experimental infection was carried out by the cohabitation method. *L. garvieae* CLFP LG1, previously isolated during a natural outbreak in rainbow trout, was grown for 24 h at 22 °C in Tryptic Soy Agar (TSA; Scharlab, Barcelona, Spain). After incubation, the cells were harvested by centrifugation (2000 × *g*), washed twice with PBS, and re-suspended in the same buffer. The absorbance at 600 nm was adjusted to 0.125 ± 0.005 in order to standardize the number of bacteria (1×10^7 CFU mL^{-1}). The bacterial suspension was diluted to a density of 1×10^4 CFU mL^{-1}, and 0.1 mL of this suspension was injected intraperitoneally into cohabitation fish. The fish were anaesthetised with tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd Vancouver, Canada) before injection. All cohabitants (8 fish for each group) were marked by clipping the adipose fin after injection, and were placed into the appropriate tank with the experimental fish. Dead fish were collected and cumulative mortality was recorded and the probiotic efficacy was calculated on the last day of the trial by relative percent survival [25]. *L. garvieae* was isolated from tissue samples of freshly dead fish on Columbia Sheep Blood Agar (BioMérieux, Marcy l'Etoile, France) at 22 °C for 48 h, and its identity was verified by a previously described PCR method [23].

2.5. Sample collection

At the end of the 21-day probiotic feeding trial, 5 fish per treatment were sacrificed by immersion in a tank containing MS-222 at a final concentration of 150 mg L^{-1} for 15 min, according to the instructions given by the Zaragoza University Ethics Committee for Animal Experimentation.

Intestine and head-kidney samples were then collected from each treatment. Subsequently, the same number of samples ($n = 5$)

were taken at the end of the experimental infection. All samples were stored separately at −80 °C until use.

2.6. Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE)

PCR-DGGE was employed to assess the potential colonization and population of the probiotics and pathogen on the distal intestinal mucosa in order to ascertain if this was an infection route for *L. garvieae* and whether the probiotic was able to colonise the mucosal surfaces. DNA was extracted from 10 (5 prior to infection with *L. garvieae* and 5 at the end of the challenge trial) intestinal samples per treatment (*Lb. plantarum*, *L. lactis* and *Leu. mesenteroides*) and the control group using QIAamp® Stool Mini Kit (Qiagen) prior to PCR amplification of V3 region of the bacterial 16S rRNA gene as previously described [26]. DGGE was performed using a Dcode Universal Mutation Detection System (Bio-Rad). Twenty μL of standardized PCR products were run on an 8% acrylamide gel with a denaturing gradient of 40–60% (where 100% denaturant is 7 M urea and 40% formamide). The gel was run at 65 V for 17 h at 60 °C in 1×TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA).

Visualization of the DGGE bands was achieved by SYBR Green staining (Molecular Probes, Eugene, OR, USA). Gels were scanned in a Bio-Rad universal hood II (Bio-Rad) and optimised for analyses by enhancing contrast and greyscale.

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

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, with some modifications. Briefly, 40 mg of tissue were homogenized in 1 mL of TRIzol and 200 μL of chloroform was added. After mixing, samples were centrifuged at 10,000 × *g* for 15 min. The upper aqueous phase was transferred in a tube containing an equal volume of isopropanol. Mixtures were thoroughly vortexed and centrifuged at 12,000 × *g* for 10 min. Supernatants were discarded and the precipitated RNA pellets were washed using 1 mL of 75% ethanol. Total RNA dissolved in diethylpyrocarbonate (DEPC) water was treated with DNAse (TURBO DNA-*free*™, Ambion) following the manufacturer's instructions, to remove the contaminating genomic DNA. RNA concentration and purity were measured spectrophotometrically (NanoDrop Technologies, Wilmigton, USA) and stored at −80 °C until use. A total amount of 1 μg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad). PCRs were performed with the SYBR green method in a iQ5 iCycler thermal cycler (Bio-Rad). Duplicate PCR reactions were carried out for each sample analyzed. The reactions were set on a 96-well plate by mixing, for each sample, 1 μL of diluted (1/20) cDNA, 5 μL of 2× concentrated iQ ™ SYBR Green Supermix (Bio-rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM forward primer and 0.3 μM of reverse primer. Table 1 presents the primer sequences used. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20s at 95 °C, 20s at 60 °C and 20s at 72 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak.

