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ORIGINAL PAPER

Live microbial feed supplement in aquaculture for improvement of stress tolerance

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Abstract Two bacterial strains, *Lactobacillus fructivorans*, isolated from sea bream (*Sparus aurata*) gut, and *Lactobacillus plantarum*, isolated from human faeces, were administered simultaneously, during sea bream development, using *Brachionus plicatilis* and/or *Artemia salina* as vectors. The probiotic treatment significantly affected gut colonization. To test the probiotic influence on stress responsiveness, sea bream fry, 47 days post-hatching (p.h.), were subjected to pH stress (from 8.6 to 6.3) and cumulative mortality, cortisol levels and HSP70 gene expression were analysed. Cortisol was selected, since under stress conditions its level increases. HSP70 was selected with consideration of its wide involvement in response to a great number of injuries, and because it protects cells probably by binding and refolding damaged proteins. The results obtained indicated that the administration of probiotic to sea bream fry induced higher HSP70 levels, indicating a greater potentiality to respond to the harmful conditions possibly present in fish farms. This hypothesis is supported by the fact that the levels of cortisol found were significantly lower ($P < 0.05$) in both groups under probiotic treatment. When pH was used as a stressor, it induced a higher cumulative mortality in the control; the mortality was found to be significantly lower in both treated groups. Interestingly, a significant increase ($P < 0.05$) in HSP70 gene expression was observed in all stressed groups. These results suggest an improvement in tolerance to acute stress of fry fed with probiotics.

Keywords Cortisol · Fish diet · HSP70 · *Lactobacillus* · Mortality · Sea bream

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Introduction

Physiological stress is one of the primary contributing factors of fish disease and mortality in aquaculture. Under natural conditions fish often experience brief periods of stress, bringing about a temporary disturbance of homeostasis (Van - Weerd and Komen 1998). This acute type of stress causes the fish to respond through two consecutive response levels. The primary response is of an endocrine nature with the main

purpose of mobilising energy. It is characterised by stimulation of the adrenergic system (leading to a rise in the catecholamines adrenaline and noradrenaline) and the hypothalamus–pituitary–inter-renal (HPI) axis (causing an increase in adrenocorticotropic hormone [ACTH] and cortisol). Catecholamines furnish energy in the short term (through glycogenolysis) whereas cortisol furnishes energy in the long term, by stimulating catabolism of glycogen, lipids and proteins. An important aspect of the secondary response is metabolic adaptation.

In intensive fish culture conditions, disturbances are usually of a prolonged nature and the accompanying chronic stress will lead to a continued loss of homeostasis, to which adaptation is not possible, or only after a long time (Schreck 1981). The stress response shifts from adaptive to maladaptive (Barton and Iwama 1991), eventually resulting in decreased disease resistance, impaired reproduction and reduced growth. Such whole body responses are regarded as the tertiary response level (Barton et al. 1987).

Most fish are very sensitive in their larval stages to any change in environmental conditions (Westernhagen 1988). Changes in water characteristics can increase the mortality rate during the first developmental stages; therefore, water quality is of special interest in aquaculture. The pH has been considered as an environmental factor that strongly affects survival during the first developmental stages (Kamler 1992). Sea water has a high buffer capacity, and the pH is maintained within a narrow range (7.5–8.4). Rearing methods can induce changes in water pH beyond the expected normal values of natural sea water. For example, the oxidation process of the fish excretory products in recirculation systems induces a pH decrease (Brownell 1980). In contrast, microalgae routinely added to rearing tanks induces an increase in pH due to their CO_2 consumption.

In addition to chemical–physical water changes, intestinal microbiota disorders caused by bacterial disease are also considered to be a major cause of mortality in most fish hatcheries. The indigenous microflora have several beneficial effects on the host. In addition to aiding in digestion and absorption of macromolecules, the flora acts as a barrier to gut pathogens by blocking their attachment to gut binding sites (Bernet et al. 1994; Coconnier et al. 1993), which is the first step of bacterial pathogenicity (Finlay and Falkow 1990). In addition to limiting pathogen attachment, several members of the indigenous microflora, including the Lactobacilli, produce bacteriocins that have antibacterial actions (Bernet-Camard et al. 1997; Daw and Falkiner 1996). Therefore, stability of the intestinal microflora is very important for the health of an organism. Currently, to protect fish against bacterial diseases, treatment with chemotherapeutic agents is used.

