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Research Article

Evaluation of Micro-Particulate Diets used in Marine Hatcheries for Seabream Larvae Reared with the Probiotic Bacterium *Bacillus* Sp, under the Prevalent Conditions in Egypt

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Abstract

The present study was carried out at the larval rearing unit of the commercial Haraz Hatchery. A feeding trial was conducted to evaluate micro-particulate diets that are used in marine hatcheries for seabream larvae. Three different experimental micro-diets were used to study the effects of micro-particulate diets on the survival and growth of *Sparus aurata* larvae. The feeding regime was started at ages raring from 80 to 100 days, and the larvae were fed (9% of their body weights). Gilthead seabream larvae were fed three different diets. The commercial diet was from Aller Feed Co. (basal micro-diet T1) and *Bacillus subtilis* was added to the basal diet to generate a bacterial diet (improved local micro - diet T2). Additionally, the *B. subtilis* bacterial diet was mixed with a mixture of the basal diet and a marine larvae micro-diet obtained from Invy Co. (improved local and imported micro - diet T3). All diets were filtered through 350- 400 micron sieves. The different treatments were performed on a large scale as an application trial in 8.5 m^3 conical-bottom circular tanks. The results showed that the addition of *B. subtilis* to the micro-particulate diets resulted in improvement of the survival rates of the seabream larvae. Additionally, the improved diets increased the total length gain of the seabream larvae. The results indicated that larvae fed on diets T3 and T2 exhibited significantly increased final weights, total weight gains and average daily weight gains. The results showed that there were no significant difference between T2 and T3 and both increased SGR and RGR values. The lowest SGR and RGR values were observed for T1.

Keywords: Gilthead seabream; Larvae; *S. aurata*; Micro-diets; *Bacillus subtilis*; Larval rearing; Feeding

Introduction

Egypt is seeking to sustainable development, for increase the total production and national income. Several projects have been carried out in different regions of Egypt. The most important of these projects are aquaculture mega projects that aim to increase fish productivity, provide good sources of protein, achieve food safety in terms of quality and quantity, reduce the import of fish and increase export leading to improvement of the national income and to an increase in the value of the Egyptian currency. Recently, large governmental mariculture projects were established in Kafr El sheikh (Gholion), East Port Said (Shark El Tafriaa Project) and the Suez Canal region (mariculture project), All of these projects require large quantities of high quality and healthy fish larvae. These projects depend on larvae imported from Greece, Italy, Saudi Arabia and other places leading to unstable environments. There is a demand for locally sourced marine fish larvae. Egyptian marine hatcheries are unable to meet the larvae requirements of mega projects because of the relatively low productivity of these hatcheries. However, these projects include large marine hatcheries, e.g., the fish hatcheries in the Gholion project were designed to produce 40 million units of marine finfish fry. In the past, fish fry were collected from the wild sources, growing them in small ponds and transferred to fish farms. This method is very dangerous and leads to overfishing, which has adverse effects on the natural environment and causes reduction in fish stocks. To preserve fish resources, hatcheries were developed, and researchers and experts devised new methods of hatching, breeding and larval rearing. Hatcheries produce larvae of the same species, size and age in the quantities needed at different times and seasons during the year.

Research efforts have led to the development of brood stock management technologies including spawning technologies [1], maturation and spawning in floating cages and tanks and larval rearing technologies. Recently, most hatcheries suffering from reduced survival rates during larval rearing, because of environmental and biological, factors that affect growth and production, Hatcheries also face challenges associated with the costs and availability of live food. Larval feeding an important factor that affects the production of marine larvae and post larvae particularly in the early larval stages [2]. In marine hatcheries worldwide, live food organisms are provided during the initial culture stages. However large-scale production of live food is expensive, time consuming and the quality of the food not satisfactory [3]. Development of artificial feeding diets is one

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of the most important research areas for intensive larval and post larval cultivation [4]. Artificial feeds include micro- bound, micro-coated and micro-encapsulated diets as microparticles or micro-diets. These micro- particulate feeds including micro-bound, micro-coated and micro-encapsulated diets protect food from degradation and nutrient dissolution. Larvae require bio-foods and co-micro diets such as Artemia powder, yeast and dried algae, additionally; growth promoters such as prebiotics prebiotic and/or probiotics may be useful for improvement of the larval micro-diet efficiency. In marine fish, the most widely used probiotic bacteria species are Bacillus sp and Lactobacillus sp. [5]. These organisms are typically delivered in feeds and/or rearing water in the form of live cultures, lyophilized cells, dead cells, disrupted cells, cell-free supernatants, and spores. Most probiotic studies have been undertaken for periods of 1week to 8 weeks to achieve disease resistance and support the immune systems in fish. We can use probiotic bacteria B. subtilis sp in Artemia enrichment then feed larvae in early stage that increase immunity.

