

Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*)

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We studied the effect of several lactic acid bacteria (LAB) on the humoral response of brown trout (*Salmo trutta*). LAB groups (*Lactococcus* (*Lc.*) *lactis* ssp. *lactis*, *Lactobacillus* (*Lb.*) *sakei* and *Leuconostoc* (*Leu.*) *mesenteroides*) were administered orally at 10^6 colony-forming units/g feed to brown trout for 2 weeks, after which fish were switched to an unsupplemented feed. Blood and intestinal samples were taken from the onset of feeding supplemented diets at 1, 2, 3 and 4 weeks. During the LAB-feeding period, *Lc. lactis* ssp. *lactis*, *Lb. sakei* and *Leu. mesenteroides* persisted in the fish intestines, but the number of LAB slowly decreased in the intestines after changing to the unsupplemented diet. Only *Lb. lactis* ssp. *lactis* and *Leu. mesenteroides* were detected at levels above 1×10^2 colony-forming units/g at the end of the fourth week. In comparison to untreated control fish, the alternative complement activity in the serum was found to be significantly greater in all LAB groups at the end of the second week. Groups supplemented with *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* exhibited an elevated level of lysozyme activity at the end of the third week, but the group supplemented with *Lb. sakei* did not exhibit any significant change in lysozyme activity. Serum immunoglobulin levels were higher compared with the control group, but there was no significant difference between the LAB and control groups.

Probiotics: Brown trout: Lactic acid bacteria: Colonisation: Complement activity: Lysozyme activity: Immunoglobulin

Probiotics have been defined as a viable microbial food supplement that beneficially influences the health of the host (Reid *et al.* 2003). Several studies on probiotics have been published over the past decade. The methodological and ethical limitations of animal studies, however, make it difficult to understand the mechanisms of action of probiotics, and only partial explanations are available. Nevertheless, some possible benefits linked to the administration of probiotics include: (1) the restoration of a normal intestinal microbiota; (2) a contribution to the elimination of pathogenic bacteria; (3) a source of nutrients and enzymatic contribution to digestion. Other benefits are still being investigated, for example reinforcement of the capacity of the intestinal barrier against exogenous antigens and enhancement of the immune response against pathogenic micro-organisms; as well as antiviral effects (Gatesoupe, 1999; Verschuere *et al.* 2000; Irianto & Austin, 2002; Balcázar *et al.* 2006a; Vine *et al.* 2006).

The lactic acid bacteria (LAB) are a group of Gram-positive rod- and coccus-shaped organisms that are non-spore-forming and non-motile; they produce lactic acid as their major end product during carbohydrate fermentation. The use of these potential probiotic cultures stimulates the growth of preferred micro-organisms that outcompete potentially harmful bacteria and reinforce the organism's natural defence mechanisms.

The introduction of a selected bacterial strain such as LAB into the food chain or as a dietary supplement has been proposed as an alternative mode of improving the health of cultured organisms (Gildberg *et al.* 1997; Robertson *et al.* 2000; Nikoskelainen *et al.* 2001).

The role of LAB in inducing overall immunity has been extensively investigated and reviewed in endothermic animals and humans (Schiffrin *et al.* 1995; Gill & Rutherford, 2001; Shu & Gill, 2002; Fooks & Gibson, 2002). Few studies have, however, been directed at the immunological enhancement of fish defence mechanisms by probiotic bacteria.

Studies by our research group have recently demonstrated that *Lactococcus* (*Lc.*) *lactis* ssp. *lactis* CLFP 100, *Leuconostoc* (*Leu.*) *mesenteroides* CLFP 196 and *Lactobacillus* (*Lb.*) *sakei* CLFP 202 isolated from the intestines of healthy salmonids exhibit high adhesion to intestinal mucus, a competitive exclusion of fish pathogens and a high degree of resistance to pH 3.0 and 10% fish bile under *in vitro* conditions (Balcázar, 2006; Balcázar *et al.* 2006b). The aims of the current study were thus to investigate whether *Lc. lactis* ssp. *lactis* CLFP 100, *Leu. mesenteroides* CLFP 196 and *L. sakei* CLFP 202 could stimulate the humoral immune response of brown trout (*Salmo trutta*), and to determine their capacity for binding intestinal mucus in these fish.

Abbreviations: CFU, colony-forming unit; LAB, lactic acid bacteria; MRS, Man, Rogosa and Sharpe.