There is a growing concern about the use of chemical compounds, not only in human medicine and agriculture but also in aquaculture. For such reason, there is a growing interest in finding other methods of preventing losses in hatcheries. One alternative strategy to the use of antimicrobial drugs that is gaining acceptance within the industry is the use of probiotic bacteria to control potential pathogens (Gomez-Gill et al. 2000).

Probiotics are usually defined as live microbial feed supplements, that are administered in such a way as to enter the gastrointestinal tract and to be kept alive; this beneficially affects the host animal by improving its intestinal microbial balance and in turn its health (Gatesoupe 1999). Most frequently, probiotics are associated with lactic acid bacteria (Nousiainen and Setälä 1993). After hatching, the larval gut is normally colonised before feeding by *Pseudomonas*, *Cytophaga* and *Flexibacter* (Ringø and Gatesoupe 1998). However, these normal microbiota can be artificially dominated by a lactic acid bacteria isolated from the fish intestines and added to the water (Sakata 1990; Strøm and Ringø 1993).

The main strategies in the use of probiotics are to isolate intestinal bacteria, with favourable properties, from mature animals and include large numbers of those bacteria in the feed for immature animals of the same species (Gomez-Gill et al. 2000). Recently, we demonstrated that treatment with a mix of *Lactobacillus fructivorans* and *Lactobacillus plantarum* in sea bream larviculture influenced gut colonisation and significantly decreased larvae and fry mortality (Carnevali et al. 2004).

In this study we monitored a possible correlation between the microflora stability and stress responsiveness of the gut. The aim of this paper was to evaluate stress responsiveness in sea bream larvae under probiotic treatment. Efficient functioning of maintenance and repair processes seemed to be crucial for both survival and physical quality of life. This was accomplished by a complex network of the so-called longevity assurance processes, which are composed of several genes termed ''vitagenes''; among these, the heat shock system, a highly conserved mechanism responsible for the preservation and repair of cellular macromolecules, such as proteins, RNAs and DNA. Recent studies have shown that the heat shock response contributes to establishing a cytoprotective state in a wide variety of disorders. HSP70 is known also to be an anti-apoptotic factor: its expression level can determine the fate of the cell in response to a stress stimulus (Beere et al. 2000; Saleh et al. 2000; Ravagnan et al. 2001; Garrido et al. 2001). In the present study, the mortality, cortisol levels and HSP70 gene expression were evaluated in larvae under pH stress to assess the effect of probiotic administration at a biomolecular level and provide information on the enhancement of stress resistance in fish.

Materials and methods

Probiotic strains

The probiotic strain used was *Lactobacillus fructivorans* previously isolated from sea bream gut. As a control of the efficiency of bacterial strain administration, *Lactobacillus plantarum*, isolated from human faeces (Silvi et al. 2003), was administered contemporaneously to the autochthonous *L. fructivorans*. Both strains were cultivated in 5-l shake flasks containing MRS (de Man, Rogosa and Sharpe) broth (Oxoid, Basingstoke, UK) at 17–20°C in aerobic conditions, inoculated with 2% inocula of deep frozen beads of both strains. After 48 h of fermentation, the *L. fructivorans* and *L. plantarum* biomass was centrifuged at 3,345× *g* (Megafuge 1.0 R; Heraeus). The identity of both strains was checked by Gram staining, isolation on MRS agar plates (Oxoid) and API 50 CHL enzymatic kit (bioMérieux, Marcy-l'Etoile, France) as reported in Carnevali et al. (2004). The strains were stored at 4°C for a maximum period of 47 days in sterile bottles, their viability being checked every 7 days during the whole storage period by bacterial count on MRS agar plates. Viability of the two strains decreased by 20.5 and 12.3% respectively from day 5 to day 47 after production, maintaining a bacterial concentration of about 10^{10} CFU/g of cell pellet.

