

# Non-specific Immune Response of Rainbow Trout (*Oncorhynchus Mykiss*) by Dietary Heat-inactivated Potential Probiotics

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**Background:** The effects of the dietary administration of two heat-inactivated whole bacteria from the *Vibrionaceae* family, singly or combined, on innate immune response of the rainbow trout were studied. The two bacteria (Pdp11 and 51M6), which were obtained from the skin of rainbow trout, showed in vitro characteristics that suggested they could be considered as potential fish probiotics. **Methods:** The fish were fed four different diets: control (non-supplemented), or diets supplemented with heat-inactivated bacteria at  $10^8$  cfu/g Pdp11,  $10^8$  cfu/g 51M6 or with  $0.5 \times 10^8$  cfu/g Pdp11 plus  $0.5 \times 10^8$  cfu/g 51M6 for 4 weeks. Six fish were sampled at weeks 1, 2, 3 and 4, and then the main humoral (natural haemolytic complement activity and serum peroxidase content) and cellular innate immune responses (leucocyte peroxidase content, phagocytosis, respiratory burst and cytotoxicity) were evaluated. **Results:** The serum peroxidase content and the natural haemolytic complement activity increased with time, reaching the highest values in the third and fourth weeks of feeding, respectively. The phagocytic ability of specimens fed the mixture of the two inactivated bacteria was significantly higher than in the controls after 2 and 3 weeks of treatment. The same activity increased significantly in rainbow trout fed the Pdp11 diet for 2 weeks or the 51M6 diet for 3 weeks. Respiratory burst activity was unaffected by all the experimental diets at all times assayed. Cytotoxic activity had significantly increased after 3 weeks in fish fed the 51M6 diet. **Conclusion:** Our results demonstrated the usefulness of incorporating inactivated probiotic bacteria into fish diets.

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## INTRODUCTION

Probiotics are defined as live microbial preparations that improve the health and well-being of the host (1-3). Recent works have studied the use of probiotics for aquacultural use (4-8). However, criteria have been limited to disease resistance and the in vitro inhibition of pathogenic bacteria while there are few data concerning their effects on the host immune responses (9-12).

Releasing the cells of live bacteria into fish pens or cages poses a potential risk to wild aquatic organisms since the bacteria may escape into the environment at large. The use of inactivated bacteria clearly solves such safety-related issues since they can no longer interact with other aquatic organisms. Probiotics have, indeed, been redefined as microbial complements, not necessarily alive, that have beneficial effects on host health (13,14). However, studies concerning the effects of dead probiotics on fish health are very scarce (15,16).

The aim of the present work was to assess the effects of the dietary administration of two heat-inactivated whole bacteria on the rainbow trout innate immune response. The two assayed bacteria (Pdp11 and 51M6) were selected as candidate probiotics from rainbow trout skin from a pool of 49 isolates taken from skin, gills and gut. They were characterized according to their adhesive ability to fish mucus, their antagonistic activity against fish pathogens, and their resistance to fish bile and mucus. In addition, Pdp11 and 51M6 inhibit adhesion of the pathogen *Photobacterium damsela* ssp. *Piscicida* to fish mucus by competitive exclusion (17).

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Although these *in vitro* characteristics suggest their potential usefulness as fish probiotics, their *in vivo* immune-modulatory properties have never been described. Therefore, the present paper evaluates the immunomodulatory effects of the dietary administration, singly or combined, of these two potential probiotics, after inactivation by heat, on the rainbow trout innate immune system.

## MATERIALS AND METHODS

### Bacteria

Two bacteria from the *Vibrionaceae* family, isolated from rainbow trout skin, Pdp11 and 51M6, were selected from a pool of 49 isolates obtained from skin, gills and intestine of rainbow trout, because their *in vitro* characteristics suggested they could be considered as potential fish probiotics (17).

Tubes containing 5 ml of trypticase soy broth (Oxoid) supplemented with 1.5% NaCl (TSBs) were inoculated with bacteria (Pdp11 or 51M6) of one colony from 24 h culture on trypticase soy agar (Oxoid) supplemented with 1.5% NaCl (TSAs) and incubated at 22°C with continuous shaking for 24–48 h. Bacterial cells were harvested in sterile phosphate-buffered saline (PBS, pH 7.4) and the number of bacterial cells present per ml of culture media of such aliquots was measured by using a Z2 Coulter Particle Counter (Beckman Coulter, USA) to adjust the required concentration. Both bacterial cultures were heat-inactivated for 1 h at 60°C.

