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Effects of Probiotic (*Bacillus* sp.) Supplementation during Larval Development of Gilthead Sea Bream (*Sparus aurata*, L.)

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Abstract

In this study, the effects of administration of commercial probiotic, *Bacillus* sp., were examined on growth parameters and digestive proteases (alkaline and acid proteases) activities in gilthead sea bream, *Sparus aurata*, during larval development until on day 40. Probiotics were supplemented in triplicate from on day 3 coinciding with starting of exogenous feeding. In the experimental group, probiotic was introduced to rotifer and tank water until 20 days after hatching. Also, control group did not receive probiotic. In experimental group, the specific activities of alkaline protease was significantly higher ($p<0.05$) in larvae compared to control group. Acid protease activity was not effected by probiotic supplementation however, it was relatively higher in experimental group than the control ($P>0.05$). Additionally, no significant differences were found in both survival (8.5% higher) and specific growth rate (0.7% higher) larvae of *S. aurata* that had probiotic supplemented by rotifer with water as compared to controls ($P>0.05$). Consequently, specific activities of alkaline and acid protease could be significantly increased by the administration of *Bacillus* sp. to live food with water the and therefore; this method would be more effective for obtaining of relatively better growth parameters and nutritional condition in *S. aurata* larvae.

Keywords: *Bacillus*, Probiotic, digestive enzyme activity, growth, survival, *Sparus aurata*.

Probiotik (Bacillus sp.) Eklenmesinin Çipura (*Sparus aurata*, L.) Larvalarında Gelişime Olan Etkileri

Özet

Bu çalışmada, çipura larva yetiştiriciliğinde 40. güne kadar ticari bir ürün olan probiotik bakteri (*Bacillus* sp.) eklenmesinin larvalarda büyüme parametreleri ve sindirim enzimleri (alkalin ve asit proteaz) üzerine olan etkileri incelenmiştir. Bütün denemeler 3 tekrarlı yürütülmüş ve probiotik eklenmesi ağız açılımı ile birlikte eş zamanlı olarak yumurtadan çıktıktan sonraki 3. günden itibaren uygulanmıştır. Deneme grubunda, probiotik bakteriler 20. güne kadar rotifer kültürüne ve tank ortamına eklenmiştir. Bunun yanında, kontrol grubuna herhangi bir uygulama yapılmamıştır. Deneme grubunda, alkalin proteaz spesifik aktivitesi kontrol grubuna göre önemli derecede yüksek bulunmuştur ($P<0,05$). Asit proteaz aktivitesi ise probiotik eklenmesinden etkilenmemiş bununla birlikte kontrol grubuna görece aktivite görece yüksek tespit edilmiştir ($P>0,05$). Ek olarak, rotifer kültürüne ve tank ortamına eklenen çipura larvalarının yaşama oranı (%8,5 daha yüksek) ve spesifik büyüme oranı (%0.7 daha yüksek) değerleri kontrol grubuna göre önemsiz farklılıklar göstermiştir ($P>0,05$). Sonuç olarak, probiotiklerin canlı yem kültürü ve tank ortamına eklenmesi alkalin ve asit proteaz aktivitesini önemli oranda artırmış, bu yüzden probiotiklerin bu yöntemle uygulanmasının büyüme parametreleri ve besinsel durumları açısından daha etkili olduğu sonucuna varılmıştır.

Anahtar Kelimeler: *Bacillus*, probiotik, sindirim enzimleri aktivitesi, büyüme, yaşama oranı, *Sparus aurata*.

Introduction

During the last decade, using of chemical drugs, especially antibiotics, have become limited due to developing of resistance by certain pathogens. Therefore, in order to control and/or elimination of using of antibiotics in aquacultural systems more efforts have been conducted for obtaining of knowledge This situation is accelerated to use alternative methods for solution of this problem. It is commonly reported that in order to optimize aquatic environment, preventing fish diseases and improving to control pathogens in culture systems, the supplementation of probiotics is the common method.