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Materials and methods

Bacterial strains

Three bacterial strains, *Lc. lactis* ssp. *lactis* CLFP 100, *Leu. mesenteroides* CLFP 196 and *Lb. sakei* CLFP 202, isolated from salmonids and genetically identified by 16S rRNA gene sequencing, were selected from a pool of 246 strains obtained from the intestinal content of healthy salmonids, because their *in vitro* characteristics suggested that they could be considered as potential fish probiotics (Balcázar, 2006; Balcázar *et al.* 2006b,c). They were grown aerobically in de Man, Rogosa and Sharpe (MRS) broth (Pronadisa, Madrid, Spain) at 22°C. Stock cultures stored at -80°C were prepared from overnight cultures grown in MRS to which 20% (v/v) glycerol (Prolabo, Fontenay, France) was added just prior to freezing.

Preparation of the feed

The three selected strains were grown overnight in MRS broth in a shaking incubator at 22°C. After incubation, the cells were harvested by centrifugation (2000g), washed twice with PBS (10 mM-Na₃PO₄, 150 mM-NaCl; pH 7.2) and resuspended in the same buffer. The absorbance at 600 nm was adjusted to 0.25 ± 0.05 in order to standardise the number of bacteria (10⁷–10⁸ colony-forming units (CFU)/ml). Dilution plating was used to verify the relationship between absorbance at 600 nm and CFU/ml.

Commercial feed (ProAqua Nutrition SA, Palencia, Spain) was taken as the basal diet to supplement the three selected strains. In order to reach a final concentration of 10⁶ CFU/g feed, bacterial suspensions were slowly added to the feed, mixing bit by bit in a drum mixer. The amount of LAB in each feed was determined by plate counting on MRS agar. The LAB concentration at 10⁶ CFU/g feed was selected because there is evidence that the best results in terms of preventing bacterial disease have been obtained using this concentration (Balcázar, 2006; Balcázar *et al.* 2006c).

Fish and experimental conditions

Brown trout (*S. trutta*) were obtained from a commercial fish farm. The fish were fed with a standard commercial feed at a rate of 1.5% of the biomass per day. The fish had not been vaccinated or exposed to fish diseases and were deemed pathogen-free by standard microbiological techniques and by a previously described multiplex PCR technique for the simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum* and *Yersinia ruckeri* (Del Cerro *et al.* 2002). The fish were acclimated for 1 week in tanks before the start of the trial. After the acclimation period, the average weight of the fish was 70 g, and the fish were divided into four 500-litre tanks, each containing 100 fish. All the fish were maintained in static aerated fresh water at 14 ± 1°C with a 25% water change every day and a 12 h dark/12 h light photoperiod during the entire trial period.

One group served as the control and was fed unsupplemented feed for the whole trial. The other three groups were fed with feed containing different LAB for 2 weeks (first and second weeks). The first group was fed a diet supplemented with 10⁶ CFU/g *Lc. lactis* ssp. *lactis*; the second group one

supplemented with 10⁶ CFU/g *Lb. sakei* and the last group a diet supplemented with 10⁶ CFU/g *Leu. mesenteroides*. The feed rate was 0.7% and 1% of the biomass during the first and second weeks, respectively. After 2 weeks of LAB-feeding, the feeds were changed to unsupplemented feed (third and fourth weeks), and the feed rate was kept at 1% of the biomass until the end of the trial.

Blood samples

Blood samples were taken from ten fish from each group at the end of weeks 1, 2, 3 and 4. The fish were killed by immersion in a tank containing tricaine methanesulfonate (MS-222; Syndel Laboratories Ltd, Vancouver, Canada) at a concentration of 150 mg/l water for 15 min. Blood was drawn from the caudal vein of the individual fish. The plasma and serum samples were separated using standard procedures. All samples were stored separately at -80°C prior to analysis. The plasma samples were used for total immunoglobulin analysis, and the serum samples for determining lysozyme and alternative complement activity.

Bacterial counts and specific identification of the lactic acid bacteria. The microbial analyses were carried out at five time points: before the trial and at the end of weeks 1, 2, 3 and 4. The fish were killed as described before and opened aseptically, and the whole intestine was removed. The intestine was dissected, and the contents were collected by carefully scraping with a rubber spatula; these were then weighed. The microbial analyses were performed by spreading appropriate dilutions in PBS from 10⁻¹ to 10⁻⁶ on a selective medium for LAB: MRS agar (De Man *et al.* 1960). The plates were incubated aerobically at 22°C for 2 days.