β-actin and 60S were used as house keeping genes in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative controls and no primer-dimer formations were observed in the control templates. The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad). Modification of gene expression is represented with respect to the control sampled at the same time of the treatment.

2.8. Statistical analysis

Survival curves were calculated using the Kaplan–Meier method and compared by the log-rank test. One-way ANOVA and Tukey's multiple comparison test were used to determine the significant variation ($P < 0.05$) in the immune response between the control and experimental group. All statistics were performed using SPSS for Windows version 15.0 (SPSS, Chicago, USA).

3. Results

3.1. Disease challenge

To investigate whether the probiotics were able to protect rainbow trout against lactococcosis infection, fish were infected with *L. garvieae* by the cohabitation method. Fish fed diets containing probiotics showed a cumulative mortality ranging from 12.5% (*Lb. plantarum*) to 32.5% (*Leu. mesenteroides*), whereas mortality was 32.5% in fish not treated with the probiotics (Fig. 1). Statistical analysis demonstrated that fish fed the diet containing *Lb. plantarum* at 36 days had significantly ($P < 0.05$) lower mortality than fish fed diets containing *L. lactis*, *Leu. mesenteroides* and the control. Relative survival value was thus 61.5% in fish fed the diet containing *Lb. plantarum* compared with the control group. External examination and bacteriological analysis of fish that died during the study revealed the presence of *L. garvieae* in all cases.

3.2. PCR-DGGE analysis

PCR-DGGE analysis revealed that the intestinal mucosa of all fish sampled was devoid of *L. garvieae*. Additionally, no detectable probiotic populations were present on the intestinal mucosal tissues in the respective groups.

3.3. Relative mRNA expression of immune-related genes

As *Lb. plantarum* was the only strain to confer protection against *L. garvieae* infection in rainbow trout, this treatment was selected to investigate the expression of immune-related genes in the head

Table 1
Primers used for detection of target genes.

Gene	GenBank accession nos	Product size	Forward primer	Reverse primer
IL-1β	AJ223954	91	ACATTGCCAACCTCATCATCG	TTGAGCAGGTCCTTGTCCTTG
IL-10	AB118099	70	CGACTTTAAATCTCCCATCGAC	GCATTGGACGATCTCTTTCTTC
TNF-α	AJ277604	75	GGGGACAAACTGTGGACTGA	GAAGTTCTTGCCCTGCTCTG
IL-8	AJ279069	69	AGAATGTCAGCCAGCCTTGT	TCTCAGACTCATCCCCTCAGT
IgT	AY870265	72	AGCACCAGGGTGAAACCA	GCGGTGGGTTCAGAGTCA
Tlr5	AB091105	89	GGCATCAGCCTGTTGAATTT	ATGAAGAGCGAGAGCCTCAG
β–actin	AJ438158	167	ACAGACTGTACCCATCCCAAAC	AAAAAGCGCCAAAATAACAGAA
60s	NM001165047	147	AGCCACCAGTATGCTAACCAGT	TGTGATTGCACATTGACAAAAA