The present work aimed to the evaluate micro-particulate diets used in marine hatcheries for seabream larval rearing with the addition of the probiotic bacteria B. subtilis sp, tested under the prevalent conditions in Egypt. Additionally, we studied the effects of the diets on survival rate, growth rate, and health and feed conversion ratio of seabream larvae.

Materials and Methods

The present study was carried out in the larval rearing unit of the commercial Haraz Hatchery. This hatchery is located in Nemra 2, Al-Qantarah Gharb, 52 Canal Road Port Said, and Ismailia, Egypt. Haraz Hatchery is a marine hatchery that produces seabream, seabass and shrimp larvae. This hatchery contains different units, namely, broodstock, larval rearing, live food culture and nursery.

Experimental larvae

Seabream (S. aurata) females weighting 1000 to 2000g/fish and males weighting 250 to 300g/fish were collected from the Mediterranean Sea transported to the hatchery and selected for use as fish broodstock. Broodfish were stocked at a density of 6 kg/m^3 with a male: female sex ratio of 1:2. The water temperature was between 14°C and 18°C. Broodstock spawning of both species was induced by controlling the photoperiod (12 h light/day to 16 h light/day). Brood fish were fed fresh natural foods such as shrimp, sardines and squids. After ovarian maturation of females, spawning occurred. Eggs were collected with a manual egg collector, with 400-micron plankton net. Management of egg and larval rearing was performed according to [6]. The eggs hatched after 48 h to 72 h. Initially, the larvae were fed rotifers (a small 100 microns to 210 microns) strain for the first two days followed a larger strain (130 microns to 340 microns). After 18 days the larvae started feeding on Artemia Franciscan (AF 300 microns to 400 microns). At 25 DAH (days after hatching) the larvae were fed EG Artemia 400 microns to 450 microns. When retched 40 days to 45 days the larvae were fed gradually with a micro diet with a protein proportion of approximately 50% to 65%. At 60 DAH the larvae were dependent on the micro diet alone, and were reared under depends on artificial lighting (1000 lux for 12h to 16 h lights daily). The water exchange was 100 % to 200% every 24 h. The larvae were maintained in this unit for 60 days. At 5 mg to 10 mg, the larvae were transferred to the nursery unit. The stocking density of the S. aurata larvae was approximately 120000 larvae per tank (4000/m^3). Feeding was performed every half hour from 8:00 to 20:00 with a micro diet, the size of which increased gradually from 80 microns to 400 microns.

Experimental facilities

In the larval raring unit the tanks were covered with black plastic sheets to control temperature, and the experiment was performed in six circular fiberglass tanks. The internal diameter of each tank was 3 m, with a depth of 1.5 m (1.2 m water column), a volume of 8.5 m^3 and a 2% slope. The size of the main drain was 4 inches (central pipe); the size of the over flow drain was 4 inches and surface drainage was 1.5 inches, and the size of the water inlet was 1.5 inches. The bottom color of the tank was white and the walls were bluish grey. Sea bream larvae can be stocked at up to 5500 larvae/tank with a stocking density of approximately 647 larvae/ m^3. Three treatments were conducted in the experiment. All tanks were provided a with source of filtered sea water obtained from the Suez Canal, Each tank was equipped with a filter on the water inlet and a surface filter, and five aeration sources were fixed on the tank wall in a circular manner for suitable distribute aeration and to help in the distribution of waste in the middle of the tank at the central drain.

Experimental design

A feeding trial was conducted to evaluate the micro-particulate diets that are used in marine hatcheries for seabream larvae from 80 days of age. By using three different treatments (two replicates for each treatment), the larvae in the six tanks were fed three different experimental diets.

Bacterial mixture preparation

The diet was prepared at the Center of Microbial Resources, Faculty of Agriculture, Ain Shams University, Shobra, Egypt. The bacterial spore stock of a B. subtilis strain isolated from a broiler gastrointestinal tract (prepared in chemically defined medium) was divided in 1ml aliquots and stored with 30% of glycerol in liquid nitrogen. B. subtilis strain (EMCC 1250) was cultured in tryptic soya broth supplemented with 2% NaCl and fermentation was stopped 26happroximately 26 h. The cells were then harvested by centrifugation and re suspended in sterilized seawater. The diet was prepared by adding B. subtilis bacteria to feed obtained from Aller Feed co. The cell density of this strain was approximately 10^7cell/g; the bacterial diet was dried for 24 h, then grinded and screening though a 350 microns to 400 microns sieve. The bacterial diet was mixed with the feed and used for larval feeding.

