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Application of multi-species of *Bacillus* in sea bream larviculture

Matteo Alessandro Avella [a], Giorgia Gioacchini [a], Olivier Decamp [b], Pavlos Makridis [c], Claudia Bracciatelli [a], Oliana Carnevali [a,*]

[a] Department of Marine Sciences, Polytechnic University of Marche, Ancona, Italy
[b] INVE Aquaculture, Bangkok, Thailand
[c] Institute of Aquaculture, Hellenic Centre for Marine Research, 71003 Iraklion Crete, Greece

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ABSTRACT

To date, probiotics can be considered a valid alternative to the use of antibiotics in aquaculture and in particular, in fish larviculture, to prevent high mortality and to improve welfare and promote growth. In the two last decades, many studies reported promising results using a single beneficial bacterial strain in the culture of many finfish species. Looking for innovative ways to improve fish larviculture, we tested a mixture of *Bacillus* probiotic bacteria in the gilthead sea bream (*Sparus aurata*) larviculture focusing on their effects on survival, growth and general welfare. The mixture, composed of three *Bacillus* strains, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* was provided via rotifers and *Artemia* nauplia and added to the water (group 1) or supplied exclusively via live prey (group 2).

The *Bacillus* mixture significantly increased growth in terms of standard length and body weight in both experimental groups, at larval and juvenile stages, 47 and 75 days after hatching, respectively, with the highest values obtained from Group 2 treatment.

In addition, the expression of genes involved in growth metabolism (Insulin-like Growth Factors I and Myostatin) and animal welfare (70 KDa-Heat Shock Protein and Glucocorticoid Receptor) were analysed. The morphometric analysis was supported by molecular results which clearly evidenced higher expression of IGFI and lower levels of myostatin in groups fed on probiotics. In addition, a better tolerance to farming conditions was also found as evidenced by the lower expression of HSP70 and Glucocorticoid Receptor, suggesting beneficial effects of the supplied *Bacillus* mixture on fish welfare.

The data generated in this study provide scientific and technical support for the implementation of sustainable development of sea bream aquaculture.

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1. Introduction

Animal gut microflora consists of hundreds of different bacterial strains, (Walker and Duffy, 1998) able to promote digestion and absorption of nutrients, to increase body resistance to infectious diseases (Tannock, 1988), to yield positive effects on growth and to improve general animal welfare (FAO/WHO, 2001). In aquaculture, captive rearing conditions generally can be sources of stress, triggering high mortality, mainly during larval rearing. In response, drugs have been widely used (SCAN, 2003), leading to very harmful environmental impacts. For this reason, the European Union placed restrictions on antibiotic use in aquaculture (European Union, 2003) and completely banned the use of antibiotics as chemotherapies and growth promoters in animal feed by 2006, while Federal and Drug Administration in the United States of America implemented new risk assessment criteria to assess "microbiological safety" of antimicrobial use in animals (US Food and Drug Administration, 2003).

Thus, to date, defining alternative strategies to support aquaculture productivity are extremely necessary. In the last decade, the scientific community carefully examined roles and effects of probiotics in aquaculture as an alternative to antimicrobial drugs, demonstrating positive effects on fish survival (Villamil et al., 2002), growth (Burr et al., 2005), stress resistance (Smith and Davey, 1993; Rollo et al., 2006), immunosystem enhancement (Erickson and Hubbard, 2000; Picchietti et al., 2007), and finally general welfare (Balcázar et al., 2006; Silvi et al., 2008).

Among probionts, *Bacillus* are gram positive, spore forming bacteria, used commercially as probiotics; since the physical and biological characteristics of the spore, *Bacillus* preparations are resistant and have a long lasting shelf life and so they can be stored inventively in a desiccated form; moreover the production cost of spores for aquaculture is very convenient (Wang et al., 2008) and the beneficial roles of their applications in the aquaculture field are well established (Gatesoupe, 1999, 2008).

Bacillus can act positively on cultured organisms by enhancing survival and growth (Gomez-Gil et al., 2000), by stimulating the digestive (Ziaei-Nejad et al., 2006) and immune systems (Gatesoupe,

* Corresponding author. Tel.: +39 071 2204990; fax +39 071 2204650.
E-mail address: o.carnevali@univpm.it (O. Carnevali).

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1999) and by improving water quality in terms of bioremediation (Kennedy et al., 1998; Moriarty, 1998). Several studies demonstrated these positive effects using a single or two probiotic strains, and just few studies described the effects of a mixture of probiotics in fish and shrimp aquaculture (Balcázar, 2003; Lara-Flores et al., 2003; Ziaei-Nejad et al., 2006). Concurrently, *Bacillus* species can be found in marine environment and are part of the microflora of several marine species (Kennedy et al., 1998; Hovda et al., 2007).

In particular, *B. subtilis* is known to produce proteases and other enzymes that enable it to contribute to the natural digestion activity of the host (Ziaei-Nejad et al., 2006) and it can be a source of micro- and macro-elements as feed (Verschuere et al. 2000). Moreover, *B. subtilis* is well known to prevent host organism gut colonization by potential pathogens through the production of antimicrobial compounds, or by actively competing for nutrients or space (Vaseeharan and Ramasamy, 2003). *B. licheniformis* has been shown to act as an antiviral (Arena et al., 2006) ,while *B. pumilus*, commonly present in marine fish microflora, (Sugita et al., 1998) has been found to produce extracellular proteases including amylase and cellulase, key enzymes involved in rohu fingerlings digestive activity (Ghosh et al., 2002). Interestingly, *B. subtilis* has been previously administered with other strains such as *B. licheniformis* or *B. pumilus* in rainbow trout, leading to higher growth and immune resistance (Raida et al., 2003;Bagheri et al., 2008). In addition, the three aforementioned species of *Bacillus* have been proposed as valid alternative to the use of antibiotics in shrimp aquaculture (Banerjee et al., 2007). All those aspects might make the *Bacillus* mixture here used as a valid growth promoter for sea bream larviculture, and for these reasons it is plausible to expect positive results from the application of a *Bacillus* mix to larviculture of commercially relevant species, such as in this case, the gilthead sea bream, *Sparus aurata.*

