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Virgibacillus proomii and *Bacillus mojavensis* as probiotics in sea bass (*Dicentrarchus labrax*) larvae: effects on growth performance and digestive enzyme activities

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Abstract This study examined the effects of two probiotics (*Virgibacillus proomii* and *Bacillus mojavensis*) on the digestive enzyme activity, survival and growth of *Dicentrarchus labrax* at various ontogenetic stages in three separate experiments. These probiotics were incorporated as single or mixed into fish feed for a period of 60 days. The growth parameters, proximate composition of whole body, digestive enzymes and gut microbiology were monitored at regular. The increments in length and weight and the survival were significantly higher ($P < 0.05$), and the values of food conversions were significantly lower ($P < 0.05$) in fishes fed the probiotic. The administration of *V. proomii* and *B. mojavensis* in diet resulted in an increase ($P > 0.05$) in body ash and protein content and in the specific activity of phosphatase alkaline and amylase in the digestive tract of all the fishes. *V. proomii* and *B. mojavensis* persisted in the fish intestine and in the feed in high numbers during the feeding period (group 1: 5.8×10^4 CFU/ml, group 2: 9.6×10^4 CFU/ml, and group 3: 9.8×10^4 CFU/ml day 60). The two probiotics *V. proomii* and *B. mojavensis* were adequate for improved growth performance and survival and for healthy gut microenvironment of the host.

Keywords Bacteria · Probiotic · *Virgibacillus proomii* · *Bacillus mojavensis* · Enzyme · *Dicentrarchus labrax*

Introduction

European sea bass, *Dicentrarchus labrax*, is a major finfish of interest in Mediterranean aquaculture; these hatchery procedures, at their beginning in the 1980s (Barnabé and Billard 1984), had greatly improved during the last two decades. Appropriate nutrition at first feeding in larvae is an important factor for successful larval and juvenile rearing (Dámaso-Rodrígues et al. 2010; Heath and Moore 1997). Marine fish larvae have generally a poorer capacity to digest and/or absorb complex nutrients in comparison with larger fish (Ronnestad and Conceicao 2005), and much higher growth rates (Conceicao et al. 1998).

This very high growth potential of fish larvae means greater requirements in terms of energy, amino acids (AAs), highly unsaturated fatty acids (HUFAs), phospholipids (PLs) and other nutrients.

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In this way, a suitable balance of lipids can help the fish achieve the so-called protein sparing effect, by means of a shift of dietary protein from energetic purposes into growth (Li et al. 2012; Ronnestad and Conceicao 2005). Taking into account these last considerations, digestion is a particularly relevant process in animal nutrition since it influences the bioavailability of nutrients needed for fish growth. The analysis of digestive enzymes is a key tool when studying the nutritional condition and adaptation of fish to dietary changes, in particular, the evolution of digestive activities during larval maturation. Therefore, the assessment of the presence and level of certain enzymatic activities may be used as a comparative indicator of the fish development rate, as well as of their further survival rate (Cara et al. 2007). Indeed, changes in enzymatic activities can be used for studying the effects of the dietary additives that might modulate the maturation process of the digestive system (Gisbert et al. 2009). In this way, dietary probiotic addition is being increasingly reported as an enzymatic contributor to digestion (Sun et al. 2013; Tinh et al. 2008).

Fish larvae are exposed to microbiota-associated disorders because they start feeding when the digestive tract is not fully developed (Ronnestad and Conceicao 2005; Stottrup and McEvoy 2003) and the immune system is still incomplete (Vadstein 1997). For this reason, probiotic treatments are particularly desirable at these stages (Tinh et al. 2008), providing a balanced gut microbiota condition (Olafsen 2001).

The gastrointestinal tract serves as a route for entry of pathogenic microorganisms (Chen et al. 2008; Ringo et al. 2004), and it is believed to be the major route for the onset of diseases like vibriosis, furunculosis, enteric septicaemia and aeromoniasis in fish (Nayak 2010). A healthy intestinal microbiota not only aids the digestive function, but also acts by inhibiting pathogens (Makridis et al. 2005; Sugita et al. 2002). In a specimen not sick, a proper balance between the intestinal microbiota and the host's control mechanism (Nayak 2010; Sansonetti 2004) occurs, and if this balance is disturbed, the pathogens can establish infections (Sekirov and Finlay 2009; Virgin 2007).

In addition, probiotic application has increased in fish aquaculture based on the beneficial effects obtained previously in livestock (Fulton et al. 2002) and humans (Gills 2003). Due to the different environmental conditions of aquatic animals, probiotics have frequently been selected from specimens and environmental autochthonous bacteria (Chabrillón et al. 2005a, b; Lauzon et al. 2010).

Since the *Bacillus* genus has not been reported as pathogens of the aquatic organisms (Moriarty 1998), its application has been promoted and more widely accepted within the aquaculture industry (Gullian et al. 2004). *Bacillus* species are able to produce antibiotics, amino acids and enzymes (Sanders et al. 2003). Consequently, *Bacillus* probiotics may have positive nutritional effects on fish.

Avella et al. (2010) demonstrated that the administration of a *Bacillus* probiotic mixture has a benefit for sea bream larvae in terms of stress response and growth.