### Fish and experimental design

One hundred specimens (65 g mean weight) of the rainbow trout (*Oncorhynchus mykiss*) obtained from a fish farm placed near Kunsan, Korea. The fish were kept in four fiber glass flow-through underground fresh water tanks (25 fish per tank) of 450 L (flow rate 1,500 L/h) at 20°C with a 12 h dark: 12 h light photoperiod and fed with a commercial pellet diet. Fish were allowed to acclimatize for 15 days before the start of feeding trials. Before sampling, they were starved for 24 h. Specimens were sacrificed by an overdose of benzocaine (4% in acetone) (Sigma), weighed and measured.

Experimental diets containing 0 (control), heat-inactivated Pdp11, heat-inactivated 51M6 or both killed bacteria, which showed no bacterial colony after inactivation, were prepared in the laboratory from the commercial pellet diet. Briefly, the normal pellet diet was crushed and mixed with tap water before adding the bacterial suspensions at the desired concentration, and then made into pellets again. The re-made pellets

were allowed to dry and were stored at 4°C until use.

The fish in each aquarium received one of the four different diets: a diet consisting of the non-supplemented commercial diet (control group); the same diet supplemented with  $10^8$  cfu/g heat inactivated Pdp11; a diet supplemented with  $10^8$  cfu/g heat-inactivated 51M6 and, finally, the fourth group received a diet supplemented with  $0.5 \times 10^8$ /g Pdp11 and  $0.5 \times 10^8$  cfu/g 51M6, both heat-inactivated. Fish were fed at a rate of 10 g dry diet kg/biomass (1%) per day for 1, 2, 3 or 4 weeks. The biomass of the fish in each aquarium was measured before the experiment and daily ration was adjusted accordingly. No mortality was observed during the experiment.

### Serum collection and leucocyte isolation

Six fish from each aquarium were randomly sampled at 1, 2, 3 and 4 weeks after the beginning of the feeding trial. Blood and head kidney (HK) samples were obtained from each specimen and several immunological parameters were determined, as described below. Blood samples were collected from the caudal vein with a 27-gauge needle, 1 ml syringe and allowed to clot at 4°C for 4 h. After centrifugation, the serum was removed and frozen at  $-80^\circ\text{C}$  until used to assess natural hemolytic complement activity and the peroxidase level. HK leucocytes were isolated under sterile conditions as described previously (18). Briefly, the head kidney was cut into small fragments and transferred to 8 ml of sRPMI medium: RPMI 1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to rainbow trout plasma osmolarity, 353.33 mOs), 100 IU/ml penicillin (Flow), 100  $\mu\text{g}/\text{ml}$  streptomycin (Gibco), 10 IU/ml heparin (Sigma) and 5% fetal bovine serum (Gibco). Cell suspensions were obtained by forcing fragments of the organ through a 100  $\mu\text{m}$  nylon mesh. After two washes (400 g, 10 min, 4°C), HK leucocytes were counted in a Neubauer chamber and adjusted to  $10^7$  cells/ml of sRPMI.

### Natural hemolytic complement activity

The activity of the alternative complement pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets (19). SRBC were washed in phenol red-free Hank's buffer (HBSS) containing  $\text{Mg}^{2+}$  and ethylene glycol-*O,O'*-bis-[2-amino-ethyl]-*N,N,N',N'*-tetraacetic acid (EGTA) and re-suspended at 3% (v/v) in HBSS. Aliquots of 100  $\mu\text{l}$  test serum as complement source, serially diluted in HBSS containing  $\text{Mg}^{2+}$  and EGTA to give final serum concentrations rang-

ing from 10% incubation for 90 min at 22°C, the samples were centrifuged at 400 g for 5 min at 4°C to remove unlysed erythrocytes. The relative hemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a spectrophotometer (BMG, Fluoro Star Galaxy). The values of maximum (100%) hemolysis were obtained by adding 100  $\mu$ l of distilled water to 100  $\mu$ l samples of SRBC and minimum (spontaneous) hemolysis was obtained from DRBC without serum.

The degree of hemolysis (Y) (percentage of hemolytic activity with respect to the maximum) was estimated and the lysis curve for each specimen was obtained by plotting Y/(1-Y) against the volume of serum added (ml) on a log scaled graph. The volume of serum producing 50% hemolysis (ACH<sub>50</sub>) was determined for each experimental group.