Probiotics have been identified as live microorganisms colonized in the intestine and/or animal originated microbial supplements could be effected fish health by improving the intestinal microbial balance. (Gatesoupe, 1999, 2007; Verschuere *et al.*, 2000; Balcázar *et al.*, 2006; Wang *et al.*, 2008; Dimitroglou *et al.*, 2011). Nowadays, probiotics in aquaculture has being commonly supplemented for controlling of disease and also rising up of feeding and husbandry parameters. As determined by several authors, *Bacillus* species bacteria are widely used as a probiotic bacteria in aquatic animal nutrition such as *Bacillus cereus, B. toyoi, B. licheniformis and B. subtilis* (Balcázar *et al.*, 2006; Ziaei-Nejad *et al.*, 2006; Wen-Ying *et al.*, 2010; Avella *et al.*, 2010; Jafaryan *et al.*, 2011; Wu *et al.*, 2012; Mohapatra *et al.*, 2012). The useful effects of these probiotics as a nutrient supplementary additive include relatively higher rates of growth feed efficiency, and also prevention of intestinal disorders and pre digestion of anti-nutritional factors present in the ingredients. Also, recent studies focused on probiotics are mainly related with resistance to some aquatic pathogens such as *Vibrio* sp. (Villamil *et al.*, 2003; Planas *et al.*, 2006), *Amyloodinium ocellatum* (Li *et al.*, 2005), *Carnobacterium* sp. (Robertson *et al.*, 2000), and microbial monitoring but some studies especially aimed on growth performance, feeding parameters, and also increase of feed efficiency with enzymatic activity in fish. Increasing efforts and interests for description of possible use of probiotics in both marine fish species such as summer flounder, *Paralichthys dentatus*, (Eddy and Jones, 2002) common dentex, *Dentex dentex*, (Hidalgo *et al.*, 2006) and Japanese flounder, *Paralichthys olivaceus*, (Taoka *et al.*, 2006) and freshwater teleosts such as rohu, *Labeo rohita* (Ghosh *et al.*, 2003), the Chinese carps, *Hypophthalmichthys molitrix* and *Ctenopharyngodon idella*, common carp, *Cyprinus carpio*, tilapia, *Oreochromis mossambica*, walking catfish, *Clarias batrachus*, the murrel, *Channa punctatus*, (Bairagi *et al.*, 2002), rainbow trout, *Oncorhynchus mykiss* (Panigrahi *et al.*, 2005), and Nile tilapia *Oreochromis niloticus* (El-Haroun *et al.*, 2006).

More studies have been focused that probiotics has been widely using for increasing both feed conversion ratio and efficiency and also digestive enzyme activities. However, little studies have been carried out based on growth performances and digestive enzyme activities to incorporate probiotics effects in marine fish cultured species *S. aurata* (Salinas *et al.*, 2005, 2006; Díaz–Rosales *et al.*, 2006; Suzer *et al.*, 2008; Avella *et al.*, 2010) freshwater carp species *Cyprinus carpio* (Wang and Xu, 2006) and *Ctenopharyngodon idella*, (Wu *et al.*, 2012) and also shrimp *Penaeus vannamei*, (Wang, 2007; Zhou *et al.*, 2009) Also, beneficial effects of probiotics are well known in aquaculture, there is limited information about influence of *Bacillus* sp. probiotics on digestive enzyme activities in gilthead sea bream (*S. aurata*), which is one of the most valuable and economic species cultured in Mediterranean aquaculture. The aim of this study is to investigate using of *Bacillus* sp. and their mix, as probiotics supplements by live food for gilthead sea bream (*S. aurata*), until 40 days after hatching (DAH) during the larval development.

