Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish

Matteo A Avella, Ike Olivotto, Stefania Silvi, Allen R. Place and Oliana Carnevali

Am J Physiol Regul Integr Comp Physiol 298:R359-R371, 2010. First published 18 November 2009;
doi:10.1152/ajpregu.00300.2009

You might find this additional info useful...

This article cites 87 articles, 13 of which can be accessed free at:
/content/298/2/R359.full.html#ref-list-1

Updated information and services including high resolution figures, can be found at:
/content/298/2/R359.full.html

Additional material and information about ***American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*** can be found at:
http://www.the-aps.org/publications/ajpregu

This information is current as of October 4, 2013.

Downloaded from on October 4, 2013

American Journal of Physiology - Regulatory, Integrative and Comparative Physiology publishes original investigations that illuminate normal or abnormal regulation and integration of physiological mechanisms at all levels of biological organization, ranging from molecules to humans, including clinical investigations. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2010 by the American Physiological Society. ISSN: 0363-6119, ESSN: 1522-1490. Visit our website at http://www.the-aps.org/.

Am J Physiol Regul Integr Comp Physiol 298: R359–R371, 2010.
First published November 18, 2009; doi:10.1152/ajpregu.00300.2009.

Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish

Matteo A Avella,[1] Ike Olivotto,[1] Stefania Silvi,[2] Allen R. Place,[3] and Oliana Carnevali[1]

[1]*Department of Marine Sciences, Università Politecnica delle Marche, Ancona, Italy;* [2]*Department of Comparative Morphology and Biochemistry, Università degli Studi di Camerino, Camerino, Italy; and* [3]*Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland*

Submitted 28 May 2009; accepted in final form 11 November 2009

Avella MA, Olivotto I, Silvi S, Place AR, Carnevali O. 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, 2010. First published November 18, 2009; doi:10.1152/ajpregu.00300.2009.—We set out to determine whether probiotic addition would improve larval development in the false percula clownfish *Amphiprion ocellaris* and to determine what molecular responses could be observed in the larvae following probiotic exposure. We supplied the probiotic bacterial strain *Lactobacillus rhamnosus* IMC 501 to clownfish larvae from the first day posthatch simultaneously by live prey and with addition to rearing water (*group 2*) and exclusively by live prey (*group 3*). We observed twofold higher body weight in both clownfish larvae and juveniles when probiotics were supplied via live prey and added to the rearing water. In addition, development was accelerated with metamorphosis occurring 3 days earlier in fingerlings treated with probiotic. Alteration in molecular biomarkers supported the faster growth observation. There was significantly increased gene expression of factors involved in growth and development (insulin-like growth factors I and II, myostatin, peroxisome proliferator-activated receptors α and β, vitamin D receptor α, and retinoic acid receptor γ) when probiotics were delivered via live prey and added to the rearing water. Moreover, probiotic treatment lessened the severity of the general stress response as exhibited by lower levels of glucocorticoid receptor and 70-kDa heat shock protein gene expression. Furthermore, an improvement of skeletal head development was observed, with a 10–20% reduction in deformities for juveniles treated with probiotic. All data suggest a potent effect on development resulting from the administration of lactic acid bacteria to larval clownfish, and this study provides a preliminary molecular entry path into the investigation of mechanisms responsible for probiotic enhancement in fish development.

metamorphosis; fish growth; nuclear receptor gene expression

THE POSITIVE EFFECTS OF PROBIOTIC administration to finfish growth and development are well documented (28, 29, 85). Generally, probiotic administration during early developmental stages is most effective, frequently resulting in greater than an order of magnitude increase in survivorship (29). The false percula clownfish (*Amphiprion ocellaris*) has a high demand for aquarium trade, but its production in captivity faces critical bottlenecks during early life stages. The stresses in captive rearing conditions frequently result in high mortality rates (10, 57) and growth abnormalities leading to high incidence of skeletal deformities (3, 24, 25, 30, 41, 42, 43). Probiotics are well known to positively impact fish welfare (40) by reducing the general stress response and promoting growth (71), as well as increasing survivorship overall (85). Among the lactic acid bacteria (LAB), *Lactobacillus rhamnosus*, a LAB species originally intended for human use, is well known to have probiotic properties in humans (64) as well as in teleosts (55). To date, although a few studies have delineated the effects of this *Lactobacillus* species on immune modulation in fish (54, 59), no studies have reported its effect on larval fish survivorship, growth, and development.

Stress responses are generally classified as primary, secondary, and tertiary (8). Primary responses include the activation of the hypothalamus-pituitary-interrenal axis with the release of cortisol in the blood stream (52). It is well established that cortisol is the main corticosteroid stress-related hormone in fish, and its effects are mediated by the intracellular glucocorticoid receptor (GR) where GR mRNA abundance is directly related to cortisol levels (1, 80). Among secondary responses, changes in heat shock proteins (HSPs) can also occur; in particular, HSP70 has been widely studied in fish, but its role in stress response to aquaculture conditions is still a matter for debate (2, 22, 25, 79, 92). Last, stress can lead to tertiary responses, which impact the whole animal's performance in terms of growth (46, 47), metabolism depletion, and mortality (86, 87). Fish growth is positively correlated to muscle growth and is controlled by extrinsic regulators such as insulin-like growth factor I (IGF-I), insulin-like growth factor II (IGF-II), and myostatin (MSTN), which are involved in fish myogenesis (5, 60, 61). These gene products have been widely used as biomarkers for muscle growth (17, 23). IGF-I and IGF-II are two polypeptides with similar structure and function to insulin. Gene expression of IGF-I and IGF-II are well known to vary during development (26, 60) and are strictly dependent on feeding regime (12, 13, 27). On the other hand, MSTN, a member of the transforming growth factor-β (TGF-β) superfamily, acts in an opposite way by inhibiting muscle cell proliferation (50). Moreover, growth metabolism is correlated to several other factors belonging to the nuclear receptor superfamily, such as peroxisome proliferator-activated receptors (PPARs), retinoic acid receptors (RARs), and vitamin D receptors (VDRs), which are natural receptors for many organic compounds and are important modulators for achieving optimal growth, correct development, and proper pigmentation in marine fish (23, 42, 59, 63, 64). These factors integrate multiple cellular signaling pathways that affect and regulate growth and organism remodeling through processes such as lipid metabolism (18), energy administration (11), fatty acid catabolism(18), epithelial cell growth and differentiation (11), calcium homeostasis, growth and bone formation (90), morphogenesis, and chondrogenesis (51). Specifically, VDRs mediate the effects of the most bioactive derivative of vitamin D,

Address for reprint requests and other correspondence: O. Carnevali, Dept. of Marine Science, Via brecce bianche 60131, Ancona, Italy (e-mail: o.carnevali@univpm.it).

 0363-6119/10 $8.00 Copyright © 2010 the American Physiological Society

1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$], by initiating a cascade of molecular events leading to bone mineralization and remodeling and by modulating cell growth and differentiation in a wide array of tissues (34). PPARα and -β are known to be involved in lipid metabolism and cell growth (9) and to be activated by fatty acids, since they are natural receptors for fatty acids (32). Moreover, fermentation products of LAB, including short-chain fatty acids and immunoregulatory molecules such as eicosanoids, are known to activate PPARs (18), and for these reasons we use them as biomarkers for checking the effects of *L. rhamnosus* on lipid and eicosanoid production.

Concerning RARs, these nuclear receptors mediate the biological effects of retinoids, derivatives of vitamin A, which are required for cellular growth and development (20). In particular, RARγ is known to be involved in correct cranial development during the early larval developmental stages (81). For these reasons, we use VDRα and RARγ as biomarkers for skeletal development, and we hypothesize that probiotic treatment could improve clownfish early development by acting on the modulation of these two nuclear receptors. We decided that using real-time PCR to measure changes in transcript abundance for the biomarkers associated with stress, changes in growth, and skeletal development was most appropriate for this study because of the costs associated with two-dimensional protein gels and the lack of commercially available antibodies for these biomarkers that we targeted.

Several probiotic strains have been used to enhance fish survivorship and growth (71, 74, 75), with several *Lactobacillus* species showing beneficial effects in fish juvenile husbandry (6, 7). Even in intensive larval rearing conditions, positive effects on survivorship and growth metabolism have been reported (15, 16).

In the current study we used probiotic treatment with the bacterial strain *L. rhamnosus* IMC 501 to modulate the growth and development of clownfish larvae and juvenile. We evaluated the response to probiotic treatment through morphometric measurements such as total length and body weight and through molecular measurements of gene expression of the biomarkers for IGFs, MSTN, PPARα, PPARβ, VDRα, RARγ, GR, and HSP70 using real-time PCR. The effect on larval metamorphosis and skeletal deformities was recorded, and the presence of *L. rhamnosus* in treated fish was quantified using real-time PCR.

MATERIALS AND METHODS

Probiotic strain. Live prey and rearing water were treated with the probiotic strain *L. rhamnosus* IMC 501, provided by Synbiotec (Camerino, Italy), following the instructions provided by the company. The bacterial preparation for administration was performed according to Carnevali et al. (16), and *L. rhamnosus* IMC 501 was used at a concentration of 10^6 colony-forming units (CFU)/ml two times a day.

The probiotic was added to water and supplied to live food (either rotifers or *Artemia*) 15 min before administration, and the mixture was incubated under low aeration. The treatment was administered twice daily. Addition to rearing water followed the instructions provided by Synbiotec at 0.01 g/l to reach a final concentration in water of 10^6 CFU/g.