#### Peroxidase content

The total peroxidase content present in serum or inside leucocytes was measured according to Quade and Roth (20). Briefly, 15  $\mu$ l of serum was diluted with 35  $\mu$ l of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS in flat-bottomed 96-well plates. Then, 50  $\mu$ l of 20 mM 3,3', 5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H<sub>2</sub>O<sub>2</sub> (Sigma) were added (both substrates of peroxidase). To estimate the leucocyte peroxidase content, 10<sup>6</sup> HK leucocytes in sRPMI per well were dispensed into round-bottomed 96-well plates. The plates were centrifuged (400 g, 10 min) and the supernatants were removed. Leucocytes were then lysed with 75  $\mu$ l of 0.02% cetyltrimethylammonium bromide (CTAB, Sigma) on a shaker at 40 cycles per min. Afterwards, 50  $\mu$ l of 10 mM TMB and 5 mM H<sub>2</sub>O<sub>2</sub> were added. Then, 150  $\mu$ l of serum was transferred from each well to new 96-well plates. In both cases, the color-change reaction was stopped after 2 min by adding 50  $\mu$ l of 2 M sulphuric acid and the optical density was read at 540 nm in a multiscan reader. Standard samples without serum or leucocytes, respectively, were also analyzed.

#### Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by rainbow trout HK leucocytes was studied by flow cytometry according to Rodriguez et al. (21). Heat-killed and lyophilized yeast cells were labeled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5  $\times$  10<sup>7</sup> cells/ml of sRPMI. Phagocytosis samples consisted of 125  $\mu$ l of labeled yeast cells and 100  $\mu$ l of HK leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centri-

fuged (400 g, 5 min, 22°C), resuspended in sRPMI and incubated at 22°C for 30 min. At the end of the incubation time, the samples were placed on ice to stop phagocytosis and 400  $\mu$ l ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40  $\mu$ l ice-cold trypan blue (0.4% in PBS). Standard samples of FITC-labelled *S. cerevisiae* or HK leucocytes were included in each phagocytosis assay. All samples were analyzed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 5000 cells, which were acquired at a rate of 300 cells/s. data were collected in the form of FSC, SSC and green fluorescence (FL1). The cytometer was set to analyze the phagocyte population identified by their high FCS and SSC. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (green-FITC fluorescent cells) within the phagocyte cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells.

#### Respiratory burst activity

Respiratory burst activity of founder kidney cells was assessed by monitoring their ability to reduce nitroblue tetrazolium (22). Briefly, leucocytes (10<sup>5</sup>) were washed one time with HBSS at 1,000 rpm for 3 min at 4°C and incubated in 100  $\mu$ l of complete media in the presence of phorbol myristate acetate (PMA, 1  $\mu$ g/ml) and NBT (mg/ml). After 1 hr at 25°C, excess amount of NBT was washed out with PBS and the leucocytes were fixed with 70% methanol. After discarding 70% methanol, the leucocytes were washed twice with PBS. The reduced formazan was solubilized with 120  $\mu$ l KOH and 140  $\mu$ l dimethyl sulphoxide (DMSO) and optical density values were read at 620 nm in an ELISA reader (ASYS HITECH, Austria).

#### Natural cytotoxic activity

The natural cytotoxic activity of rainbow trout HK leucocytes was evaluated using a flow cytometry technique based on a double-fluorescent labeling (23). Each cytotoxic assay was carried out in duplicate. L-1210 tumor cells (mouse lymphoma, ATCC CCL-219) were cultured in sRPMI-1640 culture medium. They were incubated at 37°C, with 85% relative humidity and 5% CO<sub>2</sub> atmosphere and maintained in exponential growth. Tumor cells were labeled by incubating with 10  $\mu$ g/ml of 3, 3' diocetadecyloxycarbocyanine perchlorate (DiO, Sigma) for 90 min in dark condition. After la-