Materials and Methods

Larval Rearing

Larviculture was conducted in green water recirculated system in triplicate and larvae were stocked 80 ind.l^{-1} densities at 10 m^3 cylindrical tanks and also larval rearing parameters and protocols were carried out according to Suzer *et al.* (2008). Physiochemical parameters of sea water such as temperature, dissolved oxygen, salinity, pH, ammonia and nitrite levels were recorded daily. During the larval rearing, water temperature was gradually increased between 17 and 22 °C. Moreover, oxygen saturation (>85%), salinity (38.0‰) and pH (7.7) were maintained, respectively. Ammonia and nitrite were measured and presented constant values always which were below 0.01 mg.l^{-1}. No flow rate in tanks was performed for the first 2 days of the culture period. From day 3 to 12, the tank water was partially replaced (5–7% daily) by draining a filter (200 µm). Flow rate was increased slowly from 2 to 20% with the age of the larvae. Photoperiod was set on a 24 h light cycle daily until end of algal addition for green water technique (20 DAH) and then 16-h light and 8-h dark until end of the experiment (40 DAH).

Concurrently with mouth opening from 3 to 20 DAH, newly hatched larvae were fed by two strains of rotifers (*Brachionus plicatilis* but mainly with *Brachionus rotundiformis* at a density of 10–15 individuals.ml^{-1} for sufficiently feeding of whole larvae and also they enriched with algae and enrichment media (S.presso, Artemia Systems SA, Ghent, Belgium) plus green-water composed of *Nannochloropsis* sp., *Chlorella* sp., *Tetraselmis sp.* and *Isochrysis* sp. at a density of 1.5–2 x 10^5 cells.ml^{-1}. Between 10 and 30 DAH, *Artemia* nauplii grade (AF 480 INVE Aquaculture, Ghent, Belgium) were introduced 4–7 individuals.ml^{-1} densities and then *Artemia* metanauplii were supplemented from 25 DAH until on day 40 at 2–4 individuals.ml^{-1} (EG, Artemia Systems SA, Ghent, Belgium), both enriched with Protein Selco (Artemia Systems SA, Ghent, Belgium). Larval feeding regime was summarized in Figure 1.

Experimental Design

The commercial probiotic used in this study Bactosafe (solid form) BernAqua NV, Olen, BELGIUM) contained spores of 3 species of *Bacillus* (i.e., *Bacillus subtilis, B. licheniformis* and *B. cereus*)

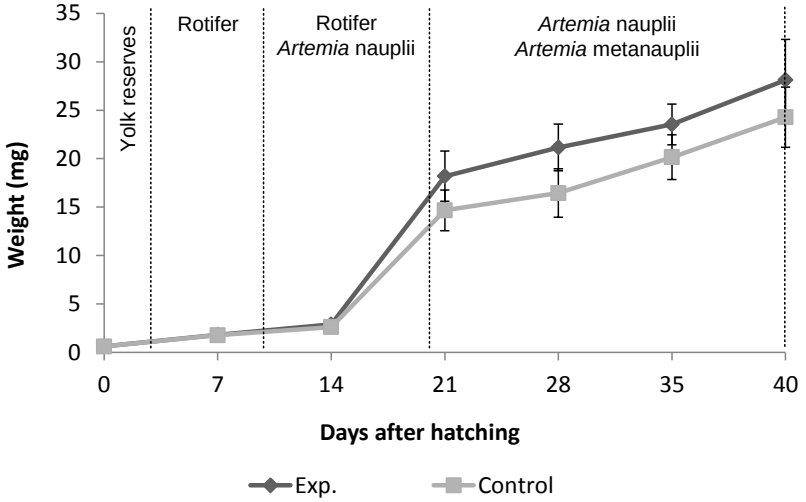

Figure 1. Growth of *S. aurata* larvae in weight up to day 40. Each mean±SD is a pool of 30 larvae (n=3).

and other bacteria *Pediococcus acidilactici.* Before administration of the probiotic, spores were rehydrated to vegetative bacteria according to manufacturer's instructions (10 ppm per m^3).

In Experiment 1, probiotics was added to both live food (rotifer) and directly larval tank until 20 DAH.

Experiment 2 was accepted as control group which had received no probiotic in either live food or tank's water.

Sampling

In order to measure of growth, larvae collected from each groups and each tanks by 7 days interval until 35 DAH and at the end of the experiment. (30 larvae/group). Specific growth rate was calculated by formulae SGR (specific growth rate) = 100 (Ln FBW−Ln IBW)/Δt, with IBW, FBW: initial, final body weight of fish (mg), Δt: time interval (day). At the end of the experiment, survival of larvae was determined by counting larvae which were remained in the tanks.