Detection of Lactobacillus rhamnosus IMC 501. A real-time quantitative PCR (QPCR) procedure was used for the quantification of *L. rhamnosus* IMC 501 in *A. ocellaris* samples by using the following primers: Y2, 5′-CCCACTGCTGCCTCCCGTAGGAGT-3′ (conserved 16S rRNA; Ref. 91), and Lr, 5′-TGCATCTTGATTTAATTTTG-3′ (*L. rhamnosus* 16S; Ref. 77). The UltraClean fecal DNA kit (MO BIO Laboratories, Carlsbad, CA) was used for DNA extraction from whole body *A. ocellaris* samples. Larvae and juveniles were surface-disinfected with benzalkonium chloride (0.1% wt/vol) for 30 s, rinsed three times in autoclaved water, and homogenized in 3 ml of reducing solution (35, 55). DNA samples (0.4 μl) were analyzed in 20-μl amplification reactions consisting of 9 μl of 2× brilliant SYBER green QPCR master mix (Stratagene) and 0.4 μl of each primer, both at concentrations of 0.3 μM, and 9.8 μl of water.

Thermal cycling for the quantification of *Lactobacillus* species consisted of an initial cycle of 95°C for 10 min and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. To determine the specificity of the amplification, analysis of the melting curve for the product was performed after the last cycle of each amplification. Real-time PCR was performed with an Mx3000 (Stratagene), and all PCRs were performed in triplicate.

Standard curve. For quantification of *L. rhamnosus* IMC 501 in a known sample, a standard curve was generated and used in subsequent analyses. A 10-fold serial dilution of a pure culture of strain *L. rhamnosus* IMC 501 was prepared in a sterile saline solution, after which triplicate aliquots of 50 μl of each dilution were spread-plated on deMan Rogosa Sharpe (MRS) medium (21). After 48 h of aerobic incubation at 37°C, colonies were counted. DNA extracted from the initial dilution series was analyzed using the real-time QPCR protocol as described above.

Quantitative data from MRS platings were subsequently used to generate a standard curve in which the bacterial cell numbers were plotted against the cycle threshold (C_T) value, being the minimal cycle number at which the fluorescence signal exceeds the threshold level. The standard curve was generated from real-time PCR analysis of DNA extracts using 16S rRNA as the target gene. A linear relation between initial bacterial concentration and C_T values was obtained in the range corresponding to 10^5-10^9 CFU ($R^2 = 0.9914$). *L. rhamnosus* was quantified by real-time PCR, interpolating the C_T values with that of a standard curve.

Animals and diet. The experimental model used in this study was the false percula clownfish, *A. ocellaris*. Clownfish pairs were kept in 200-liter tanks. Flower pots were supplied to the brood stock as a suitable substrate for egg laying. Photoperiod and temperature were maintained at a 13 h light-11 h dark and 28°C, salinity 30‰, pH 8–8.5, and NH_3 and $NO_2^- < 0.02$ parts per million (ppm). Fish were fed twice a day with commercial frozen mysis and krill (EscheMatteo, Parma, Italy). At these conditions, fish spawned every 15 days and the embryo development was 7 days (168 h) to hatch.

On the hatching day, the flower pot with the egg clutch was transferred to a 20-liter larval rearing tank presenting the same chemical-physical characteristics of the brood stock tank. A gentle air flow was provided to the egg clutch by an airstone to simulate the fanning activity of the male. The embryos were left in darkness for 50 ± 5 min, and after this period, hatching took place. After this, the airstone was removed and the flower pot was replaced in the brood stock tank.

Control and treatment tanks were set up as follows; the water in the 20-liter larval tank was gently replaced twice an hour by a dripping system and the sides of the tank were covered with black panels to reduce light reflection while the phytoplankton (*Nannochloropsis oculata*) was used (50,000 cells/ml) to condition the tank until *day 8* posthatch (PH). Larvae were subjected to an extended photoperiod (24 h light-0 h dark) (4, 56), and water quality was monitored every day with the following parameters: temperature 28°C, salinity 30‰, pH 8–8.5, and NH_3 and $NO_2^- < 0.02$ ppm.

Larvae were fed four times a day (at 8:00 AM, 12:00 PM, 5:00 PM, and 10:00 PM) on Algamac 2000 (Aquafauna Bio-Marine)-enriched rotifers (*Brachionus plicatilis*, 10 individuals/ml) from *day 1* to *5* PH and un-enriched AF430 *Artemia salina* nauplii (INVE Technologies, Dendermonde, Belgium) from *day 6* to *30* following the Oce.AN. (a

spin-off of the Polytechnic University of Marche) larval feeding protocol (Algamac 2000-enriched rotifers from *day 1* to *6* PH; un-enriched *Artemia* nauplii from *day 7* to *30* PH). All the zooplankton strains were enriched using Algamac 2000 (Aquafauna Bio-Marine, Hawthorne, CA) following the instructions provided by the company (0.5 g/10^6 rotifers and 0.2 g/10^5 *Artemia* nauplii; at 25°C for 8–12 h).

Larvae were divided into three experimental groups each of 100 (3 replicates each) and fed as follows. In the control group, probiotics were not supplied to larvae. In *group 2*, probiotics were supplied to larvae from *day 1* PH twice a day (at 8:00 AM and 5:00 PM) via live prey (incubating rotifers and *Artemia* nauplii for 15 min in a probiotic resuspended solution with a concentration of 10^6 CFU/ml marine water) and added into the rearing water (at the concentration of 10^6 CFU/ml marine water). In *group 3*, probiotics were supplied to larvae from *day 1* PH twice a day (at 8:00 AM and 5:00 PM) via live prey only. Rotifers and *Artemia* nauplii were supplied at the final concentration of 10 rotifers/ml and 5 nauplii/ml, respectively. After live prey administration, water flow was stopped for 1 h to give larvae sufficient time to catch live prey. During feeding, the oxygen level was monitored and never reached levels below 5 mg/l. To assess probiotic effects on water quality, pH, NH_3, NO_2^-, and NO_3^{2-} were checked daily.

Sampling and dissection. Larval sampling was performed on *day 5* PH (larval phase) and *day 30* PH (juvenile phase) at 8:00 AM just before feeding. For each sampling, 10 individuals were used for morphometric analyses and 10 individuals were stored at −80°C for molecular and microbiological analyses. Clownfish larvae and juveniles for both morphometric and biomolecular analyses were euthanatized using MS222 before sampling. After euthanization, larvae and juveniles for molecular analyses were immediately stored at −80°C.

During larval development, metamorphosis time was considered as the day posthatch when the first specimens showed orange pigmentation and the first white band on the head region. The percentage of metamorphosed larvae from *day 9* to *17* PH was determined for each experimental group . On *day 15* PH, survivorship was also estimated.

At the end of treatment (*day 30* PH), the remaining clownfish juveniles were anesthetized with MS222 (0.1 g/l) and then analyzed to check detectable skeletal deformities. With the use of the criteria of Villeneuve et al. (82), fish were examined under a binocular microscope (STEMI 2000) to determine spinal malformation and defective formation of the operculum, and the incidence of skeletal body malformations (operculum and vertebral column deformities) was recorded. The percent malformation in each tank was determined as follows: (number of malformed larvae/number of remaining total larvae) × 100.

Histology. Day 30 PH clownfish juveniles ($n = 10$) were fixed in 4% paraformaldehyde (PFA) and processed with standard histological techniques (66). Sagittal sections (5 μm) were stained with hematoxylin-eosin and observed under a light microscope. Fish showing correct development were compared with specimens showing skeletal alterations.

Gene expression analysis. Total RNA extraction from whole larval body was optimized with the best recovery being obtained with 30 mg of larvae using the 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 spectrophotometer, and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on a 1% agarose gel. RNA was stored at −80°C 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 of oligo d(T)$^+$ adapter primer, 5′-GACTGCAGTCGACATCGATTTTTTTTTTTTTTTTTT-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 of 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.

Real-time QPCR. Sequenced regions containing the aforementioned markers for several species closely related to clownfish as well as out-group species were aligned, and the consensus sequences was used for making primers for real-time QPCR (Table 1). Triplicate PCRs were carried out for each sample analyzed. After optimization (primer annealing temperature and cDNA dilutions), the PCRs were performed with the SYBR green method in the iQ5 multicolor real-time PCR detection system (Bio-Rad). The reactions were set up in 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 the fluorescent intercalating agent, 0.3 μM forward primer, and 0.3 μM reverse primer. The

Table 1. *Species used for making primers for real-time PCR*

Gene	Fish Sequences		Out-Group Sequence
GR	*Pimephales promelas*	AY533141	*Rattus norvegicus* AB115420
	Dicentrarchus labrax	AY619996	
	Sparus aurata	DQ486890	
HSP70	*Chrysiptera parasema*	AY129817	
	Amphiprion ocellaris	EU683309	
IGF-I	*Dicentrarchus labrax*	AY800248	*Gallus gallus* NM_001004384
	Sparus aurata	AY996779	
IGF-II	*Sparus aurata*	AY839105	*Gallus gallus* NM_001030342
	Dicentrarchus labrax	EF563836	
MSTN	*Dicentrarchus labrax*	AY839106	*Mus musculus* NM_010834
	Sparus aurata	AF258448	
PPARα	*Salmo trutta* (f. fario)	DQ139936	
	Salmo salar	NM_001123560.1	
PPARβ	*Salmo trutta* (f. fario)	DQ139937	
	Salmo salar	AM229306	
VDRα	*Dicentrarchus labrax*	AM040727	
	Danio rerio	NM_130919	
RARγ	*Dicentrarchus labrax*	AJ496181	
	Sparus aurata	EU643831	
β-Actin	*Pomacentrus molluccensis*	DQ243822	*Balaenoptera acutorostrata* AB270707
	Astronotus ocellatus	EU553593	

See text for definition of genes.

thermal profile for all reactions was 15 min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 55°C, and 20 s at 72°C. The exceptions were PPARα (15 min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 51°C, and 20 s at 72°C) and PPARβ (15 min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 49°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 a single melting curve.