Specific identification through random amplification of polymorphic DNA was carried out by amplifying 20–30 colonies randomly selected from the MRS plates of 30–300 colonies. Bacterial cultures (1.0 ml) were homogenised in 200 µl Tris-EDTA buffer (pH 8.0; 10 mM-Tris, 1 mM-EDTA) and centrifuged at 12 000 g for 1 min, pellets being extracted with InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. DNA yield and purity were determined spectrophotometrically by measuring 260 nm:280 nm absorbance ratios (Gene Quant pro RNA/DNA Calculator; Amersham Pharmacia Biotec, Cambridge, UK).

The primers used for the random amplification of polymorphic DNA analysis of bacterial DNA have been previously described (Brousseau *et al.* 1993; Johansson *et al.* 1995; Barangou *et al.* 2002). Nine-mers were randomly designed with a G + C content of 80%. The primers used in this study were ED-01 (5'-ACGCGCCCT-3') and ED-02 (5'-CCGAGTCCA-3') (Sigma St. Louis, MO, USA). A total volume of 50 µl reaction mixture used for the random amplification of polymorphic DNA PCR analysis of probiotic bacteria contained 0.2 mM-dNTP, 2.5 mM-MgCl₂, 1.0 µM of each primer, PCR buffer (10 mM-Tris-HCl [pH 8.3], 50 mM-KCl), 1 U *Taq* polymerase (Invitrogen Corp, Carlsbad, CA, USA) and 5 µl DNA template. Amplification of DNA was performed in a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Corp., Wellesley, MA, USA) with the following conditions: an initial denaturation of 10 min at 94°C; four cycles of 45 s at 94°C, 2 min at 30°C and 45 s at 72°C; 36 cycles of 15 s at 94°C, 30 s at 36°C

and 45 s at 72°C; 10 min at 72°C. The DNA amplicons were separated on a 4% (v/v) acrylamide gel and compared with a 1 kb ladder (Bio-Rad Laboratories).

Alternative complement activity. The complement activity (alternate pathway) was assayed following the procedure of Yano (1992) using rabbit erythrocytes. Briefly, the rabbit erythrocytes were washed and adjusted to 2×10^8 cell/ml in 0.01 M-ethylene glycol tetraacetic acid–Mg–gelatin veronal buffer. The 100% lysis value was obtained by lysing 100 μ l rabbit erythrocytes with 3.4 ml distilled water and measuring the optical density of haemolysate at 414 nm against distilled water. The test serum was appropriately diluted, and different volumes ranging from 0.1 to 0.25 ml were made up to 0.25 ml total volume before being allowed to react with 0.1 ml rabbit erythrocytes in test tubes. After incubating at 20°C for 90 min with occasional shaking, 3.15 ml saline solution was added to each tube, and these were centrifuged at 1600g for 10 min at 4°C. The optical density of the supernatant was measured at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50% haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH50 value = units/ml).

Lysozyme activity. The turbidometric assay method, originally described by Parry *et al.* (1965) and modified for microtitre assay by Demers & Bayne (1997) was used. Serum (25 μ l/well) was placed in triplicate in a ninety-six-well plate, and 175 μ l of a suspension of *Micrococcus lysodeikticus* (75 mg/ml in 0.1 M-phosphate buffer with 0.09% (v/v) NaCl, pH 5.8) was added. After the plate had been shaken, the decrease in absorbance at 450 nm was recorded for 5 min. Lysozyme activities were converted to lysozyme concentrations using hen egg white lysozyme as a standard.

Total immunoglobulin. Plasma total immunoglobulin was measured by the method of Siwicki & Anderson (1993). Briefly, the total protein content in the plasma was determined by a microprotein determination method (C-690; Sigma), using bovine serum albumin (Sigma) as a standard. In addition, 500 μ l of each plasma sample was mixed with an equal volume of 12% (v/v) solution of polyethylene glycol (Sigma) at 4°C, and the protein content was determined as mentioned above. The difference between the protein values of the untreated and the polyethylene glycol-treated samples corresponded to the total immunoglobulin content and was expressed in mg/ml.