Dissection of the Digestive Tract

In order to perform enzymatic analysis, groups of larvae in triplicate sampled from each tank 7 days interval in similar with above sampling. For each sampling time, total 100 larvae until 10 DAH, total 75 larvae until 20 DAH and total 50 larvae were sampled until 40 DAH. Samples were usually collected at the same hour (08:00) and depths (30-50 cm), before food distribution. Digestive tracts were used for enzymatic analysis in larvae and therefore, larvae were dissected on a glass maintained at 0 ºC for isolation of the gastrointestinal tract

Enzymatic Assays

Digestive tract samples were collected and homogenised in 5 volumes v/w of ice-cold distilled water (pH 7.0). Extracts utilized for enzyme assays were obtained after homogenization of larvae (35 mg ml^{-1}) in cold 50 mM Tris-HCl buffer, pH 7.5, followed by centrifugation (13.500x*g*; 30 min at 4 °C). Alkaline protease activity was measured using the method of Walter (1984) using casein as substrate (pH 9.0). Acid protease activity was determined using haemoglobin as the substrate in (pH 2.0) (Anson, 1938). Supernatant absorbance was detected at 280 nm. Enzymatic activities were expressed as specific activities (mU/mg $protein^{-1}$). Protein was determined by the Bradford method (Bradford, 1976). All enzymatic analyses were measured weekly at intervals until 35 DAH and at the end of the experiment and also were analyzed by Jenway 6300 UV−Visible Spectrophotometer.

Statistical Analysis

All measurements were carried out in triplicate. Results are presented as mean ± SD (*n*=30 for larval growth; *n*=3 for enzymatic analysis). Levene test was used for determination of the variance homogeneity. Significant differences at survival data and also larval growth and enzymatic activity data among experimental groups were compared by Fisher's chi-square test and by one–way ANOVA, followed by Newman–Keul's multiple range test, respectively at 5% level of significance.

Results

Larval Growth and Survival

Larval growth of *S. aurata* determined in experimental groups until 40 DAH is presented in Figure 1. In experimental group, larvae exhibited their weight by a factor more than of 20−fold multiplied from 7 to 40 DAH. At the end of the experiment, the best results on total length development and weight

were determined in Exp.1 probiotic given groups as 21.17±1.8 mm and 28.12±4.2 mg. Besides, these values were estimated as 17.03±1.2 mm and 24.28±3.1 mg for control group (Figure 1). There are no significant differences on weight and total length development in experimental and control group (P>0.05). Moreover, final survival rates as 16.7% and 8.2% and also specific growth rate were 7.8%.day^{-1} and 7.1%.day^{-1} for experimental and control group, respectively. Growth performance and husbandry parameters in experimental group was not significantly higher than control group (P>0.05).

Enzyme Activity

The specific activity of alkaline protease enzymes demonstrated exponentially increase more than 14-fold in experimental group to 28 DAH. After this date, this activity slightly declined until end of experiment. The peak of specific activity of alkaline protease enzymes was measured on day 28 in experimental group as 162.3±18.3 mU/mg protein^{-1} (Figure 2). There were significant differences between experimental and control group for alkaline protease enzyme activity (P<0.05).

In contrast to alkaline protease activity, no specific activity of acid protease was measured until 28 DAH, but after this date acid protease activity slowly increased consecutively in all groups. Especially, sudden increases were determined after 35 DAH synchronously with decreases in alkaline protease activity and also weaning by microdiet. At the end of the experiment, no significant differences were found between groups (P>0.05). The highest acid protease activity was measured on day 40 in experimental group on day 40 as 11.6±2.1 U/mg