belonging, free DiO was removed by washing three times in PBS and cell staining uniformity was examined by flow cytometry. Leucocytes ( $10^7$  cell/ml) in sRPMI (effectors) were mixed with DiO-labelled L-1210 cells ( $10^6$  cells/ml) (targets) to obtain a final effector:target ratio of 50:1. Samples were centrifuged (400 g, 1 min, 22°C) and incubated at 22°C for 3 h. Cytotoxic samples incubated for 0 h (control) were used to determine initial target viability. At the end of the incubation period, 30  $\mu$ l of propidium iodide (400  $\mu$ g/ml, PI, Sigma) was added to each sample and mixed gently before analysis in a FACScan (Becton Dickinson) flow cytometer adjusted to obtain optimal discrimination of the target cell population. Data were collected in the form of two parameter FSC, SSC, FL1 (green fluorescence, DiO) and FL2 (red fluorescence, PI) dot plots and histograms. Each analysis was performed on 3000 cells, which were acquired at a rate of 300 cell/ml. The FACS only accepted the positive FL1 region, which corresponded to DiO-labelled target cells. The percentage of dead or non-viable target cells showing green and red fluorescence was related to the cytotoxic activity of rainbow trout leucocytes. Cytotoxic activity, a parameter describing the percentage of non-viable target cells, was calculated using the formula: Cytotoxic activity (%) =  $100 \times (\% \text{ sample} - \% \text{ control}) / (100 - \% \text{ control})$ .

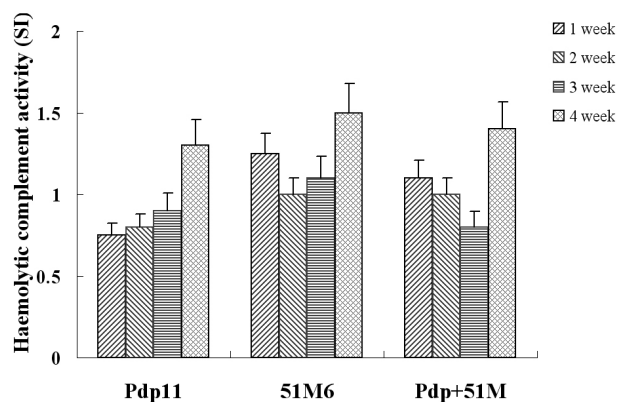
### Statistical analysis

The results are expressed as the stimulation index (mean + standard error, SE), which was obtained by dividing each sample value by the mean control value at the same sampling point for each measured parameter. Values higher than 1 reflect an increase and lower than 1 a decrease in each parameter. The data from the flow cytometric assays were analyzed using the statistical option of the Lysis Software Package (Becton Dickinson). Data were statistically analyzed by one-way analysis of variance (ANOVA) and Turkey's comparison of means when necessary. Differences were considered statistically significant when  $p < 0.05$ .

## RESULTS

### Natural haemolytic complement activity

The supplementation of rainbow trout diet with heat-inactivated Pdp11, 51M6 or both bacteria resulted in a similar pattern of effects upon natural haemolytic complement activity in serum. For all the experimental diets, the highest activity was always recorded at week 4, although the observed



**Fig. 1.** Natural haemolytic complement activity measured as optical density at 550 nm from rainbow trout specimens fed diets containing  $10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11,  $10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 or  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11 and  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 for 1 week, 2 weeks, 3 weeks or 4 weeks. Results are expressed as stimulation index (mean + SE;  $n = 6$ ) obtained by dividing each sample value by its mean control value.

differences were not statistically significant (Fig. 1).