Although beneficial effects of probiotics are well known in aquaculture, little information about the influence of probiotics on body composition, growth performance, gut microbiota modulation and digestive enzyme activity in sea bass (*D. labrax*), which is one of the most valuable cultured species in Mediterranean countries, is needed.

Therefore, this study was firstly designed to evaluate the use of bacteria (*Virgibacillus proomii* and *Bacillus mojavensis*) and their mix, as probiotic supplements in diets for sea bass (*D. labrax*) larvae until 60 dah.

Materials and methods

Bacteria strains

Two strains of bacteria: (G3) and (G27), were isolated from the local hatchery (from the intestine of *Chelon labrosus*, rotifer and *Artemia*). They have been selected as candidate probiotic after characterization of antagonism to pathogens, biofilm colonization and gnotobiotic tests (Hamza et al. 2015; in preparation). The isolated bacteria were subjected to the 16S rDNA sequence analysis and identified as *V. proomii* and *B. mojavensis* with a maximum identity ranging between 97 and 98 %. The obtained sequences were deposited in the EMBL database under the following accession numbers: LN828203 (G3) and LN828204 (G27).

Probiotic bacteria were prepared according to the method described by Nimrat et al. (2008). Briefly, each bacterial species was seeded separately into a

500-ml flask containing 200 ml of TSB and shaken at 200 rpm for 24 h (30 °C). Bacterial suspensions were centrifuged at 4500*g* for 30 min, and cell pellets were collected and washed three times with sterilized phosphate buffer saline (PBS), pH 7.2. The pellets were re-suspended in the same buffer for further use.

Live food

The rotifers *Brachionus plicatilis* counted by binocular loup (200 rotifers ml^{-1}) were cultivated on seaweed *Picochlorum* sp. (2×10^6 cells ml^{-1}) and DHA-protein Selco (INVE) following the instructions provided by the manufacturer for 24 h in tanks of 200 l at 24 °C. Rotifers were then filtered through a sieve (60 µm) and have been rinsed and transferred in buckets of 5 l (200 rotifers/ml) containing sea water and probiotic bacteria (1×10^6 CFU ml^{-1}). Rotifers have been maintained in enrichment with the bacterial suspension (*V. proomii* and *B. mojavensis*) for 3 h and filtered, washed by sea water and distributed to *D. labrax* larvae at the rate of 3–5 rotifers ml^{-1}. This dose has been previously reported as suitable by Lobo et al. (2014) and is in the range of other probiotics used in larviculture (Dias et al. 2011; Hernández-Martínez et al. 2009).

Brachionus plicatilis were obtained from the Institute of Aquaculture of the Hellenic Center for Marine research. However, *Picochlorum* sp. has been isolated at the National Institute of sciences and technology of the sea (Monastir, Tunisia).

Cysts of *Artemia salina* (AF and EG, INVE Aquaculture, Belgium) were decapsulated with NaOH and hypochlorite at a concentration of 2 g of cyst l^{-1} and incubated for 24 h at 28 °C and 35 ‰ salinity under strong illumination and aeration. The newly hatched nauplii were rinsed after hatching and enriched with the bacterial suspension (*V. proomii* and *B. mojavensis*) at a concentration of 10^6 CFU/ml^{-1} for 3 h in buckets of 5 l with aeration. These *Artemia* were added to the fish tanks after rinsing with sterilized sea water.

Diet preparation

The larvae feed composition is given in detail in Table 1. Feed was processed at the laboratory as follows: the ingredients were ground up in a laboratory grinder (Retsch®) with a 1-mm screen. The meal obtained was mixed with oil and water (30 %) in a horizontal mixer (Mainca®) until the consistency was such that it could be pelleted. Then the mixture was extruded in a meat grinder through a 3-mm die. The strands of feed were dried at 60 °C for 24 h in a drying oven (Venticell® 222) until the residual moisture content was <10 % and then ground and pelleted to a set of granulometric sizes suitable for each developmental stage (Cahu and Zambonino Infante 2001). The probiotic (*V. proomii* and *B. mojavensis*) was top-coated on the pellets using 3 % of fish oil as a carrier. The same quantity of fish oil without bacterial suspension was used for control diet. The final concentrations of probiotics were adjusted to 10^6 CFU (colony-forming units) g^{-1} of the diet in experiments, respectively, after air-drying at 45 °C. The pellets were packed in sterile polypropylene containers, stored at 4 °C for viability studies and then distributed continuously during the daylight periods with automatic feeders (9 h/day).

The probiotic concentration in the feed was systematically checked after processing by counting strains on TSB plates using serial dilution. The control diet was checked for the absence of detectable probiotic prior to use. However, no bacteria were administered to the control group.