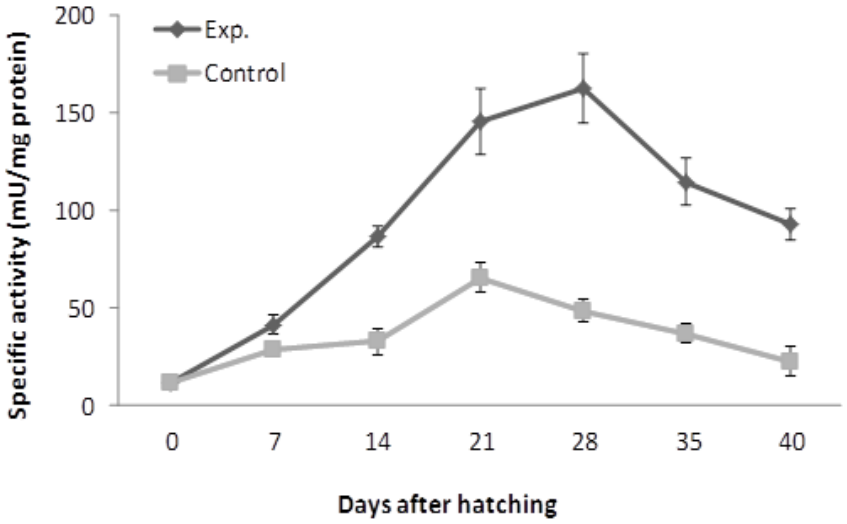

Figure 2. Specific activities of alkaline protease during larval development of *S. aurata* up to day 40. Results are expressed as means±SD (n=3).

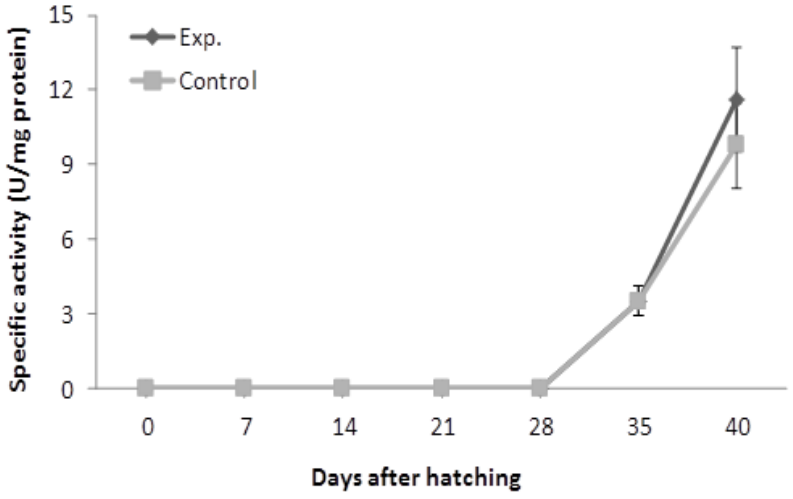

Figure 3. Specific activities of acid protease during larval development of *S. aurata* up to day 40. Results are expressed as means±SD (n=3).

protein^{-1} (Figure 3).

Discussion

Growth in all experimental groups was satisfactory but supplementation of probiotic presented slight increases in growth performance of larvae as compared to control. Moreover, similar higher profiles for SGR and survival date were recorded in experimental groups however, it is clearly determined that probiotic administration by rotifer promoted growth parameters of larvae. Also, obtained results from experimental group which probiotic was supplemented by rotifer and water presented that growth parameters were relatively higher compared to control because of the fact that administration of the probiotic significantly changed the proportion of *Bacillus* bacteria in the gut flora (Salinas *et al.*, 2005; Avella *et al.*, 2010). Similar findings were noted in Indian white shrimp, *Fenneropenaeus indicus*, (Ziaei-Nejad *et al.*, 2006), and shrimp *Penaeus vannamei*, (Wang, 2007). In these studies, it was reported that supplementation of probiotics by live food and/or extruded pellet food caused to significant increases in both growth performance and parameters in compared to basal diets and/or no probiotic administration. These findings were in agreement with freshwater species such as Nile tilapia, *Oreochromis niloticus* and carp, *Cyprinus carpio*, (Wang and Xu, 2006) and marine fish species such as red drum, *Sciaenops ocellatus*, (Li *et al.*, 2005) and Japanese flounder, *Paralichthys olivaceus*, juveniles (Taoka *et al.*, 2006).