Live food

Chlorella sp. was cultured in a Guillard's medium (Guillard 1975) at 28°C in 250-l cylindrical tanks. The algal cultures were diluted daily at a rate corresponding to 50% of the maximum growth rate (Reitan et al. 1993).

Brachionus plicatilis (100 μm maximum length) was cultured in 1600-l square tanks, and fed with *Chlorella* sp., *Saccharomyces cerevisiae* (1 g/10^{-6}) and DHA Selco at a 10:1 weight ratio (Olsen et al. 1993). The rotifers were short-term enriched for 14–16 h in 2 mg 1/10^{-1} Protein Selco (INVE Aquaculture, Marloie, Belgium) to increase their nutritional value (Øie et al. 1997).

Artemia salina (EG grade; INVE Aquaculture) cysts were decapsulated with NaOH and hypochlorite at a concentration of 100 g of cyst l^{-1}, incubated for 24 h at 28°C and 5‰ salinity under strong illumination and aeration, and fed for 3 days post-hatching (p.h.) with commercial products (oils, vitamins and essential fatty acids) at 28°C. Three-days-old *Artemia nauplii* were administered to fry.

First feeding

Sea water was UV treated and filtered with sand filter, approximately 25,000 sea bream (*Sparus aurata*) larvae being distributed in 400 l tanks, and fed as described below. Microalgae were added on day 5 p.h. to a final concentration of 1×10^6 cell/cm^3. The algae were added once a day. The water temperature in the tanks was gradually increased from 17°C in 1 day p.h. larvae to 18.5°C 5 days p.h. The flow was initiated on

day 1 and was set at 30 l h^{-1}. The tanks were aerated through air-stones at a low level. *B. plicatilis* was added to the tanks at a final population density of 3 rotifers ml^{-1}, the quantity being gradually increased until reaching a density of 15 ml^{-1} on day 26.

Treatment with *Artemia* started on day 27 p.h. at a density of 1 *nauplius* ml^{-1} and was gradually increased reaching a density of 4 ml^{-1} at the end of administration (day 47 p.h.). On day 34, concomitantly with *Artemia*, the dry food (diameter 100–1,200 μm) was administered at a final quantity of 25 g/tank given in five different times. The quantity of dry food was gradually increased reaching the final amount of 50 g/tank on day 47.

Experimental design

Three experimental groups (each in duplicate) composed of 25,000 larvae were set up: the control group, group A, and group B. Group A received a mixture of both bacterial strains via rotifers from day 5 to day 26 and from day 27 to 47, via *Artemia salina* as a vector. Group B received a mixture of both bacterial strains from days 27 to 47, and the administration was also performed via *Artemia salina* as a vector.

Bacterial mixture preparation

The two strains *L. fructivorans* and *L. plantarum*, both at a concentration of 10^{10} CFU/g of cell pellet were mixed at a proportion of 80 and 20% (w/w) respectively and added to live food (either rotifers or *Artemia salina*), 15 min before administration, the mixture being incubated under low aeration. The final concentration of bacteria in the tank was 10^5 bacteria ml^{-1}. The treatment was administered twice a day.

Microbial analysis of fry sea bream gut

Intestinal microflora were analysed at 35 days p.h., when 10 larvae were collected from each tank and starved for 2 h. The larvae were surface-disinfected with benzalkonium chloride (0.1% w/v) for 30 s, rinsed three times in autoclaved water and homogenised in 3 ml of reducing solution (Holdeman et al. 1997; Muroga et al. 1987). The homogenate was serially diluted in reducing solution, plated in duplicate on: Columbia Blood Agar (bioMérieux) for both aerobic and anaerobic total counts, MacConkey agar (Oxoid) for Enterobacteriaceae counts, Chapman agar (bioMérieux) for *Staphylococcus* counts, and MRS (Oxoid) for *Lactobacillus* counts. Incubation was performed at 17–20°C for 24–48 h and anaerobically at the same temperatures inside an anaerobic cabinet (Don Whitley Scientific, Shipley, UK). Colonies grown on MRS agar were examined microscopically and all non-spore forming straight rods were tested by API 50 CHL to identify *Lactobacillus* species. *L. fructivorans* and *L. plantarum* were detected on treated sea bream gut and on the control group using both MRS medium and API50CHL identification system.

