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

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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 survival, 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,
1,25-dihydroxyvitamin D₃ [1,25-(OH)₂D₃], 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 -δ 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 Symbiotec (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 10⁹ 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 Symbiotec at 0.01 g/l to reach a final concentration in water of 10⁶ 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'–CCCAGTGTGCTTCCCGTGGAGGAT–3' (conserved 16S rRNA; Ref. 91), and Lr-5'TGATCTGTATTTGTG–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% w/v) 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 (Ct) value, being the minimal number 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 Ct values was obtained in the range corresponding to 10⁵-10⁹ CFU (R² = 0.9914). *L. rhamnosus* was quantified by real-time PCR, interpolating the Ct 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 NH₃ and NO₂⁻ < 0.02 parts per million (ppm). Fish were fed twice a day with commercial frozen mysis and krill (EscheMatteo, Dendermonde, Belgium) from *Brachionus plicatilis* rotifers (10 individuals/ml) from *Daphnia pulex* day 1 to 5, 10 individuals/ml) from *Daphnia pulex* day 6 to 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 NH₃ and NO₂⁻ < 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 *Algalac 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

**Survivorship of *L. rhamnosus* IMC 501.** L. rhamnosus IMC 501 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 a day. Addition to rearing water followed the instructions provided by Symbiotec at 0.01 g/l to reach a final concentration in water of 10⁶ CFU/g.
spin-off of the Polytechnic University of Marche) larval feeding protocol (Algalac 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 Algalac 2000 (Aquafana 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, NH3-N, NO2 and NO3 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 euthanized 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 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 1 to day 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 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 U 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)6 adapter primer, 5'-GACTGCAGTCGACATCGATTTTTTTTTTTTTTTTTTTTTTTTTTTT-3', in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fish Sequences</th>
<th>Out-Group Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>Pinephales promelas</td>
<td>Rattus norvegicus AB115420</td>
</tr>
<tr>
<td></td>
<td>Dicentrarchus labrax</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sparus aurata</td>
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<tr>
<td>HSP70</td>
<td>Chrysiptera parasema</td>
<td>Galax gallus NM_00100438</td>
</tr>
<tr>
<td></td>
<td>Amphiprion ocellaris</td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td>Dicentrarchus labrax</td>
<td>Galax gallus NM_001030342</td>
</tr>
<tr>
<td></td>
<td>Sparus aurata</td>
<td></td>
</tr>
<tr>
<td>IGF-II</td>
<td>Dicentrarchus labrax</td>
<td>Galax gallus NM_001030342</td>
</tr>
<tr>
<td></td>
<td>Sparus aurata</td>
<td></td>
</tr>
<tr>
<td>MSTN</td>
<td>Dicentrarchus labrax</td>
<td>Mus musculus NM_010834</td>
</tr>
<tr>
<td></td>
<td>Sparus aurata</td>
<td></td>
</tr>
<tr>
<td>PPARα</td>
<td>Salmo trutta (f. fario)</td>
<td>DQ139936</td>
</tr>
<tr>
<td></td>
<td>Salmo salar</td>
<td>NM_001123560.1</td>
</tr>
<tr>
<td>PPARβ</td>
<td>Salmo trutta (f. fario)</td>
<td>DQ139937</td>
</tr>
<tr>
<td></td>
<td>Salmo salar</td>
<td>AM292036</td>
</tr>
<tr>
<td>VDRα</td>
<td>Dicentrarchus labrax</td>
<td>AM040727</td>
</tr>
<tr>
<td></td>
<td>Danio rerio</td>
<td>NM_130919</td>
</tr>
<tr>
<td>RARγ</td>
<td>Dicentrarchus labrax</td>
<td>AJ496181</td>
</tr>
<tr>
<td></td>
<td>Sparus aurata</td>
<td>EU643831</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Pomacentrus moluccensis</td>
<td>DQ243822</td>
</tr>
<tr>
<td></td>
<td>Astronotus ocellatus</td>
<td>EU553593</td>
</tr>
</tbody>
</table>

