Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression

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Abstract

In the present study a Lactic Acid Bacteria (LAB) strain *Lactobacillus delbrueckii delbrueckii*, acting as probiotic, was administered to *Dicentrarchus labrax* (European sea bass) juveniles for a short (25 days) and a long (59 days) time and the effects of the bacteria on gut colonization in sea bass juveniles cortisol level and growth were evaluated with respect to fish fed on live preys solely (control group). Measures of body weight and standard length (S.L.) were performed to assess juveniles growth, while cortisol, a hormone directly involved in stress responses, was chosen as a stress marker. In addition, the expression of two antagonistic genes involved in muscular growth (IGF-I and myostatin (MSTN) was analyzed through real-time PCR. Fish fed on live preys added with *L. delbrueckii delbrueckii* showed a high gut colonization by this Lactic Acid Bacteria strain. In the same fish a significantly (*p* < 0.05) lower cortisol level was observed with respect to those fed on live preys solely (control 5.1±0.47 ng/g; group A: 3.6±0.36 ng/g; group B: 4.5±0.48 ng/g). In addition, an increase of IGF-I transcription was observed in fish treated with LAB, being IGF-I mRNA levels six times higher in both treated groups with respect to the control (control: 1±0.01 a.u.; group A: 6.63±0.18 a.u.; group B: 6.16±0.15 a.u.). On the contrary, MSTN mRNA transcription was significantly inhibited in treated groups (control 1±0.02 a.u.; group A: 0.71±0.01 a.u.; group B: 0.26±0.003 a.u.). These results are in agreement with the increase of body weight recorded in this study. Fish fed on LAB showed 81% higher body weight in long treated group and 28% in short treated one with respect to control.

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1. Introduction

Intensive zootechny makes large use of industrial feeds and anti-microbial substances added to the normal diet of reared animals in order to promote growth.

According to the Food and Drug Administration (FDA), in the USA the quantity of antibiotics used in zootechny is ten times higher than that used for human health.

In intensive marine larviculture, the large quantity of disinfectants and drugs released into the rearing water can affect in a negative way the development of a protective gastrointestinal microflora in reared fishes (*Austin and Al-Zahrami, 1988; Strom and Ringo, 1993*). For this reason, reared larvae are exposed to intestinal flora disorders and
they are more susceptible to disease and stress (Gomez-Gil et al., 2000).

In the light of these considerations, it is necessary to provide aquaculture with alternative means that could keep a microbiologically healthy environment in the rearing tanks and, at the same time, enhance the production and the economical profits from this activity.

Some studies suggest that bacterial probiotics could answer the requirement of controlling potential pathogens and protecting fish health, preventing them from disease (Gatesoupe, 1989, 1991; Maeda and Liao, 1992; Nogami and Maeda, 1992; Strøm and Ringø, 1993; Carnevali et al., 2004).

Some of the numerous disease that can rise inside the rearing tanks are favoured by a stress state of the reared animals due to high stocking densities, handling and poor water quality.

These stressors can sharply affect cortisol plasma levels in fish (Patiño et al., 1986; Small, 2004) and since cortisol is the principal corticosteroid hormone in teleost fish, this is generally considered harmful for immune system and in more general terms for animal’s welfare. In addition, in fish, as observed in mammals, cortisol exerts proteolytic effects (Barton et al., 1987; Andersen et al., 1991; Vijayan et al., 1997) since glucocorticoid synthesis may induce catabolic and anabolic effects which delay somatic growth (Tomas et al., 1979; Ma et al., 2003). Thus, it can be assumed that animals showing lower cortisol levels, at the same time, present higher growing rates. Starting from these information, it is clear how the evaluation of cortisol levels in reared fish is a common and important practise to estimate animal’s welfare (Tripathi and Verma, 2003).