Statistical analysis

ANOVA and Duncan's multiple range tests were used to determine whether there were significant differences ($P < 0.05$) between treatments. All statistics were performed using SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Microbiological observations

Before the trial, the fish had no detectable *Lc. lactis* ssp. *lactis*, *Lb. sakei* and *Leu. mesenteroides* in their intestines. The number of viable LAB increased rapidly in all LAB groups,

ranging from 2.9×10^5 to 1.2×10^6 CFU/g at the end of the first week and from 2.4×10^6 to 4.9×10^7 CFU/g at the end of the second week (Fig. 1). Moreover, the microbiological analysis of the organs (liver, kidney, spleen) revealed that it was safe to administer LAB to the fish. Following replacement of the LAB feed with unsupplemented feed, the number of LAB decreased slowly in all LAB groups. Only *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* could be detected at levels above 1×10^2 CFU/g at the end of the fourth week. It is important to point out that the control group had a low number of LAB (< 40 CFU/g), especially *Carnobacterium maltaromaticum* and *Lactobacillus curvatus*, at the end of the first week, but this amount fell to levels below the detection limit at the end of the second week.

Complement activity

The alternative complement activity (ACH 50) in the serum was significantly ($P < 0.05$) greater in all LAB groups at the end of the second week, but there was no significant difference ($P > 0.05$) between the LAB groups (Fig. 2). The ACH 50 level of the LAB groups remained higher than that of the control group at the end of the third week, after the LAB groups had received the unsupplemented diet for a week, but the difference was no longer statistically significant ($P > 0.05$). At the end of the fourth week, the ACH 50 value returned to the same level as in the control group.

Lysozyme activity

The serum lysozyme activity was significantly higher ($P < 0.05$) in the *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* groups at the end of the third week compared with the control group. However, only the *Lc. lactis* ssp. *lactis* group showed a statistical difference at the end of the fourth week (Fig. 3). It is important to point out that the group supplemented with *Lb. sakei* during the first 2 weeks did not exhibit any significant change in lysozyme activity compared with the control group throughout the trial.

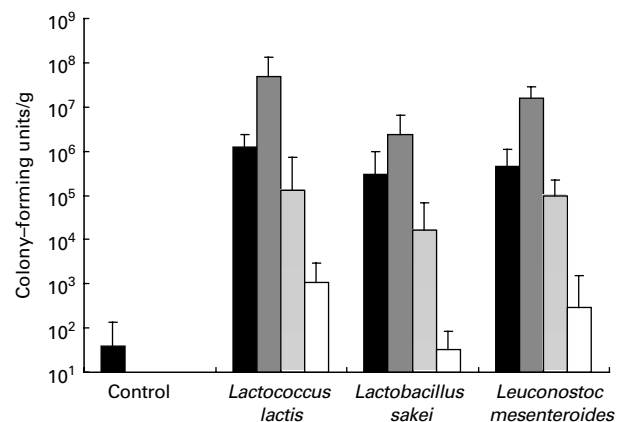


Fig. 1. The lactic acid bacteria (LAB) content of fish intestine at different time points in brown trout fed diets supplemented or not supplemented with probiotics. LAB counts were performed by spreading dilutions on MRS agar, and species were identified through random amplification of polymorphic DNA analysis. Values are means with standard deviations represented by vertical bars for ten fish. Week 1 (■); week 2 (□), week 3 (▣), week 4 (□).

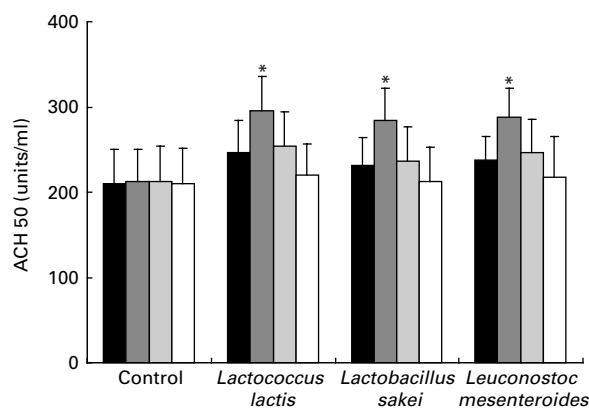


Fig. 2. Serum alternative complement activity (ACH 50) in brown trout fed diets supplemented or not supplemented with probiotics. Values are means with standard deviations represented by vertical bars for ten fish. *Mean values were significantly different: $P < 0.05$. Week 1 (■); week 2 (□), week 3 (▨), week 4 (□).