pH stress

A container with 100 l of salted water (salinity37‰) was prepared for the pH tolerance stress. The pH has been lowered to 6.3, adding CO_2 for 30 min. Six tanks of 50 cm in diameter with a small-meshed net in one side to allow water changes were settled. On day 47, 200 larvae maintained at pH 8.6 were caught from each experimental group and divided into two groups of 100 larvae each. All groups were moved to the small containers as follows: tanks 1 and 2 from the control group; tanks 3 and 4 from group A; and tanks 5 and 6 from group B. This experiment was performed in triplicate. The fry were exposed to 6.3 pH for 60 min and mortality was monitored in each container every 5 min. During the pH stress temperature, oxygen and pH were monitored every 5 min.

Since dead larvae were first observed after 30 min, in the further experiment for the evaluation of cortisol levels and HSP70 gene expression, samples were taken after 25 min of exposure. The larvae sampled were kept in liquid nitrogen until their assessment.

RNA extraction and RT-PCR

Total RNA was extracted from 100 mg of total body tissue using TRIzol RNA isolation reagent

(Invitrogen), based on the acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987) following the manufacturer's protocol. Final RNA concentrations were determined by optical density measurement at 260 nm and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on a 1% agarose gel.

Ten micrograms of total RNA were denatured at 70°C for 10 min and converted into cDNA at 42°C by Superscript II RT (Invitrogen) using an Adapter-oligo dT (5′-GACTCGAGTCGACAT CGATTTTTTTTTTTTTTTT-3′) as a primer. Finally, the samples were heated at 70°C to terminate the reaction.

The resulting cDNA was subsequently amplified with 5 units of *Taq* DNA polymerase (Dynazyme) in20 μl of master mix containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTPs and HSP70 (forw: 5′-CAATGACTCCCAGCGGCA-3′ rew: 5′-GGTGATGGAGGTGTAGAAGTC-3′) or *β*-actin (forw: 5′-TTCCTCGGTATGGAGTCCT-3′ rew: 5′-TGGGGCAATGATCTTGATCCT-3′) primers (50 pmol).

Preliminary experiments were conducted in order to determine the optimal number of PCR cycles and the amount of cDNA template needed to ensure that the reactions were in the exponential phase. PCR amplification for *β*-actin was carried out for 27 cycles with the following profile: denaturation at 94°C for 20 s, primer annealing at 56°C for 30 s and primer extension at 72°C for 30 s. HSP70 was carried out for 30 cycles with the following profile: denaturation at 94°C for 1 min, primers annealing at 58°C for 1 min and primer extension at 72°C for 1 min.

Cloning and sequencing

Polymerase chain reaction product obtained with specific HSP70 primers was cloned as already described in Carnevali and Maradonna (2003). Briefly, the PCR product was purified using the PCR purification kit (Qiagen) and then cloned into the p-GEM T easy vector (Promega), following the manufacturer's protocol. The plasmid was transformed into DH5α cells by the TransformAid kit (MBI Fermentas). Several positive clones were analysed by PCR and restriction cutting in order to verify the presence of the insert and then sequenced using an ABI model 310 DNA sequencer (Perkin-Elmer, Oak Brook, IL, USA).

HSP70 gene expression quantification

The DNA fragments from PCR were loaded in a TAE agarose gel and then blotted onto a nylon membrane (Nytran Super charge, Schleicher & Schuell). After the procedure was run, the gel was denatured by washing twice for 30 min in a denaturing buffer (3M NaCl, 0.4 M NaOH). Next, the gel was washed in alkaline transfer buffer (3 M NaCl, 8 mM NaOH, 2 mM sarkosyl) for 15 min and then transferred for 1 h to the same alkaline buffer. After blotting, the membrane was neutralised in 1× neutralising buffer (5 × 1 M phosphate buffer at pH 6.8, diluted at 1 × before using) for 5 min, following which the DNA was linked by UV light.