Additional biomarkers such as Insulin-like Growth Factors and myostatin maybe useful in estimating growth rates. IGF-I a polypeptide with a similar structure and biological function to insulin, is commonly used at this purpose; this factor is synthesized virtually in all tissues and can act in an endocrine as well as in a paracrine/autocrine way (Kelley et al., 2000) promoting muscle and cartilage growth (Funkenstein et al., 1989) and differentiation in a variety of systems.

Since it has been evaluated that IGF-I plasma level is correlated to growth rates in different animals such as cattle (Breier et al., 1988), red deer (Suttie et al., 1991), sheep (Gatford et al., 1996), pigs (Owens et al., 1999) and Guinea pigs (Sohlstrom et al., 1998), it can be stated that IGF-I levels may provide an indication of relative growth (Beckman et al., 2004; Dyer et al., 2004).

However, recently it was demonstrated that muscular growth is regulated by the interplay of positive and negative signals: among negative signals, MSTN was identified. MSTN is a member of the Transforming Growth Factor-β (TGF-β) super family. MSTN, also termed growth and differentiation factor-8 (GDF-8), was first characterized in mouse where it is expressed during embryogenesis in developing somites (McPherron et al., 1997), and in adult skeletal muscle. Reduction of myostatin gene expression in mice resulted in a highly muscled phenotype, with both muscle hypertrophy and hyperplasia (McPherron et al., 1997). Fish MSTN gene sequence was first cloned in Danio rerio by McPherron and Lee (1997) but studies on MSTN gene regulation started only recently (Ostbye et al., 2001; Rescan et al., 2001; Terova et al., 2006).

The main goal of the present study was to elucidate the effects of Lactobacillus delbrueckii delbrueckii administration in Dicentrarchus labrax (European sea bass) juveniles on their cortisol level and growth by integrating both macroscopic (body weight and S.L) and molecular data (total body cortisol level, IGF-I and MSTN gene expression).

2. Materials and methods

2.1. Probiotic strain

The isolation of lactobacilli from adult sea bass gut was performed using the methods reported by Carnevali et al. (2004). The probiotic strain used was L. delbrueckii delbrueckii (AS13B).

The strain identification based on biochemical profile has been confirmed using molecular methods. The sequence of 16S rDNA of strain has been determined. DNA extraction was carried out by using the DNeasy Tissue kit (Qiagen, Milan, Italy). PCR reaction has been prepared using primers P0 and P6 corresponding to positions 27 (forward) and 1495 (reverse) of Escherichia coli 16S rDNA following Birmeta et al. (2004). After determining the occurred amplification by electrophoresis, the PCR products were purified by QIAquick PCR purification kit (Qiagen, Milan, Italy) and then sequenced. The sequences obtained have been compared to sequence information available in the GenBank DNA database.

Among Lactobacillus spp. isolated from adult sea bass gut, L. delbrueckii delbrueckii showed the best capacity to survive the typical rearing conditions and it was then chosen as the strain to administer during sea bass larvae rearing. The isolation and cultivation of strain and the bacterial preparation for administration were performed according to Carnevali et al. (2004).

2.2. First feeding

Seawater, at a salinity of 36 psu, was UV treated and filtered with sand filter. The water temperature in the larval
tanks was 17 °C and was gradually increased, between days 1 and 11 p.h. up to 18.5 °C and maintained constant during the remaining period of the experiment. The water, in the larval tank, was replaced 5 times a day by a dripping system and was gently aerated through air stones. *Brachionus plicatilis* were added to the tanks at a final density of 3 rotifers ml⁻¹, the quantity being gradually increased until reaching a density of 15 ml⁻¹ at day 30.

Feeding with *Artemia* started on day 30 p.h. at a density of 1 ml⁻¹ and was gradually increased reaching a density of 15 ml⁻¹ at the end of administration. On day 42, concomitantly with *Artemia*, dry food (Trouwit, Hendrix, 100–1200 μm) was administered at a final quantity of 25 g tank⁻¹ in five times.