A relative quantification of cDNA was made using β-actin as a reference transcript (internal standard). Pfaffl's mathematical model (62) was applied to determine the ratio between the different expression of the target gene (GR, HSP70, IGF-I, IGF-II, MSTN, PPARα and PPARβ, VDRα, and RARγ) in the treated and control group and the different expression of the standard gene (β-actin) in the treated and control group. The following equation was used to express the relative expression ratio: $(E_{target})^{\Delta CP_{target}}/(E_{standard})^{\Delta CP_{standard}}$, where E_{target} is the real-time PCR efficiency of target gene transcript (IGF-I, IGF-II, MSTN, PPARα and PPARβ, VDRα, or RARγ), $E_{standard}$ is the real-time PCR efficiency of the standard gene transcript (β-actin), ΔCP_{target} is the crossing point deviation of the control minus that of the sample for the target gene transcript, and $\Delta CP_{standard}$ is the crossing point deviation of the control minus that of the sample for the standard gene transcript. Modification of gene expression is represented with respect to the control, which is assumed to have the value of 1 AU (arbitrary unit). With the use of Pfaff's mathematical model, the cDNA levels of the genes were quantified in each group and at each time of sampling. To test real-time PCR efficiency, serial dilutions of cDNA, each in triplicate, were amplified by real-time PCR using the specific primers for target genes and the standard gene. Log cDNA dilution was plotted vs. ΔCP, and the efficiencies (E) were calculated according to $E = 10^{(-1/\text{slope})}$.

Table 2 shows primers sequences used in the present study (at a final concentration of 10 pmol/μl), partial sequence accession numbers deposited in GenBank, and the sequence identity score reported by BLAST at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis. Results are means ± SD. The significance of differences was determined using a one-way ANOVA, followed by Tukey's or Bonferroni's test for multigroup comparisons, using the statistical software package SigmaStat 3.1 (Systat Software). A P value <0.05 was regarded as statistically significant.

Table 3. *Retention of Lactobacillus rhamnosus IMC 501 in Amphiprion ocellaris larvae and juveniles*

	Control Group	*Group 2*	*Group 3*
Larvae (5 days)	0.0 ± 0.0^{a}	$1.9\times10^{8} \pm 1.1\times10^{8\ b}$	$6.7\times10^{7} \pm 1.3\times10^{7\ c}$
Juveniles (30 days)	0.0 ± 0.0^{a}	$4.7\times10^{8} \pm 2.8\times10^{8\ e}$	$2.1\times10^{7} \pm 0.8\times10^{7\ f}$

Values are means ± SD. Control group clownfish larvae were fed a standard diet without probiotic supply. In g*roup 2* fish, probiotic was delivered through live prey and added in water. In *group 3*, probiotic was delivered through live prey. Counts were colony-forming unites (CFU)/g of larvae or juveniles. $^{a,b,c,d,e,f}P < 0.05$, letters indicate statistical significance.

RESULTS

Retention of L. rhamnosus 501 in clownfish larvae and juveniles. Clownfish microbiota was affected by *L. rhamnosus* administration. In the control group, no *L. rhamnosus* were detected, whereas in both experimental groups, significant numbers of *L. rhamnosus* were detected in both larval and juvenile stages. In particular, the best treatment for *L. rhamnosus* retention was observed in *group 2*, where probiotic was delivered via both live prey and addition to the rearing water (Table 3). Moreover, in *group 2*, no effects on water quality (concerning pH, NH_3, NO_2^-, and NO_3^{2-}) were observed upon probiotic administration.

Survivorship. Probiotic treatment significantly increased survivorship in both *group 2* and *group 3* treatments (Fig. 1). In particular, the best survivorship was achieved in *group 2*, with a survival of 80 ± 3%, whereas in *group 3*, a survival of 70 ± 4% was observed. In particular, the high mortality observed at *day 3* PH in prior studies (88) was not observed in any of the probiotic-treated groups (*groups 2* and *3*).

Growth rate measurements. Probiotic treatment was able to accelerate development, since in both *group 2* and *group 3* clownfish, larvae metamorphosed 3 days (*day 9* PH) earlier than larvae in the control group (*day 12* PH) (Fig. 2). On *day 5* in *groups 2* and *3*, larvae showed a significantly ($P < 0.05$)

Table 2. *Primers sequences, GenBank partial sequence accession nos., and percent sequence identity for biomarker genes used in this study*

Gene	Primer Ssequence	GenBank Accession No.	Sequence Identity
GR	F: 5′-CGGTCACTGCTACGTCTTCA-3′ R: 5′-CCTCCCAGCACACAGGTAAT-3′	EU722335	89% with *Dicentrarchus labrax* GR
HSP70	F: 5′-ACGGAGAGTCGATTTCGATG-3′ R: 5′-GAAGGACATCAGCGACAACA-3′	EU683309	90% with *Paralichthys olivaceus* HSP70
IGF-I	F: 5′-AGTGCGATGTGCTGTATC-3′ R: 5′-CAGCTCACAGCTTTGGAAGCA-3′	EF620549	90% with *Lates calcarifer* IGF-I
IGF-II	F: 5′-CGGCAGAAACGCTATGTGGA-3′ R: 5′-TGCTGGTTGGCCTACTGAAA-3′	EF620550	87% with *Mugil cephalus* IGF-II
MSTN	F: 5′-TTTTGAGCAAACTGCGAATG-3′ R: 5′-CACGTCGTACTGGTCGAGAA-3′	EF620551	97% with *Dicentrarchus labrax* MSTN
PPARα	F: 5′-TTCAGCGACATGATGGAGCC-3′ R: 5′-CAGTTTCTGCAGCAGATTGG-3′	EF620552	87% with *Dicentrarchus labrax* PPARα
PPARβ	F: 5′-AGGAGATAGGGGTACACGTG-3′ R: 5′-CAGGAACTCCCGGGTCACAA-3′	EF620553	93% with *Dicentrarchus labrax* PPARβ
VDRα	F: 5′-ACGTGAAGGCCCAGTAACAC-3′ R: 5′-CGACTCAGGTGAGTGGTTGA-3′	EU676177	96% with *Dicentrarchus labrax* VDRα
RARγ	F: 5′-ATGTGAAGGAGGAGGTGGTG-3′ R: 5′-GGTTTCTTGGTGAGCTTTGC-3′	EU676178	96% with *Salmo salar* RARγ
β-Actin	F: 5′-GAAAATCATCCAATTGCTGGATG-3′ R: 5′-CTTCCCACGCAAGG ACAGA-3′	EF620554	93% with *Pomacentrus molluccensis* β-actin

F, forward primer; R, reverse primer.

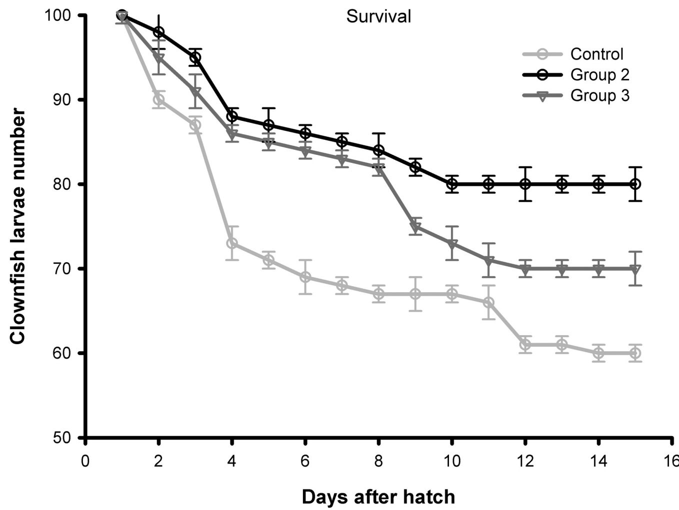
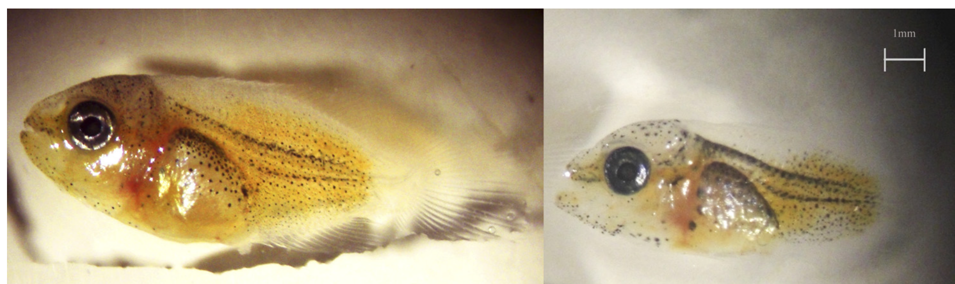


Fig. 1. Survivorship. Clownfish survivorship curves (control, *group 2*, and *group 3*) for 15 days posthatch (PH). Probiotic was administered with either live feed alone (*group 3*) or live feed and addition to the rearing water (*group 2*). The highest survivorship was obtained in *group 2* (80%), where *Lactobacillus rhamnosus* was delivered via live prey and addition to the rearing water.

higher body weight (*group 2*, 18.4 ± 0.1 mg; *group 3*, 9.2 ± 0.1 mg) with respect to those of the control group (5.6 ± 0.1 mg), and in particular on *day 5* PH, *group 2* showed almost three times higher body weight than the control group (Fig. 3). On *day 30* PH, only *group 2* juveniles showed significantly ($P < 0.05$) higher body weight (116.4 ± 0.1 mg) with respect to the other experimental groups (control group, 45.5 ± 0.05 mg; *group 3*, 45.6 ± 0.1 mg) (Fig. 3). Standard length (distance from the tip of the snout to the point of notochord flexion, as mentioned in Ref. 36) was not significantly affected by probiotic treatment in the larval stage (*day 5*: control group, 5.8 ± 0.02 mm; *group 2*, 5.73 ± 0.1 mm; *group 3*, 5.7 ± 0.1 mm) (Fig. 3), whereas juveniles from *group 3* showed a significantly greater standard length (*day 30*: control group, 11.40 ± 0.1 mm; *group 2*, 11.35 ± 0.1 mm; *group 3*, 12.60 ± 0.1 mm) (Fig. 3).