Upon probionts administration, there are many ways to determine the effects of supplied probiotics to fish larvae, such as determining gut epithelia colonization, testing the effects on nutritional contributions of supplied bacteria, checking immune stimulatory function (Tinh et al., 2008). Moreover, by using a molecular approach, we already shown in previous studies that the use of *Lactobacillus* spp. could influence the gene expression of factors related to stress response (Rollo et al., 2006), welfare and growth metabolism (Avella et al., 2010; Carnevali et al., 2004, 2006; Merrifield et al., 2010). In particular, concerning stress response, stressors can evoke physiological response such as changes in glucocorticoids and Heat Shock Proteins (HSPs) levels (Barton, 2002). In particular, cortisol, the main glucocorticoid/stress hormone in teleost is known to increase in stressful conditions (Carnevali et al., 2006) and to be strictly related with Glucocorticoid Receptor gene expression (Mommsen et al., 1999; Vijayan et al. 2003). Among HSPs, 70 kDa-Heat Shock Protein (HSP70) is known to increase in stressful conditions, including improper aquaculture conditions (Delaney and Klesius, 2004). Moreover we previously reported that probiotic administration to sea bream fingerlings led to lower levels of HSP70 (Rollo et al., 2006). For these reasons we hypothesized that *Bacillus* mixture administration would lead to lower levels of Glucocorticoid Receptor (GR) and HSP70. While considering larval growth, we focused on Insulin-like Growth Factor I (IGFI) and Myostatin (MSTN). In particular, since fish growth is positively related to muscle growth and, in a molecular point of view, is controlled by intrinsic factors such as IGFI and MSTN, factors strictly involved in fish myogenesis (Ayson et al., 2002; Patruno et al., 2006; Peterson et al., 2004) and widely used as markers for muscle growth (Duan and Plisetskaya, 1993; Chauvigné et al, 2003), we hypothesize a positive relation between probiotic administration, growth and higher levels of the above mentioned markers.

Using a morphometric and a molecular approach, we aimed to determine the effects of a mixture of probiotics composed of three *Bacillus* strains, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* on survival, welfare and growth in gilthead sea bream larval rearing. This study could contribute to implement knowledge to develop sustainable and competitive seabream aquaculture.

2. Materials and methods

2.1. Experimental model

A rearing experiment with gilthead sea bream larvae (*S. aurata*) was run in the facilities of Hellenic Centre for Marine Research, Institute of Aquaculture, Iraklion, Crete. Gilthead sea bream larvae were reared in 500 L tanks until 33 days post hatch (ph). Each pair of tanks was supported by a biofilter unit of 1 m^3. An air-lift pump within each unit supplied a continuous movement of water even when the water supply from the biofilter unit was very low (Papandroulakis et al., 2002).

During the first phase of the rearing, a re-circulation system was used, where gilthead sea bream were stocked in six 500 L tanks at a stocking density of 50 g embryos per tank. The tanks were kept under 24 h light regime from days 5–33. The larvae were fed rotifers, *Brachionus plicatilis* (days 5–30 ph), and enriched *Artemia* metanauplii (days 24–75 ph), while microalgae *Chlorella minutissima* (days 5–33 ph) were added daily in the tanks. The rearing followed the protocol described by Papandroulakis et al. (2002).

2.2. Hatching and larval rearing

The bacteria were supplied in powder form by Inve Technologies. The powder contained 5×10^{10} spores g^{-1}, and the amount needed each day was weighed, added to seawater at 1 g L^{-1}, and kept under continuous stirring at room temperature (20–22 °C) for a minimum of 4 h. From day 3 ph to the end of the experiment, this bacterial suspension was either daily added directly to water (100 mL per 500-L tank, final concentration in seawater 10^4 bacteria mL^{-1}) and through bioencapsulation in live food, or administered only by live food. In the latter case, rotifers and *Artemia* were concentrated in seawater and an equal volume of bacterial suspension was added (final concentration in bacterial suspension was 2.5×10^8 bacteria mL^{-1}). This short-term incubation lasted for 30 min. Bacteria bionencapsulated in live food were given in one of the three feedings of each day (midday feeding) and in particular from day 5 to day 25 ph via rotifers, from day 26 via *Artemia* metanauplii and from day 33 ph, and via *Artemia* metanauplii and artificial diet (every 20 min from 8:00 to 20:00) until the end of the experiment. Rotifers and *Artemia* metanauplii with bioencapsulated bacteria were rinsed before administration.

2.3. Experimental design

Larvae were divided into three experimental groups in each treatment and each rearing tank was stocked with approximately $50\pm1\times10^3$ larvae (3 replicates each) and fed as follows:

Control group: probiotics were not supplied to larvae; group 1: probiotics were supplied to larvae via live prey (rotifers and *Artemia* nauplii) and added to the rearing water; group 2: probiotics were only supplied to larvae via live prey (rotifers and *Artemia* nauplii). On day 33, 300 ± 30 larvae from each tank were moved to another rearing system of 50-L tanks for the second phase of the experiment with a flow through water supply where the fish remained until the end of the experiment. At 33, 47 and 75 days ph, sea bream larval survival was estimated. For morphometric and molecular studies, larval sampling was performed on day 47 ph (larval phase) and on day 75 ph (juvenile phase). For each sampling, 30 ± 3 specimens were used for morphometric analysis and 30 ± 3 specimens were stored at −80 °C for molecular analysis till assay.

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2.4. Microbiological sampling

Samples were taken from rotifers and *Artemia* metanauplii after bioencapsulation and rinsing as well as of non-treated rotifers and *Artemia*. Live food organisms were rinsed thoroughly with sterile seawater, homogenized in glass homogenizers, and plated on tryptic soy agar dishes added with 2% NaCl (TSAS), and thiosulfate citrate bile salts (TCBS) agar. The dishes were incubated at 20 °C, and the numbers of colonies were counted seven days after plating.

To test the efficacy of the probiotic mixture supplied, the heterotrophic aerobic gut microflora of the larvae was examined on days 6, 19, 26, 40, 47 and 62 ph . At each sampling point, the larvae from each tank were anaesthetized with metomidate (20 mg L^{-1}), surface-disinfected with benzalkonium chloride (0.1% w/v) for 30 s, washed in autoclaved seawater, and homogenized in 5 ml of autoclaved seawater (Makridis et al., 2000). Serial dilutions of the homogenates were plated on TSAS and TCBS dishes. The dishes were incubated at 20 °C, and the numbers of colonies were counted seven days after plating.

2.5. Morphometric analysis

Larvae on days 47 and 75 post hatch (ph) were sampled and anesthetized with MS222 (Sigma Aldrich) (1 mg/l) and total length (TL) and body weight (BW) were recorded using the balance OHAUS Explorer E11140 accurate to 0.1 mg and micrometric microscope STEMI 2000.

2.6. Biomolecular analysis

2.6.1. RNA extraction and cDNA synthesis

Total RNA extraction from whole larval body was optimised, the best recovery being obtained with 30 mg of larvae, using Minikit RNAeasy® (Qiagen) extraction kit following the manufacturer's protocol. Total RNA extracted was eluted in 15 μL of RNAse-free water. Final RNA concentrations were determined by Thermo Scientific NanoDropTM 1000 Spectrophotometer, and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stirred at −80 until use.