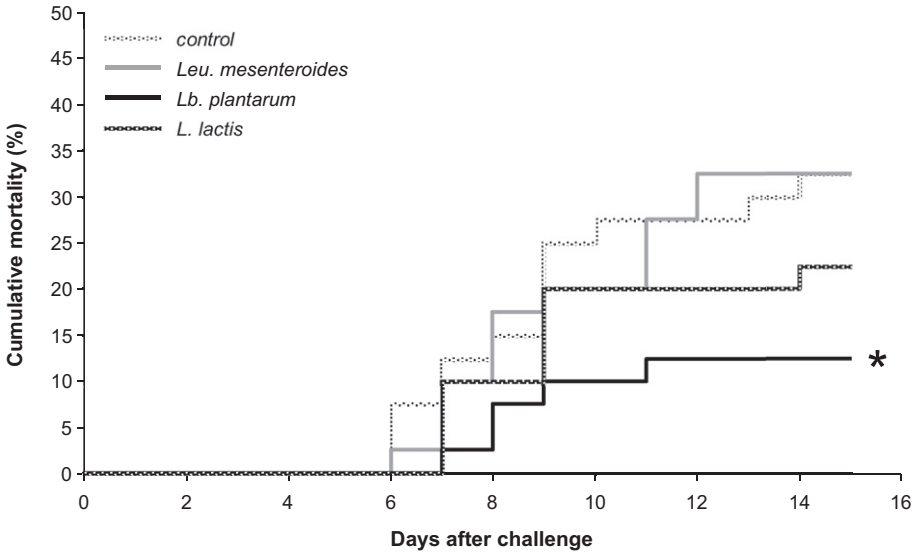

Fig. 1. Mortality curves of rainbow trout challenged with *L. garvieae* and treated with probiotics. Mortality between groups was compared using the Kaplan–Meier method. The asterisk indicates significant difference from the control ($P < 0.05$).

kidney (IL-1β, IL-10 and TNF-α) and the intestine (IL-8, IgT and Tlr5).

In the head kidney (Fig. 2), IL-1β, IL-10 and TNF-α gene expression in fish fed *Lb. plantarum* were significantly ($P < 0.001$) up-regulated prior to infection with *L. garvieae* compared to the control group. After the cohabitation, IL-1β and TNF-α gene expression were up regulated in the control group compared to pre-infection levels. In contrast however, IL-1β and TNF-α levels in the probiotic group were significantly lower than pre-infection levels with IL-1β expression significantly lower ($P < 0.001$) compared with the control group. However, IL-10 gene expression in the probiotic group was significantly ($P < 0.001$) higher after infection than in the control group.

In the intestine (Fig. 3), IL-8 gene expression was significantly ($P = 0.002$) up-regulated in the probiotic group prior to infection. After the infection, Tlr5 anf IgT levels were up-regulated in the intestine of fish from the control group whereas only IL-8 and IgT were up-regulated in the probiotic group; Tlr5 mRNA levels remained unaffected in the probiotic group post infection. After infection, IgT and IL-8 gene expression were significantly higher ($P < 0.001$) in the probiotic group than in the control group. In contrast, Tlr5 gene expression in the probiotic group was significantly ($P < 0.001$) lower after infection than in the control group.

4. Discussion

To date the beneficial effects of probiotic administration against *L. garvieae* infection in rainbow trout have been demonstrated with dietary *Aeromonas sobria* GC2 [27], *Leu. mesenteroides* CLFP 196 and *Lb. plantarum* CLFP 238 [22]. The present study confirmed the benefits of *Lb. plantarum* against *L. garvieae* infection as a significant reduction of cumulative mortality. In contrast, *L. lactis* and *Leu. mesenteroides* were ineffective at reducing *L. garvieae* induced mortalities.

To evaluate whether probiotic treatment had an effect on the expression of immune-related genes, four cytokines (IL-1β, IL-8, IL-10 and TNF-α), Tlr5 and IgT were examined using real-time PCR. In the present study, IL-1β, IL-10 and TNF-α gene expression were significantly up-regulated in the head kidney of fish fed dietary *Lb. plantarum* CLFP 3 compared to the control group. These findings