Molecular alterations: changes in biomarkers for stress, growth promotion, and development. Changes in transcript abundance for the various biomarkers support the changes in morphometric measurements observed. On *day 5* PH, a significant reduction in the stress biomarker GR was observed in both groups treated with probiotics, whereas no significant differences were detected between the treatments. This difference persisted at *day 30* PH, with a significant reduction observed in both *groups 2* and *3* compared with control. *Group 3* juveniles at *day 30* PH exhibited significantly lower GR transcript levels than *group 2* juveniles (Fig. 4). Similar responses in HSP70 transcript levels were observed between treatments and controls on both *days 5* and *30* PH, with *group 2* individuals showing the lowest gene expression. At the juvenile stage, no significant differences were detected in HSP70 expression between *groups 2* and *3* (Fig. 4).

Concerning growth factor biomarkers (Fig. 5), on *day 5* PH, a significant increase in IGF-I and IGF-II transcript abundance was observed in clownfish fed probiotics (*groups 2* and *3*). In contrast, MSTN transcript abundance was significantly lower in *groups 2* and *3* with respect to the control group at *day 5* PH. *Group 3* MSTN transcript abundance was significantly lower than that in *group 2*. In juveniles (*day 30* PH), the IGF-I and MSTN gene expression showed the same trend observed in larvae (Fig. 5). PPAR gene expression (Fig. 6) on *day 5* PH in larvae fed probiotics delivered by live prey and rearing water (*group 2*) showed a significant increase for both subtypes α and β with respect to the control group and *group 3*, as similarly observed for IGF gene expression. On *day 30* PH, a significantly higher PPARα gene expression was observed in both groups treated with probiotic, with the highest levels in *group 2* (Fig. 6). Surprisingly, the subtype β was not affected in *group 2*, and an unexpected lower expression was observed in *group 3* (Fig. 6). For VDRα and RARγ, a significant transcript abundance increase was detected only in *group 2*, whereas no



	Control group	Group 2	Group 3
Day 9 ph	0	3 ± 2 b	3 ± 2 b
Day 10 ph	0	27 ± 4 c	28 ± 6 c
Day 11 ph	0	39 ± 3 d	37 ± 3 d
Day 12 ph	3 ± 1 a	68 ± 3 e	66 ± 5 e
Day 13 ph	29 ± 4 b	94 ± 2 g	91 ± 3 g
Day 14 ph	41 ± 4 f	100 ± 2 h	100 ± 1 h
Day 15 ph	72 ± 6 e	100 h	100 h
Day 16 ph	93 ± 4 g	100 h	100 h
Day 17 ph	100 h	100 h	100 h

Fig. 2. Metamorphosis time. Two representative photos show clownfish larvae on *day 9* PH. The specimen at *left* had completed metamorphosis, shown by the orange pigmentation and the first white band detectable on the head region, and was treated with the probiotic *L. rhamnosus* IMC 501 from the first day PH. The specimen at *right* belongs to the control group, and metamorphosis is shown incomplete at *day 9* PH. The percentage of metamorphosed clownfish larvae from each experimental group are indicated. [a,b,c,d,e,f,g,h]$P < 0.05$, letters indicate statistical significance.

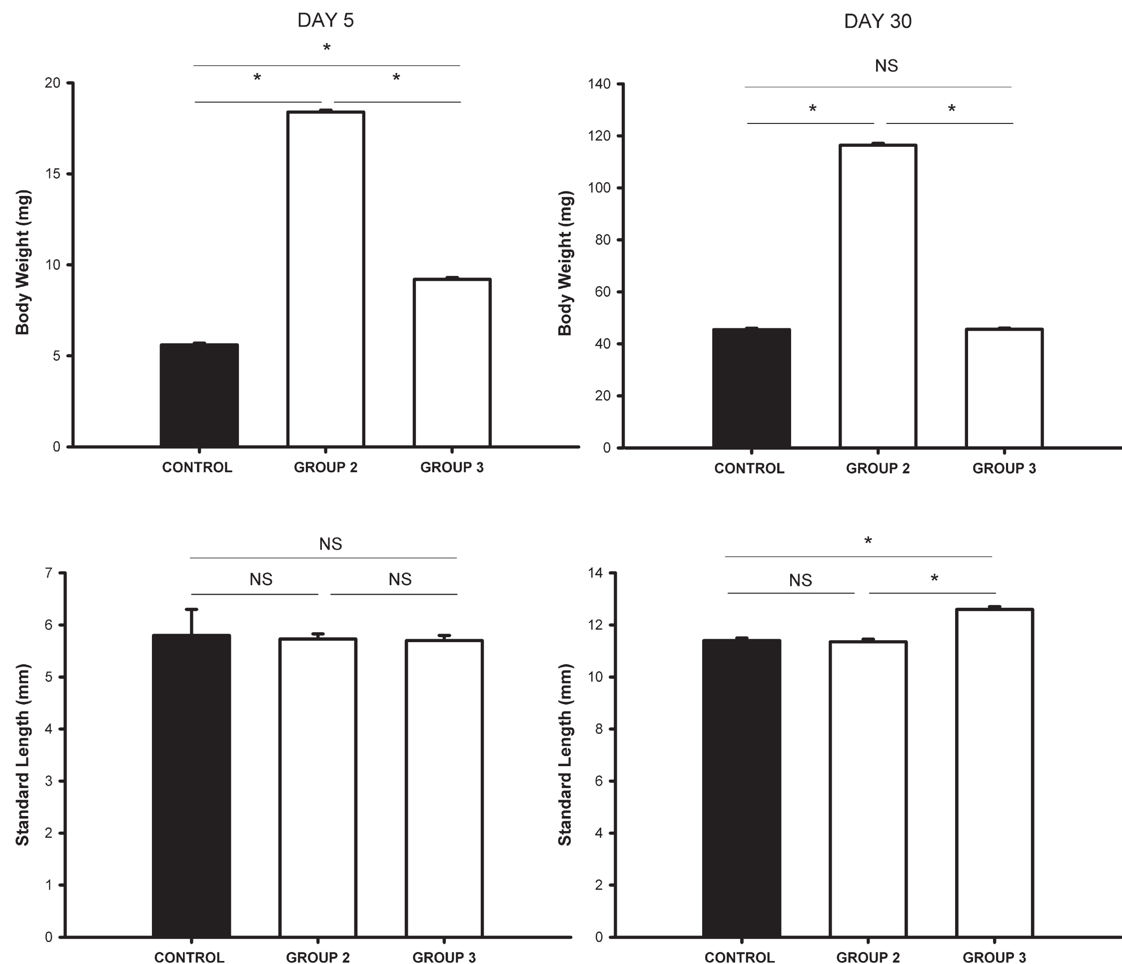


Fig. 3. Morphometric effects (body length and weight) of probiotic exposure. On both 5 and 30 days PH, body weight was significantly affected by *L. rhamnosus* administration, particularly in *group 2*, where a value of ~3-fold higher body weight with respect to the control group was registered. Also, *group 3* body weight was positively affected by probiotic treatment, although not as intensely as observed in *group 2*. Thirty-day probiotic treatment with *L. rhamnosus* had no effect on clownfish standard length (NS, no significant difference). $^*P < 0.05$ compared with standard diet (control group).

significant differences were detected between the control group and *group 3* (Fig. 6).

Skeletal deformities. On *day 30* PH, clownfish treated with probiotic presented a significantly lower level of skeletal deformities (in this instance, only defective operculation has been found) with respect to the control group. In the control group, 41.33 ± 2.31% of the specimens exhibited skeletal abnormalities. Among experimental groups, *group 2* displayed the lowest level (19.43 ± 0.71%) of deformities, with *group 3* displaying 31.01 ± 3.08% of specimens with skeletal deformities.

Histology. Histological sections of clownfish juveniles (*day 30* PH) showed skeletal alterations on the cranium and on the operculum (Fig. 7). In our case, no deformity along the vertebral columns was detected, but deformation of the skull was observed. The structures affected in the head were those related to the opercular complex and the neurocranium.

Histology of the head region at the stage of development analyzed (30 days PH) showed altered orientation of the opercular fold. In malformed specimens, the succession of operculum tissue layers (the 4 dermal bony plates) appeared regular, but changes in the mutual arrangement of the skull parts involved were clearly distinguishable (e.g., eye, brain). Both the ocular and neurocranial cavities appeared to be misshapen with a resultant atypical placement of the eyes. Also visible by gross examination, as well as histologically, was a misshapening of the jaw that changed both the shape and area of the oral opening. The magnitude of oral deformity varied from fish to fish as well as along the oral opening of each individual deformed fish.

DISCUSSION

In this study we have established the beneficial role of *L. rhamnosus* administration on the early developmental stages of the false percula clownfish, *A. ocellaris*. Newly hatched marine fish larvae, before metamorphosis, have a rudimentary digestive systems (19, 31, 66), and our hypothesis was that probiotic administration during the early larval development period could improve larval vitality, perhaps through early maturation of the gastrointestinal tract. The beneficial effect of the probiotic administration persisted throughout the developmental process, even resulting in accelerated metamorphosis to the juvenile stage.