The variation in HSP70 mRNA expressions was evaluated by semiquantitative PCR using *β*-actin as an internal standard. At the amplification conditions described above, the parallel amplification efficiency was obtained at 30 and 27 cycles for HSP70 and *β*-actin respectively. After homologous hybridisation of Southern blots (standard buffer: 5× SSC, 1× blocking reagent, 0.1% sarkosyl, 0.02% SDS, at maximum stringency conditions), the PCR products were visualised by chemiluminescent detection and autoradiography. The films were scanned using a laser scanner (Sharp Electronics, Milan, Italy) and then subjected to densitometric analysis by ImageQuant software v. 1.2 (Molecular Dynamics; Amersham Biosciences, Sunnyvale, CA, USA).

Cortisol analysis

Cortisol extraction was performed in whole-body juveniles. Samples from different groups were weighed, homogenised and extracted with 4 volumes of dichloromethylene for 30 s. The dichloromethylene fraction was collected while the remaining fraction was extracted again as described above; this step was repeated three times. The collected fractions were pooled, dried and re-dissolved in one volume of EIA buffer.

The analysis of cortisol levels was performed using Cortisol EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) using a standard curve in the range of 7.8–1,000 pg/ml, in accordance to kit instructions. The assay sensitivity 2 ng/tube and the inter- and intra-assay coefficients of variation were 6.3 and 4.4% respectively. To validate cortisol assay, parallelism between the standard curve and serial dilution of the extracted solution was performed.

Statistic

Data presented in this paper are in the form mean ± SD of means. Results were examined by one-way ANOVA followed by the Student–Newman–Keuls test or the Student's *t* test as appropriate, using a statistical software package, Stat View 512+TM (Brain Power, Calabasas, CA, USA). A *P* value of 0.05 was used as the limit of statistical significance. The statistic analysis of cumulative mortality was performed using the Siegel–Tukey test.

Results

Microbial analysis of guts from the sea bream fry

The microbial analysis at 35 days p.h. revealed that both treated groups had a significantly different bacteria colonisation compared with the control group (Fig. 1). Total anaerobes and aerobes significantly decreased in both treated groups, as well as the Enterobacteriaceae and *Staphylococcus* spp. *Lactobacillus* were not found in the control group, while groups A and B presented values of 4.51 ± 0.06 log CFU/g of larvae and 4.87 ± 0.08 log CFU/g of larvae respectively.

Mortality

The oxygen assay showed a concentration of 8–10 ppm throughout the experiment. The temperature showed a constant value of 18.6°C.

Mortality was monitored for 60 min. The first larvae deaths occurred after 30 min of exposure to pH stress. The results indicated that the control group showed the highest cumulative mortality, which was almost double compared with the two treated groups (Fig. 2).

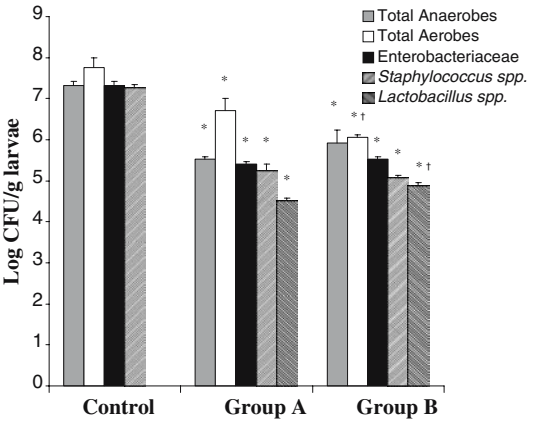

Fig. 1 Bacterial counts, at 35 days post-hatching (p.h.), of total anaerobes and aerobes, Enterobacteriaceae, *Staphylococcus* and *Lactobacillus* from the gut of sea bream fry of the control group, of group A fed on a diet integrated with probiotics- from days 5 to 47, and group B fed on a diet integrated with probiotics from days 27 to 47. *Significantly different from control group ($P < 0.05$, Student's *t* test). †Significantly different from group A ($P < 0.05$, Student's *t* test)