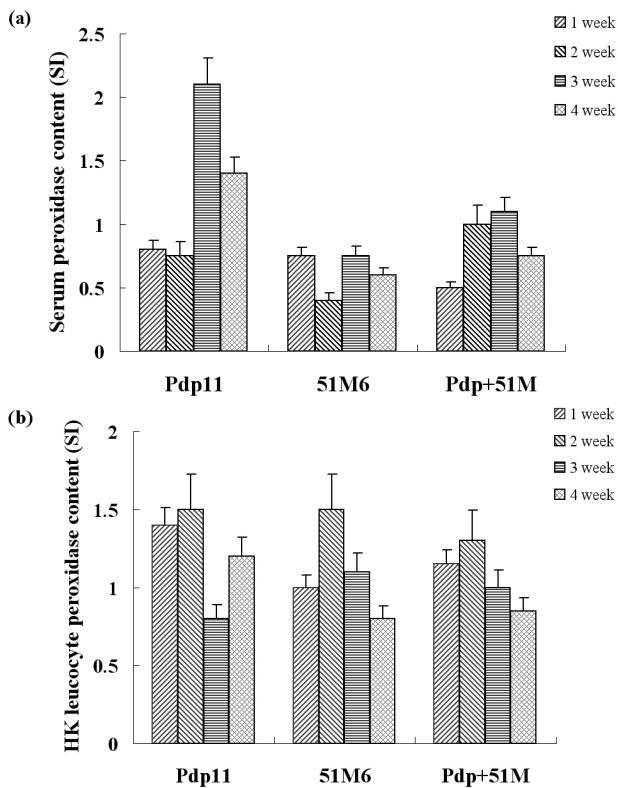
### Peroxidase content

Serum peroxidase content from fish fed the bacteria-supplemented diets was not statistically different from that of control fish at any given time of the trial (Fig. 2A). In general, the lowest serum peroxidase contents were found at weeks 1 and 2 for all the experimental diets. The highest peroxidase content values were reached after the third week, especially in the group that received the diet containing Pdp11.

Supplementation of rainbow trout diet with heat-inactivated Pdp11, 51M6 or both bacteria did not result in significant variations in the peroxidase content of the head kidney leucocytes (Fig. 2B). The highest values were observed after the second week, although the differences were not statistically significant.

### Phagocytic activity

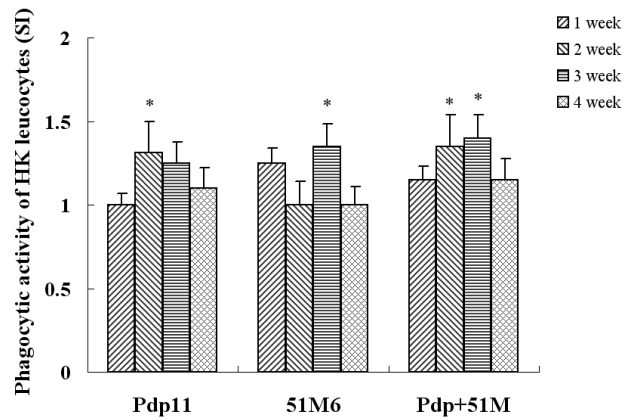
Phagocytosis was the most affected activity of all the assayed immune parameters. The phagocytic ability of head kidney leucocytes was significantly increased after 2 weeks feeding with the Pdp11 supplemented diet, although this activity fell in a time-dependent manner after 3 or 4 weeks (Fig. 3). Similar results were obtained from the group fed the 51M6 supplemented diet, although the increases were not statistically significant until the third week of the treatment. However, when fish received the diet supplemented with



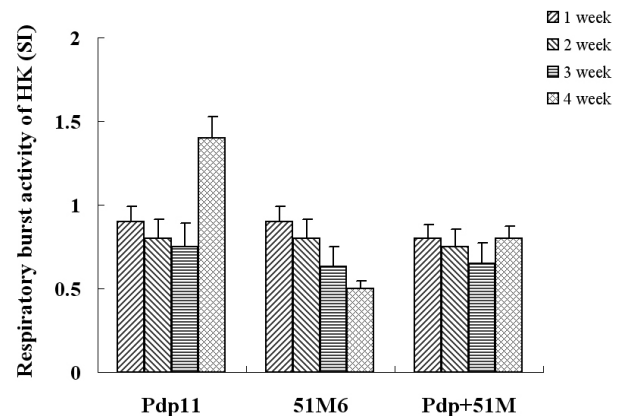
**Fig. 2.** Serum peroxidase content (a) and head kidney leucocyte peroxidase content (b) measured as the optical density at 450 nm from rainbow trout specimens fed diets containing  $10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11,  $10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 or  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11 and  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 for 1 week, 2 weeks, 3 weeks, or 4 weeks. Results are expressed as stimulation index (mean + SE;  $n=6$ ) obtained by dividing each sample value by its mean control value.

both bacteria, increases were already statistically significant after 2 weeks of feeding and remained statistically higher than the control at week 3. No statistically significant differences were detected in the phagocytic ability of specimens fed any experimental diet for 4 weeks with respect to that of the control group.

No statistically significant differences were found in the phagocytic capacity of leucocytes between the control and any of the inactivated bacteria-supplemented diets at any time (data not shown). Although not significant, the highest values were recorded from the groups fed the two individual bacteria-supplemented diets, Pdp11 and 51M6, in the second and first weeks of treatment, respectively.