Through microbiota analysis, the best retention of *L. rhamnosus* was achieved when probiotics were delivered via live

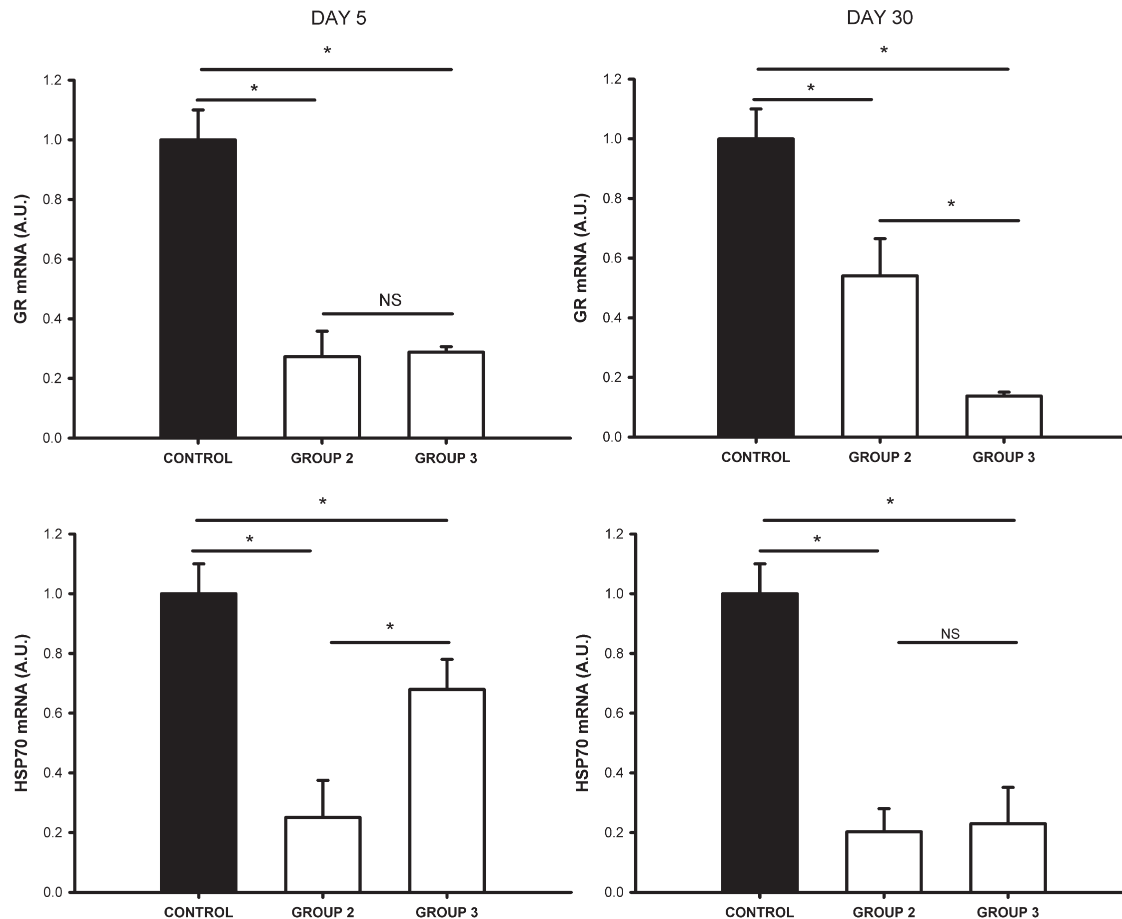


Fig. 4. Stress response biomarker effects. A significant downregulation of glucocorticoid receptor (GR) and 70-kDa heat shock protein (HSP70) gene expression was observed in clownfish treated with probiotic on both *days 5* and *30* PH. In particular, on *day 30* PH, *group 3* showed the lowest level of GR gene expression. On *day 5* PH, *group 2* showed the lowest level of HSP70 gene expression, whereas no significant differences were observed on *day 30* PH between *group 2* and *3* treatments. $*P < 0.05$ compared with standard diet (control group).

prey and addition to rearing water. Administration through prey and rearing water provides larvae two routes of exposure, immersion and feeding (40), as well as the additional benefits provided by live feed itself. The positive effects of the probiotic strain *L. rhamnosus* were clearly evident; in particular, stress tolerance to common rearing conditions as well as growth and development were significantly improved. Stress usually occurs in intensive larval rearing systems and must be dealt with by several adaptive responses that are ultimately detrimental for fish growth, behavior, development, and survivorship (8). During the initial response of the organism, plasma cortisol levels increase, leading to negative effects on fish survival and growth (8). We have previously demonstrated in sea bass larvae that cortisol levels can be reduced by *Lactobacillus delbrueckii delbrueckii*, *L. plantarum*, and *L. fructivorans* treatment, improving larval fish welfare (15, 65). In the present study we observed that *L. rhamnosus* also was able to decrease GR gene expression in both clownfish larvae and juveniles. Since it is known that GR and cortisol are correlated (80), the decrease in GR transcript is consistent with a reduction in stress through administration of probiotics.

Previous observations have indicated that high mortality in clownfish during early larval development is associated with malnutrition (88). We found that treatment with probiotics resulted in lower mortality during the most critical period of clownfish development. We attribute this reduction in mortality to a role for *L .rhamnosus* in feeding initiation.

We also detected a significant decrease of HSP70 gene expression in larvae and juveniles treated with *L. rhamnosus*. The role of HSP70 in stress response is still controversial. For example, in the Atlantic salmon (*Salmo salar*), HSP70 has been found to be expressed and/or translated after exposure to stress conditions (65, 69, 70). Moreover, many studies report that stress induced by food restriction leads to HSP70 being upregulated in juveniles of the carp (*Labeo rohita*) (89) and in the gilthead sea bream (*Sparus aurata*) and in rainbow trout (*Oncorhynchus mykiss*) early stages (14). Although a few studies bring into question the suitability of HSP70 as a biomarker for the fish stress response (37, 38), what we could observe in our study was that a standard diet plus probiotic administration can lead to a decrease of HSP70 gene expression, which correlates with an increase in survivorship, growth, and development.

We have previously shown in sea bass (15) that a supply of *L. delbruekii delbruekii* during larval stages enhanced larval and juvenile body weight. In the current study, *L. rhamnosus* improved clownfish body weight at both larval and juvenile stages, whereas standard length was significantly increased

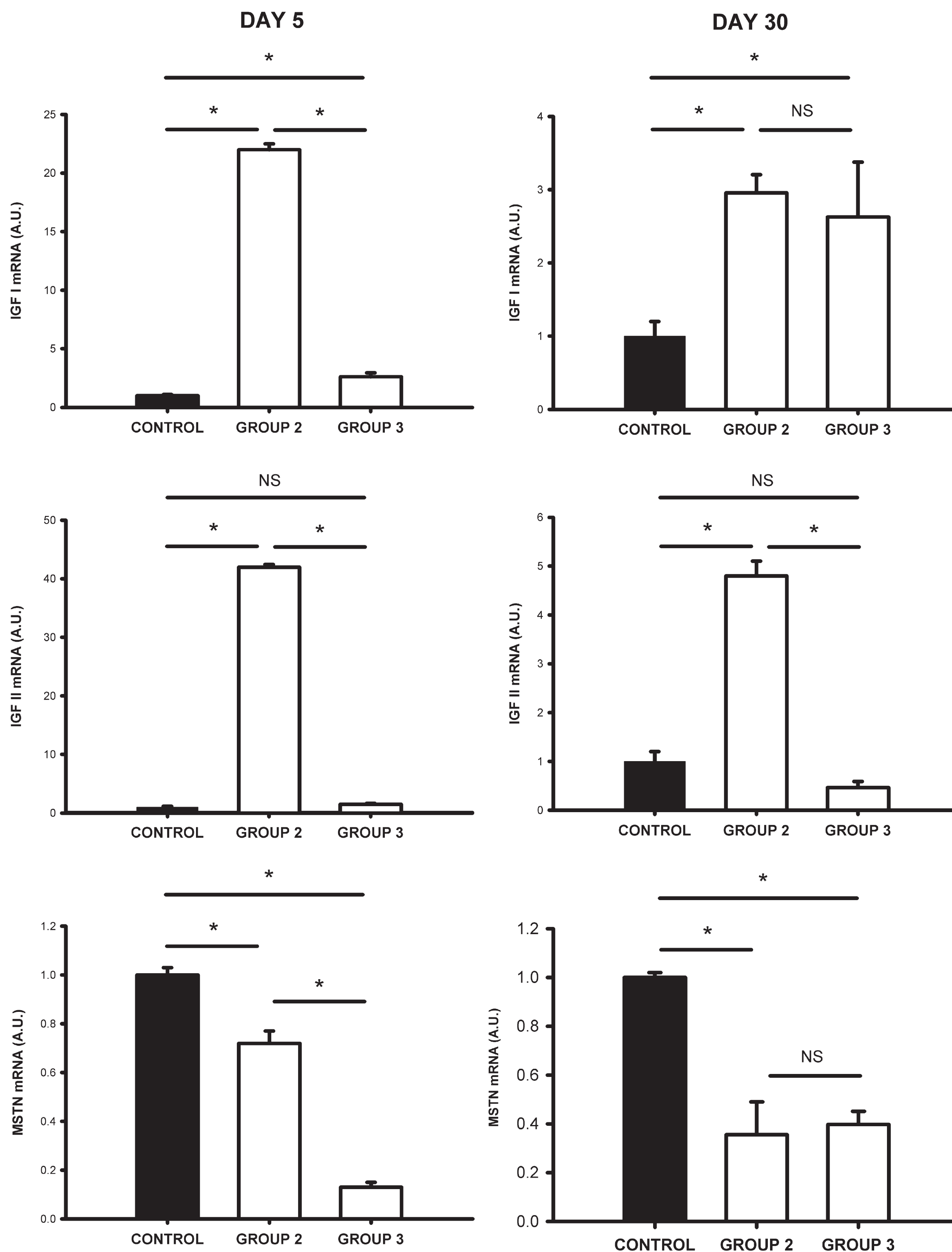


Fig. 5. Growth response biomarker effects. On *day 5* PH, an increase in gene expression for insulin-like growth factor I (IGF-I) and IGF-II was observed in *group 2* fish with little alteration in *group 3* fish. At *day 30* PH, in both *groups 2* and *3*, IGF-I gene expression was about 3 times higher with respect to the control group, whereas for IGF-II, only *group 2* gene expression was positively affected. A significant downregulation of myostatin (MSTN) gene expression was observed in clownfish treated with probiotic on both *days 5* and *30* PH. $^*P < 0.05$ compared with standard diet (control group).

only on *day 30* PH in *group 3*. Furthermore, clownfish larvae metamorphosed 3 days earlier than the control group, when probiotics were administered. In this regard it is well established that probiotics deliver to intestinal mucosal cells several molecules such as proteins, mineral salts, free fatty acids, and vitamins (39, 44, 73, 84) useful for development, growth, and general welfare of the host organism. The enhancement of larval growth in terms of body weight, standard length, and metamorphosis time can be attributed to the probiotics administration. Tovar-Ramirez et al. (74, 75) showed that the live