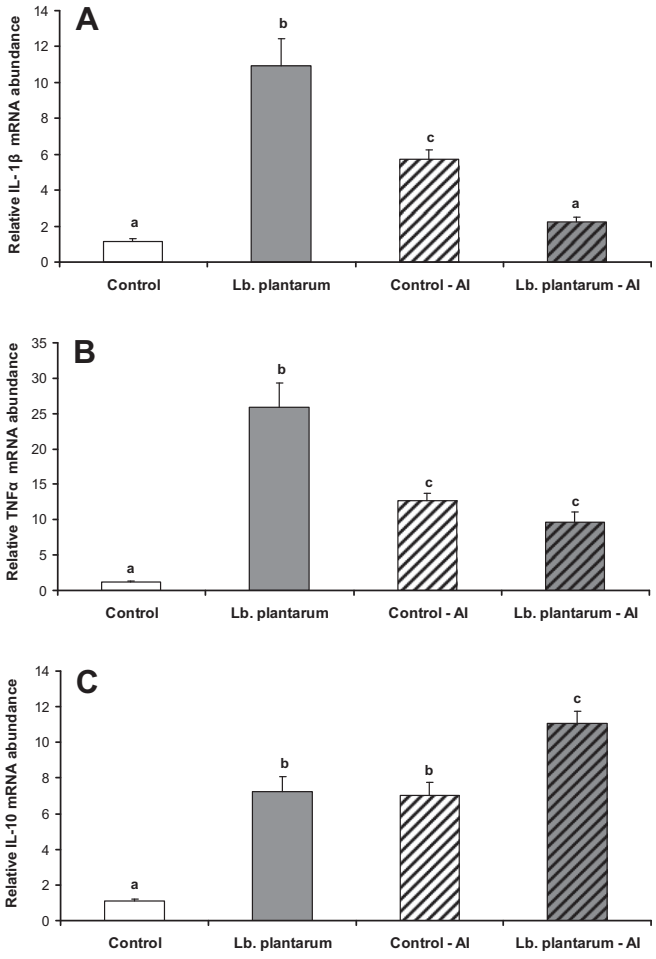

Fig. 2. IL-1β (A), TNF-α (B) and IL-10 (C) mRNA quantification in the kidney of control and fish fed diet containing *Lb. plantarum* CLFP 3 before and after *L. garvieae* infection (-AI). Values with different superscript letters are significantly different ($P < 0.05$).

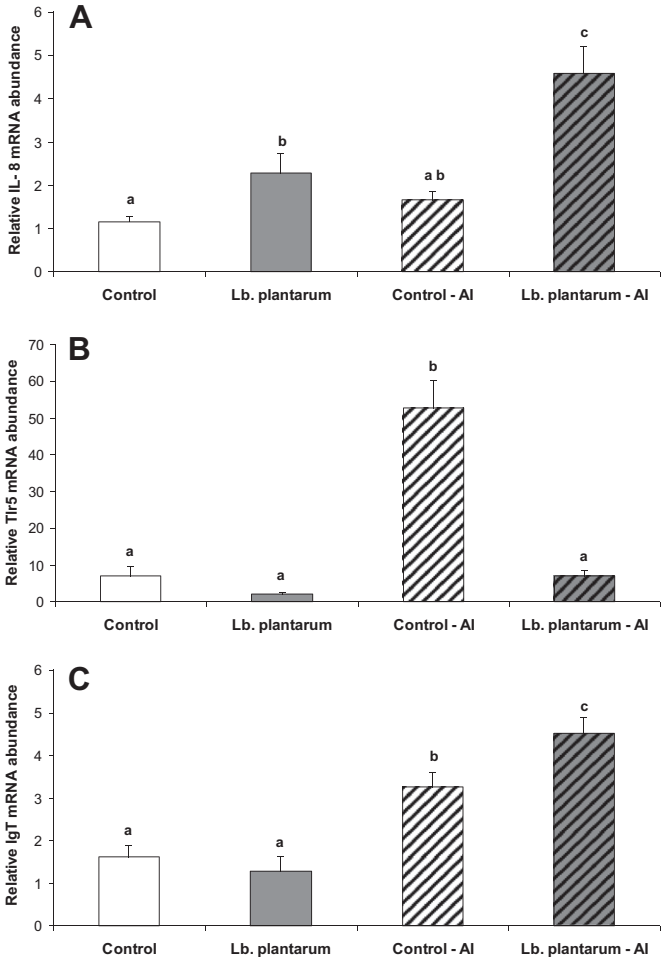

Fig. 3. IL-8 (A), Tlr5 (B) and IgT (C) mRNA quantification in the intestine of control and fish fed diet containing *Lb. plantarum* CLFP 3 before and after *L. garvieae* infection (-AI). Values with different superscript letters are significantly different ($P < 0.05$).