Table 1 Main ingredients of the experimental diet (Torrecillas et al. 2007)

Ingredients	Diet (% dry weight)
Fish meal[a]	51.50
Soybean meal	9.78
Wheat	7.50
Wheat gluten	7.50
Corn meal	6.53
Fish oil[b]	12.69
Fats and oils	2.03
Mineral mix[c]	1.43
Vitamin mix[d]	1.03
Antioxidant (BHT)	0.01

[a] Peruvian fish meal (65 % protein)

[b] Peruvian fish oil

[c] TROUW Seabream/Seabass (0.8 g), choline chloride (0.17 g) and inositol (0.06 g)

[d] TROUW Seabream/Seabass (1 g) calcium carbonate (0.2 g), potassium monophosphate (0.19 g) and NaCl 97 % (0.04 g)

Larval rearing

European sea bass larvae *D. labrax* were provided by the National Institute of Science and Technology of the sea, Centre de Monastir of Tunisia. The larva was reared till 60 days post-hatching dph. The larvae were distributed into ten 1000-l conical fibre glass tanks at 1 dph, with an initial stocking density of 80 larvae l^{-1}. The running water was not recycled in the rearing unit, and the water flow rate was progressively increased from 15 L h^{-1} at 5 dph to 35 l h^{-1} at 40 dph. The temperature was progressively increased from 17 °C at 1 dph to 20 °C at 25 dph. Salinity was 35 g L^{-1} throughout the trial. The larvae were kept in the dark until 4 dph, and then photoperiod was maintained at 18–6 light/dark from 4 dph onwards. The light intensity was progressively increased from 10 to 200 lux between 4 and 25 dph and then kept constant (Ben Khemis et al. 2006).

Mouth opening occurred at 4 dph; the first feeding live food, both rotifers and *Artemia*, was utilized. *B. plicatilis* was added to the tanks at a final density of 3 rotifers ml^{-1}, the quantity being gradually increased until a density of 15 ml^{-1} was reached at day 17. Feeding with *Artemia* AF started at day 16 dph and EG at day 26 at a density of 1 ind. ml^{-1} and increased gradually, reaching a density of 15 ind. ml^{-1} at the end of administration (day 40 p.h.). At day 38, concomitantly with *Artemia*, the dry food was administered at a final quantity of 25 g tank-1 given at five different times. The larvae were fed in large excess by using belt feeders for 18 h/day. The particle size was 120–200 μm from 10 to 20 dph and then 200–400 μm afterwards.

The first tank contained larvae fed without probiotic (control treatment), and the other tanks contained larvae enriched with *V. proomii* (G3: group 1) or *B. mojavensis* (G27: group 2) alone or in mixture (K: group 3).

Growth and survival

For growth studies, thirty specimens from each triplicate were weekly and randomly sampled. These samples were anesthetized with ice-cold sea water and then fixed with formaldehyde (4 % in phosphate-buffered solution pH 7.4) and kept refrigerated until analysis at the end of the experiment. Fish total length was measured on photographs which were taken using a digital camera (Nikon Coolpix 4500), and measurements were taken later using image analysis software ImageJ 1.29. An object micrometre was photographed with each set of photographs to avoid errors due to the autofocus of the camera. Drained weights of fixed larvae and juveniles were measured immediately after photographing. Survival was checked along the experiment. Specific growth rate was calculated by formula $SGR = 100\ (\text{Ln FBW} - \text{Ln IBW})/\Delta t$, with IBW and FBW: initial and final body weights of fish (mg), and Δt: time interval (day). At the end of the experiment, larval survival was determined by counting the larvae remaining in the tanks.

Analysis of body composition

For the study of larval and fry body composition, five samples were randomly collected from each rearing tank on days 1 (225), 20 (75), 40 (30) and 60 (15), the numbers in parenthesis being the total number of specimens sampled per replicate. Fish samples were washed several times with distilled water prior to being frozen at −80 °C, until analysis. Total soluble protein was determined following the method of Bradford (1976). Total lipid content was assessed by extraction with chloroform/methanol 2:1 as described by Bligh and Dyer (1959) modified by Fernández-Reiriz et al. (1989) and gravimetrically determined after centrifugation.

Monitoring of bacteria

Six larvae from each batch were sampled and analysed for digestive microbiota. Prior to grinding and homogenization, the sea bass larvae were rinsed with sterilized distilled water, washed with 7.00 % alcohol and then rinsed again with sterilized distilled water to remove all external bacteria. All samples were diluted serially with sterilized normal saline solution (0.85 % w/v NaCl). Total counts of bacteria were determined by plating on tryptic soy agar (with 1.5 % w/v NaCl). Probiotic bacteria, *V. proomii* and *B. mojavensis* samples were cultured on TSB agar. The number of colonies (from TSB agar) on each plate was counted after incubation for 72 h at 37 °C. The number of colonies from TSA on each plate was counted after incubation for 48 h at 24–25 °C. All analyses were conducted on days 10, 20, 30 and 60.

Enzymatic assays

For enzymatic assay, larvae were sampled on days 0, 20, 40 and 60. Samples corresponded to 120, 70, 60 and 40 larvae were collected from each enclosure, respectively. They were stored immediately at −80 °C pending assay. Before homogenizing in ice-cold distilled water, larvae were vortexed in 500 µl ice-cold distilled water for 30 s to obtain released enzyme (supernatant S1). This supernatant contained the secreted pancreatic enzymes, i.e. trypsin and amylase (Ma et al. 2005). The larvae were then homogenized in 1–2 ml ice-cold distilled water, depending on the weight of the sample, with a homogenizer (polytron, PT-MR 2100) for 30 s and then centrifuged at 3300×g for 3 min. This supernatant (S2) was used to analyse unreleased pancreatic enzymes (trypsin and amylase) and intestinal enzymes (alkaline phosphatase [AP], aminopeptidase N [AN] and leucine–alanine peptidase [Leu-Ala]). Amylase and trypsin activities were assayed according to Métais and Bieth (1968) and Holm et al. (1988), respectively. The brush border membrane enzymes, alkaline phosphatase and aminopeptidase were assayed according to Bessey et al. (1946) and Maroux et al. (1973), respectively. Assay of the cytosolic leucine–alanine peptidase was performed using the method of Nicholson and Kim (1975). Activities were measured as µmol of substrate hydrolysed per min per mg protein, at 37 °C for AP, Leu-Ala and AN, and at 25 °C for trypsin (Zambonino-Infante and Cahu 1994). Amylase activity represented the equivalent enzyme activity required for hydrolysing 1 mg of starch in 30 min at 37 °C (Zambonino-Infante and Cahu, 1994). Enzymatic activities were expressed as specific activities (mU/mg protein^{-1}).