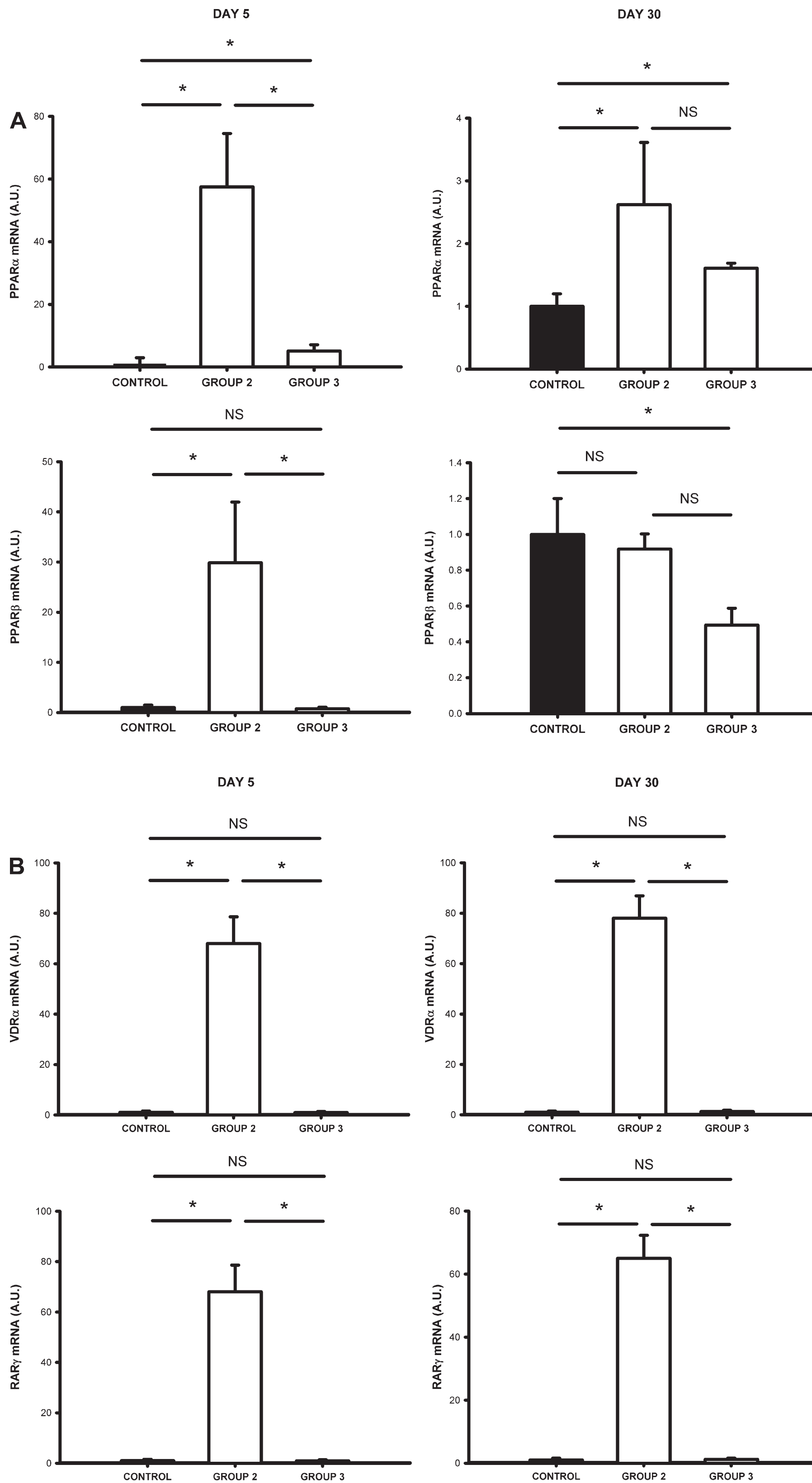


Fig. 6. Metabolic response biomarker effects. As with IGF gene expression, on *day 5* PH, an increase in gene expression for peroxisome proliferator-activated receptor-α (PPARα) and PPARβ was observed in *group 2*. In *group 3*, just PPARα gene expression was significantly enhanced, whereas no significant effects were registered for PPARβ. On *day 30*, PPARα gene expression was upregulated in both *groups 2* and *3*, with a further and significant increased expression in *group 2*. Concerning PPARβ, no significant differences were observed in *group 2*, whereas a significant and unexpected lower expression was observed in *group 3*. Vitamin D receptor (VDRα) and retinoic acid receptor (RARγ) gene expression on both 5 and 30 days PH was significantly modulated by probiotic treatment in *group 2*, whereas no significant differences were detected between the control group and *group 3*. $*P < 0.05$ compared with standard diet (control group).

yeast *Debaryomyces hansenii*, intended as a probiotic, could accelerate larval digestive system development by delivery of polyamines to the sea bass digestive tract. Whether *L. rhamnosus* provides similar growth-promoting factors is unclear at this time, but clearly probiotic administration is able to accelerate clownfish development.

Although the positive role of probiotics in the culture of marine organisms is well established (48, 78, 83), few studies

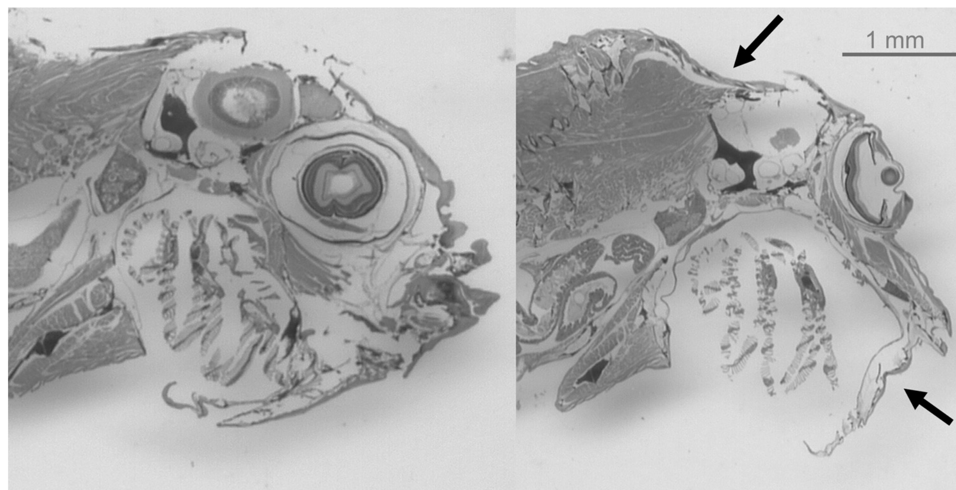


Fig. 7. Histological analysis. Two representative histological sections show clownfish juveniles on *day 30* PH. The specimen at *left* shows a correct skeletal development. The specimen at *right* displays clear alterations in the operculum refolding and in the cranium (see arrows).

have evaluated the effect of these beneficial bacteria on gene expression modulation for growth-related factors. Their effect on the modulation of nuclear receptor genes related, in particular, to fatty acid, retinoic acid, and vitamin D metabolism such as PPARα, PPARβ, VDRα, and RARγ are unknown. In our study, the IGF and nuclear receptor genes were extensively modulated by the probiotic treatment. In particular, in larvae, unexpected higher expression of both IGF-I and IGF-II was detected in *group 2* when *L. rhamnosus* was administered via live prey and addition to the rearing water. This alteration is correlated with a threefold higher body weight registered in the same experimental group with respect to the control. In juveniles, significantly higher IGF-I and IGF-II transcript levels were observed, although the relative increase was not as great as observed in larvae. On this matter we can assume that probiotic treatment was able to act positively on clownfish growth in a similar fashion to what we previously suggested for the European sea bass (15).

Concerning nuclear receptors, a gene expression trend similar to that of the IGFs was observed. This may be due to the fact that *Lactobacillus* fermentation products include short-chain fatty acids and immunoregulatory molecules able to activate PPARs (18), and for this reason it is plausible to attribute the higher PPAR gene expression to the probiotic activity. Moreover, since PPARα and -β are involved in several biological processes such as skeletal development during ontogenesis (11), it is possible to suppose a positive correlation between these systems and the higher growth. Along with the PPARs, the nuclear receptors RARγ and VDRα are essential factors for many developmental processes, including cellular proliferation, differentiation, and metabolism. In particular, RARs are the main vitamin A bioactive form receptors and are involved in several developmental processes during the early ontogeny and morphogenesis of vertebrates (20), and the disruption of the retinoid pathway is known to increase the occurrence of skeletal malformations (33). Villeneuve et al. (81) detected RARγ in the jaws and branchial arches of European sea bass larvae at *day 5* PH, proposing that this isoform was essential for the correct head development during the early larval developmental stages. At *day 37* PH, RARγ was detected in the nuclei of the cells of pectoral fins, vertebrae, and muscle, suggesting a role in the general development of sea bass larvae. In our study, in both larval and juvenile clownfish, RARγ gene expression was seriously affected when probiotics were delivered by live prey and through the rearing water as similarly observed for IGFs and PPARs. The vitamin D endocrine system is involved in a wide array of biological processes, such as bone metabolism, cell proliferation, and cell differentiation (76). Vitamin D action is mediated by the VDR, a receptor belonging to the nuclear receptor superfamily, like PPARs and RARs. In fish, vitamin D can stimulate calcium absorption in the intestine (72), and its receptor (VDR) is an important factor for calcium homeostasis and skeletal formation (49), and particularly notable for this study, it has been demonstrated that VDR plays an essential role in calcium absorption in the intestine (58). In our study the gene expression of VDRα was positively stimulated by probiotic administration solely in *group 2*. For these reasons, considering the RARγ and VDRα gene expression results, we hypothesize a positive relationship between *L. rhamnosus* administration and general body development, not only in the muscle mass as indicated by IGFs and MSTN gene expression, but also in the skeletal system development as further supported in *group 2*, where the lowest incidence of skeletal deformities was observed.