are in agreement with previous studies that have shown that the supplementation of probiotic bacteria increases the expression of pro-inflammatory cytokines, including IL-1β and TNF-α in rainbow trout [16,28]. The present study reports for the first time the up-regulation of rainbow trout IL-10, which is considered to have regulatory roles in immune responses [29], gene expression in response to probiotic feeding. Interestingly, the probiotic group displayed higher IL-10 gene expression after *L. garvieae* infection, both compared to the control group and compared to the pre-infection probiotic levels. This supports the findings from mice studies which show that probiotic strains displaying potential to induce higher levels of IL-10 offer good protection against *in vivo* diseases [30].

It is considered that resident mucosal immune cells in the intestine of fish may modulate the local immune site by secreting various cytokines and immune-regulatory substances. The intestinal epithelium may therefore play a key role in the initiation and regulation of mucosal immunity to bacteria by interacting with immune cells of the gut-associated lymphoid tissue, lamina propria lymphocytes and intraepithelial lymphocytes [31]. Previous studies have shown that the expressions of IL-8, as well as other cytokines, are elevated in the proximal intestine of rainbow trout when infected by *A. salmonicida* [15]. Similarly, *L. garvieae* infection in the present study induced a higher IL-8 mRNA abundance compared to pre-infection levels, but the differences were not significant. Compared to the control levels however, IL-8 gene expression was significantly up-regulated in the intestine of fish fed dietary *Lb. plantarum* both prior and after *L. garvieae* infection. *L. garvieae* infection in the present study also induced elevated IgT mRNA abundance levels in both the control and *Lb. plantarum* fed fish. In contrast to the effect on IL-8 in the present study, probiotic feeding had no effect on intestinal IgT mRNA levels in the absence of the bacterial challenge; however, post challenge with *L. garvieae*, IgT gene expression was significantly higher in the probiotic group than in the control group. Our results also demonstrated that Tlr5 gene expression in fish fed dietary *Lb. plantarum* CLFP 3 was not induced in the intestine, neither before nor after infection with *L. garvieae*. However, Tlr5 gene expression in the control group was significantly higher after infection. Although *L. garvieae* is not a flagellated bacterium, and Tlr5 is involved in the recognition of flagellin, previous studies have suggested that some pathogen-derived factors other than flagellin may contribute to its activation [32,33].

PCR-DGGE analysis revealed no detectable probiotic populations present on the intestinal mucosal tissues in the respective groups. Previous studies however have shown that the probiotic species used in the present study can populate the gastrointestinal tract of trout fed supplemented diets [20,21]. The present study indicates that these populations may be primarily luminal (allochthonous) populations which do not establish a resident population present on the epithelium itself. Despite this however, the immune-regulatory genes in the intestine were significantly affected by the application of dietary *Lb. plantarum* CLFP 3. These findings indicate that the direct association of the probiotic-epithelium is not always a prerequisite to induce localised effects which ultimately induce systemic immune responses. Therefore, probiotic candidates which do not show positive selection attributes (i.e. adhesion to epithelial cells, adhesion to mucus and growth within mucus, pathogen antagonism etc) in preliminary *in vitro* assays should not necessarily be dismissed as candidate strains. Alternative methods, such as the assessment of relevant immune-regulatory gene expression of intestinal mucosal cells and lymphocytes after exposure to probiotic cell wall components and extracellular products, could offer a viable *in vitro* method to be used in preliminary selection criteria.