### 2.3. Experimental design

European sea bass were hatched at Panittica Pugliese Fish-farm (Torre Canne di Fasano, BR, Italy) and reared for a period of time of 70 days. Larvae, from each group, were reared in 400-l fiberglass conical shaped tanks. The tanks were lightened by incandescent lights (3×36 W) suspended 40 cm on the water surface and light reflection was reduced by the grey colour of the tank.

Water chemical–physical characteristics in the rearing tanks were as follows: pH 8.6, salinity 36 psu, temperature ranged from 17 °C at hatching and was gradually increased between days 1 and 11 p.h. up to 18.5 °C and maintained constant during the remaining period of the experiment.

After hatching, in accordance to the farmer’s rearing protocol, larvae were subjected to 24 h of dark until day 11 p.h. From day 12, the hours of light were increased gradually, reaching a 12L/12D photoperiod by day 35 p.h.

Three experimental groups, control, group A and group B (Table 1), each in duplicate and composed of 40,000 larvae of *D. labrax*, were distributed in 400 l tanks. Group A received the bacterial probiotic at the same concentration of group A, from days 30 to 70 with *Artemia* solely as carrier. In the control group no bacteria were added (Table 1).

### 2.4. Sampling, and gut microbial analysis

70 days p.h. juveniles from each group were collected into a new 30 l tank containing MS222 (Sigma, 100 mg/l). After being anaesthetized 150 juveniles from each group were sampled and rapidly transferred in liquid N. The samples were stored at −80 °C until total RNA and cortisol extraction were performed. 100 larvae from each tank were measured both in standard length and weight.

The gut microbial analysis was performed on juveniles 70 days p.h.; the analysis was carried out as reported by Carnevali et al. (2004). MRS (de Man, Rogosa, Sharpe) agar (Oxoid) has been used for *Lactobacillus* counts in all experimental groups. All plates have been incubated in aerobic conditions for 48 h at 17–20 °C. Bacteria from colonies grown on MRS agar were examined microscopically. All Gram positive straight rods, non-spore forming, catalase negatives and non-motiles were selected and tested by API 50 CHL to identify *Lactobacillus* species.

### 2.5. RNA extraction

Total RNA was prepared from pools of whole larvae and extracted using RNeasy kit (Qiagen). Total RNA was quantified based on absorbance at 260 nm and its integrity was verified by ethidium bromide staining of the ribosomal RNA bands on a 1% denaturing agarose gel.

### 2.6. Cortisol analysis

Cortisol extraction was performed in whole-body juveniles. Samples from different groups were weighed and homogenized and extracted with 4 vol. of dichloro methane for 30 s. The dichloro methane fraction was collected while the remaining fraction was extracted again as described above; this step was repeated three times. The collected fractions were pooled, dried and re-dissolved in 1 vol. of EIA buffer. The analysis of cortisol levels was performed using Cortisol EIA kit (Cayman Chemical Company) using a standard curve in the range of 7.8–1000 pg ml⁻¹, in accordance to the kit instructions. The assay sensitivity 2 ng tube⁻¹ and the inter- and intra-assay coefficients of variation were 6.3% and 4.4%, respectively. To validate cortisol assay, parallelism between the standard curve and serial dilution of the extracted solution was performed.

### Table 1

<table>
<thead>
<tr>
<th>11–29 days p.h.</th>
<th>30–45 days p.h.</th>
<th>46–70 days p.h.</th>
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<td>Control</td>
<td>Rotifers</td>
<td><em>Artemia</em></td>
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<tr>
<td>Group A</td>
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<tr>
<td>Group B</td>
<td>Rotifers</td>
<td><em>Artemia</em></td>
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Note: Group A received probiotics via rotifers from days 11 to 29 and via *Artemia* nauplii from days 30 to 70. Group B received probiotic starting from days 30 to 70 with *Artemia* as carrier. In the control group no bacteria were added.
2.7. cDNA synthesis

Total RNA (5 μg) isolated from sea bass juveniles was reverse transcribed using oligo dT as a primer (0.5 ng), Superscript-II® RT (Invitrogen), 1 μl dNTPs (10 mM), 4 μl Buffer 5×, 2 μl DTT (0.1 M), RNAse OUT (40 U/μl). RT reaction was performed in a final volume of 20 μl. Cycling conditions were: 70 °C for 5 min, 42 °C for 50 min and 70 °C for 5 min.