The greater effectiveness seen in delivering probiotic by both live food and rearing water addition might be due to the higher retention of *L. rhamnosus* observed. *Lactobacillus* is not readily retained in the gastrointestinal tract and needs to be continually readministered. Similar results were previously obtained in gilthead sea bream, *S. aurata* (71), where significant increases in both growth performance and survival were found when probiotic was administered by both live food and water addition. The two different retention values of *L. rhamnosus* affected metamorphosis time in a similar fashion, suggesting that the physiological mechanism involved in this developmental process might be similarly affected by the two effective probiotic quantities.

Conclusions. Our data suggest that the probiotic strain *L. rhamnosus* can significantly improve the false percula clownfish welfare in terms of survivorship, growth, and development, especially when the probiotic is delivered via live prey and added to the rearing water. The molecular results together with the higher body weight, earlier metamorphosis, and lower

deformity incidence suggest a role for *L. rhamnosus* IMC 501 in accelerating the early and correct development for this species.

Perspectives and Significance

In summary, the present study underlines the beneficial effects of probionts to host physiology, especially in fish early development. Data presented are a starting point for a number of studies on the application of probiotic use during early life stages of vertebrates to clarify their effects on growth and development. Whether administration of probiotics in early life affects later developmental milestones such as gonad differentiation and maturation or progression to puberty and aging must await future studies.

ACKNOWLEDGMENTS

We thank Chiara Piccinetti, Sabina Oliveri, and Giorgia Gioacchini for help during treatments and analyses. Special thanks go to Ernest Williams, Center of Marine Biotechnology, University of Maryland Biotechnology Institute, for welcome help during the writing of this article. This is contribution 09-xxx from the Center of Marine Biotechnology.

The probiotic product *L. rhamnosus* IMC 501 was kindly provided by SYNBIOTEC (Camerino, Italy).

GRANTS

Funding for this study was provided by the "Fondi di Ateneo 2006" to O. Carnevali.

REFERENCES

1. **Acerete L, Balasch JC, Castellana B, Redruello B, Roher N, Canario AV, Planas JV, Mackenzie S, Tort L.** Cloning of the glucocorticoid receptor (GR) in gilthead seabream (*Sparus aurata*). Differential expression of GR and immune genes in gilthead seabream after an immune challenge. *Comp Biochem Physiol* 148: 32–37, 2007.
2. **Ackerman PA, Iwama GK.** Physiological and cellular stress responses of juvenile rainbow trout to vibriosis. *J Aquat Anim Health* 13: 173–180, 2001.
3. **Alfonso JM, Montero D, Robaina L, Astorga N, Izquierdo MS, Gines R.** Association of lordosis-scoliosis-kyphosis deformity in gilthead sea bream (*Sparus aurata*) with family structure. *Fish Physiol Biochem* 22: 159–163, 2002.
4. **Avella MA, Olivotto I, Gioacchini G, Maradonna F, Carnevali O.** The role of fatty acids enrichments in the larviculture of false percula clownfish *Amphiprion ocellaris*. *Aquaculture* 273: 87–95, 2007.
5. **Ayson FG, de Jesus EGT, Moriyama S, Hyodo B, Funkenstein B, Gerter A, Kawauchi H.** 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, 2002.
6. **Balcázar JL, de Blas I, Ruiz-Zarzuela I, Vendrell D, Calvo AC, Márquez I, Gironés O, Muzquiz JL.** Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout (*Salmo trutta*). *Br J Nutr* 97: 522–527, 2007.
7. **Balcázar JL, de Blas I, Ruiz-Zarzuela I, Vendrell D, Gironés O, Muzquiz JL.** Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). *FEMS Immunol Med Microbiol* 51: 185–193, 2007.
8. **Barton BA.** Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol* 42: 517–525, 2002.
9. **Bedu E, Desplanches D, Pequignot J, Bordier B, Desvergne B.** Double gene deletion reveals the lack of cooperation between PPARα and PPARβ in skeletal muscle. *Biochem Biophys Res Commun* 357: 877–881, 2007.
10. **Benetti DD, Sardenberg B, Welch A, Hoenig R, Refik Orhun M, Zink I.** Intensive larval husbandry and fingerling production of cobia *Rachycentron canadum*. *Aquaculture* 281: 22–27, 2008.
11. **Burdick AD, Kim DJ, Peraza MA, Gonzalez FJ, Peters JM.** The role of peroxisome proliferator-activated receptor-δ/β in epithelial cell growth and differentiation. *Cell Signal* 18: 9–20, 2006.
12. **Cameron C, Moccia RD, Azevedo PA, Leatherland JF.** Effect of diet and ration on the relationship between plasma GH and IGF-1 concentrations in Arctic charr, *Salvelinus alpinus* (Linnaeus). *Aquac Res* 38: 877–886, 2007.
13. **Cameron C, Moccia RD, Leatherland JF.** Growth hormone secretion from the Arctic charr (*Salvelinus alpinus*) pituitary gland in vitro: effects of somatostatin-14, insulin-like growth factor-I, and nutritional status. *Gen Comp Endocrinol* 141: 93–100, 2005.
14. **Cara JB, Aluru N, Moyano FJ, Vijayan MM.** Food-deprivation induces HSP 70 and HSP 90 protein expression in larval and gilthead sea bream and rainbow trout. *Comp Biochem Physiol B* 142: 426–431, 2005.
15. **Carnevali O, De Vivo L, Sulpizio R, Gioacchini G, Olivotto I, Silvi S, Cresci A.** 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, 2006.
16. **Carnevali O, Zamponi MC, Sulpizio R, Rollo A, Nardi M, Orpianesi C, Silvi S, Caggiano M, Polzonetti AM, Cresci A.** Administration of probiotic strain to improve sea bream wellness during development. *Aquac Int* 12: 377–386, 2004.
17. **Chauvigné F, Gabillard JC, Weil C, Rescan PY.** 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, 2003.
18. **Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ.** Nuclear receptors and lipid physiology: opening the X-files. *Science* 30: 1866–1870, 2001.
19. **Chen BN, Qin JG, Kumar MS, Hutchinson WG, Clarke SM.** Ontogenetic development of digestive enzymes in yellowtail kingfish *Seriola lalandi* larvae. *Aquaculture* 260: 264–271, 2006.
20. **Ciguère V.** Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signalling. *Endocr Rev* 15: 61–79, 1994.
21. **De Man JC, Rogosa M, Sharpe ME.** A medium for the cultivation of lactobacilli. *J Appl Microbiol* 23: 130–135, 1960.
22. **Delaney MA, Klesius PH.** Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture* 236: 633–644, 2004.
23. **Duan C, Plisetskaya EM.** Nutritional regulation of insulin-like growth factor-I mRNA expression in salmon tissues. *J Endocrinol* 139: 243–252, 1993.
24. **Fernández I, Hontoria F, Ortiz-Delgado JB, Kotzamanis Y, Estévez A, Zambonino-Infante JL, Gisbert E.** Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of vitamin A enriched rotifers (*Brachionus plicatilis*). *Aquaculture* 283: 102–115, 2008.
25. **Forsyth RB, Candido EPM, Babich SL, Iwama GK.** Stress protein expression in coho salmon with bacterial kidney disease. *J Aquat Anim Health* 9: 18–25, 1997.
26. **Funes V, Asensio E, Ponce M, Infante C, Cañavate JP, Manchado M.** Insulin-like growth factors I and II in the sole *Solea senegalensis*: cDNA cloning and quantitation of gene expression in tissues and during larval development. *Gen Comp Endocrinol* 149: 166–172, 2006.
27. **Gabillard JC, Kamagar BB, Montserrat N.** Coordinated regulation of the GH/IGF system genes during refeeding in rainbow trout (*Oncorhynchus mykiss*). *J Endocrinol* 191: 15–24, 2006.
28. **Gatesoupe FJ.** The use of probiotics in aquaculture. *Aquaculture* 180: 147–165, 1999.
29. **Gatesoupe FJ.** Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. *J Mol Microbiol Biotechnol* 14: 107–114, 2008.
30. **Gavaia PJ, Dinis MT, Cancela ML.** Osteological development and abnormalities of the vertebral column and caudal skeleton in larval and juvenile stages of hatchery-reared Senegal sole (*Solea senegalensis*). *Aquaculture* 211: 305–323, 2002.
31. **Gisbert E, Piedrahita RH, Conklin DE.** Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus*) with notes on feeding practices. *Aquaculture* 232: 455–470, 2004.
32. **Grimaldi PA.** Regulatory functions of PPARβ in metabolism: implications for the treatment of metabolic syndrome. *Biochim Biophys Acta* 1771: 983–990, 2007.
33. **Haga Y, Suzuki T, Kagechika H, Takeuchi T.** A retinoic acid receptor-selective agonist causes jaw deformity in the Japanese flounder, *Paralichthys olivaceus*. *Aquaculture* 221: 381–392, 2003.