Total RNA, was treated with DNAse (10 UI at 37 °C for 10 min, MBI Fermentas). A total amount of 5 μg of RNA was used for cDNA synthesis, employing 0.5 μg oligo d(T) + adapter primer, 5′-GACTG-CAGTCGACATCGATTTTTTTTTTTTTTTT-3′, in a buffer containing 50 mM Tris–HCl (pH 8,3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 40 units RNAse OUT (Invitrogen) and 200 units of Superscript II RT (Invitrogen, Life technologies, Milan, Italy). Cycling conditions were: 70 °C for 5 min, 42 °C for 52 min and 72 °C for 15 min.

2.6.2. Primer design

Specific amplification primers were designed using the Beacon Designer Software (PREMIER Biosoft International, Palo Alto, CA). The following primers were used at final concentration of 10 pmol/μl.

β-ACT: (For:5′-TGCGTGACATCAAGGAGAAG-3′;Rev:5′-CAG-GACTCCATACCGAGGAA-3′);

IGF I: (For:5′-TCTTCAAGAGTGCGATGTGC-3′;Rev:5′-ACAGCTTTG-GAAGCAGCACT-3′);

MSTN:(For:5′-AAGCGCTACAAGGCCAACTA-3′;Rev:5′-CGACTCAA-GAGCATCCACAA-3′);

GR: (For: 5′-CGGTCACTGCTACGTCTTCA-3′; Rev: 5′-CCTCCCAGCA-CACAGGTAAT-3′);

HSP70: (For:5′-CAATGACTCCCAGCGGCA-3′;Rev:5′-GGTGATG-GAGGTGTAGAAGTC-3′);

2.6.3. Real time PCR

Triplicate PCR reactions were carried out for each sample analysed. After Real time conditions optimisation, the PCRs were performed with SYBR green method in iQ5 multicolor Real time PCR Detection system (Bio-Rad). The reactions were set on a 96-well plate by mixing, for each sample, 1 μl of diluted (1/20) cDNA, 5 μL of 2× concentrated SYBR Green PCR Master Mix (Bio-Rad) containing SYBR Green as fluorescent intercalating agent, 0.3 μM forward primer and 0.3 μM of reverse primer. The thermal profile for all reactions was 15 min 95 °C and then 45 cycles of 20 s at 95 °C, and 20 s at 60 °C and 20 s at 72 °C. The fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak.

2.6.4. Quantification of cDNA

A relative quantification of cDNA was made using β-Actin (β-Act) as reference gene (housekeeping gene). The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad). Modification of gene expression is represented with respect to the control sampled at the same time of the treatment.

2.6.5. Data analysis

Survival, morphological and molecular data were analysed using one way ANOVA, followed by Tukey test, with a statistical software package, SigmaStat 3.1 (Systat software Inc.). A probability of 0.05 was utilized to account for the statistical difference between the means.

3. Results

3.1. Survival and microbiological results

On days 33, 47 and 75 ph, no significant differences in terms of survival have been estimated among the experimental groups.

The heterotrophic microbiota of the live food was examined before and after bioencapsulation in rotifers and *Artemia*. The total counts and numbers of presumptive *Vibrio* were significantly lower in treated *Artemia* when compared to control *Artemia* (Table 1), whereas in the case of rotifers lower counts were measured only on TCBS medium. On the contrary in fish samples, no significant differences were shown in the total counts and numbers of presumptive *Vibrio* among the different treatments (Table 2).

3.2. Morphometric approach

Sea bream larvae (47 ph) and juveniles (75 ph) fed on live prey treated with *Bacillus* strains, showed significant growth increases in both standard length and body weight compared to the control group (Table 3). In particular, on day 75 ph, group 2 showed a further and significant increase in growth rate.

Table 1
Number of colony-forming units per rotifer or *Artemia* determined after plating in tryptic soy agar added salt (TSAS) and TCBS agar.

	TSAS	TCBS	TCBS as percentage of total bacteria
Control rotifers	780 ± 2	13 ± 7^{a}	1.6
Enriched rotifers	1430 ± 460	47 ± 5^{b}	3.2
Control Artemia	2150 ± 90^{a}	750^{a}	35
Enriched Artemia	780 ± 40^{b}	260^{b}	33

Different superscript letters (a, b) indicate statistical significance between control rotifers vs. enriched rotifers or control *Artemia* v.s enriched *Artemia*.

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Table 2
Numbers of colony-forming units ($\times 10^3 \pm$SEM) per fish determined after plating in tryptic soy agar added salt (TSAS) and TCBS agar.

Days post hatch	Control		Group 1		Group 2	
	TSAS	TCBS	TSAS	TCBS	TSAS	TCBS
6	0.6 ± 0.3	0.08 ± 0.02	1.7 ± 0.6	0.08 ± 0.01	1.6 ± 1.0	0.15 ± 0.1
19	15.0 ± 8.9	3.9 ± 3.5	15.8 ± 11.7	1.4 ± 0.9	16.9 ± 3.1	5.9 ± 3.1
26	41.0 ± 9.5	0.9 ± 0.8	38.7 ± 10.7	0.2 ± 0.06	34.2 ± 1.2	1.2 ± 1.0
40	36.9 ± 3.6	2.2 ± 0.4	50.2 ± 30.2	1.1 ± 0.6	52.0 ± 27	8.3 ± 4.7
47	430 ± 230	2.2 ± 1.6	680 ± 470	15.4 ± 15.2	320 ± 11	1.7 ± 0.9
62	250 ± 10	52.2 ± 7.2	245 ± 25	53.5 ± 12.5	250 ± 90	146 ± 58

3.3. Molecular approach–mRNA analysis by Real-time PCR

The sequences of the PCR products obtained with β-Act, IGFI, MSTN and GR, specific primers provided an identity of 99 % with the sequences available in GenBank (accession nos: β-ACT AF384096, IGF-I EF563837, MSTN AF258448 and GR DQ486890). For HSP70, a partial nucleotide sequence was obtained and deposited in GenBank (nos. EU805481), and the novel gilthead sea bream sequences of the PCR products obtained provided 91% identity with *Paralichthys olivaceus*.

The housekeeping gene here used was β-Act. There is contradictory support for β-Act use as a quantitative reference for RT-PCR due to evidence of directional shifts in expression under differing experimental conditions and tissues (Radonić et al., 2004). More recently β-Act has been both advocated (Infante et al., 2008) and rejected (Toegel et al., 2007) as a quantitative reference for Real-time PCR. Similarly to what reported by Fernandes and collaborators (2008) who suggested β-Act as suitable reference genes for Real time PCR studies of the development of the Atlantic halibut, in our experimental model the β-Act gene expression in the larval stages considered, did not vary significantly and was considered in the present study as a suitable housekeeping gene.