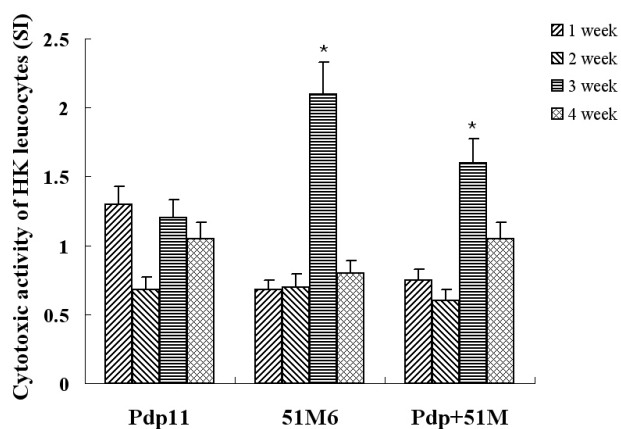
**Fig. 3.** Phagocytic ability of head kidney leucocytes from rainbow trout specimens fed diets containing  $10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11,  $10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 or  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11 and  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 for 1 week, 2 weeks, 3 weeks or 4 weeks. Results are expressed as stimulation index (mean + SE;  $n=6$ ) obtained by dividing each sample value by its mean control value. Symbol \* denotes statistically significant differences ( $p < 0.05$ ) with respect to the control group.



**Fig. 4.** Respiratory burst activity of head kidney from rainbow trout specimens fed diets containing  $10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11,  $10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 or  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11 and  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 for 1 week, 2 weeks, 3 weeks or 4 weeks. Results are expressed as stimulation index (mean + SE;  $n=6$ ) obtained by dividing each sample value by its mean control value.

### Respiratory burst activity

No statistically significant differences were found in the respiratory burst activities of rainbow trout head kidney leucocytes between the control and inactivated bacteria-supplemented diets at any time of the experiment (Fig. 4). The greatest increase in this parameter occurred at the fourth week in the group that received the heat-inactivated Pdp11 supplemented



**Fig. 5.** Cytotoxic activity of head kidney leucocytes from rainbow trout specimens fed diets containing  $10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11,  $10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 or  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated Pdp11 and  $0.5 \times 10^8$  cfu  $g^{-1}$  heat-inactivated 51M6 for 1 week, 2 weeks, 3 weeks or 4 weeks. Results are expressed as stimulation index (mean+SE; n=?6) obtained by dividing each sample value by its mean control value. Symbol \* denotes statistically significant differences ( $p < 0.05$ ) with respect to the control group.

diet.

### Natural cytotoxic activity

The dietary administration of heat-inactivated Pdp11 did not cause any significant variations in the cytotoxic activity of HK leucocytes. Fish fed either of the two diets containing heat-inactivated 51M6 showed no significant differences in the cytotoxic activity of HK leucocytes during the first 2 weeks of treatment but administration of these two diets for 3 weeks significantly increased the cytotoxic activity of HK leucocytes, although such enhancement was not observed after 4 weeks of treatment (Fig. 5).

## DISCUSSION

Studies on how the fish immune system is affected upon probiotic stimulation are scarce. In this work, we have evaluated both the humoral and cellular innate immune responses of rainbow trout fed for 1, 2, 3 or 4 weeks with heat-inactivated bacteria-supplemented diets.

Despite the existence of previous reports revealing the significant positive effect of different probiotic bacteria in the level of complement (24), all the experimental diets in this study led to increased complement activity although differences were not statistically significant compared with the control group. The finding that the highest values were reached

at the end of the experiment agrees with results found by Panigrahi et al. (24) in rainbow trout fed live *Lactobacillus rhamnosus*.

The other humoral immune parameter that was assayed, the peroxidase content in serum, was not significantly affected by any experimental diet at any time of the trial. The highest levels of peroxidase were observed during the third week, especially in rainbow trout that received the diet containing Pdp11. On the other hand, the peroxidase content of leucocytes responded in different ways, with values that peaked in the second week in the groups fed the single bacterial diets, although the differences with respect to control group were not statistically significant. Nevertheless, Salinas et al. (12) reported a significant decrease in the leucocyte peroxidase content in the third week in fish fed live *Lactobacillus delbrückii* ssp. *lactis* and *Bacillus subtilis*-supplemented diets.