2.8. Real-time PCR

After reverse transcription target cDNA was amplified by real-time PCR using Cromo4 Real Time (MJ Research) apparatus and the SYBR Green Master Mix 2× (Finzyme). Reactions took place in a final volume of 10 l containing 0.2 μl primers 0.3 μM. Oligo nucleotide primers were designed using the specific electronic program Primer3 according to the sequences of *D. labrax* (GenBANK accession nos. AJ579342, AY839105, AY839106 and AY148350). Target gene chosen was IGF-I (For: 5′-TACAGCTATGGCCTCAAT-3′; Rew: 5′-TTGGCAGGTGACAGTACAT-3′); MSTN (For: 5′-TCTTGGAGCACAAGTGATG-3′; Rew: 5′-CACGTCGTACTGGTC GAGAA-3′); as internal standard β-actin was chosen (For: 5′-GACGCTGATGACGATG-3′; Rew: 5′-GATACGTACCACACCCACC-3′) as suggested by *Caelers et al.* (2004). Cycling conditions consisted of 45 cycles with a first step of denaturation at 95 °C for 15 min and a final extension of 72 °C for 5 min. The annealing temperature was optimized for each couple of primers: 57 °C for 20 s IGF-I, 55 °C for 20 s MSTN and 58 °C for 20 s actin. A melting curve from 70 °C to 95 °C was performed to test the specificity of primers and the product of the PCR reaction was sequenced using ABI 310 DNA sequencer (Perkin-Elmer, Oak Brook, IL, USA), after purification with MinElute kit (Qiagen).

2.9. Quantification of cDNA

A relative quantification of cDNA was made using β-actin as reference gene (internal standard). *Pfaffl’s (2001)* mathematical model was applied to determine the ratio between the different expressions of target gene in treated and in control group and the different expressions of standard gene in treated and control group. The equation express the so called relative expression ratio:

\[
\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_{P,\text{target}}(\text{control-sample})}}{(E_{\text{standard}})^{\Delta C_{P,\text{standard}}(\text{control-sample})}}
\]

where \( E_{\text{target}} \) is the real-time PCR efficiency of the target gene transcript (IGF and MSTN); \( E_{\text{standard}} \) is the real-time PCR efficiency of a standard gene transcript (β-actin); \( \Delta C_{P,\text{target}} \) is the Crossing Point deviation of the control-sample of the target gene transcript; \( \Delta C_{P,\text{standard}} \) is the Crossing Point deviation of the control-sample of the standard gene transcript (*Pfaffl, 2001*). 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 standard gene. Log cDNA dilution was plotted versus \( \Delta C_{P} \) and the efficiencies were calculated according to \( E=10^{\frac{-1}{\text{slope}}} \) (*Rasmussen, 2001*).
2.10. Statistical analysis

The data on bacterial counts and cortisol levels were analyzed by Two-way ANOVA test followed by Tukey’s test, to determine the effect of treatment. The level for accepted statistical significance was $p < 0.05$. The data obtained by real-time were analyzed by Two-way ANOVA test followed by Dunnett’s multiple comparison test to determine the effect of the treatment. The level for accepted statistical significance was $p < 0.05$.