See text for definition of genes.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>GenBank Accession No.</th>
<th>Sequence Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>F: 5'-CGGTCATTGCGAGCCCTTTCA-3'</td>
<td>EU722335</td>
<td>89% with Dicentrarchus labrax GR</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTCGGAAGCAGGATGTAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP70</td>
<td>F: 5'-ACGGAGAGTGTATGTTGACT-3'</td>
<td>EU683309</td>
<td>90% with Paralichthys olivaceus HSP70</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAAGGACATCCAGGGACAACA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-I</td>
<td>F: 5'-AGTGCGATGTGCTATTAC-3'</td>
<td>EF620549</td>
<td>90% with Latice calcarifer IGF-I</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CACGTCACAGTCTGGAAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-II</td>
<td>F: 5'-CGGAGAAACGCCATGTCTGG-3'</td>
<td>EF620550</td>
<td>87% with Mogil cephalus IGF-II</td>
</tr>
<tr>
<td></td>
<td>R: 5'-TGCGTTGGTGGTCTACGAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSTN</td>
<td>F: 5'-TGTCTGAGAATAGATAG-3'</td>
<td>EF620551</td>
<td>97% with Dicentrarchus labrax MSTN</td>
</tr>
<tr>
<td></td>
<td>R: 5'-ACAGGCTGCTGCTGGAAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARα</td>
<td>F: 5'-TTTGGACACAGAATGGCATG-3'</td>
<td>EF620552</td>
<td>87% with Dicentrarchus labrax PPARα</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CACGCTGCTGCTGGAAC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARβ</td>
<td>F: 5'-AGGGATGATGGTCACCTGCT-3'</td>
<td>EF620553</td>
<td>93% with Dicentrarchus labrax PPARβ</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GAAGGAACTCCCGCTGCTGCAAA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDRα</td>
<td>F: 5'-AGCGAGCCACGCCAGAAAAC-3'</td>
<td>EU676177</td>
<td>96% with Dicentrarchus labrax VDRα</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AGGTGTCTGGTGAAGGTCATG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RARγ</td>
<td>F: 5'-ATCGAGAGGAGAGGGTCATG-3'</td>
<td>EU676178</td>
<td>93% with Salmo salar RARγ</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GGTTGTCTGGTGAAGGTCATG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: 5'-GAAATCATCCATCTGCTGTCAG-3'</td>
<td>EF620554</td>
<td>93% with Pomacentera molluccensis β-actin</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CTTCGCAAAGGCAAGGACAGA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.
higher body weight (group 2, $18.4 \pm 0.1$ mg; group 3, $9.2 \pm 0.1$ mg) with respect to those of the control group (5.6 \pm 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 \pm 0.1 mg) with respect to the other experimental groups (control group, 45.5 \pm 0.05 mg; group 3, 45.6 \pm 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 \pm 0.02 mm; group 2, 5.73 \pm 0.1 mm; group 3, 5.7 \pm 0.1 mm) (Fig. 3), whereas juveniles from group 3 showed a significantly greater standard length (day 30: control group, 11.40 \pm 0.1 mm; group 2, 11.35 \pm 0.1 mm; group 3, 12.60 \pm 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 $\alpha$ and $\beta$ with respect to the control group and group 3, as similarly observed for IGF gene expression. On day 30 PH, a significantly higher PPAR$\alpha$ gene expression was observed in both groups treated with probiotic, with the highest levels in group 2 (Fig. 6). Surprisingly, the subtype $\beta$ was not affected in group 2, and an unexpected lower expression was observed in group 3 (Fig. 6). For VDR$\alpha$ and RAR$\gamma$, a significant transcript abundance increase was detected only in group 2, whereas no
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 opercular 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...
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. delbrueckii delbrueckii* 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...
only on day 30 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. 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).
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...
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 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.

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 probiotics 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.

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