Phagocytic activity was significantly enhanced in rainbow trout fed Pdp11 at week 2 but the enhancement occurred a week later in the groups fed 51M6 or the mixture of bacteria. These results suggest that in order to enhance phagocytic activity by using dietary 51M6, longer administration periods are required than when Pdp11 supplemented diets are provided. Increased phagocytic activity in the head kidney leucocytes of fish fed live probiotic bacteria-supplemented diets (24,25) and killed bacteria-supplemented diets has been reported by other authors (15,16). However, *Lactococcus lactis* supplementation did not affect the phagocytic activity of turbot head kidney macrophages after 1 week of daily administration (26). rainbow trout fed live *L. delbrückii* ssp. *lactis*, *B. subtilis*, or both in equal amounts, showed a more sustained stimulatory effect in their phagocytic activity of head kidney phagocytes, the mixture of both bacteria again having the greatest stimulatory effect (12).

Although phagocytic activity showed a significant increase, no statistically significant differences were found in the respiratory burst activity of rainbow trout head kidney leucocytes between control and treated fish at any time of the experiment. Supplementation with Pdp11 provoked the greatest stimulation after 4 weeks of administration.

These results lead us to conclude that the bacterium with the greater stimulatory effect on the rainbow trout innate immune system is Pdp11, since specimens fed this bacteria possessed higher levels of serum peroxidase and their HK leucocytes showed higher levels of phagocytic and respiratory burst activities. Despite the fact that Pdp11 and 51M6 belong

to the same family (*Vibrionaceae*), their immunostimulant effects are rather different, with 51M6 mainly affecting cytotoxic activity. On the other hand, the immunomodulatory effects caused by Pdp11 administration were faster than those prompted by 51M6. This time-course difference was well illustrated by the changes recorded in the phagocytic activity of HK leucocytes from each of these experimental groups. While, phagocytic ability was significantly greater with respect to the control group after the second week of being fed Pdp11 and the mixture of the two bacteria, it was not until week 3 that a stimulatory response was observable in the group fed 51M6. More importantly, the combination of both bacteria resulted in higher phagocytic activities at week 3, probably due to the presence of 51M6.

The present study investigated the effects of two mono-strain probiotics and one multistrain formulation. Timmerman et al. (27) concluded that multistrain probiotics (more than one strain of the same species or closely related species) and/or multispecies probiotics could be more effective and more consistent than that of a monostrain probiotic, since mixed cultures may contain bacteria that complement each other's health effect and thus have synergistic probiotic properties. The two bacteria assayed in the present work had no synergistic immunostimulatory effects, which could be due to the close systematic relationship between them. On the contrary, Salinas et al. (12) reported synergistic effects between *Lactobacillus* spp. and *Bacillus* spp., which do not belong to the same family.

Finally, it is worth pointing out that most of the innate immune parameters assessed were enhanced in the third week of the trial, and so longer periods of feeding are not necessary. This agrees with Salinas et al. (12) and could be a consequence of the way of administration. It is known that the most effective method for administering immunostimulants to fish is by injection, whereas the efficacy of oral and immersion methods decreases with long term administration (27).

To conclude, the present results indicate that oral administration of heat-inactivated bacteria has stimulatory effects on the innate immune system of the rainbow trout, with Pdp11 being more effective than 51M6. It is clear that bacteria isolated from fish surfaces, such as Pdp11 and 51M6, are capable of modulating the rainbow trout innate immune system. The possibility of more potent effects being observed when these bacteria are administered as live cells awaits confirmation.