Previous studies have indicated that the gastrointestinal tract can be a port of entry for *L. garvieae* [1]. The present study however revealed no detectable *L. garvieae* levels present from the intestinal mucosal samples which indicates that this was not the primary infection route for *L. garvieae* during the cohabitation challenge. These findings, along with the gene expression analyses, indicate that host immunological responses were responsible for mediating elevated diseases resistance of the *Lb. plantarum* fed fish in the present study, not probiotic antagonism and competition as has previously been observed with these strains *in vitro* [19].

5. Conclusions

Fish fed diet containing *Lb. plantarum* had significantly lower mortality than fish fed diets containing *L. lactis*, *Leu. mesenteroides* and the control. PCR-DGGE analysis revealed no detectable populations present on the intestinal mucosal tissues in the respective groups. In the head kidney, IL-1β, IL-10 and TNF-α gene expression were significantly up-regulated by *Lb. plantarum*. Moreover, IL-10 gene expression was also significantly higher in the *Lb. plantarum* group after *L. garvieae* infection. In the intestine, IL-8 was up-regulated in the probiotic group prior and after infection, but only after the infection was IgT significantly elevated. In contrast, Tlr5 gene expression in the probiotic group was lower after infection than in the control group. These findings indicate that host

immunological responses were responsible for mediating elevated diseases resistance of the *Lb. plantarum* fed fish.