34. **Haussler MR, Whitfield GK, Haussler CA, Hsieh J, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW.** The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J Bone Miner Res* 13: 325–349, 1998.
35. **Holdeman VL, Cato EP, Moore WEC.** *Anaerobe Laboratory Manual* (4th ed.). Blacksburg, VA: Virginia Polytechnic Institute and State University, 1977.
36. **Howe JC.** Standard length: not quite so standard. *Fish Res* 56: 1–7, 2002.
37. **Iwama GK, Afonso LOB, Todgham A, Ackerman P, Nakano K.** Are hsps suitable for indicating stressed states in fish? *J Exp Biol* 207: 15–19, 2004.
38. **Iwama GK, Vijayan MM, Forsyth RB, Ackerman PA.** Heat shock proteins and physiological stress in fish. *Am Zool* 39: 901–909, 1999.
39. **Kailasapathy K, Chin J.** Survival and therapeutic potential of probiotic organisms with reference to *Lactobacillus acidophilus* and *Bifidobacterium* spp. *Immunol Cell Biol* 78: 80–88, 2000.
40. **Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L.** Probiotics in aquaculture: the need, principles and mechanisms of action and screening processes. *Aquaculture* 274: 1–14, 2008.
41. **Koumoundouros G, Gagliardi F, Divanach P, Boglione C, Cataudella S, Kentouri M.** Normal and abnormal osteological development of caudal fin in *Sparus aurata* L. fry. *Aquaculture* 149: 215–226, 1997.
42. **Koumoundouros G, Maingot E, Divanach P, Kentouri M:.** Kyphosis in reared sea bass (*Dicentrarchus labrax* L.): ontogeny and effects on mortality. *Aquaculture* 209: 49–58, 2002.
43. **Koumoundouros G, Oran G, Divanach P, Stefanakis S, Kentouri M.** The opercular complex deformity in intensive gilthead sea bream (*Sparus aurata* L.) larviculture. Moment of apparition and description. *Aquaculture* 156: 165–177, 1997.
44. **Kullberg MC.** Immunology: soothing intestinal sugars. *Nature* 453: 602–604, 2008.
45. **Lall SP, Lewis-McCrea LM.** Role of nutrients in skeletal metabolism and pathology in fish—an overview. *Aquaculture* 267: 3–19, 2007.
46. **Ma K, Mallidis C, Artaza J, Taylor W, Gonzalez-Cadavid N, Bhasin S.** Characterization of 51-regulatory region of human myostatin gene: regulation by dexamethasone in vitro. *Am J Physiol Endocrinol Metab* 281: E1128–E1136, 2001.
47. **Ma K, Mallidis C, Bhasin S, Artaza J, Gonzalez-Cadavid N, Arias J, Salehian B.** Glucocorticoid-induced skeletal muscle atrophy is associated with upregulation of myostatin gene expression. *Am J Physiol Endocrinol Metab* 285: E363–E371, 2003.
48. **Macey BM, Coyne VE.** Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. *Aquaculture* 245: 249–261, 2005.
49. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schülz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM.** The nuclear receptor superfamily: the second decade. *Cell* 83: 835–839, 1995.
50. **McPherron AC, Lawler AM, Lee SJ.** Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. *Nature* 387: 83–90, 1997.
51. **Mendelsohn C, Lohnes D, Décimo D, Lufkin T, LeMeur M, Chambon P, Mark M.** Function of the retinoic acid receptors (RARs) during development. II. Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120: 2749–2771, 1994.
52. **Mommsen TP, Vijan MM, Moon TW.** Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev Fish Biol Fish* 9: 211–268, 1999.
53. **Muroga K, Higashi M, Keitoku H.** The isolation of intestinal micro-flora of farmed red sea bream (*Acanthopagrus schlegeli*) at larval and juvenile stages. *Aquaculture* 65: 79–88, 1987.
54. **Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius E.** Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish Shellfish Immunol* 15: 443–452, 2003.
55. **Nikoskelainen S, Salminen S, Bylund G, Ouwehand AC.** Characterization of the properties of human- and dairy-derived probiotics for prevention of infectious diseases in fish. *Appl Environ Microbiol* 67: 2430–2435, 2001.
56. **Olivotto I, Cardinali M, Barbaresi L, Maradonna M, Carnevali O.** Coral reef fish breeding: the secrets of each species. *Aquaculture* 224: 69–78, 2003.
57. **Palazzi R, Richard J, Bozzato G, Zanella L.** Larval and juvenile rearing of common sole (*Solea solea* L.) in the Northern Adriatic (Italy). *Aquaculture* 255: 495–506, 2006.
58. **Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN, Goltzman D.** Inactivation of the 25-hydroxyvitamin D 1α-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. *J Biol Chem* 279: 16754–16766, 2004.
59. **Panigrahi A, Kiron V, Puangkaew J, Kobayashi T, Satoh S, Sugita H.** The viability of probiotic bacteria as factor influencing live yeast on European sea bass the immune response in rainbow trout *Oncorhynchus mykiss*. *Aquaculture* 243: 241–254, 2007.
60. **Patruno M, Maccatrozzo L, Funkenstein B, Radaelli G.** Cloning and expression of insulin-like growth factors I and II in the shi drum (*Umbrina cirrosa*). *Comp Biochem Physiol* 144: 137–151, 2006.
61. **Peterson BC, Waldbieser GC, Bilodeau L.** 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, 2004.
62. **Pfaffl MV.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
63. **Rainuzzo JR, Reitan KI, Olsen Y.** The significance of lipids at early stages of marine fish: a review. *Aquaculture* 155: 103–115, 1997.
64. **Reid G.** The scientific basis for probiotic strains of *Lactobacillus*. *Appl Environ Microbiol* 65: 3763–3766, 1999.
65. **Rollo A, Sulpizio R, Nardi M, Silvi S, Orpianesi C, Caggiano M, Cresci A, Carnevali O.** Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiol Biochem* 32: 167–177, 2006.
66. **Sarasquete MC, Polo A, Yúfera M.** Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L. *Aquaculture* 130: 79–92, 1995.
67. **Sargent J, McEvoy L, Estevez A, Bell G, Bell M, Henderson H, Tocher D.** Lipid nutrition of marine fish during early development: current status and future directions. *Aquaculture* 179: 217–229, 1999.
68. **Sargent JR, McEvoy LA, Bell JG.** Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture* 155: 85–101, 1997.
69. **Smith TR, Tremblay GC, Bradley TM.** Characterization of the heat shock protein response of Atlantic salmon (*Salmo salar*). *Fish Physiol Biochem* 20: 279–292, 1999.
70. **Smith TR, Tremblay GC, Bradley TM.** Hsp70 and a 54 kDa protein (osp54) are induced in salmon (*Salmo salar*) in response to hyperosmotic stress. *J Exp Zool* 284: 286–298, 1999.
71. **Suzer C, Çoban D, Kamaci HO, Saka S, Firat K, Otgucuoğlu Ö, Küçüksari H.** *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, 2008.
72. **Suzuki T, Suzuki N, Srivastava AS, Kurokawa T.** Identification of cDNAs encoding two subtypes of vitamin D receptor in flounder, *Paralichthys olivaceus*. *Biochem Biophys Res Commun* 270: 40–45, 2000.
73. **Teusink B, Smid EJ.** Modelling strategies for the industrial exploitation of lactic acid bacteria. *Nat Rev Microbiol* 4: 46–56, 2006.
74. **Tovar-Ramirez D, Zambonino Infante J, Cahu C, Gatesoupe FJ, Vàzquez-Juarez R.** Influence of dietary live yeast on European sea bass (*Dicentrarchus labrax*) larval development. *Aquaculture* 234: 415–427, 2004.
75. **Tovar-Ramirez D, Zambonino J, Cahu C, Gatesoupe FJ, Vàzquez-Juàrez R, Lésel R.** Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* 204: 113–123, 2002.
76. **Uitterlinden AG, Fang Y, van Meurs JBJ, Pols HAP, van Leeuwen JPTM.** Genetics and biology of vitamin D receptor polymorphisms. *Gene* 338: 143–156, 2004.
77. **Verdenelli MC.** *Assessment of Novel Probiotic Lactobacillus Strains From Human Feces for the Production of Functional Foods* (PhD thesis). Camarino, Italy: Università degli Studi di Camerino, 2007.
78. **Verschuere L, Rombaut G, Sorgeloos P, Verstraete W.** Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev* 64: 655–671, 2000.
79. **Vijayan MM, Pereira C, Forsyth RB, Kennedy CJ, Iwama GK.** Handling stress does not affect the expression of hepatic heat shock protein 70 and conjugation heat-shock-cognate hsc71 gene from rainbow trout. *Eur J Biochem* 204: 893–900, 1997.

80. **Vijayan MM, Raptis S, Sathiyaa R.** Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. *Gen Comp Endocrinol* 132: 256–263, 2003.
81. **Villeneuve L, Gisbert E, Cahu CL, Le Gall MM, Zambonino-Infante JL.** Expression and localization of some retinoid receptors during European sea bass (*Dicentrarchus labrax*) larvae development. *Aquaculture* 242: 537–551, 2004.
82. **Villeneuve L, Gisbert E, Zambonino-Infante JL, Quazuguel P, Cahu CL.** Effect of nature of dietary lipids on European sea bass morphogenesis: implication of retinoid receptors. *Br J Nutr* 94: 877–884, 2005.
83. **Waché Y, Auffray F, Gatesoupe FJ, Zambonino J, Gayet V, Labbé L, Quentel C.** Cross effects of the strain of dietary *Saccharomyces cerevisiae* and rearing conditions on the onset of intestinal microbiota and digestive enzymes in rainbow trout, *Oncorhynchus mykiss*, fry. *Aquaculture* 258: 470–478, 2006.
84. **Walker A.** Milk and two oligosaccharides. *Nat Rev Microbiol* 7: 483, 2009.
85. **Wang Y, Li J, Lin J.** Probiotics in aquaculture: challenges and outlook. *Aquaculture* 281: 1–4, 2008.
86. **Wedemeyer GA, Barton BA, McLeay DJ.** Stress and acclimation. In: *Methods for Fish Biology*, edited by Schreck CB and Moyle PB. Bethesda, MD: American Fisheries Society, 1990, p. 451–489.
87. **Wedemeyer GA, McLeay DJ.** Methods for determining the tolerance of fishes to environmental stressors. In: *Stress and Fish*, edited by Pickering AD. New York: Academic, 1981, p. 247–275.
88. **Wilkerson JD.** *Clownfishes. A Guide to Their Captive Care, Breeding and Natural History* (1st ed.). Shelburne, VT: Microcosm, 2001, p. 183–207.
89. **Yengkokpam S, Pal AK, Sahu NP, Jain KK, Dalvi R, Misra S, Debnath D.** Metabolic modulation in *Labeo rohita* fingerlings during starvation: Hsp70 expression and oxygen consumption. *Aquaculture* 285: 234–237, 2008.
90. **Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S.** Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. *Nat Genet* 16: 391–396, 1997.
91. **Young JP, Downer HL, Eardly BD.** Phylogeny of the phototrophic *Rhizobium* strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. *J Bacteriol* 173: 271–277, 1991.
92. **Zarate J, Bradley TM.** Heat shock proteins are not sensitive indicators of hatchery stress in salmon. *Aquaculture* 223: 175–187, 2003.