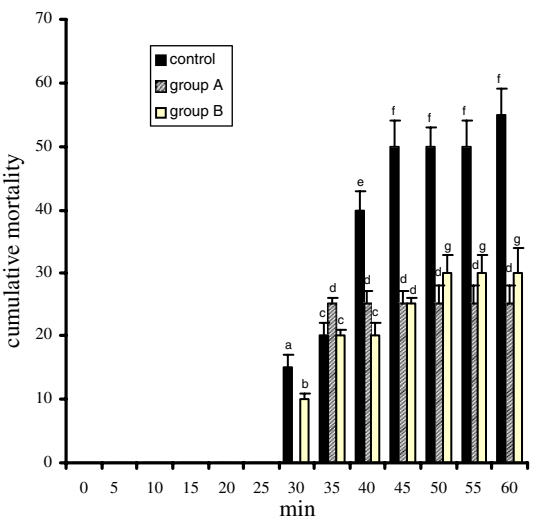

Fig. 2 Cumulative mortality induced by pH stress in the control group, in group A fed on a diet integrated with probiotics from days 5 to 47, and group B fed on a diet integrated with probiotics from days 27 to 47. The results are the average of three replicates. Values with different lower case letters indicate statistical significance ($P < 0.05$)

Group A showed a slightly lower mortality than group B. This difference was not statistically significant indicated that the administration of probiotics raised the tolerance to pH stress in sea bream larvae.

Cortisol levels

Figure 3 shows the cortisol levels before and after exposure to pH stress. In normal rearing conditions (before exposure to pH 6.3) cortisol levels in treated groups were significantly lower compared with the control group. After exposure to pH stress, cortisol levels significantly increased in all groups. The control group showed the highest cortisol concentration.

Gene expression

The sequences of the PCR products obtained with HSP70 specific primers and submitted to Genbank provided an identity of 91%, with *Paralichthys olivaceus* HSP70 (accession number AB010871) (Fig. 4).

HSP70 is a constitutive protein and its messenger was detected in all groups before exposure to stress conditions, although the levels were lower in the treated groups. The analysis of HSP70 gene expression showed a significant increase after pH stress in all groups tested, but the highest levels were found in the two treated groups (Fig. 5). The β-actin gene expression did not show any changes in all the experimental groups analysed.

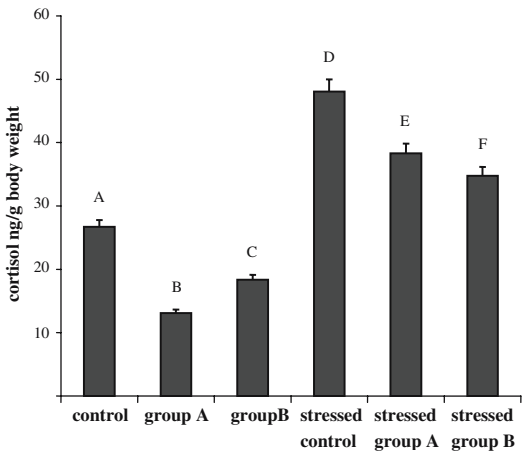

Fig. 3 Levels of cortisol in the control group, in group A fed on a diet integrated with probiotics from days 5 to 47, and group B fed on a diet integrated with probiotics from days 27 to 47, before and after exposure to pH stress. Data are expressed as mean ± SD; values with different letters indicate statistical significance ($P < 0.05$)

Discussion

In aquaculture, pH variations are caused by utilisation of artificial diets, organism density, algae administration etc.. Changes in pH may affect the efficiency of production and provoke significant economic loss. In the present study, the results achieved suggested that probiotics had a beneficial effect on larvae exposed to pH stress conditions.