The Real Time PCR results showed lower levels of HSP70 and GR gene expression in fish fed on live prey treated with probiotic at both days 47 and 75 ph (Figs. 1 and 2). In particular, on day 47 ph, a significant lower HSP70 gene expression level was registered in groups 1 and 2 (Fig. 1a) with respect to the control group; on day 75 ph, groups 1 and 2 showed significantly lower HSP70 gene expression (Fig. 1b), and in particular group 2 evidenced a further and significant lower gene expression level with respect to the control. Considering GR, a significant reduction of gene expression was measured in groups 1 and 2 on both days 47 and 75 ph (Fig. 2).

Concerning MSTN, on day 47 ph, a significant lower gene expression was observed in both groups 1 and 2, with a further and significant lower expression evidenced in group 1 with respect to the control group (Fig. 3a); on day 75 too, a significant lower gene expression was observed in both treatments, but in this case, the lowest MSTN gene expression was observed in group 2 (Fig. 3b).

Table 3
Gilthead sea bream total length (mm) and body weight on days 47 and 75 post hatch. Averages in the same row with different superscript letters are significantly different ($P<0.05$).

Total length (mm)			
	Control	Group 1	Group 2
Day 47	$10 \pm 0{,}1^{a}$	$11{,}1 \pm 0{,}2^{b}$	$10{,}7 \pm 0{,}05^{c}$
Day 75	$22{,}3 \pm 0{,}1^{a}$	$23{,}7 \pm 0{,}2^{b}$	$25{,}9 \pm 0{,}05^{c}$
Body weight (mg)			
Day 47	$56{,}1 \pm 0{,}2^{a}$	$56{,}2 \pm 0{,}1^{a}$	$77{,}6 \pm 0{,}2^{b}$
Day 75	$272{,}6 \pm 3^{a}$	$347{,}4 \pm 2^{b}$	$496{,}6 \pm 4^{c}$

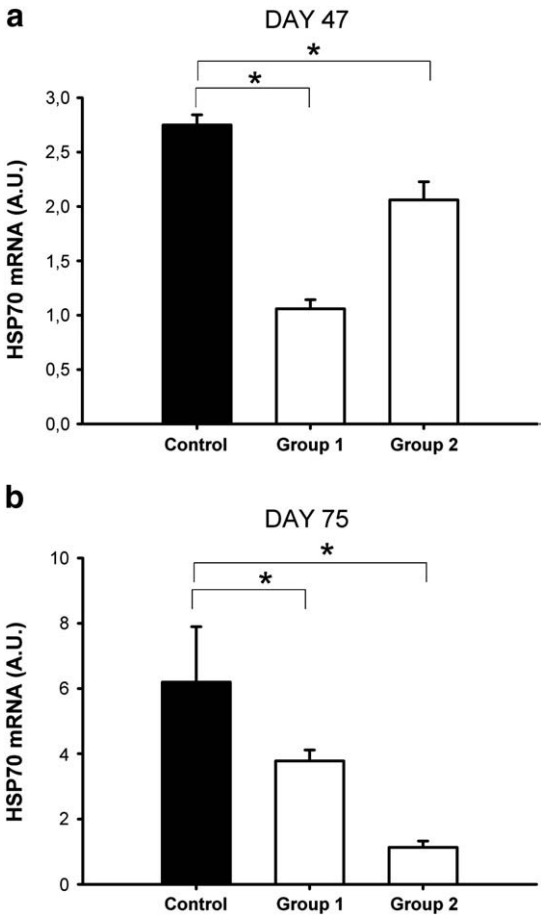


Fig. 1. HSP70 gene expression at 47 (a) and 75 (b) days ph respectively. Lower levels were registered in treated groups, suggesting a positive role of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* administration on cellular stress response. Asterisk, $P<0.05$ compared among experimental groups.

For IGFI gene expression, on both days 47 and 75 ph, we observed significantly higher mRNA levels in groups 1 and 2 and the highest levels of IGFI gene expression with respect to the control have been always observed in group 2 (Fig. 4).

4. Discussion

Probiotic application to marine organisms aims to increase seafood supply and safety, to control the proliferation of harmful microorganisms, and to develop new drugs. Managing microflora in aquaculture to enhance animal welfare, using beneficial bacterial strains, can be considered a biotechnological tool for the development of sustainable and environmental friendly aquaculture. Generally, in aquatic environments, the microflora community is composed of many bacterial strains that continually change in structural composition and related functions. Thus, supplying a mixture of beneficial strains able to act and interact under a variety of conditions such as occur in captive rearing, plausibly implies that among the bacteria mixture supplied, a beneficial bacterium would dominate the environmental and the gut microflora (Verschuere et al., 2000).

In this particular instance, we supplied a mixture of three *Bacillus* strains, *B. subtilis*, *B. licheniformis* and *B. pumilus*, which probiotic effects have been previously demonstrated when administered to cultured organisms (Banerjee et al., 2007; Cladera-Olivera et al., 2004; Decamp et al., 2008; Li et al., 2007; Raida et al., 2003); moreover, these bacteria were selected since their well known probiotic ability and additionally, they do not act in antagonist way each another (Le Duc et al., 2004).

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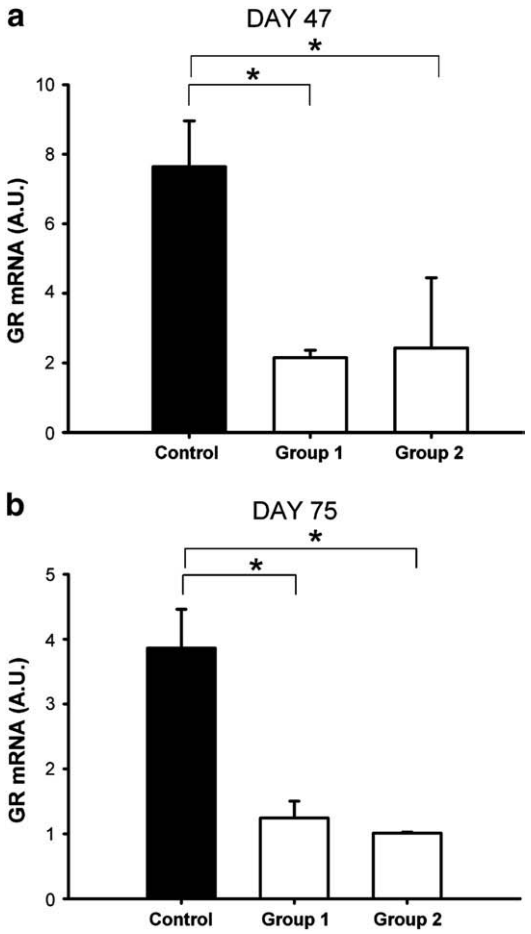


Fig. 2. GR gene expression at 47 (a) and 75 (b) days ph respectively. Probiotic treatment lead to significant GR gene expression lower levels in both groups 1 and 2 with respect to the control group; no significant differences have been detected between the two different kinds of probiotic treatments (group 1 and group 2). Asterisk, $P<0.05$ compared among experimental groups.