Statistical analysis

Results were given as mean ± SD ($n = 30$ for larval growth; $n = 5$ for enzymatic analysis). The variance homogeneity of the data was performed using Levene's test. Survival data were compared by Fisher's Chi-square test, and also larval growth and enzymatic activity data were compared by one-way ANOVA, followed by Newman–Keul's multiple range test when significant differences were found at a 0.05 level. All measurements were taken in triplicate. Statistical analyses were performed by STATVIEW software.

Results

Larval growth and survival

Growth of sea bass larvae in groups during the 60-day period of study is described in Fig. 1. In all experimental groups, larvae multiplied their weight by a factor more than 50-fold from day 1 to day 60. At the beginning of the experiment, initial larval weight was 0.90 ± 0.01 mg. Specific growth rates in groups were calculated as 6.91 % day^{-1} for group 1, 7.03 % day^{-1} for group 2, 7.09 % day^{-1} for group 3 and 6.54 % day^{-1} for control group. At the end of the experiment, the best results on total length development and weight were determined in group 3 as 19.45 ± 2.2 mm and 59.12 ± 2.3 mg.

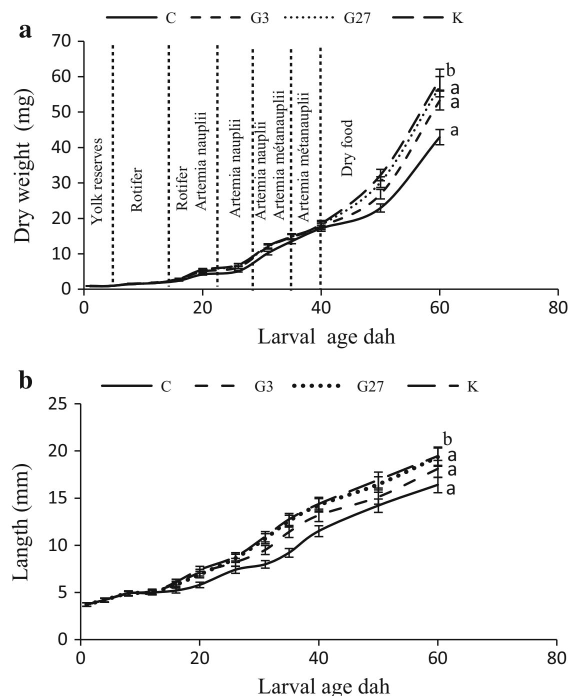

Fig. 1 Feeding protocol and larval growth in **a** dry weight (mg) and **b** total length (mm) of Dicentrarchus labrax larvae. C: control, G3: group 1, G27: group 2, and K: group 3. Results are expressed as mean ± SD ($n = 3$). Values with different (*a*–*b*) superscript letters indicate difference related to development day ($P < 0.05$)

Besides, these values were estimated as 18.1 ± 2.3 mm and 53.23 ± 2.1 mg for group 1, 19.35 ± 1.6 mm and 57.14 ± 1.4 mg for group 2, and 16.4 ± 1.5 mm and 42.9 ± 2.7 mg for control group (Fig. 1). There were no significant differences on weight and total length development in group 1, group 2 and group 3, whereas significant differences were found in group 3 and control group ($P < 0.05$). Moreover, final survival rates were determined as 72.4, 83.8, 89.1 and 57.1 % for experimental groups 1, 2 and 3 and control group, respectively (Fig. 1).

Analysis of body composition

With regard to larval body composition, a clear increment in larval individual nutrient content with age was found after three treatments. For instance, at day 60 after hatching, larvae groups 1, 2 and 3 contained, respectively, 24.16, 26.8 and 27.23 mg protein larva^{-1} compared with 20.23 mg larva^{-1} in control fish. Similarly, at 60 dah group 1, 2 and 3 was also significantly richer in fat content contained