3. Results

3.1. Microbial gut analysis

Microbial analysis showed evident gut colonization of *D. labrax* juveniles by *L. delbrueckii delbrueckii* in treated groups. In fact, at the end of the experimentation (70 days p.h.) group A and group B viable count of *L. delbrueckii delbrueckii* was 4.35 log CFU/g larvae, in group A and 5.68 log CFU/g larvae in group B while in the control was significantly lower ($p < 0.05$) showing 0.45 log CFU/g larvae (Fig. 1). These values of viable counts represent the 96% (group A) and 95% (group B) of the total LAB. The control group showed a lower percentage of *L. delbrueckii delbrueckii* (10%) with a simultaneous presence of other LAB species.

3.2. Cortisol

Cortisol levels obtained from 70 days p.h. larvae whole-body homogenates were significantly ($p < 0.05$) lower in group A (3.6 ±0.36 ng g$^{-1}$) than in control (5.1 ±0.47 ng g$^{-1}$). Group B showed cortisol levels of 4.5±0.48 ng g$^{-1}$ (Fig. 2).

3.3. Standard length and body weight

On day 70 p.h., a significant difference ($p < 0.05$) between body weights among the groups was observed with treated groups showing higher increase in body weight with respect to control (group A: 85± 5.36 mg, group B: 60±6.7 mg, control: 47± 4.69 mg) (Fig. 3). Measures of standard length (S.L.) didn’t show any significant difference among group A (21.9 ±2.99 mm), group B (20.1 ±3.27 mm), and control (20.2 ±3.43 mm) (Fig. 4).
group B (19.7 ± 2.55 mm) and control (16.4 ± 2.7 mm) (Fig. 4).

3.4. Real-time PCR amplification efficiencies and linearity

Real-time PCR efficiencies (E) for investigated transcripts were calculated as shown in Fig. 5. The efficiencies were: 2.00 for β-actin, 2.00 for IGF-I, and 1.72 for MSTN with a linearity of r > 0.98 (r, Pearson correlation coefficient). Application of Pfaffl’s mathematical model, that takes into account the real amplification efficiency, to quantify target gene cDNA, had let us have a realistic result.

3.5. IGF-I and MSTN cDNA levels

The sequences of the PCR products obtained with IGF-I and MSTN specific primers provided an identity of 95% and 97% respectively with the sequences available in GenBank (nos. AJ579342, AY800248 IGF-I; no. AY839106 MSTN).

Using the Pfaffl’s mathematical model, the cDNA levels of the three genes were quantified in each group. Considering cDNA levels as control 1 ± 0.01 arbitrary units (a.u.), the relative ratio of IGF-I in group A (6.63 ± 0.18 a.u.) and in group B (6.16 ± 0.15 a.u.) was six times more than in control group (Fig. 6).

MSTN cDNA levels were lower in groups feed on probiotics than in control group, with relative expression ratio of 0.71 ± 0.01 a.u. in group A, 0.26 ± 0.003 a.u. in group B and 1 ± 0.02 a.u. in control (Fig. 7).

4. Discussion

Since the first application of probiotics in aquaculture, a growing number of scientific papers have dealt with this subject and have demonstrated the validity of their use to control potential pathogens and to increase the survival rates and welfare of reared fish larvae (Gatesoupe, 1991; Nogami and Maeda, 1992; Carnevali et al., 2004). The present study provided evidences on the effects of probiotic treatment both on welfare and growth in European sea bass, one of the most important farmed species for the European ichthyic market.

Results from microbial analysis evidenced that, in groups treated with L. delbrueckii delbrueckii, intestinal population of Lactobacillus was composed of the majority by L. delbrueckii delbrueckii, confirming that the technique of probiotic administration using zooplankton as carrier was effective.

Generally, the analysis of cortisol levels and body weight carried out in this study gave the evidence that the groups fed live preys added with L. delbrueckii delbrueckii showed a better tolerance to captive rearing conditions. This was evidenced by the lower cortisol levels detected in the treated experimental groups with respect to control. It is well known that cortisol plays an adaptive function against stressors since it regulates metabolic energy, hydro-mineral balance, oxygen uptake, and immune competence (Wendelaar Bonga, 1997). On the other hand, elevated cortisol levels are deleterious to fish determining growth suppression (Pickering et al., 1987).