Bioencapsulation of bacteria in *Artemia* was able to reduce the total bacterial load and the numbers of presumptive *Vibrio* per *Artemia*. Presumptively, treating *Artemia* nauplii with *Bacillus* mixture could be a useful tool to improve the safety of live food administered since it is well established that high density of *Vibrio* bacteria, commonly associated to *Artemia* nauplii (Benetti et al., 2008), may have negative effects on fish larval welfare. The addition of *Bacillus* bacteria in the live food diet did not have any effect on the total counts on fish gut heterotrophic bacteria and presumptive *Vibrio*. However, the species composition of the gut microbiota was not studied, so possible effects on qualitative aspects may not be excluded.

Concerning the molecular approach, our previous study showed that *Lactobacillus* administration in sea bream larviculture was able to increase larval and juveniles body weight but not standard length (Carnevali et al., 2004). On the contrary, the *Bacillus* mixture here used, increased both body weight and standard length in sea bream larvae and juveniles. On this matter, fish growth is strictly related to environmental conditions, especially in aquaculture, where un-proper captive rearing conditions induce stress and may negatively affect fish welfare. Previous results showed that stressful conditions at cellular level activate the HSPs gene expression, and in particular HSP70 which is involved in stress response (Forsyth et al., 1997; Ackerman and Iwama, 2001), protecting cells by binding and refolding damaged proteins (Feder and Hofmann, 1999). Our results demonstrate that sea bream larvae treated with probiotics showed lower HSP70 gene expression levels and for this reason we suppose a positive role of this mix of *Bacillus* reducing the severity of cellular stress.

In addition, generally, aquaculture conditions also induce physiological stress, leading to higher glucocorticoids levels in fish (Fagundes and Criscuolo Urbinati, 2008). On this regard, stress can activate the nervous system and trigger a humoral reaction cascade along the hypothalamo-pituitary-interrenal axis that ultimately results in the rapid release of corticosteroid stress hormones, the glucocorticoids. The physiological effects of glucocorticoids are regulated by the cellular Glucocorticoid Receptor (GR), a ligand-inducible transcription factor that can activate or repress target genes. Glucocorticoid-mediated gene induction, can influence a variety of physiological functions related to metabolism, immunity, behavior, osmoregulation, and cardiovascular transport. It was demonstrated in the rainbow trout *Oncorhynchus mykiss* that cortisol treatment significantly increased GR mRNA levels (Mommsen et al., 1999) and furthermore, Vijayan et al. (2003) found in the same species a positive relation between cortisol and GR gene expression levels. Our previous study reported that *Lactobacillus deulbrekii deulbrekii* treatment was able to enhance European seabass welfare as suggested by the lower levels of cortisol detected in treated fingerlings (Carnevali et al., 2006); moreover, we previously evidenced in rainbow trout that immunostimulants too seemed to be able to increase fish welfare; in fact, in this case, a significant lower levels of GR was evidenced (Gioacchini et al., 2008, 2010). Our hypothesis was that also a mix of *Bacillus* species could positively act on fish welfare, by decreasing GR levels. Supporting our previous findings, the present study showed that sea bream larvae treated with *Bacillus* mixture present a significant reduction of GR. On the basis of these data we hypothesize that the probiotics supplied during sea bream larval development

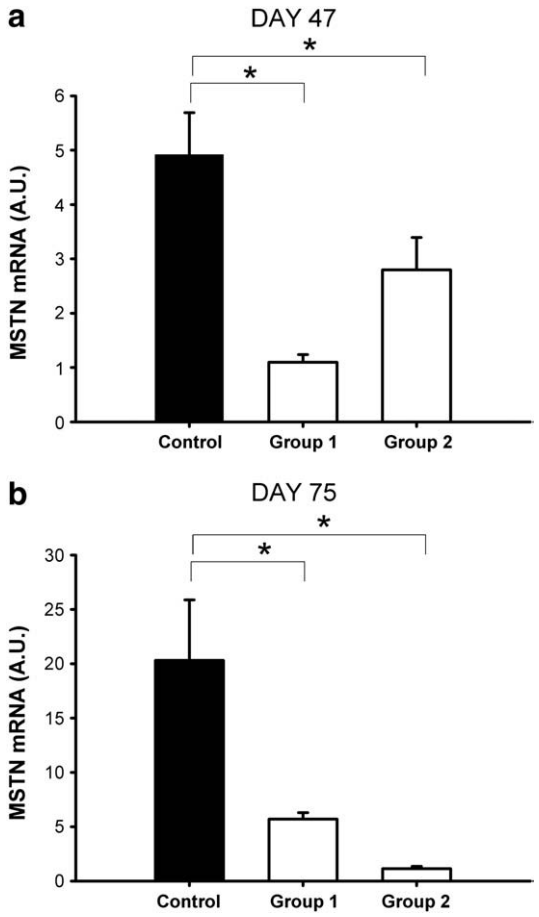


Fig. 3. MSTN gene expression at 47 (a) and 75 (b) days ph respectively. Larvae and juveniles treated with probiotics showed significant lower levels of MSTN gene expression. The lowest MSTN gene expression was evidenced in group 1 on day 47, while on day 75 in group 2. Asterisk, $P<0.05$ compared among experimental groups.