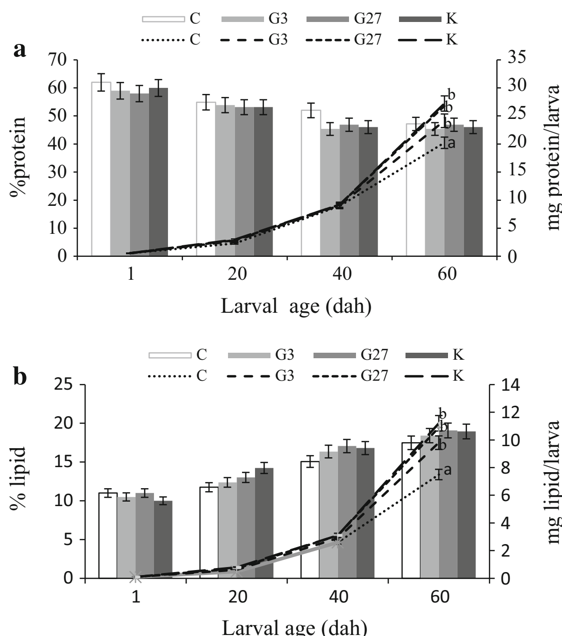

Fig. 2 Protein (**a**) and lipid (**b**) contents (g × 100 g^{-1} dry weigh and mg × larvae-1) of *Dicentrarchus labrax*. C: control, G3: group 1, G27: group 2, and K: group 3. Results are expressed as mean ± SD ($n = 3$). Values with different (*a*–*b*) superscript letters indicate difference related to development day ($P < 0.05$)

respectively (9.8, 10.9 and 11.2 mg larva^{-1} compared with 7.5 mg larva^{-1} in control (Fig. 2).

Bacterial study

Virgibacillus proomii and *Bacilus mojavensis* successfully colonized the intestinal tract of larvae (Table 2). Total bacterial counts among probiotic-supplemented groups were significantly different from total bacterial counts in controls in digestive tract of larvae ($P < 0.05$). For the digestive tracts of larvae in which the mean of total bacterial counts among probiotics administered groups was more than that of control ($P < 0.05$). However, the flora in probiotic treatments was significantly different from the flora in control ($P > 0.05$). On the other hand, *bacillus* colonization was detected the more dominant in experimental groups; however, no colonization was observed in the digestive tract of sea bass larvae in control group (Table 2).

Enzyme activity

The specific activity of trypsin demonstrated an exponential increase in all experimental groups to 20 dah. After this date, this activity has declined to increase again at the end of the experience. The highest specific activity of trypsin was determined in group 2 as 105.62 ± 13.2 mU/mg protein^{-1} (Fig. 3). Trypsin activity was significantly higher in group 2 and group 3 compared to group 1 and control group ($P < 0.05$); however, no differences were found between group 3 and group 2 ($P > 0.05$).

Amylase specific activity slowly increased to 20 dah and then slightly decreased until the end of the experiment. The highest specific activity of this enzyme was found at 20 dah in group 2 as 2.99 ± 0.08 mU/mg protein^{-1} (Fig. 3). The specific activity of amylase in group 2 and group 3 larvae was significantly different among group 1 and control group ($P > 0.05$), although there were no differences between these groups ($P > 0.05$).

Aminopeptidase (LAP) specific activity showed similar pattern for trypsin; sudden increase was observed at 20 dah, followed by slight decline and increase until the end of the experiment. The peak of lipase activity was measured in group 2 on day 20 as 35.13 ± 3.2 mU/mg protein^{-1} (Fig. 3).

Aminopeptidase specific activity in group 2 and group 3 was significantly higher than in group 1 and

Table 2 Total bacteria count and *V. proomii* and *B. mojavensis* count in digestive tract of *D. labrax*. Results are expressed as mean ± SD ($n = 3$)

Groups	dah	Total counts 10^6 CFU/ml	TSB counts	Bacillus counts (10^4 CFU/ml)
Control	10	43 ± 1.6[a]	–	0
	20	2900 ± 0.98[a]	–	0
	30	28,000 ± 22.3[a]	–	0
	60	32,000 ± 10.5[a]	–	0
Group 1	10	3.7 ± 0.17[b]	–	0
	20	0.29 ± 0.23[b]	+	2.7 ± 0.05[b]
	30	0.12 ± 0.1[b]	+	3.6 ± 0.23[b]
	60	0.10 ± 0.005[b]	+	5.8 ± 0.13[b]
Group 2	10	5.1 ± 0.2[b]	–	0
	20	1.9 ± 0.1[b]	+	3.4 ± 0.32[b]
	30	0.26 ± 0.09[b]	+	5.6 ± 0.18[b]
	60	0.22 ± 0.02[b]	+	9.7 ± 0.14[b]
Group 3	10	4.4 ± 0.2[b]	–	0
	20	26 ± 0.14[b]	+	3.8 ± 0.15[b]
	30	30 ± 0.17[b]	+	5.9 ± 0.21[b]
	60	37 ± 0.1[b]	+	9.8 ± 0.03[b]

Values with different (*a*–*b*) superscript letters indicate difference related to development day ($P < 0.05$)