Preparation of micro-particulate diets

Three micro-particulate treatments were used. The commercial diet obtained from Aller Feed Co is used in most marine hatcheries and was used as the basal diet in the present work.

1. Treatment 1: micro-diet from Aller Co. (basal diet T1)
2. Treatment 2: bacterial mixture mixed with the basal diet to obtain a bacterial cell density of approximately 5×10^5 cells /g, by adding 5% of the bacterial mixture. The diet contained approximately 5 g of bacterial mixture for every 95g of basal diet (improved diet T2).
3. Treatment 3: bacterial mixture mixed with a mixture of the basal diet and marine larval micro diet (Invy Co.). To obtain a bacterial cell density about 5×10^5 cells /g, the bacterial mixture was added at 5%. The diet approximately 5 g of bacterial mixture for every 95 g of the feed that contained 75% basal diet and 25% Invy Co. (improved mixed diet T3). All the diets were mixed well and screened through the 350-400 micron sieves

Analytical methods

Proximate analysis of the feed was performed by standard methods for determination of the moisture, dry matter, crude protein, crude fat, ash and nitrogen free extract levels (AOAC, 1990), at the feeding Laboratory of the Fish Farming and Technology Institute (Table 1).

The water quality used in the experiment is shown in Table 2 in terms of physicochemical parameters (APHA, 2005).

Table 1: Chemical compositions of the experimental diets.

Experimental micro-diets	Dry matter %	% based on DM				
		CP	EE	CF	NFE	Ash
Basal micro-diet (T1)	93.64	65.72	13.29	6.75	5.12	9.12
Improved local micro-diet (T2)	93.64	65.72	13.29	6.75	5.12	9.12
Improved local and imported micro- diet (T3)	93.92	65.53	15.55	6.51	4.24	8.17

CP, EE, CF and NFE indicate crude protein, ether extract, crude fiber and nitrogen free extract, respectively.

Table 2: Physicochemical Parameters of the water used in the present study.

Parameter	Concentration
Salinity (ppt)	42
Temperature (°C)	21 ± 2
pH	8.7 ± 0.2
Dissolved oxygen (ppm)	5.7 ± 1
Carbonate (mg/L)	38
Bicarbonate (mg/L)	95
Total alkalinity (mg/L)	133
Electrical conductivity (mm hos/cm)	61.4
Total dissolved solids (mg/L)	39296
Total suspended solids (mg/L)	4
NH_3 (mg/L)	0.68

Sample storage

At the end of the experiment, samples were preserved in glycerine 80% (a sticky substance) which does not freeze and preserves larvae without changing the shape. The larvae were placed in a small plastic packet, immersed in glycerin, covered with a plastic sheet stored in a freezer.

Antibiotic Sensitivity Test

The B. subtilis Strain was maintained on nutrient agar slants in a refrigerator. The bacterial strain was tested for susceptibility to a panel of six antimicrobial agents: neomycin, chloramphenicol, gentamicin, tobramycin, tetracycline and erythromycin. Testing was performed by use of a broth micro dilution method as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1990).Two or three identical isolated colonies of B. subtilis were picked up and placed in sterile saline solution. A sterile cotton swab was dipped in the solution, excess liquid was removed from the swab and the surface of an agar plate was streaked with the swab in three directions (the agar surface must be dry). After the plates had dried, antibiotic discs were placed on the surfaces of the plates. Each plate was incubated at 37°C for 18h to 24 h. After incubation the inhibition zones around the antibiotic discs were measured carefully in millimetres using a measuring scale and compared with the values in the Kirby-Bauer chart, the results were interpreted as susceptible, intermediately susceptible or resistant.

Estimation of growth parameters and survival rates

Growth parameters were estimated according to the following equation

Total Weight Gain (TWG): TWG= W_1 - W_0

W_1= average final weight

W_0= average initial weight

Specific Growth Rate (SGR) was calculated as the percentage increase in weight per larvae per day using the following equation, SGR = Lin W_1 - Lin W_0/ feeding days × 100

W_1 = larval weight at the end of the experiment

W_0 = larval weight at the start of the experiment

Relative Growth Rate (RGR)

RGR = final weight - initial weight/initial weight × 100

Feed Conversion Ratio (FCR) = feed intake/weight gain

Survival % was calculated as the number of harvested larvae/ number of stock larvae × 100

Statistical analysis

All data were statistically analyzed using SPSS for Windows (2013). The results are presented as the mean ± SE (for larval growth parameters, survival and food conversion ratio). The degree of significance between the mean correlations was also calculated for some of the measured parameters. Additionally, the SD was calculated for total weight and total length. All statistical analyses were performed according to [7], and differences were subjected to Duncan's multiple range test.