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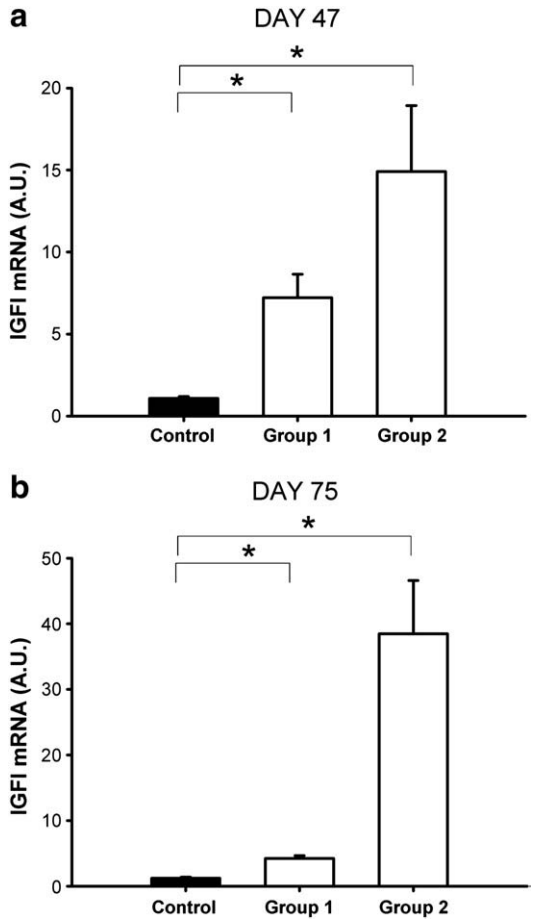


Fig. 4. IGFI gene expression at 47 (a) and 75 (b) days ph respectively. Higher levels of IGFI have been observed on both day 47 and 75 ph always in group 2, where also the highest BW and SL have been detected. Asterisk, $P<0.05$ compared among experimental groups.

could lead to a better stress response in intensive rearing culture conditions. By the way, stressors can harm animal growth and several studies demonstrated the relation between glucocorticoids and muscle growth at the molecular level; for example it is known that glucocorticoids increase the abundance of MSTN mRNA in mammals muscle cells (Ma et al., 2001, 2003). MSTN is a member of the TGF- family and is a negative factor able to inhibit myoblast proliferation; on the contrary, IGFI is a polypeptide which can act in an endocrine–paracrine/autocrine way, promoting muscle and cartilage growth and differentiation, positively affecting fish growth (Perrot et al., 1999). Moreover, IGFI gene expression is known to be inhibited by glucocorticoids in teleosts (Kajimura et al., 2003). So we hypothesize that a stressor like the intensive larval rearing conditions could evoke a physiological response in sea bream larvae, inducing higher GR levels and consequently, higher MSTN and lower IGFI levels with a consequent further inhibition of muscle growth.

In our study we hypothesize that the *Bacillus* mixture supplied during sea bream early development, acted positively on fish welfare by improving tolerance to rearing conditions with consequent decrease of HSP70 and GR gene expression. Moreover, we related the lower GR gene expression detected, with the significant MSTN gene expression decrease and the significant increase of IGFI gene expression. Since it is well known that IGFI positively acts on muscle cell proliferation, while MSTN inhibit muscle growth (Duan and Plisetskaya, 1993; Chauvigné et al, 2003; McPherron et al., 1997), we suggested that the interplay between the higher IGFI and the lower MSTN gene expression, could imply higher sea bream larval growth rate, as also supported by morphometric results. The highest growth registered upon probiotic delivery might be also related to the ability of the *Bacillus* species here used to deliver digestive enzymes, able to improve host digestion (Ghosh et al., 2002, Nagano and To, 2000; Ziaei-Nejad et al., 2006; Bagheri et al., 2008) or to provide extra micro- and macro-elements useful for fish larval growth (Verschuere et al. 2000).

The highest growth registered upon probiotic delivery might be also related to the ability of the *Bacillus* species here used to deliver digestive enzymes, able to improve host digestion (Ghosh et al., 2002, Nagano and To, 2000). However, concerning group 1 and group 2, even if the probiotic treatment here used lead to a mostly similar GR gene expression response, the highest BW, TL and IGFI gene expression were evidenced in group 2. For this reason we could assume that probiotic treatment could act on fish growth not only via GR levels reduction and for this reason the effects of *Bacillus* administration on growth metabolism should be deeper investigated.

However, Suzer et al. (2008) reported different responses in gilthead sea bream survival and growth, depending on the amount of supplied probiotic, and the way of administration. In our case, the best results concerning growth and stress response were found in group 2 where probiotics were delivered only by live food. Group 1 showed intermediary performances in terms of growth and stress response, suggesting that the probiotic amount provided only via live prey can positively affect sea bream growth and welfare. Apparently, probiotic administration to water may negatively interfere with the beneficial effects induced by administration via live prey, as observed in group 1. The lower performances observed in group 1 with respect to group 2, may be related with the increase of bacterial load in the water.

5. Conclusions

The molecular approach here used better elucidated the positive effects of the probiotics on the gilthead sea bream larviculture. Data obtained in this study showed the benefit of the administration of *Bacillus* probiotic mixture in terms of stress response and growth. Considering the low cost of production of *Bacillus* spores for aquaculture which offer convenience and cost benefits to farm operators and on the basis of the data here obtained, we suggest the administration by live preys of a mixture composed by *B. subtilis*, *B. licheniformis* and *B. pumilus* as a valuable feed additive in sea bream larviculture.

The results here achieved can provide new insight to the emerging blue biotechnology, for a further increase of productivity and competitiveness of the aquaculture industry.

Contributors

Matteo Alessandro Avella: carried out molecular analysis, interpreted the data, performed statistical analysis and drafted the manuscript (Introduction, Materials and methods—Morphometric and biomolecular analysis, morphometric and biomolecular results, Discussion and conclusions).

Giorgia Gioacchini: evaluated Real time PCR results and plotted graphs.

Olivier Decamp: designed the study.

Pavlos Makridris: carried out Morphometric and microbiological analyses, interpreted morphometric data, drafted part of the paper (Materials and methods—experimental model, hatching and larval rearing, probiotic strain).

Claudia Bracciatelli: carried out molecular analyses and participated to the interpretation of molecular data.

Oliana Carnevali: conceived of the study, participated in its design and coordination and helped to draft the manuscript.

All authors read and approved the final manuscript.

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References

Ackerman, P.A., Iwama, G.K., 2001. Physiological and cellular stress responses of juvenile rainbow trout to vibriosis. J. Aquat. Anim. Health 13, 173–180.

Arena, A., Maugeri, T.L., Pavone, B., Iannello, D., Gugliandolo, C., Bisignano, G., 2006. Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant *Bacillus licheniformis*. Int. Immunopharmacol. 6, 8–13.

Avella, M.A., Olivotto, I., Silvi, S., Place, A.R., Carnevali, O., 2010. Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 298, R359–R371.

Ayson, F.G., de Jesus, E.G.T., Moriyama, S., Hyodo, B., Funkenstein, B., Gerter, A., Kawauchi, H., 2002. Differential expression of insulin-like growth factor I and II mRNAs during embryogenesis and early larval development in rabbitfish, *Siganus guttatus*. Gen. Comp. Endocrinol. 126, 165–174.

Balcázar, J.L., 2003. Evaluation of probiotic bacterial strains in *Litopenaeus vannamei*. Final report, National Center for Marine and Aquaculture Research, Guayaquil, Ecuador.