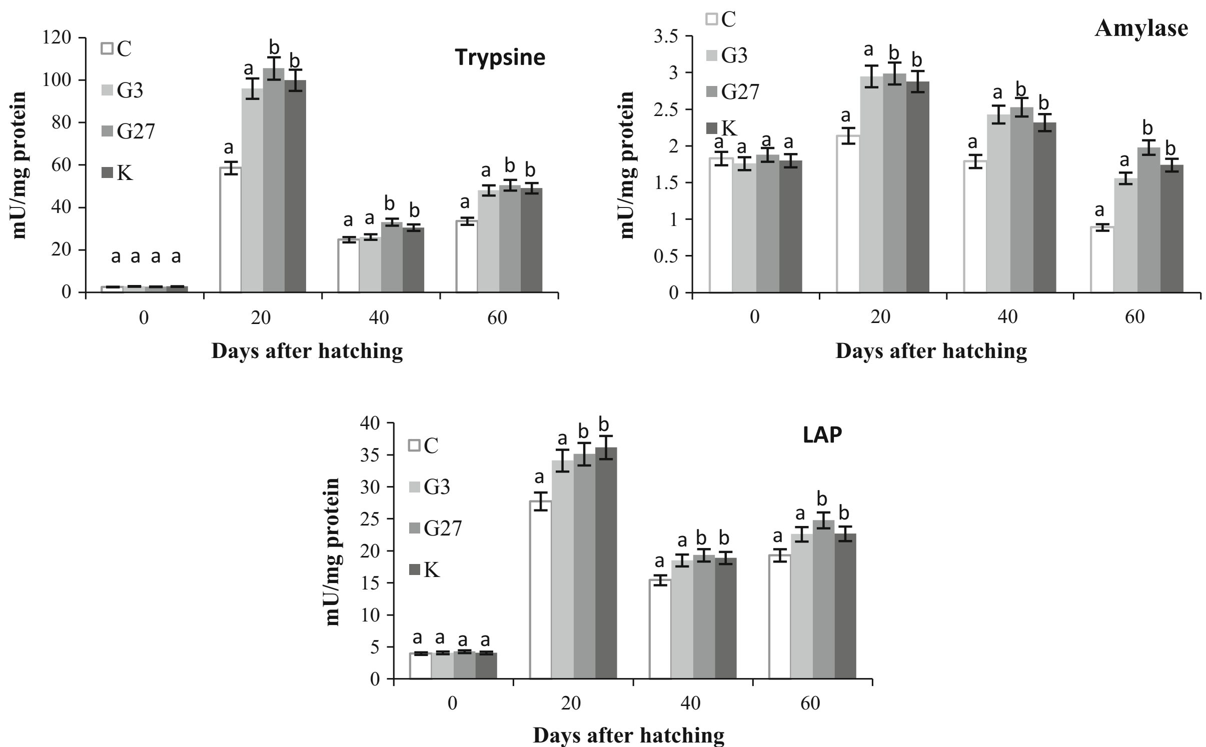

Fig. 3 Specific activities of pancreatic enzymes, trypsin, amylase and aminopeptidase (LAP), during larval development of *Dicentrarchus labrax* up to day 60. Results are expressed as mean ± SD ($n = 3$). Values with different (*a*–*b*) superscript letters indicate difference related to development day ($P < 0.05$)

control group ($P < 0.05$), but there were no differences between the latter groups ($P > 0.05$).

Specific activities of intestinal enzymes, AP and Leu-Ala peptidase, demonstrated opposite pattern among the experimental groups. Until the third week, AP specific activities fluctuated insignificantly in all experimental groups ($p > 0.05$); however, sharp increases in AP were measured significantly in group 2 and group 3 at 40 dah ($P < 0.05$) and continued to the end of the experiments. The highest specific activity of AP was determined on day 60 in group 2 as 847.16 ± 49.7 mU/mg protein^{-1} (Fig. 4).

In contrast to AP activity, at the beginning of the experiments specific activity of Leu-Ala peptidase was relatively higher during the first three weeks, but after this date it slightly declined consecutively in all experimental groups. In particular, sharp decreases were measured after 40 dah synchronously with increase in AP activity. At the end of the experiment, Leu-Ala enzymatic activities in group 2 and group 3 were significantly higher than in group 1 and control group ($P < 0.05$), although there were no differences between the latter groups ($p > 0.05$). The peak of Leu-Ala peptidase activity was measured in group 2 on day 20 as 460.18 ± 38.7 mU/mg protein^{-1} (Fig. 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 on both welfare and growth in European sea bass, one of the most important farmed species for the European ichthyic market.

Larval development in all groups was satisfactory, but probiotic treatments demonstrated significant increases in growth performance of larvae. Besides, similar significant rises were recorded in SGR and survival parameters in experimental groups. But it is clearly determined that probiotic supplementation by live and inert food enhanced husbandry parameters of larvae. These findings were in agreement with the study on Indian white shrimp, *F. indicus* (Ziaei-Nejad et al. 2006), and shrimp, *P. vannamei* (Wang 2007). Authors reported that probiotic supplementation into diet (live food and/or extruded pellet food) resulted in significant increases in both growth performance and husbandry parameters compared to basal diets and/or no supplemented groups. Similar finding were recorded in freshwater species such as Nile tilapia *O. niloticus* and carp, *C. carpio* (Wang and Xu 2006), and marine fish species such as red drum, *Sciaenops ocellatus* (Li et al. 2005), and Japanese flounder, *P. olivaceus*, juveniles (Taoka et al. 2006).