At the same time, at molecular level, the expression of genes involved in muscular growth was also positively affected by bacterial integrators confirming a beneficial role of probiotics on the whole metabolism. In fact, groups fed on probiotics showed significantly higher IGF-I expression with respect to control group. IGF-I is extremely important for the regulation of the establishment and the maintenance of differentiated cell functions via endocrine and paracrine, autocrine signaling (Lackey et al., 1999), as well as the promotion of cellular proliferation and differentiation in many systems (Le Roith et al., 2001). The comparison of cortisol levels and IGF-I expression evidenced a negative relation between this hormone and IGF-I; these data are in accord to that obtained in human by Rosmond et al. (1995).

IGF axis is involved in growth promotion and in particular in myoblast proliferation. However, recently it was demonstrated that muscular growth is regulated by the interplay of positive and negative signals represented by the IGF system and MSTN.

In particular, MSTN depresses both the number of myoblasts which reach terminal differentiation and division and the degree of fiber enlargement (Bass et al., 1999). Two distinct isoforms were identified in trout (Biga et al., 2004) while so far only one MSTN gene has been identified in sea bass (Terova et al., 2006). At gene level, the presence of GH-responsive elements in the MSTN promoter region was evidenced in salmonids (Roberts and
Goetz, 2003), and suggests that at the same time GH can down-regulate MSTN gene transcription and stimulate the IGF-I gene expression. Moreover, Ma et al. (2003) demonstrated that human myostatin promoter contains a number of glucocorticoid responsive elements (GREs) suggesting that myostatin expression is mediated via glucocorticoid receptors and proposing a possible up-regulation of myostatin expression through a glucocorticoid receptor-mediated pathway. Similar GREs were also identified in the salmonid myostatin from Roberts and Goetz (2003).

However, contrasting results are available on the role of glucocorticoid on the modulation of MSTN gene expression: while several studies have reported a glucocorticoid on the modulation of MSTN gene expression in mammals (Lang et al., 2001; Ma et al., 2003) in fish, a down- and up-regulation of MSTN by cortisol, depending on the species, has been evidenced. However, further research is necessary to determine whether MSTN expression in fish muscle cells is directly or indirectly altered by glucocorticoids. In fact, in the brook trout, the promoter region of the MSTN does contain a glucocorticoid regulatory element (Roberts and Goetz, 2003) indicating that the MSTN gene of brook trout is under glucocorticoids control. On the contrary, in channel catfish, exogenous glucocorticoids decrease the expression of MSTN (Weber et al., 2005) and Rodgers et al. (2003) reported a down-regulation of MSTN by cortisol treatment in tilapia. In addition, in zebrafish, chronic stress induced a general depression of muscle protein synthesis not related to myostatin levels but the cortisol levels were not tested (Vianello et al., 2003).

In the present study the results obtained suggested that higher cortisol levels maybe associated with the up-regulation of MSTN gene transcription indicating an interplay among IGF-I, cortisol and MSTN in controlling body growth. Further studies are also necessary to optimize the conditions of probiotic administration in order to maximize the beneficial effects, minimize the costs and promote a fast and easy-managing of probiotics in marine aquaculture.

5. Conclusion

The data here presented showed that the use of *L. delbrueckii* delbrueckii as probiotic had positive effects on welfare and growth of sea bass juveniles. Probiotics decreased cortisol levels of treated animals and affected the transcription of two genes involved in the regulation of body growth, IGF-I and MSTN. In particular, IGF-I transcription was increased and MSTN transcription was inhibited in treated groups. All the mentioned changes resulted in a sharp increase of body weight of treated animals.

These results maybe of great importance for the development of an environment-friendly aquaculture as well as a valid alternative to the use of drugs and antibiotics in marine aquaculture.

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**References**