Balcázar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Múzquiz, J.L., 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114, 173–186.

Bagheri, T., Hedayati, S.A., Yavari, V., Alizade, M., Farzanfar, A., 2008. Growth, survival and gut microbial load of rainbow trout (*Onchorhynchus mykiss*) fry given diet supplemented with probiotic during the two months of first feeding. Turk. J. Fish. Aquat. Sci. 8, 43–48.

Banerjee, S., Devaraja, T.N., Shariff, M., Yusoff, F.M., 2007. Comparison of four antibiotics with indigenous marine *Bacillus* spp. in controlling pathogenic bacteria from shrimp and *Artemia*. J. Fish Dis. 30, 383–389.

Barton, B.A., 2002. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integ. Comp. Biol. 42, 517–525.

Benetti, D.D., Sardenberg, B., Welch, A., Hoenig, R., Refik Orhun, M., Zink, I., 2008. Intensive larval husbandry and fingerling production of cobia *Rachycentron canadum*. Aquaculture 281, 22–27.

Burr, G., Gatlin, D., Ricke, S., 2005. Microbial ecology of the gastrointestinal tract of fish and the potential application of prebiotics and probiotics in finfish aquaculture. J. World Aquacult. Soc. 36, 425–436.

Carnevali, O., Zamponi, M.C., Sulpizio, R., Rollo, A., Nardi, M., Orpianesi, C., Silvi, S., Caggiano, M., Polzonetti, A.M., Cresci, A., 2004. Administration of probiotic strain to improve sea bream wellness during development. Aquacult. Int. 12, 377–386.

Carnevali, O., De Vivo, L., Sulpizio, R., Gioacchini, G., Olivotto, I., Silvi, S., Cresci, A., 2006. Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. Aquaculture 258, 430–438.

Chauvigné, F., Gabillard, J.C., Weil, C., Rescan, P.Y., 2003. Effect of refeeding on IGFI, IGFII, IGF receptors, FGF2, FGF6, and myostatin mRNA expression in rainbow trout myotomal muscle. Gen. Comp. Endocrinol. 132, 209–215.

Cladera-Olivera, F., Caron, G.R., Brandelli, A., 2004. Bacteriocin-like substance production by *Bacillus licheniformis* strain P40. Lett. Appl. Microbiol. 38, 251–256.

Decamp, O., Moriarty, D.J.W., Lavens, P., 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquac. Res. 39, 334–338.

Delaney, M.A., Klesius, P.H., 2004. Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). Aquaculture 236, 633–644.

Duan, C., Plisetskaya, E.M., 1993. Nutritional regulation of insulin-like growth factor-I mRNA expression in salmon tissues. J. Endocrinol. 139, 243–252.

Erickson, K.L., Hubbard, N.E., 2000. Probiotic immunomodulation in health and disease. J. Nutr. 130, 403–409.

European Union, 2003. Commission Press Room, 2003. Council and Parliament prohibit antibiotics as growth promoters: Commissioner Byrne welcomes adoption of Regulation on feed additives (press release, 7/22/03, Brussels, DN:IP/03/1058).

Fagundes, M., Criscuolo Urbinati, E., 2008. Stress in pintado (*Pseudoplatystoma corruscans*) during farming procedures. Aquaculture 276 (1–4), 112–119.

FAO/WHO, 2001. Report on Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. 1–4 October 2001, Cordoba, Argentina. Available at: ftp://ftp.fao.org/ es/esn/food/probio_report_en.pdf (Accessed 23 August 2007).

FDA, 2003. Guidance for Industry #152: Guidance on Evaluating the Safety of Antimicrobial New Animal Drugs with regard to their Microbiological Effects on Bacteria of Human Health Concern. Available at www.fda.gov/cvm.

Feder, M.E., Hofmann, G.E., 1999. Heat shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61, 243–282.

Fernandes, J.M.O., Mommens, M., Hagen, O., Babiak, I., Solberg, C., 2008. Selection of suitable reference genes for real-time PCR studies of Atlantic halibut development. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 150, 23–32.

Forsyth, R.B., Candido, E.P.M., Babich, S.L., Iwama, G.K., 1997. Stress protein expression in coho salmon with bacterial kidney disease. J. Aquat. Anim. Health 9, 18–25.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147–165.

Gatesoupe, F.J., 2008. Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. J. Mol. Microbiol. Biotechnol. 14, 107–114.

Gioacchini, G., Smith, P., Carnevali, O., 2008. Effects of Ergosan on the expression of cytokine genes in the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to enteric red mouth vaccine. Vet. Immunol. Immunopathol. 123, 215–222.

Gioacchini, G., Lombardo, F., Avella, M.A., Olivotto, I., Carnevali, O., 2010. Welfare improvement using alginic acid in rainbow trout (*Oncorhynchus mykiss*) juveniles. Chem. Ecol. 26, 111–121.

Gomez-Gil, B., Roque, A., Turnbull, J.F., 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture 191, 259–270.

Ghosh, K., Sen, S.K., Ray, A.K., 2002. Characterization of bacilli isolated from the gut of rohu, *Labeo rohita* fingerlings and its significance in digestion. J. Appl. Aquacult. 12, 33–42.

Hovda, M.B., Sivertsvik, M., Lunestad, B.T., Lorentzen, G., Rosnes, J.T., 2007. Characterisation of the dominant bacterial population in modified atmosphere packaged farmed halibut (*Hippoglossus hippoglossus*) based on 16S rDNA-DGGE. Food Microbiol. 24, 362–371.

Kajimura, S., Hirano, T., Visitacion, N., Moriyama, S., Aida, K., Grau, E.G., 2003. Dual mode of cortisol action on GH/IGF-I/IGF binding proteins in the tilapia, *Oreochromis mossambicus*. J. Endocrinol. 178, 91–99.

Kennedy, S.B., Tucker, J.W., Neidig, C.L., Vermeer, G.K., Cooper, V.R., Jarrell, J.L., Sennett, D.G., 1998. Bacterial management strategies for stock enhancement of warm water marine fish: a case study with common snook (*Centropomus undecimalis*). Bull. Mar. Sci. 62, 573–588.

Infante, C., Matsuoka, M.P., Asensio, E., Canavate, J.P., Reith, M., Manchado, M., 2008. Selection of housekeeping genes for gene expression studies in larvae from flatfish using real-time PCR. BMC Mol. Biol. 9, 28.

Le Duc, H., Hong, H.A., Barbosa, T.M., Henriques, A.O., Cutting, S.M., 2004. Characterization of *Bacillus* probiotics available for human use. Appl. Environ. Microb. 70, 2161–2171.

Lara-Flores, M., Olvera-Novoa, M.A., Guzman-Mendez, B.E., Lopez-Madrid, W., 2003. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). Aquaculture 216, 193–201.