Immunoglobulin level

The plasma total immunoglobulin level in the LAB groups was found to be higher than that of the control group, especially in the groups that received diets containing *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* (Fig. 4). There was, however, no significant difference ($P > 0.05$) between the LAB and control groups.

Discussion

In the present study, we observed a correlation between colonisation with probiotic bacteria and non-specific humoral responses such as alternative complement pathway activity and lysozyme activity in brown trout. Previous studies comparing animals raised without exposure to any micro-organisms with animals that had been colonised with components of the microbiota have revealed a range of host functions affected by indigenous microbial communities. For example, the complex microbial ecology of the intestinal tract provides both nutritional benefit and protection against pathogens, and is vital in modulating interactions with the

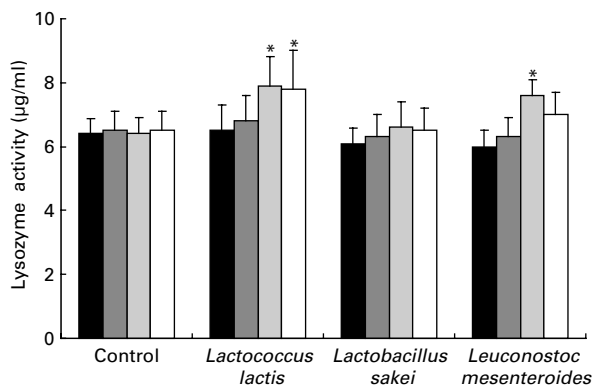


Fig. 3. Serum lysozyme activity in brown trout fed diets supplemented or not supplemented with probiotics. Values are means with standard deviations represented by vertical bars for ten fish. *Mean values were significantly different: $P < 0.05$. Week 1 (■); week 2 (□), week 3 (▨), week 4 (□).

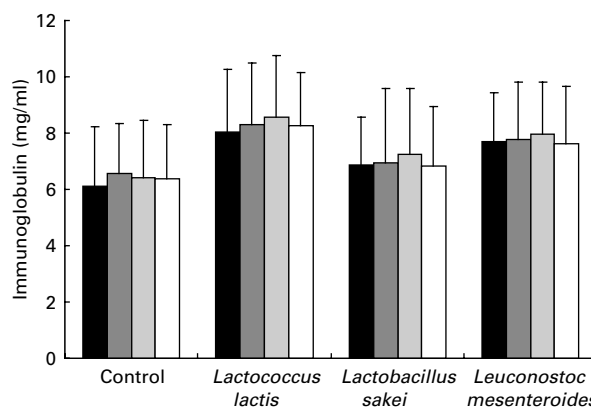


Fig. 4. Plasma total immunoglobulin (Ig) in brown trout fed diets or not supplemented with probiotics. Values are means with standard deviations represented by vertical bars for ten fish. Mean values were not significantly different: $P > 0.05$. Week 1 (■); week 2 (□), week 3 (▨), week 4 (□).

environment and the development of beneficial immune responses. This is because the microbiota directs the assembly of gut-associated lymphoid tissue (Cebra, 1999), helps to educate the immune system (Kelly *et al.* 2004), affects the integrity of the intestinal mucosal barrier (Hooper *et al.* 2003), modulates the proliferation and differentiation of its epithelial lineages (Bry *et al.* 1996), regulates angiogenesis (Stappenbeck *et al.* 2002), modifies the activity of the enteric nervous system (Husebye *et al.* 1994) and plays a key role in extracting and processing nutrients consumed in the diet (Hooper *et al.* 2002).

After 1 week of LAB-feeding in the present study, we were able to detect high amounts of LAB in the intestine, which demonstrates that *Lc. lactis* ssp. *lactis*, *Lb. sakei* and *Leu. mesenteroides*, isolated from the endogenous microbiota of salmonids, have a strong ability to adhere to and survive in the intestinal mucus. At the beginning of the study, we observed that all the fish were colonised with several *Aeromonas* species, particularly *Aeromonas hydrophila*, which constitutes a large component of the resident microbiota of freshwater fish (Gonzalez *et al.* 1999). LAB supplementation, however, demonstrated an ability to antagonise the resident microbiota, since the increase in LAB observed was possibly the result of a fall in intestinal pH induced by lactic acid or other fermented products produced by LAB strains. After changing the LAB-containing feed to unsupplemented feed, the number of LAB decreased slowly in the intestine, and only *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* were detected at levels above 1×10^2 CFU/g at the end of the fourth week.