Results and Discussion

Survival %

The data in Table 3 show the survival percentages of the seabream Sparus aurata ML (metamorphosed larvae) fed a micro-particulate local diet without B. subtilis (basal micro-diet T1), a basal diet incorporated with B. subtilis (improved local micro-diet T2) and Aller + Invey with B. subtilis (improved local + imported micro-diet T3). The results indicated that ML fed diets containing Bacillus sp. (T2, T3) had significantly higher ($P \leq 0.05$) survival rates than those fed the diet without B. subtilis (T1).

The improvement in survival % may be due to the probiotic bacterium B. subtilis sp. which produces antimicrobial compounds that inhibit the growth of pathogenic microorganisms leading to a favorable environment and habitat for stable larvae growth and to improvement of larval health [8]. Demonstrated (for marine larvae) enhancement of the quality of farming water after the addition of Bacillus spp. as probiotic. Other studies have shown an increase in immune response with the use of probiotics for different species, such B. subtilis and Pseudomonas aeruginosa for Labeo rohita [9]. These results are consistent with those obtained by [10], who demonstrated the effect of B. subtilis. Against Vibrio harveyi and the White Spot Syndrome Virus (WSSV) and with the results of [11], who used B. subtilis with gilthead seabream.

Length measurements

The data in Table 4 show the effects of the micro-diet treatments on the lengths of seabream ML. The seabream ML fed improved local diets and exported micro-diets containing B. subtilis sp. (T3, T2) exhibited significantly higher ($P \leq 0.05$) total length gains than those fed the basal micro-diet (T1).

The results showed an increase in size over time for the control and two probiotic treatments with significant differences between the means of the improved diets and the control diet. The increase in total length gain may be caused by the addition of the probiotic bacteria B. subtilis sp. to the diet that help in improve larval growth. The above results are consistent with those obtained by [12], for sea bass and by [13], for gilthead seabream. It has been suggested that the dose of the administered probiotic is the limiting factor for achievement of the optimal benefits of the probiotic. Probiotics can also be used to promote the growth of aquatic organisms, directly or via. the absorption of nutrients.

Changes in body weight

The data in Table 5 show the final weight, total weight gain and average daily weight gain of the seabream larvae (ML) fed on diets T1, T2 and T3. The results indicated that the ML fed diets T3 and T2 which contained Bacillus species. Exhibited significantly higher ($P \leq 0.05$) final weights, total weight gains and average daily weight gains than those fed the basal micro-diet which did not contain Bacillus species.

The favorable increase in total weight gain may have been caused by improvement of digestion in the presence of the probiotic bacterium B. subtilis which helped improve the digestibility of the feed and increased feed absorption by secreting enzymes that affect growth and weight gain. According to [10,14], the use of probiotics in diets can help to maintain the balance of the intestinal flora of animals, preventing digestive tract diseases, improving the digestibility of feed, leading to increased use of nutrients and improving the zoo technical performance of animals. Several studies have shown that the bacteria of the genus Bacillus. Secrete exoenzymes (proteases, lipases and carbohydrates) that can help improve digestion and nutrient absorption, resulting in improved use of food and animal growth [15].

Growth performance

The data in Table 6 show the SGR, RGR and FCR of seabream larvae fed micro- particulate diets. Results showed that there were no significant ($P \geq 0.05$) differences between T2 and T3 with increased SGR and RGR values. Additionally, the results showed that there were no significant ($P \geq 0.05$) differences between T2 and T3 in terms of FCR.

Some studies have focused on growth promotion of fish by probiotic supplementation [16], and shrimp usage [17], and have also recorded variations in growth performance parameters with different dietary treatments. Furthermore, growth promoters, antibiotics, synthetic compounds, trace minerals, vitamins and enzymes) were have been used as f additive to improve animal performance, feed efficiency and economic returns. These results are consistent with those obtained by El-Dakar and Gohar (2004) [17], for M. japonicus post larvae and for juvenile Penaeus monodon [18], using Bacillus S11 as a probiotic in the feed.