However, probiotics enhance nutrition by synthesis of essential nutrients (vitamins and short-chain fatty acids) and enzymes (amylase and protease), by

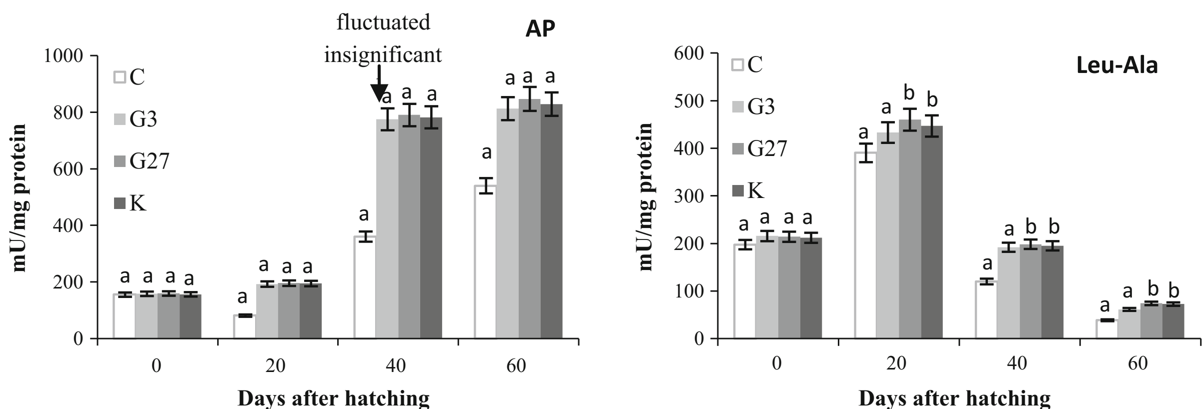

Fig. 4 Specific activities of intestinal enzymes, alkaline phosphatase (AP) and leucine leucine–alanine (Leu-Ala) peptidase, during larval development of *Dicentrarchus labrax* up to day 60. Results are expressed as mean ± SD ($n = 3$). Values with different (*a–b*) superscript letters indicate difference related to development day ($P < 0.05$)

detoxifying the potentially harmful compounds in feed and by denaturing the potentially indigestible components in the diet (Gatesoupe 1999). The enhanced nutrient and enzyme levels by probiont addition led to increased food digestion and absorption which in turn led to better growth of the fishes ingesting the probiotic cells. Goldin (1998) stated that both the increased digestibility of nutrients and infection control through antagonistic effects were responsible for weight gain. This phenomenon was operated by substitution of depressive microbial agents which hinders growth. In the absence of antigenic stimulus provided by pathogenic bacteria, the free plasma cells in the mucosa are reduced, resulting in better absorption and utilization of the nutrients. In this study, *V. proomii* and *B. mojavensis* administration in diet increases fish survival by activating their immune defences by enhancing their leucocyte phagocytic activity (Salinas et al. 2005). These properties are the cause of weight increase, greater survival and improved SGR values of *D. labrax*.

Several authors pointed out that one of the main modes of action and beneficial effects of probiotics in aquacultured organisms is enhancement of nutrition of host species through the production of supplemental digestive enzymes and higher growth and feed efficiency, prevention of intestinal disorders and pre-digestion of antinutritional factors present in the ingredients (Thompson et al. 1999; Verschuere et al. 2000). In detail, after transition through the stomach, they germinate in the intestine and use a large number of sugars (carbohydrates) for their growth and produce a range of relevant digestive enzymes (amylase, protease and lipase) (El-Haroun et al. 2006). However, in aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty 1998). In the current study, we administered *Virgibacillus promii* and *B. mojavensis* in live and inert food; thus, it was clearly determined where probiotics colonized and worked effectively in terms of growth, survival and digestive enzyme activities in different environments (by live food and/inert food). Obtained data strongly presented that more effective results were taken from live food treatments due to colonization in live food guts (rotifer and *Artemia*) and transition to larval fish intestine by digestion. Similar results were estimated in the same experimental design in some marine organisms. As described by some studies, all the probiotic-supplemented diet resulted in an increase in growth, SGR and survival, showing that the addition of probiotics increased the growth performance of shrimps *P. vannamei* and *F. indicus* (Ziaei-Nejad et al. 2006; Wang 2007). Also, these results were in agreement with Ghosh et al. (2003) for Indian carp *L. rohita*, red drum, *S. ocellatus* (Li et al. 2005), Japanese flounder, *P. olivaceus*, juveniles (Taoka et al. 2006) and common carp *C. carpio* (Wang and Xu 2006).

Also, the effective strain colonization of sea bass larval gut was monitored and confirmed the suitability of zooplankton carriers and inert diet, as well as the ability of G3 and G27 to modify gut microflora during development (Carnevali et al. 2006; Silvi et al. 2008). The probiotic strains *V. proomii* and *B. mojavensis* were detected in groups 1, 2 and 3 since the 11th dph gradually increased until the end of the treatment. On the other hand, influences of probiotics on immune responses and bacterial loading in aquatic organisms and environments were well documented (Gatesoupe 1999, 2002, 2007). As pointed out by several authors, administration of probiotics by live food and inert diet dramatically decreased bacterial activity in some teleosts (Salinas et al. 2005, 2006; Díaz-Rosales et al. 2006), *P. dentatus* (Eddy and Jones 2002), *Scophthalmus maximus* (Planas et al. 2006) and *Salmo salar* (Robertson et al. 2000). These findings were parallel with the obtained results from this study that bacterial activity was significantly decreased in experimental groups than control (Table 1). A protocol of early probiotic administration (started during gut metamorphosis) was chosen, showing that *V. proomii* and *B. mojavensis* had significant and positive effects on growth and welfare of sea bass larvae and post-larvae (Carnevali et al. 2006). It is therefore possible that these two strains produced substances that inhibited the growth of the Gram-negative bacteria (Gatesoupe 1997). *Bacillus* is able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics (Moriarty 1998). Many different antibiotic compounds (polymyxin, bacitracin, bacillin and gramicidin) are produced naturally by *Bacillus* sp., and other bacteria are unlikely to have resistance genes to all of the antibiotics produced by the *Bacillus* probionts, especially if they had not been exposed to the *Bacillus* previously (Katz and Demain 1977).