In cultured species, it is well known that one of the main beneficial effects of probiotics is enhancement of nutritional conditions of host species which is accelerated of production of supplemental digestive enzymes, prevention of gastrointestinal obstacles and anti-nutritional factors present in the ingredients (Gatesoupe, 1999, 2007; Thompson *et al.*, 1999; Balcázar *et al.*, 2006). Concisely, after transition through the stomach, in order to maintain growth and promote of intestinal enzymes (amylase, protease and lipase) probiotics colonize in the intestine and utilize by a large number of carbohydrates (Gatesoupe, 1999; El-Haroun *et al.*, 2006). However in aquaculture, probiotics could be supplemented as both food supplement and additive to the culture environment (Ziaei-Nejad *et al.*, 2006; Suzer *et al.*, 2008; Avella *et al.*, 2010). In this study, *Bacillus* sp. probiotics were administered by live food (rotifer) and tank water, thus, it was clearly determined that probiotics colonized and worked effectively along the intestine and also accelerated larval growth, survival and especially digestive enzyme activities. Moreover, according to results, it is strongly presented that relatively better results for growth performance and enzymatic activity were obtained by rotifer supplementation due to colonization in live food guts and transition to larval fish gut by digestion process. It is well recorded that all the probiotic-supplemented diet and/or addition of probiotics to diet caused to better growth, SGR and survival parameters by the same experimental design in some marine organisms such as shrimps *Penaeus vannamei* and *Fenneropenaeus indicus* (Ziaei-Nejad *et al.*, 2006; Wang, 2007). Also, similar results were reported by Ghosh *et al.* (2003) for Indian carp *Labeo rohita*, red drum, *Sciaenops ocellatus*, (Li *et al.*, 2005) and Japanese flounder, *Paralichthys olivaceus*, juveniles (Taoka *et al.*, 2006) common carp *C. carpio* (Wang and Xu, 2006). In addition to these beneficial effects, it is clearly estimated that supplementation of probiotics by live food and/or culture water presented relatively better parameters on immune responses and for decline of bacterial activity in some teleosts such as *S. aurata* (Salinas *et al.*, 2005; 2006; Díaz–Rosales *et al.*, 2006), *Paralichthys dentatus* (Eddy and Jones, 2002), *Scophthalmus maximus* (Planas *et al.*, 2006), and *Salmo salar* (Robertson *et al.*, 2000) and also bacterial loading of aquatic organisms and environments (Gatesoupe, 1999, 2007; Balcázar *et al.*, 2006; Wang *et al.*, 2008).

As described by several authors, *Bacillus* bacteria have been widely used as probiotics and also especially *B. subtilis*, a gram-positive non-pathogenic, spore-forming bacterium, it has been widely using for oral bacterial therapy, prophylaxis of gastrointestinal disorders, improving culture environmental quality and the promote the survival of cultured organisms (Irene *et al.*, 2005; Wang, 2007; Avella *et al.*, 2010; Wen-Ying *et al.*, 2010; Mohapatra *et al.*, 2012). In addition to these, particularly, *B. subtilis* is known to produce digestive proteases and other enzymes that enable it to contribute to the natural digestion activity of the cultured species and it can be a source of micro and macro-elements as feed (Verschuere *et al.* 2000; Ziaei-Nejad *et al.*, 2006; Jafaryan *et al.*, 2011). It is commonly known that the knowledge on level of activity of digestive enzymes could be used as a descriptive tool to estimate growth of the fish larvae, digestive capacity and food preferences, as well as their further survival rate for cultured species. Also, determination of digestive enzyme activities could be better for identifying nutritional capabilities of these organisms during early ontogeny under culture conditions (Ueberschaer, 1993; Kolkovski, 2001; Zambonino Infante and Cahu, 2001; Suzer *et al.*, 2008). In this study, *Bacillus* administration as probiotics is caused to significant increases in the specific activity of alkaline protease which is effectively works in alkaline pH ranges in gut of digestive tract of larvae. It is thought that this phenomenon could be related with gut colonization of *Bacillus* sp. which is caused to enhanced digestion and absorption of food, which in turn contributed to the accelerated survival and growth in *S. aurata* larvae. The promoted growth performance of larvae could be originated from improved digestive enzyme activities induced by the probiotics along the intestine. During the early ontogeny of Sparids,