Microbial analysis at 35 days p.h. showed a significantly different bacteria colonisation of the probiotically treated groups. As previously reported by Carnevali et al. (2004), at 35 days p.h. the probiotic strains produced an intestinal colonisation that affected other genera present in the gut. In fact, the increased presence of Lactobacilli in the larvae gut seemed to have an effect on the decrease in potentially pathogenic bacterial groups such as Enterobacteriaceae and *Staphylococcus* (Ringø 1999). Previous results of studies on the survival and growth of larvae reared with a diet enriched with probiotics, indicated that larvae were in good physiological condition (Carnevali et al. 2004).

In the present work, the same rearing protocol using a diet enriched with probiotics was used and the larvae were subjugated to pH stress. After the stress test, cumulative mortality, monitored within 1 h exposure to pH 6.3, was significantly higher in the control group than in the treated ones ($P < 0.05$). This result indicated that, under pH stress, probiotic administration decreased larval losses.

The hormonal assay was made 47 days p.h. before and after pH stress. Levels of cortisol suggested a better tolerance of normal rearing conditions in larvae under probiotic treatment: before pH stress, levels of cortisol in groups A and B were significantly lower compared with the control ones. Lower levels of cortisol in the treated groups seemed to have a very interesting

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P.olivaceous      GACTCCCAGCGCCAGGCCACCAAGGATGCAGGCACTATCTCTGGCCTCAATGTTTTGCGT
S.aurata          ------------------------------------CTCTGGCCTCA-TGTTCTGCGC
                                                       *********** **** ****

P.olivaceous      ATCATCAATGAACCAACTGCTGCTGCCATCGCCTATGGTTTGGACAAGAAGGTTGGATCA
S.aurata          ATCATCAACGAGCCAACTGCTGCTGCCATTGCTTATGGATTGGACAAAAAGGTTGGGTCT
                  ******** ** ***************** ** ***** ******** ******** **

P.olivaceous      GAAAGGAACGTCCTCATCCTCGATCTTGGTGGTGGCACCTTTGATGTGTCCATCTTGACC
S.aurata          GAAAGGAACGTTCTTATCTTTGATCTTGGTGGAGGCACCTTTGACGTGTCAATTTTGACC
                  *********** ** *** * *********** *********** ***** ** ******

P.olivaceous      ATTGAGGATGGCATCTTTGAGGTCAAGTCCACCGCTGGAGATACTCATCTTGGTGGGGAA
S.aurata          ATTGAGGATGGCATCTTTGAGGTAAAGTCCACTGCCGGAGATACCCATCTTGGTGGGGAA
                  ********************** ******** ** ******** ***************

P.olivaceous      GATTTCGACAACCGCATGGTCAACCACTTCATCGCTGAGTTCAAGCGCAAGTACAAGAAA
S.aurata          GATTTCGACAACCGCATGGTCAACCACTTCATCGCAGAGTTCAAGCGCAAGTACAAGAAG
                   ************************************************************

P.olivaceous      GACATCAGCGACAACAAGAGAGCTGTCCGTCGTCTGCGCACCGCTTGTGAGAGGGCAAAG
S.aurata          GACATCAGCGACAACAAGAGAGCTGTGCGTCGTCTGCGCACCGCCTGTGAAAGGGCAAAG
                  ************************** ***************** ***** *********

P.olivaceous      CGCACATTGTCTTCCAGCACCCAGGCCAGCATCGAAATCGACTCCCTGTATGAGGGAGTT
S.aurata          CGCACCCTGTCTTCCAGCACCCAGGCCAGCATTGAAATTGACTCTCTGTATGAGGGAGTT
                  *****  ************************* ***** ***** ***************

P.olivaceous      GACTTTTACACCTCCATCACCAGGGCTCGCTTTGAGGAGCTCAATGCTGACCTCTTCCGT
S.aurata          GACTTCTACACCTCCATCACCAAG-----------------------------------
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Fig. 4 Nucleotide alignment (CLUSTAL W1.81) between *P. olivaceus* HSP70 and *S. aurata* HSP70. *Star* indicates nucleotide homology

correlation with the presence of Lactobacilli in the same groups, suggesting, once more, that a major intestinal microflora stability due to the probiotic treatment enhanced fish wellness. These results agree with the study by Schaedler and Dubos (1962), which demonstrated that mice under stressful conditions showed a significant decrease in Lactobacilli concentration.