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## REFERENCES

1. Gatesoupe FJ: The use of probiotics in aquaculture. *Aquaculture* 189;147-165, 1999
2. Verschuere L, Rombaut G, Sorgeloos P, Verstraete W: Probiotic bacteria as biological control agents in aquaculture. *Microbiol & Mol Biol Rev* 64;655-671, 2000
3. Schrezenmeir J, de Vrese M: Probiotics, prebiotics and symbiotics - approaching a definition. *The Amer J of Clin Nut* 73;361-364, 2001
4. Bly JE, Quiniou SMA, Lawson LA, Clem LW: Inhibition of *Saprolegnia* pathogenic for fish by *Pseudomonas fluorescens*. *J of Fish Dis* 20;35-40, 1997
5. Gómez-Gil B, Roque A, Tumbull JF: The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. *Aquaculture* 191;259-270, 2000
6. Spanggaard B, Huber I, Nielsen J, Sick EB, Pipper CB, Martinussen: The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. *Environ Microbiol* 3;755-765, 2001
7. Raida MK, Larsen JL, Nielsen ME, Buchmann K: Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). *J of Fish Dis* 26;495-498, 2003
8. Al-Harbi AH, Naim Uddin M: Seasonal variation in the intestinal bacterial flora of hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia. *Aquaculture* 229;37-44, 2004
9. Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius EM: Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish & Shellfish Immunol* 15;443-452, 2003
10. Villamil L, Figueras A, Planas M, Novoa B: Control of *Vibrio alginolyticus* in *Artemia* culture by treatment with bacterial probiotics. *Aquaculture* 219;43-56, 2003
11. Panigrahi A, Kiron V, Puangkaew J, Kobayashi T, Satoh S, Sugita H: The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 243;241-254, 2005
12. Salinas I, Cuesta A, Esteban MA, Meseguer J: Dietary administration of *Lactobacillus delbrückii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish & Shellfish Immunol* 19; 67-77, 2005
13. Naidu AS, Bidlack WR, Clemens AR: Probiotic spectra of lactic acid bacteria (LAB). *Critic Rev in Food Sci & Nut* 39;13-129, 1999
14. Salminen S, Ouwehand AC, Benno Y, Lee YK: Probiotics:

- how should they be defined? Trends in Food Sci Technol 10;107-110, 1999
15. Irianto A, Robertson PA, Austin B: Oral administration of formalin-inactivated cells of *Aeromonas hydrophila* A3-51 controls infection by atypical *A. salmonicida* in goldfish, *Carassius auratus* (L.). J of Fish Dis 26;117-120, 2003
  16. Irianto A, Austin B: Use of dead probiotic cells to control furunculosis in rainbow trout, *Onchorhynchus mykiss* (Walbaum). J of Fish Dis 26;59-62, 2003
  17. Chabrillón M, Rico RM, Balebona MC, Morifiño M: Adhesion to sole *Solea senegalensis* Kaup, mucus of microorganisms isolated from farmed fish, and their interaction with *Photobacterium damsela* subsp. *Piscicida*. J of Fish Dis 28;229-237, 2005
  18. Esteban MA, Mulero V, Muñoz J, Meseguer J: Methodological aspects of assessing phagocytosis of *Vibrio anguillarum* by leucocytes of gilthead seabream (*Sparus aurata* L.) by flow cytometry and electron microscopy. Cell & Tissue Research 293;133-141, 1998
  19. Ortuño J, Esteban MA, Mulero V, Meseguer J. Methods for Studying the Haemolytic, Chemoattractant and Opsonic Activities of Seabream (*Sparus aurata* L.) Serum, Methodology in Fish Diseases Research. Albion Press, Aberdeen, United Kingdom; 1998
  20. Quade MJ, Roth JA: A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet Imm & Immunopathol 58;239-248, 1997
  21. Rodríguez A, Esteban MA, Meseguer J: Phagocytosis and peroxidase release by seabream (*Sparus aurata* L.) leucocytes in response to yeast cells. Anatomical Record A 272; 415-423, 2003
  22. Secombes CJ, Chung S, Jeffries AH: Superoxide anion production by rainbow trout macrophages detected by the reduction of ferricytochrome C. Dev Comp Immunol 12; 201-206, 1998
  23. Cuesta A, Esteban MA, Meseguer J: Natural cytotoxic activity of gilthead seabream (*Sparus aurata* L.) leucocytes. Assessment by flow cytometry. Vet Imm & Immunopathol 71;161-171, 1999
  24. Panigrahi A, Kiron V, Kobayashi T, Puangkaew J, Satoh S, Sugita H: Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *Lactobacillus rhamnosus* JCM 1136. Vet Immunol & Immunopathol 102;379-388, 2004
  25. Irianto A, Austin B: Use of probiotics to control furunculosis in rainbow trout *Oncorhynchus mykiss* (Walbaum). J of Fish Dis 25;333-342, 2002
  26. Villamil L, Tafalla C, Figueras A, Novoa B: Evaluation of immunomodulatory effects of lactic acid bacteria in turbot (*Scophthalmus maximus*). Clin & Diag Lab Immunol 9;1318-1323, 2002
  27. Timmerman HM, Koning CJM, Mulder L, Rombout FM, Beynen AC: Monostrain, multistrain and multispecies probiotics - a comparison of functionality and efficacy. Inter J of Food Microbiol 96;219-233, 2004