Antibiotic sensitivity result

Table 7 shows sensitivity of the B. subtilis strain to various antibiotics. This result showed excellent actively all B. subtilis against almost of antibiotics with only erythromycin exhibiting some activity against B. subtilis.

Bacillus species. Produce antimicrobial compounds that inhibit the growth of pathogenic microorganisms (Ragione and Woodward, 2003) [19]. It has been speculated that commensal microorganisms

Table 3: Survival % of sea bream metamorphosed larvae fed on three different micro diets (means ± standard errors).

Experimental micro-diets	Stock number	Harvest number	Survival %	Mortality %
T1	5500 ± 0.0^a	4471 ± 54.0^a	81.3 ± 1.00^b	18.7 ± 1.00^a
T2	5500 ± 0.0^a	4851 ± 13.0^b	88.2 ± 0.25^a	11.8 ± 0.25^b
T3	5500 ± 0.0^a	4760 ± 19.5^b	86.5 ± 0.35^a	13.5 ± 0.35^b

Values with the same superscripts in the same column are not significantly different ($P \leq 0.05$).

Table 4: Total lengths of sea bream metamorphosed larvae fed three different experimental micro diets (means ± standard errors).

Experimental micro-diets	Initial length mm/ML	Final length mm/ML	Total length gain mm/ML
T1	12.1 ± 0.36^a	19.8 ± 1.1^b	7.7± 0.8^b
T2	12.1 ± 0.36^a	20.9 ± 0.5^{ab}	8.8 ± 0.2^b
T3	12.1 ± 0.36^a	22.0 ± 0.7^a	9.9 ± 0.4^a

Values with the same superscripts in the same column are not significantly different ($P \leq 0.05$).

Table 5: Total weight and average daily weight gains of sea bream metamorphosed larvae fed on three different micro-diets (means ± standard errors).

Experimental micro-diets	Initial weight mg/ML	Final weight mg/ML	TWG mg/ML	DWG mg/ML
T1	59.50 ± 1.95^a	156.97 ± 2.69^b	97.47 ± 1.02^b	4.87 ± 0.05^b
T2	59.67 ± 1.69^a	173.66 ± 5.39^a	113.99 ± 3.82^a	5.70 ± 0.19^a
T3	59.67 ± 3.93^a	167.75 ± 2.49^{ab}	108.08 ± 1.40^a	5.40 ± 0.07^a

Values with the same superscripts in the same column are not significantly different ($P \leq 0.05$).

Table 6: Specific growth rate, relative growth rate and feed conversion ratio of sea bream larvae fed on three different experimental micro-diets (means ± standard errors).

Experimental micro-diet	SGR	RGR	FCR
T1	4.85 ± 0.04^b	163.82 ± 4.70^b	1.87 ± 0.03^b
T2	5.35 ± 0.02^a	191.03 ± 2.76^a	1.36 ± 0.02^a
T3	5.15 ± 0.03^a	181.13 ± 4.90^a	1.49 ± 0.04^a

Values with the same superscripts in the same column are not significantly different ($P \leq 0.05$).

can act as reservoirs of resistance genes. This concern is highlighted by the fact that Bacillus species. Present in a number of commercially available probiotic feed supplements for both humans and animals have been shown to be resistant to several antibiotics, such as chloramphenicol, tetracycline, erythromycin, lincomycin, penicillin, and streptomycin. Probiotics have received much attention as alternatives to antibiotics. The use of some antibiotics, such as Virginiamycin, spiramycin and bacitracin, has been banned in many European countries. The appearance of drug resistant strains of antibiotics has also led to increased research on alternative methods of disease control [13, 20,21]. These aspects led us to use probiotic B. subtilis to increase larval resistance to disease and improve the immune system instead of using antibiotics that have harmful side effects on the marine environment and on the health of, aquatic animals and human health after long periods of use in addition, antibiotic residues also have adverse effects.

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Table 7: Results of the antibiotic sensitivity tests of antibiotics against *Bacillus subtilis*.

Antibiotic	Inhibition Zone mm	Susceptible	Intermediate	Resistant	Results
Neomycin (N10)	15	≥ 26	23-25	≤ 22	Resistant
Chloramphenicol (C30)	15	≥ 26	23-25	≤ 22	Resistant
Gentamicin (CN10)	17	≥ 26	23-25	≤ 22	Resistant
Tobramycin (CN10)	18	≥ 26	23-25	≤ 22	Resistant
Tetracycline (TE30)	23	≥ 26	23-25	≤ 22	Intermediate
Erythromycin (E15)	29	≥ 26	23-25	≤ 22	Susceptible

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