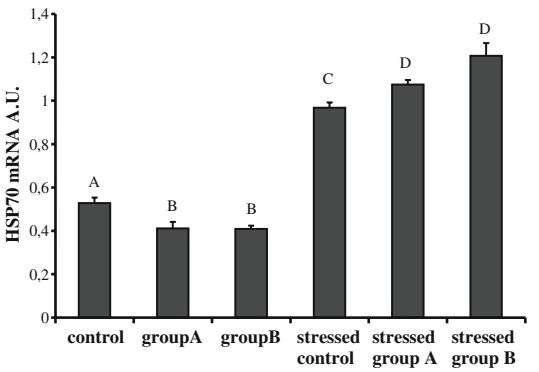

Fig. 5 Levels of HSP70 gene expression in the control group, in group A fed on a diet integrated with probiotics from days 5 to 47, and in group B fed on a diet integrated with probiotics from days 27 to 47, before and after exposure to pH stress. Data, normalised using β-actin, are expressed as mean ± SD; values with different letters indicate statistical significance ($P < 0.05$)

After pH stress, as expected, cortisol levels increased in all groups. High levels of cortisol reduce fish resistance against pathogen organisms and the cause of this higher vulnerability could be the immunosuppressive effect of cortisol that seems to affect antibody production (Ellsaesser and Clem 1986a, 1986b; Tripp et al. 1987). These results indicated that probiotic treatment enhanced larvae tolerance to normal rearing conditions, since both treated groups showed lower levels of cortisol and thus better survival.

Molecular assays drew the same conclusions; in fact, levels of HSP70 mRNA before pH stress were significantly higher in the control group, suggesting again that probiotic treatment enhanced tolerance to common rearing conditions.

The effects of pH stress were very interesting. The increase in HSP70 levels was markedly higher in the treated groups. It is known that HSP70 guarantees appropriate protection of protein structures, strengthens the immune system and stops apoptotic mechanisms (Young

1990; Beere et al. 2000; Saleh et al. 2000; Ravagnan et al. 2001). Previous studies on thermal stress reported a positive correlation between the amount of HSP70 and survival (Norris et al. 1995), and enhanced thermal tolerance by increasing the capacity to repair cellular proteins (Nakano and Iwama 2002). Furthermore, studies in fish have shown that in response to stressors cortisol attenuates HSP70 response (Basu et al. 2001; Boone and Vijayan 2002). Similar results were observed in the present study, in which stressed animals the maximal levels of cortisol were associated with minimal levels of HSP70. These results confirmed the possibility that the neuroendocrine and cellular stress responses are functionally related. Since the treated groups showed a higher survival under pH stress, we hypothesised that in the probiotic-treated groups, the cellular response, in terms of HSP70 levels, provided a better response to pH stress. However, further studies are necessary to elucidate the mechanism by which probiotics modulate cortisol levels as well as HSP70 gene expression.

The administration of probiotics, as proved here, enhanced the resistance of the organisms to chemical–physical changes and to pathogen infections, as previously observed in other species (Westerdahl et al. 1991; Havenaar et al. 1992; Salminen et al. 1998), suggesting that probiotics might be a good alternative to the use of antibiotics in aquaculture.

The results obtained in this study represent the first step towards the development of an environmentally friendly aquaculture. Moreover, since the products of aquaculture are devolved to human consumption, the use of probiotics as a live microbial feed supplement may represent a guarantee of a safer product for human health.

The relevance of this study is that different from most of the work so far carried out on the use of probiotics, which has only been at a macroscopic level, this study is the first example of the evaluation of probiotic effects on stress at a biomolecular level. The correlation between Lactobacilli colonisation and cortisol levels was very interesting. Molecular data that confirm the positive effects of the probiotic treatment were supported by macroscopic results, such as mortality. The present study represents the beginning of the accurate scientific research, which in our opinion is necessary to widen knowledge in this field and to find the answers to many questions that today remain unsolved.

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