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References

[1] Vendrell D, Balcázar JL, Ruiz-Zarzuela I, de Blas I, Gironés O, Múzquiz JL. *Lactococcus garvieae* in fish: a review. Comp Immunol Microbiol Infect Dis 2006;29:177–98.
[2] Eldar A, Ghittino C. *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases. Dis Aquat Org 1999;36:227–31.
[3] Teixeira LM, Merquior VL, Vianni MC, Carvalho MG, Fracalanzza SE, Steigerwalt AG, et al. Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalo with subclinical mastitis and confirmation of *Lactococcus garvieae* as a senior subjective synonym of *Enterococcus seriolicida*. Int J Syst Bacteriol 1996;46:664–8.
[4] Fefer JJ, Ratzan KP, Sharp SE, Saiz E. *Lactococcus garvieae* endocarditis: report of a case and review of the literature. Diagn Microbiol Infect Dis 1998;32:127–30.
[5] Gomes S, Afonso A, Gartner F. Fish vaccination against infections by Streptococcal species and the particular case of Lactococcosis. RPCV 2006;101:25–35.
[6] Bednarska M, Bednarski M, Polechonski R. Current problems of streptococcal infections in fish. Med Weter 2007;63:783–5.
[7] Balcázar JL, de Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Múzquiz JL. The role of probiotics in aquaculture. Vet Microbiol 2006;114:173–86.
[8] Dimitroglou A, Merrifield DL, Carnevali O, Picchietti S, Avella M, Daniels C, et al. Microbial manipulations to improve fish health and production - A Mediterranean perspective. Fish Shellfish Immunol 2011;30:1–16.
[9] Reid G, Sanders ME, Gaskins HR, Gibson GR, Mercenier A, Rastall RA, et al. New scientific paradigms for probiotics and prebiotics. J Clin Gastroenterol 2003; 37:105–18.
[10] Irianto A, Austin B. Probiotics in aquaculture. J Fish Dis 2002;25:633–42.
[11] Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RTM, Bøgwald J, et al. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 2010;302:1–18.
[12] Pérez T, Balcázar JL, Ruiz-Zarzuela I, Halaihel N, Vendrell D, de Blas I, et al. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal Immunol 2010;4:355–60.
[13] Secombes CJ, Wang T, Hong S, Peddie S, Crampe M, Laing KJ, et al. Cytokines and innate immunity of fish. Dev Comp Immunol 2001;25:713–23.
[14] Gioacchini G, Smith P, Carnevali O. Effects of Ergosan on early immune response of rainbow trout (*Oncorhynchus mykiss*) juveniles to enteric red mouth (ERM) vaccine. Vet Imm Path 2008;123(3–4):215–22.
[15] Mulder IE, Wadsworth S, Secombes CJ. Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*. Fish Shellfish Immunol 2007;23:747–59.
[16] Kim DH, Austin B. Cytokine expression in leucocytes and gut cells of rainbow trout, *Oncorhynchus mykiss* Walbaum, induced by probiotics. Vet Immunol Immunopathol 2006;114:297–304.
[17] Raida MK, Buchmann K. Development of adaptive immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum) surviving an infection with *Yersinia ruckeri*. Fish Shellfish Immunol 2008;25:533–41.
[18] Zhang YA, Salinas I, Li J, Parra D, Bjork S, Xu Z, et al. IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat Immunol 2011;11: 827–36.
[19] Raida MK, Buchmann K. Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. Dev Comp Immunol 2009;33:35–45.
[20] Pérez-Sánchez T, Balcázar JL, García Y, Halaihel N, Vendrell D, de Blas I, et al. Identification and characterization of lactic acid bacteria isolated from rainbow trout Oncorhynchus mykiss (Walbaum) with inhibitory activity against *Lactococcus garvieae*. J Fish Dis. (2011), doi:10.1111/j.1365-2761.2011.01260.x.
[21] Balcázar JL, de Blas I, Ruiz-Zarzuela I, Vendrell D, Calvo AC, Márquez I, et al. Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). Br J Nutri 2007;97: 522–7.
[22] Vendrell D, Balcázar JL, de Blas I, Ruiz-Zarzuela I, Gironés O, Múzquiz JL. Protection of rainbow trout (*Oncorhynchus mykiss*) from lactococcosis by probiotic bacteria. Comp Immunol Microbiol Infect Dis 2008;31:337–45.
[23] Zlotkin A, Eldar A, Ghittino C, Bercover H. Identification of *Lactococcus garvieae* by PCR. J Clin Microbiol 1998;36:983–5.
[24] Del Cerro A, Márquez I, Guijarro JA. Simultaneous detection of *Aeromonas salmonicida, Flavobacterium psychrophilum* and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. Appl Environ Microbiol 2002;68:5177–80.
[25] Amend DF. Potency testing of fish vaccines. Dev Biol Stand 1981;49:447–54.
[26] Merrifield DL, Davies SJ, Guroy D, Guroy B, Emery MJ, Llewellyn C, et al. Assessment of *Chlorogloeopsis* as a dietary supplement for red tilapia (*Oreochromis niloticus*). Aquaculture 2010;299:128–33.
[27] Brunt J, Austin B. Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 2005; 25:333–42.
[28] Panigrahi A, Kiron V, Satoh S, Hirono I, Kobayashi T, Sugita H, et al. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev Comp Immunol 2007;31:372–82.
[29] Zhang Z, Swain T, Bøgwald J, Dalmo RA, Kumari J. Bath immunostimulation of rainbow trout (*Oncorhynchus mykiss*) fry induces enhancement of inflammatory cytokine transcripts, while repeated bath induce no changes. Fish Shellfish Immunol 2009;26:677–84.
[30] Foligne B, Nutten S, Grangette C, Dennin V, Goudercourt D, Poiret S, et al. Correlation between *in vitro* and *in vivo* immunomodulatory properties of lactic acid bacteria. World J Gastroenterol 2007;13:236–43.
[31] Kagnoff MF, Eckmann L. Epithelial cells as sensors for microbial infection. J Clin Invest 1997;100:6–10.
[32] Tsoi S, Park KC, Kay HH, O'Brien TJ, Podor E, Sun G, et al. Identification of a transcript encoding a soluble form of toll-like receptor 5 (TLR5) in Atlantic salmon during *Aeromonas salmonicida* infection. Vet Immunol Immunopathol 2006;109:183–7.
[33] Zhang Z, Niu C, Storset A, Bøgwald J, Dalmo RA. Comparison of *Aeromonas salmonicida* resistant and susceptible salmon familias: a high immune response is beneficial for the survival against *Aeromonas salmonicida* Challenger. Fish Shellfish Immunol 2011;31:1–9.