At the end of the study, we observed, using microbiological and biochemical methods, that the intestinal microbiota in all groups was represented by motile Gram-negative rods (*Aeromonas* species) and Gram-positive micro-organisms, including coryneforms, micrococci and bacilli. The probiotic culture must therefore be administered continuously to fish to obtain a balance between the probiotic micro-organisms and the resident microbiota in the digestive tract, and thereby to exert its beneficial health effect. This result is in agreement with those of previous studies. Nikoskelainen *et al.* (2003) found that adding *Lactobacillus rhamnosus* ATCC 53 103 to the diet resulted in a high number of this strain in the intestines of rainbow trout,

but the number decreased when the diet was changed to unsupplemented feed. Direct comparisons between these probiotics are, however, difficult because these strains are functionally different and may be differently affected by various factors such as genetics, nutrition and the environment.

The level of alternative complement pathway activity, which is antibody independent, is very high in fish serum compared with mammalian serum (Yano, 1996), suggesting that this pathway is more important in fish than in mammals. The complement proteins have multifunctional roles in the defence against micro-organisms, ranging from the opsonisation, lysis and killing of bacteria, to chemotaxis and anaphylaxis (Goldstein, 1988). In the present study, the activation of the alternative complement system pathway at the end of the second week in all LAB groups may be attributed to the supplemented probiotics, confirming the benefit for the non-specific humoral defence system. This result is in agreement with observations by Nikoskelainen *et al.* (2003) that elevated levels of complement activity resulted from feeding *Lb. rhamnosus* to rainbow trout.

Lysozyme is a humoral non-specific defence protein widely distributed in nature, including fish (Lie *et al.* 1989). Although its exact physiological role is not yet understood, there is a general acceptance that lysozyme is involved in the defence against micro-organisms. Lysozyme hydrolyses *N*-acetylmuramic acid and *N*-acetylglucosamine, which are constituents of the peptidoglycan layer of bacterial cell walls. Lysozyme activity in fish serum has been reported to increase after injecting a bacterial product (Chen *et al.* 1996), in response to bacterial infection (Møyner *et al.* 1993) and after probiotic supplementation (Panigrahi *et al.* 2004).

Panigrahi *et al.* (2004) showed significantly higher serum lysozyme activity in rainbow trout fed with *Lb. rhamnosus* JCM 1136 at 10^9 CFU/g feed for 30 d. In this study, the groups that had been supplemented with *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* during the first 2 weeks exhibited an elevated level of lysozyme activity at the end of the third week, but only the *Lc. lactis* ssp. *lactis* group maintained an increased activity level at the end of the fourth week. In contrast, the *Lb. sakei* group did not exhibit any significant change in lysozyme activity compared with the control during the trial. Variations in lysozyme activity appear to be related to the ability of probiotic strains to adhere to the intestinal mucus. We have previously shown that *Lc. lactis* ssp. *lactis* and *Leu. mesenteroides* can be recovered in higher numbers than *Lb. sakei* from the intestinal contents, suggesting that the bacteria can proliferate in the intestinal mucus, where they can interact with epithelial cells.

Immunoglobulins are well recognised to provide disease protection in animals and humans, and several studies have demonstrated the effect of LAB on enhancing immunoglobulin. The oral administration of LAB has been found to increase immunoglobulin A in the intestine and protect mice against *Salmonella typhimurium* (Perdigon *et al.* 1990). In addition, the administration of *Lb. rhamnosus* at 2.8×10^8 CFU/g into the diet of rainbow trout has been reported to increase the level of immunoglobulin after 1 week (Nikoskelainen *et al.* 2003). We did not examine the specific antibody response, but we observed that probiotic feeding resulted in higher total immunoglobulin levels in brown trout, although the differences were not statistically significant.

In conclusion, the ability of *Lc. lactis* ssp. *lactis*, *Lb. sakei* and *Leu. mesenteroides* to modify the intestinal microbiota and stimulate the humoral immune response as potential probiotic strains was elucidated in the present study. The selected probiotic strains of fish origin are safe and capable of surviving and temporarily colonising the fish intestinal mucus, as well as antagonising the resident microbiota. They could therefore be a useful alternative to chemotherapeutic treatment to promote fish health, since the high consumption of chemotherapeutic agents in aquaculture can alter the intestinal microbiota and induce resistant populations of bacteria, with unpredictable long-term effects on public health.

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