digestive system where the probiotics could be worked effectively and because gram-positive bacteria especially members of the genus *Bacillus* could be secreted a wide range of exoenzymes (Ziaei-Nejad *et al.*, 2006; Avella *et al.*, 2010; Wu *et al.*, 2012; Mohapatra *et al.*, 2012). Furthermore, it is clearly determined that the administration methods strongly affected the success of gut colonization of the probiotics. In this study, administration of *Bacillus* probiotics to *S. aurata* larvae presented relatively better increased specific activities of alkaline protease in the larval gastrointestinal tract. Therefore, although *Bacillus* sp. supplementation significantly affected alkaline protease activity along the intestine, no significant differences were determined for acid protease activity. For this reason, specific activity of alkaline protease in experimental group was enhanced by probiotics supplementation. Similar findings are well noted by Avella *et al.* (2010) administered the mixture, composed of four *B. subtilis*, *B. strains*, *B. licheniformis*, and *B. pumilus* was provided via rotifers and *Artemia* nauplii and added to the water or supplied exclusively via live prey in sea bream larviculture. They noted that the *Bacillus* sp. mixture significantly increased growth in terms of growth parameters, feeding efficiency and metabolism at larval and juvenile stages of *S. aurata* (Avella *et al.*, 2010). In our previous study, we supplemented *Lactobacillus* spp., probiotics under similar experimental conditions in larvae of *S. aurata* until 35 DAH, significant increases were found in both growth performance and digestive enzyme activities in larvae which probiotics had been supplemented by live food (rotifer and *Artemia*) and live food with water (Suzer *et al.*, 2008). On the other hand, in order to establish successfully gut colonization, gastrointestinal pH could be stated in alkaline ranges between 8.0 and 10.0. This phenomenon is evidenced in this study that probiotic supplementation did not affect acid protease activity due to acidic pH ranges (1.5-2.0) after formation of functional stomach on day 38 in sea bream larvae (Suzer *et al.*, 2007). Additionally, the mixture, composed of three probiotic bacteria *Bacillus* sp., *Lactobacillus* sp. *and Saccharomyces cerevisiae* were provided by iso-nitrogenous and iso-caloric diets in rohu (*Labeo rohita*) fingerlings. It is reported that the fish fed combination of three probiotics at equal proportion presented significantly higher growth, protein efficiency ratio, nutrient retention and digestive enzyme activities over other treatment groups (Mohapatra *et al.*, 2012). These results are parallel with this study. Furthermore, similar results were recorded in grass carp, *Ctenopharyngodon idella*, juveniles and *B. subtilis* was provided as probiotic in different concentrations. Although, there were no significant differences in both growth parameters and feed conversion ratio as similar as in this study, it is interestingly recorded that digestive enzyme activities did not increase after long-term feeding with *B. subtilis* (56 days), but was still higher than that of control fish (Wu *et al.*, 2012). It is thought that it could be related with not efficiently colonization of *B. subtilis* in fish gut during the experiments. Also, the other reason might be evaluated that it is better to supplement not only one probiant but also mixtures of *Bacillus* spores (more than 3 species) by the diet. It is clearly thought that supplementation of probiotics effectively improve the digestive process in larvae of *S. aurata* by relatively increase on beneficial microorganisms and their microbial enzyme activity, establishment of microbial balance and enhance on the digestibility and absorption of food and feed utilization along the intestine.

In conclusion, this is the first study to examine the supplementation of *Bacillus* sp. probiotics and also its effects on husbandry parameters and digestive enzyme activities in *S. aurata* larvae. It is clearly estimated that administration of probiotic by live food (rotifer), could be resulted in relatively higher growth parameters, feed efficiency and also significant improvement in digestive alkaline protease activity. Further studies should be conducted on effects of *Bacillus* spp. as probiotic supplementation by both live food and/or in water on specific immune response, disease resistance and other biochemical parameters in cultured fish species.

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