Li, K., Zheng, T., Tian, Y., Xi, F., Yuan, J., Zhang, G., Hong, H., 2007. Beneficial effects of *Bacillus licheniformis* on the intestinal microflora and immunity of the white shrimp, *Litopenaeus vannamei*. Biotechnol. Lett. 29, 525–530.

Ma, K., Mallidis, C., Artaza, J., Taylor, W., Gonzalez-Cadavid, N., Bhasin, S., 2001. Characterization of 51-regulatory region of human myostatin gene: regulation by dexamethasone in vitro. Am. J. Physiol. 281, E1128–E1136.

Ma, K., Mallidis, C., Bhasin, S., Artaza, J., Gonzalez-Cadavid, N., Arias, J., Salehian, B., 2003. Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. Am. J. Physiol. 285, E363–E371.

Makridis, P., Fjellheim, J.A., Skjermo, J., Vadstein, O., 2000. Colonization of the gut in first feeding turbot by bacterial strains added to the water or bioencapsulated in rotifers. Aquacult. Int. 8, 367–380.

McPherron, A.C., Lawler, A.M., Lee, S.-J., 1997. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 387, 83–90.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302, 1–18.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fisher 9, 211–268.

Moriarty, D.J.W., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351–358.

Nagano, H., To, K.N., 2000. Purification of collagenase and specificity of its related enzyme from *Bacillus subtilis* FS-2. Biosci. Biotechnol. Biochem. 64, 181–183.

Papandroulakis, N., Divanach, P., Kentouri, M., 2002. Enhanced biological performance of intensive sea bream (*Sparus aurata*) larviculture in the presence of phytoplankton with long photophase. Aquaculture 204, 45–63.

Patruno, M., Maccatrozzo, L., Funkenstein, B., Radaelli, G., 2006. Cloning and expression of insulin-like growth factors I and II in the shi drum (*Umbrina cirrosa*). Comp. Biochem. Physiol. 144, 137–151.

Perrot, V., Moiseeva, E.B., Gozes, Y., Chan, S.J., Ingleton, P., Funkenstein, B., 1999. Ontogeny of the Insulin-like Growth Factor system (IGF-I, IGF-II, and IGF-1R) in gilthead seabream (*Sparus aurata*): expression and cellular localization. Gen. Comp. Endocrinol. 116, 445–460.

Peterson, B.C., Waldbieser, G.C., Bilodeau, L., 2004. IGF-I and IGF-II mRNA expression in slow and fast growing families of USDA103 channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol. 139, 317–323.

Picchietti, S., Mazzini, M., Taddei, A.R., Renna, R., Fausto, A.M., Mulero, V., Carnevali, O., Cresci, A., Abelli, L., 2007. Effects of administration of probiotic strains on GALT of larval gilthead seabream: immunohistochemical and ultrastructural studies. Fish Shellfish Immun. 22, 57–67.

Radonić, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., Nitsche, A., 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 313, 856–862.

Raida, M.K., Larsen, J.L., Nielsen, M.E., Buchmann, K., 2003. Enhanced resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum), against *Yersinia ruckeri* challenge following oral administration of *Bacillus subtilis* and *B. licheniformis* (BioPlus2B). J. Fish. Dis. 26, 495–498.

Rollo, A., Sulpizio, R., Nardi, M., Silvi, S., Orpianesi, C., Caggiano, M., Cresci, A., Carnevali, O., 2006. Live microbial feed supplement in aquaculture for improvement of stress tolerance. Fish Physiol. Biochem. 32, 167–177.

Author's personal copy

SCAN, 2003. Opinion of the Scientific Committee on Animal Nutrition on the criteria for assessing the safety of microorganisms resistant to antibiotics of human clinical and veterinary importance. European Commission Health and Consumer Protection Directorate-General.

Silvi, S., Nardi, M., Sulpizio, R., Orpianesi, C., Caggiano, M., Carnevali, O., Cresci, A., 2008. Effects of addition of *Lactobacillus delbrueckii* subsp *delbrueckii* on gut microbiota composition and contribution to the well-being of the European sea bass (*Dicentrarchus labrax* L.). Microb. Ecol. Health D 20, 53–59.

Smith, P., Davey, S., 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. J. Fish Dis. 16, 521–524.

Sugita, H., Hirose, Y., Matsuo, N., Deguchi, Y., 1998. Production of the antibacterial substance by bacillus species strain NM12, an intestinal bacterium of Japanese coastal fish. Aquaculture 165, 269–280.

Suzer, C., Çoban, D., Kamaci, H.O., Saka, S., Firat, K., Otgucuoğlu, Ö., Küçüksari, H., 2008. Lactobacillus spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: effects on growth performance and digestive enzyme activities. Aquaculture 280, 140–145.

Tannock, G.W., 1988. The normal microflora: new concepts in health promotion. Microbiol. Sci. 5 (1), 4–8.

Tinh, N.T.N., Dierckens, K., Sorgeloos, P., Bossier, P., 2008. A review of the functionality of probiotics in the larviculture food chain. Mar. Biotechnol. 10, 1–12.

Toegel, S., Huang, W., Piana, C., Unger, F.M., Wirth, M., Goldring, M.B., Gabor, F., Viernstein, H., 2007. Selection of reliable reference genes for qPCR studies on chondroprotective action. BMC Mol. Biol. 8, 13.US Food and Drug Administration, 2003. Guidance for Industry #152: evaluating the safety of antimicrobial new animal drugs with regard to their microbiological effects on bacteria of human health concern. Available online at http://www.fda.gov/cvm/guidance/fguide152.pdf (accessed 8/22/2005).

Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett. Appl. Microbiol. 36, 83–87.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. R. 64 (4), 655–671.

Vijayan, M.M., Raptis, S., Sathiyaa, R., 2003. Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen. Compar. Endocrinol. 132, 256–263.

Villamil, L., Tafalla, C., Figueras, A., Novoa, B., 2002. Evaluation of immunomodulatory effects of lactic acid bacteria in turbot (*Scophthalmus maximus*). Clin. Diagn. Lab. Immunol. 9 (6), 1318–1323.

Walker, W.A., Duffy, L.C., 1998. Diet and bacterial colonization: role of probiotics and prebiotics. J. Nutr. Biochem. 9, 668–675.

Wang, Y., Li, J., Lin, J., 2008. Probiotics in aquaculture: challenges and outlook. Aquaculture 281, 1–4.

Ziaei-Nejad, S., Rezaeib, M.H., Takamic, G.A., Lovettd, D.L., Mirvaghefia, A., Shakourie, M., 2006. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture 252, 516–524.