It is well known that digestive enzyme activity can be used as an indicator of larval food acceptance and to

some extent serve as an indicator for the digestive capacity in relation to the type of feed offered (Ueberschär 1993). Moreover, the assessment of the presence and level of activity of digestive enzymes may be used as a comparative indicator of the rate of development of the fish larvae, food acceptance, digestive capacity, as well as their further survival rate (Ueberschär 1995).

Treatment of *V. proomii* and *B. mojavensis* as probiotics to larvae resulted in an increase in the specific activity of all measured digestive enzymes in the digestive tract of larvae. The digestive system of *D. labrax* is activated particularly in the early stages of larval development where the probiotics would have the greatest effect and because Gram-positive bacteria, particularly members of the genus Lactobacillus, do secrete a wide range of exoenzymes (Moriarty 1996, 1998). Besides, enzymatic activities could not be distinguished due to enzyme synthesized by the shrimp and activity due to enzyme synthesized by the bacteria. In the current study, administration of probiotics to *D. labrax* larvae resulted in an increase in the specific activities of digestive enzymes in the larval digestive tract. This result could be related to enhanced digestion and increased absorption of food, which in turn contributed to the improved survival and growth in *D. labrax* including improved feeding parameters and specific growth rate (SGR).

The enhanced growth performance of larvae might be due to increasing digestive enzyme activity induced by the probiotics. Furthermore, bacteria secrete a wide range of exoenzymes (Moriarty 1996, 1998). Another contribution is that probiotic administration could enhance activities of digestive enzymes. Therefore, enzymatic activities in all experimental groups were enhanced by probiotic supplementation. But administration type of probiotics to larval fish strongly affected the enzymatic activity as determined for larval development. Specific activities of pancreatic enzymes, trypsin and lipase, and intestinal enzymes, AP and Leu-Ala peptidase, were significantly different in all experimental groups. Enzymatic profile of Exp. 2 and Exp. 3 demonstrated remarkably better activities than that of the Exp. 1 and control groups. Administration of probiotics by live food showed more effective expressions on digestive enzymes due to bacterial colonization in the larval gut. It is thought that probiotics influence digestive processes by enhancing the population of beneficial microorganisms and microbial enzyme activity, improving the intestinal microbial balance and consequently improving the digestibility and absorption of food and feed utilization. Also, it was expected that relatively better results on growth performance and husbandry parameters could enhance specific activities of digestive enzymes. These results were in agreement with the study of Tovar-Ramírez et al. (2004), who studied with live yeast *Debaryomyces hansenii* in *D. labrax* larvae. He has recorded the improvement on survival, growth parameters, digestive enzyme activities (trypsin, amylase and lipase) and decline in mortality and malformed larvae. Moreover, Wang and Xu (2006) investigated the effects of *Bacillus* sp. probiotics on growth parameters and protease, amylase and lipase specific activities in C. carpio juveniles and recorded that mean digestive enzyme activities of all probiotics treatment groups were significantly different ($P < 0.05$) to that of the control. The decline in specific enzyme activities of these digestive proteases during larval ontogeny of *D. labrax* could be basically explained by the normal increase in tissue proteins in growing larvae, which reflects anatomical and physiological changes in fish larvae, and does not correspond to a lowering in the amount of digestive enzymes or dietary shifts (Kamac et al. 2010). It is reported that the fluctuations in specific enzyme activities covered the period of morphological differentiation in the digestive tract and associated glands (Zambonino Infante and Cahu 2001). After the formation of gastric glands, the digestive system became functional and the specific activities of these digestive enzymes remained constant, while the total enzyme activities increased gradually with age.

Conclusions

It could be concluded that supplementation of probiotic (*V. proomii* and *B. mojavensis*) to live food (rotifer and *Artemia*) and inert diet, followed by feeding, was an effective means by which to deliver the probiotic to the sea bass larvae. This is the first study to investigate the effects of *V. proomii* and *B. mojavensis* probiotics on husbandry parameters and digestive enzyme activities in *Dicentrarchus labrax* larvae. A supplementation of probiotic to live food (rotifer and *Artemia*), followed by feeding, was an effective means by which to deliver the probiotic to the *D. labrax* larvae. As a result, supplementation of *D. labrax* diet with the

proper density of *V. proomii* and *Bacillus mojavesnsis* probiotic could be beneficial for growth and survival of larvae, especially in fast-growing conditions, where it would be essential to stimulate the precocious maturation of digestive system (Wache et al. 2006). No clear effect of probiotic on diversity of *D. labrax* fry intestine flora was detected, but high rate of probiotic bacteria colonization was observed. Since the results might be affected by the rearing conditions (Spanggaard et al. 2000), we suggest the effects of these probiotic to be tested in other locations.

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